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RESEARCH ARTICLE

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New set of microsatellite markers for the walnut hybrid progeny Mj209xRa and assessment of its transferability into *Juglans* genus

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Abstract

Aim of the study: The research was aimed to design microsatellite markers for genotyping and differentiation of trees from the walnut hybrid progeny Mj209×Ra. As a secondary objective, the transferability and classificatory capacity of some of these loci were assessed for *Juglans* genus.

Area of study: The most widely spread walnut hybrid progeny used in Europe for wood production was used. Pure species from *Juglans* genus as Arizona black walnut (*J. major* (Torrey) Heller) and European or common walnut (*J. regia* L.), as well as a different hybrid Mj209xRa lots, were also included.

Materials and methods: Genomic DNA from a hybrid tree was used for the construction of libraries enriched with dinucleotides repeats (CA/GA). From approximately 700 fragments containing SSR regions, 18 loci were finally selected for the genetic characterization. Eight of these genomic microsatellite markers were used to assess their transferability into *Juglans* genus.

Main results: Despite the high degree of kinship of the hybrid progeny, it was possible to differentiate random trees with a low probability of error. Markers also allowed to differentiate unambiguously between Arizona black walnut and European walnut. They were even able to discriminate two hybrid Mj209×Ra lots with a high degree of certainty.

Research highlights: This new set of microsatellites might be considered a complement for the markers published up to date to perform studies into Juglandaceae family.

Additional keywords: Juglandaceae; wood production; genotyping; genotype identification; simple sequence repeats; SSR.

Authors´ contributions: AC, IM and VC constructed the libraries and designed primers. AC, JQ and RJLM conducted the evaluation of markers. RJLM drafted the manuscript and performed the statistical analysis. LG lead the team and the design of experiments. The authors have read and approved the manuscript.

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Introduction

Species from Juglans genus are worldwide distributed. Palynological evidences have shown that extinct genres from Juglandaceae have lived, at least during the Late Eocene, in Europe (Manchester, 1989), while genetic data have confirmed the presence of common walnut in glacial refugia in the Balkans and western Europe, included Spain (Pollegioni *et al.*, 2017). Current distribution of walnut in Europe resulted from the combined effects of expansion/contraction from multiple refugia after the Last Glacial Maximum and its human exploitation over the last 5,000 years (Pollegioni *et al.*, 2017). Walnuts are prized by the quality of their edible nuts, and the aesthetic and mechanical properties of their timber have contributed to make them important suppliers of fine heartwood (Phelps *et al.*, 1983) and sliced veneer (Wiedenbeck *et al.*, 2004) for the manufacture of furniture and ornamental objects.

While several varieties have been obtained for nut production, there are few progenies and genotypes available specifically for wood production (Victory *et al.*, 2004). Some programs have been initiated worldwide to address this situation (Bey & Williams, 1975; Aletà *et al.*, 2004; Woeste & McKenna, 2004;

Clark & Hemery, 2010); however, the plantations' origin is mostly from unselected material (Fady *et al.*, 2003; Jacobs & Davis, 2005). In the USA, for example, only 1% of all black walnut cubic foot volume comes from plantations (Shifley, 2004), as a partial consequence of the lack of varieties dedicated to timber production.

American black walnut (*Juglans nigra* L.) has better suitability for silviculture than European or common walnut (*J. regia* L.); however, some problems of adaptability to specific places (Woeste & McKenna, 2004) might hinder its use. The use of hybrids in agriculture and forestry is a common practice that has led to an increase in the yield of crops and, in general, to introduce important characters in the offspring. From the late XIX century onwards, some interspecific hybrids of walnut have been obtained, being Paradox the most conspicuous; although, it and its clones are mainly used as rootstocks for their high vigour and tolerance to soil pests (Baumgartner *et al.*, 2013). Other hybrids, obtained from the mating between black walnuts from Rhysocaryon section and European walnuts, known as *Juglans x intermedia* Carr., have been obtained for timber production. Among them, the hybrid Mj209×Ra has shown outstanding characteristics for timber production, and also adaptability to some European conditions (Aletà *et al.*, 2003; Clark & Hemery, 2010). However, these seed progenies are affected by a high phenotypic variability, which reduces the industrial value of their commercial plantations. The way in which this variability is managed and understood might contribute to the establishment of highly productive exploitations. Therefore, solid and suitable markers are needed for the genetic characterization of Mj209xRa progenies as well as for the genotyping and differentiation of individual trees.

The microsatellite markers are an important source of genetic information for a wide variety of purposes (Glenn & Schable, 2005; Selkoe & Toonen, 2006), from genotype identification to the study of the flow of genes and populations as well as for assisting in genetic improvement. The first library enriched with microsatellites for the Juglandaceae family was constructed from *J. nigra* L. trees (Woeste *et al.*, 2002). These SSR markers have been successfully used to analyse the genetic structure of natural populations (Victory *et al.*, 2006; Aradhya *et al.*, 2007), to characterize germplasm collections (Dangl *et al.*, 2005) and to differentiate varieties and species (Robichaud *et al.*, 2006; Ross-Davis & Woeste, 2008; Pollegioni *et al.*, 2009). However, microsatellites from American black walnut have failed to separate closely related species as *J. regia* L. and *J. sigillata* Dode (Wang *et al.*, 2008; Gunn *et al.*, 2010). Due to their limited applicability on some species and objectives, new SSR genomic libraries have been also published for *J. regia* L. (Zhang *et al.*, 2010; Chen *et al.*, 2014), *J. cathayensis* L. (Dang *et al.*, 2015) and *J. hopeiensis* Hu (Hu *et al.*, 2015), contributing in this way to increase the availability of microsatellite markers for Juglandaceae family.

The hybrid Mj209×Ra, also named NG209xRa or Garavel, results from the mating between the Arizona black walnut Crêt de Cognin (*J. major* (Torrey) Heller) and, basically, the variety Franquete (*J. regia* L.) (Becquey, 1997). While the female parent is always defined as Mj209, the name of the male parent is usually defined by the species, i.e. *J. regia*, suggesting that several genotypes might be used in the mating. Nevertheless, the Mj209xRa progeny is usually affected by a high degree of kinship, which might hamper the use of some of the published microsatellites for discriminating among individual trees with reasonably high probability. Previous screening with microsatellites used by Ross-Davis & Woeste (2008) showed a limited discrimination capability for the Mj209×Ra progeny, due to low polymorphism (data not published). Other authors have also failed to differentiate close related walnut species (Wang *et al.*, 2008; Gunn *et al.*, 2010) and have observed low polymorphisms using markers from the *J. nigra* library (Chen *et al.*, 2014). Thus, as Merritt *et al.* (2015), have stated that when primers from related taxa are not conserved, a *de novo* development on a species-by-species basis is often required. Therefore, aimed to differentiate close-related trees from the Mj209xRa progeny, a new set of genomic microsatellite markers was designed and is here presented. As a side objective, the transferability of these loci into *Juglans* genus was also assessed.

Materials and Methods

Isolation, cloning and sequencing of microsatellite loci

For the construction of libraries, genomic DNA of a tree from the hybrid progeny Mj209×Ra was used. The hybridization capture strategy recommended by Glenn & Schable (2005) to obtain DNA libraries highly enriched with microsatellite loci was followed; although some modifications were introduced. After several evaluations, restriction enzymes *BamHI*, *EcoRI* and *HindIII* showed the best results for DNA digestion (fragments sizing ≈300bp). For ligation, a linker (SuperSNX) was used, including *BamHI* restriction site (bold letters):

SuperSNX24 Forward: 5'- GTTTA**GGATCC**AGCTAGCAGAATC

SuperSNX24+4P Reverse: 5'- pGATTCTGCTA-GCT**GGATCC**TAAACAAAA

These primers, once ligated to the fragmented DNA, served as template for PCR amplifications. The same cycle proposed by Glenn & Schable (2005) was used, except that the annealing temperature was reduced to 48°C (instead of 60°C) due to the introduction of a different restriction site (*BamHI*). To capture fragments with microsatellite sequences, 2 types of biotinylated oligonucleotides were selected: $(GA)_{15}$ and $(CA)_{15}$. Separate reactions were prepared for each oligonucleotide and 50µL of beads (Dynabeads M280-Streptavidin, Thermofisher) per reaction were used. Once the enriched fragments were recovered and the beads eliminated, those were ligated to plasmids (pUC19) to transform competent colonies of *Escherichia coli* (strain DH5α). Recombinant vectors were purified with QIAprep Spin Miniprep kit (Qiagen). Each strand was sequenced at least once; in case of discrepancies this step was repeated.

Primers design, assessment and final election

The interpretation of sequences was made with AUTO-ASSEMBLER software. For the edition and design of the primers, BIOEDIT and PRIMER 3.0 software were used, respectively. The markers took the name from the initials of "Walnut hybrid", followed by a capital letter, depending whether they came from libraries CA, letter A, or GA, letter B, ended by a three-cypher number. Primers with length between 18 and 22bp were selected. The size of the repeated motifs was set up between 100bp and 350bp. For PCR amplifications, theoretical annealing temperature for each pair of primers was used. The first evaluation of the amplifications was made using agarose gel (Metaphor 3%). For the final adjustment and genotyping, primers were labelled with fluorophores.

PCR amplification and genotyping

Approximately 100mg of fresh young leaves (starting material), were grinded in liquid nitrogen and conserved at -80°C until their utilization. DNA extractions were performed with DNeasy Plant Mini kit (Qiagen). The quality of genomic DNA was assessed in agarose gel (0.8%, TBE buffer) and quantified by UV spectrophotometry (Nanodrop ND-1000, NanoDrop Technologies). For PCR amplifications, volumes of 10µl, containing 1µl 10× reaction buffer (1× was 75 mM Tris-HCl, pH 9, 50 mM KCl, 2 mM $MgCl_2$ and 20 mM $(NH_4)_2SO_4$), 10 ng genomic DNA, 0.5 µM each primer, 200 µM each dNTP, and 0.4 units *Taq* DNA polymerase (Biotools B&M Labs, Spain) were used. PCR amplifications were performed with an initial step of 5 min at 94°C, followed by 30 cycles of 94°C for 30s, 30s to the annealing temperature for each pair of primers and 72°C for 30s. Afterwards, an additional extension step was performed to 60°C for 45 min, following Victory *et al.* (2006). Forward primers were labelled with fluorophores 6-FAM, PET, VIC and NED (Applied Biosystems, USA) and PCR products were fractionated by capillary electrophoresis using an ABI 3730 Analyser (Applied Biosystems). Fragment sizes were assessed with the Peak Scanner 1.0 software (Applied Biosystems). To ensure consistent results, three amplifications per sample were performed.

Plant materials

The target sample was walnut trees from the hybrid progeny Mj209×Ra, belonging to the selection program of Bosques Naturales S. A. (Spain) for timber production. The seed trees were purchased from Payre nursery (L'Albenc, France) and planted in 1999 at Villanueva de la Vera municipality (Extremadura, Spain). For genotyping and the assessment of discriminative ability of markers, 50 trees were randomly selected from this lot [Mj209×Ra (I)]. Trees from this lot were labelled with letter D.

To determine the potentiality and transferability of the new set of markers, 20 trees from a different lot of the hybrid [Mj209×Ra (II)] (Arboforest nursery, Catalonia, Spain under licence of Vilmorin S. A., France), as well as 10 Arizona black walnuts (*J. major* (Torrey) Heller) and 10 European walnuts (*J. regia* L.), also from the germplasm collection property of Bosques Naturales S. A., were used. Trees from Mj209xRa (II) lot were labelled with letter H.

Statistical analysis

Estimation of the number of alleles per locus, allelic frequencies, observed and expected heterozygosity, and unbiased or random probability of identity (PID) were calculated using IDENTITY 1.0 software (Wagner & Sefc, 1999). As the random probability to match two identical unrelated trees is lower than the same probability for siblings (PIDsib) (Woods *et al.*, 1999; Waits *et al.*, 2001), the use of PID is a clear overestimation of the discriminative value of markers for the target hybrid progeny. The PIDsib is a conservative estimator for the Mj209×Ra progeny as it is formed by half-siblings. Nevertheless, it could be considered a closer expression of the real differentiation capacity than PID (Waits *et al.*, 2001).

A dissimilarity matrix for the sample formed by 60 trees from both hybrid lots, Mj209xRa (I) and

Mj209xRa (II) (20 trees each), as well as Arizona black walnuts and European walnuts (10 trees each), was constructed using DARwin software, version 6.0.17 (Perrier & Jacquemoud-Collet, 2006). The properties of the matrix were calculated, and a hierarchical tree was generated by the unweighted neighbour joining (UNJT) method. Data were bootstrapped 100 times to obtain the consensus tree.

Results

Primers design, optimization of PCR conditions and final selection of markers

After the digestion of genomic DNA, fragments containing redundant motifs between 100 and 300bp were selected for the identification of both microsatellites and flanking (primers) regions. The purpose was to create enriched libraries containing the dinucleotide repetitions CA/GA. Transformed *E. coli*, theoretically containing segments of genomic DNA, were cultured in selective LB-medium. Then, positive colonies were picked up and around 700 of them were sequenced. Although biotinylated dinucleotides CA/GA were used to capture the microsatellites, approximately 20% of fragments contained repetitions of trinucleotides AAT/ATG.

The edition of sequences showed that 51% out of 700 inserts combined both microsatellites (SSRs) and potentially suitable flanking regions. Hence, 360 primer pairs were designed and the average length of SSRs and the individual annealing temperature (for each pair) were estimated. When necessary the annealing temperatures were adjusted to improve the resolution of PCR products and/or to remove unspecific bands.

To determine the best concentration of the DNA template for the PCR, several quantities were assessed. The objective was to use the lowest DNA quantity that would allow (1) suitable conditions for amplification, promoting in this way (2) the obtention of scorable products. 10ng of DNA was the minimum quantity that fulfilled the above-mentioned conditions, minimizing the need to use greater quantities of fresh tissue.

Primers were then tested using trees from the hybrid progeny Mj209×Ra (I). Loci that did not amplify in the sample were rejected. Markers yielding inconsistent PCR products or those with allelic frequencies close to 1 were also discarded, as well as mono and dimorphic markers and those with more than 2 alleles per locus.

In this optimization step, 85 markers (24% of total) were selected. A further selection was performed, for which each forward primer was labelled with fluorophores. Afterwards, 61 primer pairs were rejected as they could not amplify or either rendered non-scorable products. Finally, 24 markers (Table 1) were selected for genotyping a sample formed by 18 trees from Mj209×Ra (I) progeny. After this second approach, 6 markers (WhA103, WhA201, WhA222, WhA230, WhB105 and WhB224) were also discarded as they combined low polymorphism, reduced heterozygosity, or lack of it, and poor discriminative capacity.

From the initial bulk of 360 designed primer pairs, 18 (5%) were selected based on of their potential utility for genotyping and for their capacity to differentiate trees from progeny Mj209xRa (I). Using the classification proposed by Weber (1990) and Morgante & Olivieri (1993), different types of repetitions were found, including perfect microsatellites (41.6%), as WhA221, composite microsatellites (37.5%), as WhA103, and imperfect microsatellites (20.8%), as marker WhA201 (Table 1); 80% of the microsatellites correspond to dinucleotide repetitions. CT was present in 66.7% of loci, followed by CA (50%), AG/GA/GT (22.2%) and AT (16.7%) repetitions.

Genotyping trees from Mj209×Ra (I) progeny

As expected, all 18 loci amplified in the sample formed by 50 trees from the hybrid progeny Mj209×Ra (I) (Table 2) after the previous selection of markers. Different polymorphisms were observed among markers, ranging from 3 to 6 alleles per locus, with an average of 4.2 alleles/locus. A total of 75 different alleles were detected, with the smallest allele (127bp) for locus WhB216 and the biggest (248bp) for locus WhB206a. The most informative loci were WhB107, WhB120 and WhB206a (all from GA library), with 6 alleles each.

Most of alleles showed frequencies below 0.5; although 4 of them (WhA123, WhB107, WhB111 and WhB222) registered slightly higher frequencies. Considering the hybrid state of the progeny, this suggests that (1) some of these alleles might be present in both parents and/or (2) few progenitors participated in the mating. Coincidently, 3 of these loci (WhA123, WhB107, and WhB111) showed heterozygosity close to 0. On the other side, 15 markers registered observed heterozygosities above 0.326.

WhB227a had the highest differentiation capacity and locus WhA123 was the less discriminative. The combined random probability of identity (PID) was as low as 10^{-14}, whereas the same probability for siblings (PID_{sib}) was higher, about 10^{-6} (Table 2). Nonetheless, with this set of microsatellites, the genetic profile of every individual tree was established. At the same time, these loci were suitable to discriminate unambiguously two aleatory half-sibling trees (data

Table 1. Primer sequences, repeated motifs per locus and optimal annealing temperature (TO).

Locus	Primer Sequence 5'→ 3'[(a)]	Repeat motif	TO (°C)
WhA103	F: AAGAGGGTGATTCCTCAC R: GTAAAGCACAGTTTCATGTAAG	$(TA)_5(CA)_{34}$	60
WhA123	F: CACCTTTGCATCTTTTGTTCAC R: AGGGTTTTCAAGTGTCGTTTTC	$(CA)_{26}(TA)_2$	60
WhA128	F: TGGAAGTGAAGCAATATGTTTC R: CCTTTTGGAATCGTACTACAAA	$(AT)_4(GT)_{12}$	60
WhA201	F: GCATCCAAAGGTAAGTTAGAAG R: TGGACCCTAGAACACAACA	$(G)_{19}TT(GT)_2T(GT)_2$	55
WhA214	F: TAGCAGCGTCCCTATAACTG R: GCACTCTCGAATTGTCAAAC	$(CT)_9(CA)_3CC(CA)_{17}$	55
WhA221	F: AGCCACGATACAAACACAAAC R: AGGATAAAATGCTTGCGAATAG	$(CA)_9$	58
WhA222	F: AACACCACCACCACTAAGTTC R: TTCTGCTCTTCCCTCACTAAG	$(CA)_{12}$	58
WhA227	F: GGTAGGGTCATTGGAACATAG R: GGCGTACTCTGGAGACATATAG	$(CT)_{15}(CA)_{14}$	58
WhA230	F: AGTGGCAATCTCAAGGAAG R: TCTCCGTTCATCTCATTCTC	$(GT)_{12}(GA)_{17}GCAAA(G)_3$	55
WhA243	F: TTGCATCCAAATAGCTGTCC R: TTGCTGGGAACTACTGCATC	$(CA)_6CG\ (CA)_{11}$	55
WhB102	F: TGACATGGTGTTCATAACTCAG R: GTCAACAGGCAAAGAACTAGAC	$(CT)_{17}(CCCT)_3$	55
WhB105	F: TAGCCTCGTTCCAGGTTCTATA R: CCTAGAGGTAGATGCGGTAGAG	$(CT)_5CCACGTTAT(CT)_{23}$	62
WhB107	F: TAGCCAACCTTTGTTTGACTG R: TCGGACACAACTTACAACCAC	$(CT)_{27}$	60
WhB111	F: GCCTGTGGTCAGTGTTCA R: GACGGGGAAGAAATCAAG	$(CT)_{22}$	55
WhB120	F: AGGTGGGAATATGACACTGTAC R: AATCGTGGTGCTTATTAGATTC	$(GA)_{15}$	55
WhB135	F: CATTTCAGCCTCTTGAAGTTC R: ATAGCGTAACCAGCTCCTTG	$(GT)_7$	55
WhB206a	F: ATCGTCTCTCTCTCAGTGTCTC R: AACCGTCTATATCAGTCTCAGC	$(CT)_{18}$	60
WhB210	F: GCAAGCACGATAGAAAGGAC R: GGGATACCTCTGATGCCTAAG	$(CT)_{25}$	60
WhB216	F: TCCCTTTAGCATCTATGAACTG R: GATGGTGAATGTTCCTGTACTG	$(CT)_{14}(CA)_{14}$	57
WhB222	F: TAACATACACACGCAAACACAG R: GAGGGCACACCCACTAAG	$(CA)_2(GA)_{21}$	55
WhB224	F: CCAGCAGGAGAGTCTTCG R: AGCACACCATAGAGAGAAAACA	$(CT)_{14}$	55
WhB227a	F: ATGCGTGCTTATCTGTTTAGC R: TCAAGTGTGGATGGGACTATC	$(CT)_{26}(CA)_9$	55
WhB233	F: GGACTACGCGGATTTAGTG R: CTGGTCCCCGTTTAGGTA	$(GT)_7(AG)_{15}$	55
WhB236	F: CAGGTCCTCCTTTCTCTTTC R: GCCTCTTCGTATCTGTTTCTC	$(CT)_{23}$	55

[a]F = forward primer; R = reverse primer.

Table 2. Overall allelic richness (A), allelic size, allelic frequency (AF), expected (H_{Exp}) and observed (H_{Obs}) heterozygosity, random probability of identity (PID) and for siblings (PID_{Sib}) of 18 SSR loci assessed in 50 individual trees of Mj209×Ra (I) progeny.

Locus	A	Size (bp)[a]	AF[b]	H_{Exp}-H_{Obs}	PID	PID_{Sib}
WhA123	5	138-168	0.020-0.550	0.544-0.020	0.301	0.610
WhA128	3	148-202	0.290-0.400	0.659-0.580	0.190	0.488
WhA214	4	183-212	0.010-0.490	0.622-1.000	0.212	0.528
WhA221	3	196-208	0.230-0.500	0.624-1.000	0.212	0.526
WhA227	3	155-167	0.280-0.440	0.649-0.560	0.196	0.499
WhA243	3	157-172	0.260-0.480	0.634-0.520	0.205	0.515
WhB102	3	189-211	0.190-0.500	0.617-1.000	0.219	0.532
WhB107	6	171-205	0.010-0.550	0.565-0.020	0.269	0.589
WhB111	4	181-195	0.020-0.560	0.565-0.020	0.284	0.602
WhB120	6	165-197	0.010-0.290	0.552-1.000	0.105	0.408
WhB135	3	184-191	0.170-0.460	0.753-1.000	0.220	0.526
WhB206a	6	148-248	0.010-0.250	0.622-0.340	0.171	0.489
WhB210	4	195-221	0.020-0.480	0.663-1.000	0.296	0.595
WhB216	4	127-151	0.130-0.390	0.714-1.000	0.134	0.441
WhB222	5	130-155	0.010-0.551	0.608-0.326	0.238	0.547
WhB227a	5	197-239	0.040-0.260	0.767-1.000	0.094	0.397
WhB233	4	150-171	0.180-0.330	0.790-1.000	0.116	0.419
WhB236	4	160-174	0.220-0.280	0.748-1.000	0.111	0.412
A	75				7.1753×10^{-14}	4.1624×10^{-6}

[a]range of alleles. [b]lowest and uppermost frequencies.

not shown) in the sample of 50 trees from the hybrid lot Mj209xRa (I).

Assessing the transferability of de novo markers into Juglans genus

Despite not being possible to get access to the parents of hybrid progeny, relative trees, representative for the species involved in the mating (i.e. *J. major* and *J. regia*), were selected for the assessment of transferability and the classificatory ability of markers. A different lot of hybrid Mj209xRa (II) was also included.

To facilitate the automation of genotyping, two groups of multiplexed preparations (group 1 WhA123-VIC, WhA214-PET, WhA221-6-FAM, WhB216-NED, and group 2 WhB107-VIC, WhB120-6-FAM, WhB-236-PET, WhB227a-NED) were formed. These 8 markers that combined, in general, high polymorphism, discriminative capacity and observed heterozygosity, were used to determine their transferability to other walnut taxa (Table 3). The exception were loci WhA123 and WhB107, with reduced H_{Obs} and PID; however, as 5 and 6 alleles have been previously registered, respectively, it would be reasonable to expect a greater differentiation power in different taxa. Results showed that greater heterozygosity for pure species was observed for both loci. Nevertheless, for the second hybrid lot (Mj209xRa II) they were completely homozygous.

Scorable products were obtained for all taxa and most of the loci, with variable polymorphisms, ranging from 1 allele for *J. regia* with marker WhA221, up to 8 alleles for marker WhA214 observed for the hybrid lot Mj209xRa (II). *J. major, J. regia* and Mj209xRa (I) had similar allelic richness; whereas the greatest polymorphism was registered for the second hybrid lot Mj209xRa (II) (Table 3). Arizona black walnuts and European walnuts have 17 and 18 private alleles, respectively, sharing 6 common alleles. Most of the alleles (79.7%) observed in the hybrid progenies were present also in the pure species, although the lot Mj209xRa (II) has 13 private alleles not registered before. Variable informativeness was found for each locus in the different taxa. For *J. major* the most polymorphic locus was marker WhB107; for

Table 3. Number of observed alleles, and overall allelic richness (A) for all taxa assessed.

	WhA123	WhA214	WhA221	WhB107	WhB120	WhB216	WhB227a	WhB236	A
J. major	**129***	**189**	**202**	191	**165**	**141**	**197**	**160**	
	138	**212**	**208**	**197**	**184**	**144**	**203**	**166**	
	140			201		149		168	23
	168			**203**					
				205					
J. regia	140	**183**	**196**	191	**172**	**125**	**217**	168	
	168	**185**		**193**	**176**	**127**	**235**	**172**	24
	179			201	**180**	149	**239**	**174**	
	190				**197**			**184**	
Mj209xRa (I)	140	183	196	191	165	127	197	160	
	168	189	202	201	172	141	203	166	
		212	208		180	149	217	172	27
					184	**151**	235	174	
							239		
Mj209xRa (II)	140	185	196	191	165	127	197	160	
	168	189	202	193	**167**	**137**	**200**	166	
		199	**204**	201	172	141	203	168	
		203	208		180	144	217	172	41
		206	**210**		184	**146**		174	
		212	**212**		197	149		**176**	
		214							
		222							

*Bold-numbers refer to private alleles.

J. regia were loci WhA123, WhB120, and WhB236; for Mj209xRa (I) was locus WhB227a, whereas for Mj209xRa (II) was locus WhA214.

Once the successful transferability of these markers had been demonstrated, their capacity for genetic classification was also assessed. The calculation of the matrix of distances revealed that the average distance between genotypes was 0.66716. The minimum distance (d=0.0625) was observed between trees D23 and D34, from Mj209xRa (I); while the greatest differences were observed between Arizona black walnuts and European walnuts as well as between most of the hybrids (14 out of 20) from Mj209xRa (II) and the European walnuts.

After the construction of the dendrogram, three main clusters were formed (Fig. 1). The cophenetic correlation (r=0.956) indicated a fair fit of cluster analysis. Most of the genotypes (93%, 56 out of 60) were grouped into two clusters; while two putative hybrids, one for each hybrid lot, were separated in a third group. All Arizona black walnuts and most of the Mj209xRa (II) hybrids (14 out of 20) were grouped in cluster I. The second cluster was more variable. While most of the hybrids from Mj209xRa (I) (14 out of 20) were grouped in a separate cluster (II-a), in turn, the II-b was split into two groups. In II-b-1 appear, together with 2 trees from the hybrid lot Mj209xRa (II), an independent monophyletic cluster gathering all the European walnuts. A last cluster, the II-b-2, resemble the cluster III, resulting a mix of trees from both hybrid lots.

Discussion

Construction of the enriched libraries with microsatellites and characterization of the selected markers

In this work the design of specific microsatellite markers to discriminate Mj209xRa hybrid walnut genotypes was addressed, as well as the transferability of these markers to other *Juglans* species.

Specific microsatellite enriched libraries were constructed. Notwithstanding biotinylated CA and GA oligonucleotides were used for the construction of the libraries, approximately 20% of the recovered markers contain the trinucleotide motifs AAT/ATG. Using a similar approach, Woeste *et al.* (2002) also found

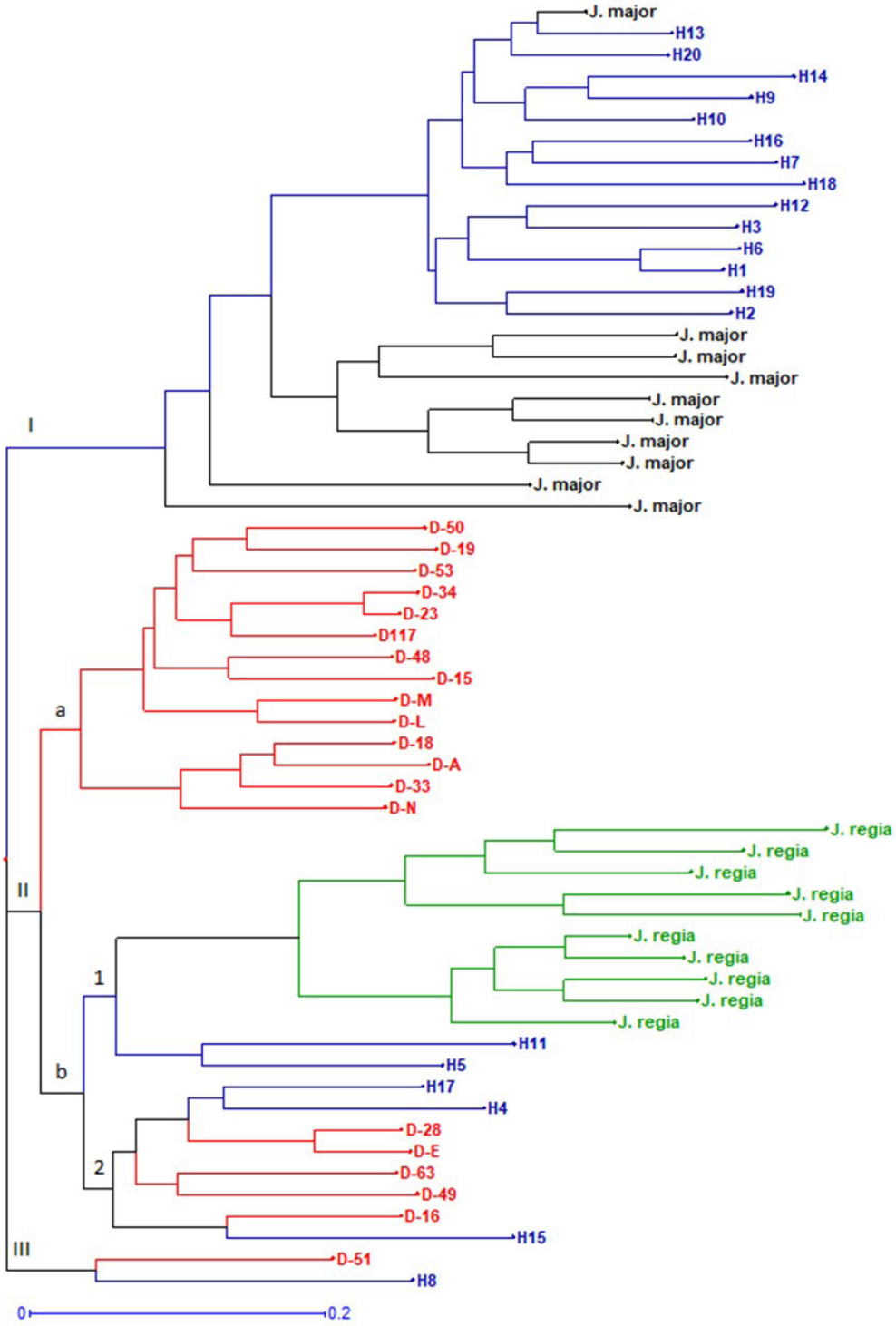

Figure 1. Unweighted neighbour joining dendrogram based on the dissimilarity distances of the allelic profiles of each individual tree. Cophenetic correlation (r) 0.956. Trees from the Mj209xRa (I) lot are labelled with letter D, while those from Mj209xRa (II) lot are labelled with letter H.

different repeated motifs, although their purpose was to create GA/CT enriched libraries. For the dinucleotide microsatellites (80% of total), variations in the type of repetition were also observed, since CT was the most repeated motif and AT was the less frequent. For some plants AT was the most abundant repeated motif (Morgante & Olivieri, 1993); although other repetition profiles, as GA_n (Merritt *et al.*, 2015), are also frequent in plant kingdom. Similarly to hybrid Mj209xRa, for *J. nigra* L., 66% of the microsatellite sequences contained perfect $(GA/CT)_n$ repeats (Woeste *et al.*, 2002). In *J. regia* L. TC was predominant, followed by

the repetitions GC and CA (Zhang *et al.*, 2010). While for *J. hopeiensis* Hu (Hu *et al.*, 2015) the dominant repeated motif in EST-SSRs was A/T (44.3%), followed by AG/CT (32.8%), AAG/CTT (4.7%), AT/TA (3.6%), and AC/GT (2.7%). Although some differences in the proportions are observed into *Juglans* genus, CT/TC, AG/GA and CA are among the most frequent repetitions; as was observed for the hybrid Mj209xRa.

Genotyping trees from Mj209×Ra (I) progeny and the assessment of transferability into Juglans genus

After the rigorous selection of markers for genotyping, scorable PCR products from all loci were obtained. Considering the origin of the Mj209xRa (I) progeny, most of the markers offered reasonably high polymorphisms, averaging 4.2 alleles/locus; with observed heterozygosity above 0.5, in general. For a bigger hybrid population (n=205), Pollegioni *et al.* (2009) found slightly better figures for polymorphism and heterozygosity using 10 microsatellites from *J. nigra*; however, the probability of identity for siblings was higher than the calculated for the lot Mj209xRa (I) with the new set of microsatellites (7.0×10^{-3} vs 4.2×10^{-6}). Therefore, the usefulness of these 18 *de novo* developed microsatellites is reflected in their capacity to establish the genetic profile of all trees assessed, as well as to differentiate random genotypes, even those differing in only one allele, as were trees D23 and D34.

Transferability of microsatellites within the same family has been widely documented; however, sometimes it must be assessed in a species-to-species base. Thus, like for the hybrid Mj209xRa (I), scorable PCR products were obtained for all selected loci in all Arizona black walnuts, common walnuts, and hybrids tested. Despite these microsatellites being specifically designed for the lot Mj209×Ra (I), a higher polymorphism was observed for hybrid trees from Mj209×Ra (II). Even, when less trees for the pure species (10 vs 20 each) were used, allelic numbers like those observed for hybrid lot Mj209×Ra (I) were registered. Other authors, as Ross-Davis & Woeste (2008), using loci developed for *J. nigra*, have also found higher polymorphisms for *J. ailanthifolia* and *J. major*, than for the American black walnuts included in their study. However, this apparent contradiction might open the opportunity to use markers outside the species or progeny for which they were created.

These microsatellite loci also confirmed their high classification capacity, when trees from different species and walnut hybrids were included in the study. Thus, the Arizona black walnuts and European walnuts were unambiguously separated, registering the greatest distances between them (ranging from 0.875 to 1.000). Even, the two hybrid lots were differentiated with some degree of certainty. Interestingly, trees from the Mj209xRa (I) lot were closer to *J. regia* than to *J. major.* While the putative hybrids (14 out of 20) from the lot Mj209xRa (II) were separated in a cluster along with the *J. major* trees. In general, trees from Mj209xRa (I) have smooth pale-grey coloured logs, with vigorous ramifications, resembling Persian walnuts; whereas those from Mj209xRA (II) have mostly grey-dark trunks, with the bark furrowed into thin ridges in the first third of the trunk, like black walnuts. This behaviour is in concordance with previous descriptions of walnut hybrid progenies that exhibit characteristics from both parents (Becquey, 1997; Hrib *et al.*, 2002; Clark & Hemery, 2010). Once nurseries usually practice selection on seed progenies, driving it towards the production of plants with phenotypic characteristics either like black or like common walnut, it might be in concordance with the representation in the dendrogram.

Conclusions

Since some published microsatellite markers for *J. nigra* were not suitable to differentiate close related trees from the hybrid lot Mj209×Ra (I), a new set of microsatellites was designed specifically for this purpose. Thus, the use of 18 of these *de novo* markers allowed to differentiate random individuals from a sample formed by 50 trees, with a calculated probability of identity as low as 4.1624×10^{-6}.

This set also proved to be transferable to other walnut taxa from *Juglans* genus, obtaining scorable and polymorphic PCR products for most of the loci assessed. Once the genetic distances were calculated, *J. major* was separated unambiguously from *J. regia*. It was even possible to discriminate two different lots from hybrid Mj209xRa with a high degree of certainty.

Therefore, the results here presented point out the potential usefulness of this set of genomic SSR markers inside the Juglandaceae family, becoming a suitable complement for the markers published up to date. Besides, the observed classificatory capacity might turn this set into a powerful tool for nurseries and genetic programs, where Mj209xRa progenies are being used.

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