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Research Article

Assessing Microsatellite Markers from *Juglans nigra* L. for Differentiation of Trees from the Walnut Hybrid Progeny Mj209xRa

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Abstract

Timber from walnut is highly prized by its aesthetical, mechanical and physical properties. Although several varieties for fruit production have been obtained, few genotypes and progenies exist for wood production. The French hybrid progeny Mj209xRa has showed great adaptability to European conditions, and its superiority regarding other hybrids and pure species, becoming the most valuable and widely used progeny for timber production. Although the great phenotypic variability that affects seed plantations reduces their industrial exploitation, it is a suitable opportunity for the selection of outstanding genotypes. Since this progeny is formed by half-siblings, the use of solid markers, as microsatellites, might help to characterize the genetic variability and to differentiate close related individuals. Recently have been published microsatellite markers for this progeny; however, because of the great degree of kinship of Mj209xRa offspring, might be necessary more markers to differentiate individuals with a high certainty degree. Therefore, the suitability of microsatellite markers from American black walnut (*Juglans nigra* L.) for genetic differentiation of trees from the walnut hybrid progeny Mj209xRa was assessed. Genomic DNA from 50 trees from the hybrid progeny Mj209xRa were used. For PCR amplifications, 10 microsatellite markers were selected, and statistical analysis were performed to determine their individual and combined differentiation capacity. PCR products from all 10 loci were obtained, although for 2 of them the amplifications were non-scorable. In general, reduced polymorphisms (3 alleles/locus) as well as lower heterozygosities and combined discriminative power (PID_{sib} in the order of 10^{-2}) were registered. Hence, this set of SSR markers was not suitable to differentiate putative hybrid trees from the Mj209xRa progeny. However, 3 loci can be used as a complement for other microsatellite sets, increasing thus the discriminative capacity of the existing markers.

Keywords: *Timber Production; Genotyping; Genotype Identification; Simple Sequence Repeats; SSR*

Introduction

Walnuts are reputed producers of fine-heartwood timber. Although wood from walnuts has good mechanical and physical properties, it is mainly prized by its aesthetical characteristics. For this reason, the markets of timber are highly variable, ranging the prices of green wood between 1500.00 and 6000.00 $/mbf (Doyle scale) [1]. Timber from American black walnut (*J. nigra* L.) is by far the most commercialized worldwide [2]. It is obtained mainly from natural stands in the USA. While in Europe, problems associated with its adaptability have confined the establishment of artificial exploitations to few locations. Since common walnut (*J. regia* L.), a species native from central Asia and Europe, has a limited industrial use, most of timber commercialized in EU proceed from importations. Nevertheless, the interest for walnut timber, moved to establish in Europe, between 1985 and 2001, around 100 000ha with seedlings from different origin [3].

Once there were available only varieties for fruit production, most of these exploitations were planted with unselected material [4], being reduced their commercial value.

During the early 80's of the last century, several initiatives started in Europe, with the main goal to assess genotypes and progenies for timber production [5]. From them, the offspring Mj209xRa, resulted of the mating between the Arizona black walnut Cre t de Cognin (*J. major* (Torrey) Heller) and, basically, the variety Franquete (*J. regia* L.) [6], has showed its great adaptability to the European conditions, as well as its superiority to other hybrid progenies and pure species, included the American black walnut [7-10]. Thus, thousand hectares of this hybrid progeny, mainly from seed materials, have been established across Europe for timber production. However, plantations from seed materials are affected by a high phenotypic variability, becoming then difficult their management, and reducing their industrial profitability.

Few is known about the genetic of the Mj209xRa progeny, and how can be managed the phenotypic variability that affect it. Therefore, the characterization of this variability might be considered an important step toward the creation of varieties for timber production, and the establishment of highly productive plantations. The first SSR genomic markers for the provenance Mj209xRa were recently published [11], allowing the identification and differentiation of random trees with a low probability of error. Since this progeny is affected by a high degree of kinship, the assessing of microsatellites from other sources might contribute to strength the classificatory capacity of the existing markers. Although it is difficult to know the effective number of loci needed for genetic differentiation of close related individuals, it has been determined that the most polymorphic loci produce more precise estimates of genetic distances [12]. In consequence, sets of loci with low polymorphisms might require a greater number of markers to increase their discriminative power. Using specific SSRs have been allowed to differentiate between random trees, even different lots, from Mj209xRa progeny; however, low allelic richness have been registered [11].

Aim of the Study

Thus, aimed to increase the pool of microsatellite markers useful to perform both studies and routine tasks for the hybrid progeny Mj209xRa, the discriminative capacity of some SSR markers from *J. nigra* L. was assessed.

Materials and Methods

Plant materials

Fifty (50) putative walnut trees from the hybrid progeny Mj209×Ra, from the selection program of Bosques Naturales S. A. (Spain) for timber production, were used. The seed trees were purchased from Payre nursery (L'Albenc, France) and planted in 1999 at Villanueva de la Vera municipality (Extremadura, Spain).

Conditions for PCR amplification and genotyping

Ten (10) primers designed for American black walnut [13] (Table 1) were assessed to determine their suitability for genotyping and genetic differentiation of trees from the Mj209xRa progeny. Fresh young leaves were collected from field growing trees and were individually 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 10 µl, containing 1 µl 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 minutes 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 minutes [14]. The first evaluation of amplifications was performed in agarose gels (Metaphor 3%), being selected 17 trees for this purpose. For the final adjustment and genotyping, 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.

Statistical analysis

Estimation of the number of alleles per locus, the allelic frequencies, the observed and the expected heterozygosity and the unbiased or random probability of identity (PID) and the same probability for siblings (PID_{sib}) were calculated using IDENTITY 1.0 software [15]. As the PID to match two identical unrelated trees is lower than the PID_{sib} [16,17], the use of PID is a clear over estimator of the real discriminative capacity of markers for the Mj209×Ra progeny, formed by half-siblings; whereas the PID_{sib} can be considered a conservative estimator. Nevertheless, PID_{sib} could be considered a closer expression of the real differentiation capacity than PID [17].

A dissimilarity matrix for the sample formed by 50 trees was constructed using DARwin software, version 6.0.17 [18]. The properties of the matrix were calculated and a hierarchical tree was generated by the unweighted neighbor joining (UNJT) method. Data were bootstrapped 100 times to obtain the consensus tree.

Results and Discussion

Despite PCR products from all loci were obtained, two of markers showed altered amplification profiles. In a first approach, locus WGA142 consistently rendered more than 3 bands for all genotypes. Whereas WGA033, after the labelling with fluorophores, not showed peaks in the expected allelic ranges. Therefore, these loci were discarded to continue with genotyping. The adjustment of conditions also included the determination of the minimal quantity of DNA for amplifications (10 ng), as well as the optimal annealing temperature for each primer, although for loci WGA004, WGA033, WGA082 [19], and WGA090 [14] those reported in the original papers were used.

Locus	Primer Sequence 5'→ 3'[a]	Species	Authors	Length (bp)	Temperature (°C)	Sum of the Reported
WGA004	F: TGTTGCATTGACCCACTTGT R: TAAGCCAACATGGTATGCCA	*J. nigra*	Woeste., *et al*. (2002)	241	45	4 (8)[b]
WGA033	F: TGGTCTGCGAAGACACTGTC R: GCATCGTCATTACCTGCTCA	*J. nigra*	Woeste., *et al*. (2002)	230	45	11 (9)
WGA082	F: TGCCGACACTCCTCACTTC R: CGTGATGTACGACGGCTG	*J. nigra*	Woeste., *et al*. (2002)	175	45	11 (9)
WGA090	F: CTTGTAATCGCCCTCTGCTC R: TACCTGCAACCCGTTACACA	*J. nigra*	Victory., *et al*. (2006)	132 - 190	55	28 (1250)
WGA142	F: CATATTCCCGGTGATTTTGG R: TGACCACAAATCGGAGATGA	Several species from Juglandaceae	Ross-Davis and Woeste (2008)	128 - 218	60	107 (486)
WGA147	F: TGGAACTTGTTCTGTGCGAG R: CCGAGTCCCCTTCACATCTA	Several species from Juglandaceae	Ross-Davis and Woeste (2008)	171 - 221	60	69 (486)
WGA148	F: GGTGAACTCCCATAGGGGTA R: CCAATGCTACTTGCAGAACC	Several species from Juglandaceae	Ross-Davis and Woeste (2008)	230 - 282	60	73 (486)
WGA204	F: GGGTCTCGCCTTCTTTTCTT R: CACAGAGAGAAGCACGGGTA	Several species from Juglandaceae	Ross-Davis and Woeste (2008)	166 - 200	60	74 (486)
WGA221	F: CGACTGCGAAGCCTTTGTAT R: TGGGCATCACACCTACGTTA	Several species from Juglandaceae	Ross-Davis and Woeste (2008)	204 - 247	60	54 (486)
WGA256	F: TGAAGACAACAAAACTGCGC R: CCGGCATTGTTTCTGAAAAT	Several species from Juglandaceae	Ross-Davis and Woeste (2008)	205 - 249	60	91 (486)

Table 1: *Information of the selected primers according to the first time that they were reported.*

[a]: F = Forward Primer; R = Reverse Primer.

[b]: In parenthesis, the number of individuals assessed.

The observed heterozygosity showed great variations (Table 1). Three loci (WGA090, WGA147 and WGA148) were completely homozygous; while markers WGA082 and WGA221 had values of H_{Obs} close to 0.50, probably conditioned by the combined presence of highly frequent alleles (> 0.50) with rare alleles (< 0.1). Although markers WGA004 and WGA256 also bear putative rare alleles, allelic frequencies below 0.50 assure high values for the expected and the observed heterozygosity. Whereas for locus WGA204, although the allele 176 was present in all genotypes (frequency 0.50), the observed heterozygosity was the highest registered; suggesting that this allele is present in only one of parent.

Locus	A	Size (bp)[a]	AF[b]	H_{Exp}-H_{Obs}	PID	PID_{Sib}
WGA040*	3	231-261	0.013-0.470	0.617-0.940	0.223	0.532
WGA082	4	171-189	0.020-0.560	0.598-0.420	0.221	0.557
WGA090	2	134-180	0.480-0.520	0.500-0.000	0.375	0.657
WGA147	2	183-191	0.120-0.880	0.211-0.000	0.645	1.105
WGA148	2	247-261	0.500-0.500	0.500-0.000	0.375	0.656
WGA204	3	168-187	0.250-0.500	0.625-1.000	0.211	0.525
WGA221*	3	227-234	0.070-0.670	0.496-0.440	0.304	0.680
WGA256	5	224-289	0.020-0.280	0.757-0.960	0.103	0.405
A	24				2.9387×10^{-5}	2.0435×10^{-2}

Table 2: *Overall allelic richness (A), allelic size, allelic frequency (AF), frequency of null alleles (N), expected (H_{Exp}) and observed (H_{Obs}) heterozygosity, random probability of identity (PID) and for siblings (PID_{Sib}) of 8 SSR loci assessed in 50 individual trees of Mj209×Ra progeny.*

a: Range of alleles.

b: Lowest and uppermost frequencies.

**: Locus bearing null alleles.*

Most of loci amplified in all 50 individuals assessed. The exception was markers WGA004 and WGA221, once their PCR products for 1 (D13) and 2 trees (D18 and D34), respectively, were not obtained. Hence, a total of 24 different alleles were observed, averaging 3 alleles/locus. A higher polymorphism was recorded for the same 50 trees using 8 of loci designed for this progeny, registering 13 more alleles (37) and 4.2 alleles/locus in average [11]; revealing some limitations of markers from *J. nigra* L. for genotyping and differentiation of trees from Mj209xRa progeny.

Different polymorphisms were registered for all loci, ranging from the dimorphic markers (WGA090, WGA147, and WGA148) to those with 4 (WGA082) and 5 alleles (WGA256). For similar size samples from pure species, higher allelic richness has been recorded using a variable number of markers from the *J. nigra* library [19]. In *J. regia* L., for a sample formed by 44 genotypes, have been registered up to 74 alleles with 14 of these microsatellites [20]; while for *J. cinerea* L. [13] and *J. nigra* L. [14] were observed in average 13 and 23 alleles, respectively. In general, there was a low allelic richness for the target hybrid sample with the 8 loci assessed, averaging 3 alleles/locus. This result shows a reduced possibility to use this set for the genetic differentiation of random trees from the hybrid Mj209xRa, as was also reflected in the high values calculated for PID (10^{-5}) and PID_{sib} (10^{-2}). For natural populations of animals, was suggested that a PID in the order of 10^{-2} might be reasonable low to differentiate individuals [17]. However, for close related populations, lower PIDs might be necessaries, as was demonstrated in dendrogram (Figure 1).

After the construction of the dissimilarity matrix, 1225 values were calculated, ranging the distances from 0 to 0.9375, with a mean distance between genotypes of 0.40429. Whereas genotype D13 registered the greatest differences (above 0.50) regarding most of trees (44 out of 50); DA and D49 as well as D50 and D23 showed the same allelic profiles, respectively. Even though the putative hybrids were classified in 3 main clusters (r = 0.869).

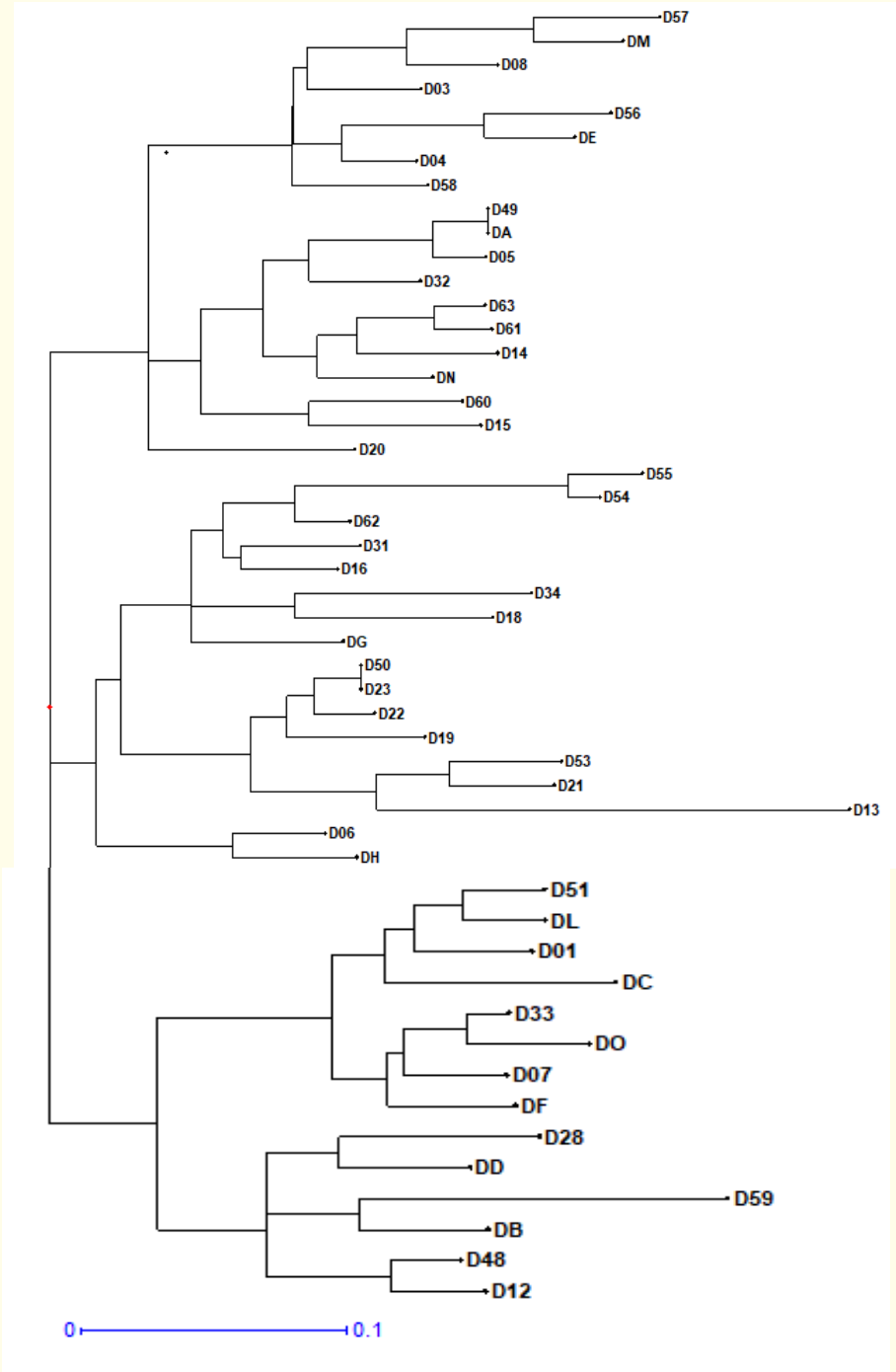

Figure 1: *Unweighted neighbor joining dendrogram based on the dissimilarity distances of the allelic profiles of each individual tree. Cophenetic correlation (r) 0.869.*

For free-ranging bears have been determined that 4 to 6 loci are required in practice to declare a match between random individuals [16]. Considering that the hybrid progeny Mj209xRa was obtained from the addressed mating of only a female parent (Mj209) and likely few unknown male parents, the probability to find trees with the same allelic profiles would be higher; making then necessary the use of a higher

number of loci (> 6) to differentiate two random trees with a reasonably high certainty. In natural close-related populations of *Petunia*, was found that 14 EST-SSR were necessary to discriminate among 4 wild taxa [21]; whereas for *J. hopeiensis* Hu was necessary the use of up to 25 microsatellite markers to perform genetic studies in natural populations from this species [22].

In a simulation (data not shown), loci WGA004, WGA204 and WGA256 were added to the eighteen loci specifically designed for Mj209xRa progeny [11]. Using the same 50 trees, the PID_{sib} was reduced from 10^{-7} to 10^{-6}; being thus effectively augmented the differentiation capacity because of the joint use of 21 markers.

Conclusion

The transferability of SSR markers depends of several factors as the grade of relationships between the target sample and the species for which these were designed; the genetic structure of assessed population; as well as the number of available and/or used markers, amongst the most important. The results here presented have showed that, although markers from American black walnut are useful for genotyping trees from Mj209xRa progeny, have limitations for their genetic differentiation probably because the high degree of kinship of this progeny. However, three of these markers can be used as a complement for the loci specifically designed for the hybrid Mj209xRa. Because of the importance of the walnut progeny Mj209xRa for timber production, it might be profitable to assess the functionality of more markers from other sources.

Conflict of Interest

The authors have declared that no competing interests exist.

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Bibliography

1. Wiedenbeck J., *et al*. "Defining hardwood veneer log quality attributes". General Technical Report NE-313. Newtown Square, PA: US Department of Agriculture, Forest Service, Northeastern Research Station (2004): 36.
2. Cassens DL. "Factors affecting the quality of walnut lumber and veneer". Proceedings of the 6th Walnut Council Research Symposium. Black Walnut in a New Century. Lafayette, EUA (2004): 161-167.
3. Ducci F and Malvotti ME. "Recent developments in the selection and breeding programme on Juglans spp. in Italy". Cost Action E42 Growing Valuable Broadleaved Tree Species. WG1 Workshop on Genetics Leuven, Belgium (2006).
4. Fady B., *et al*. "Walnut demonstrates strong genetic variability for adaptive and wood quality traits in a network of juvenile field tests across Europe". *New Forests* 25.3 (2003): 211-225.
5. Jay-Allemand C., *et al*. "Walnut trees for woodland use in Mediterranean countries: current situation and prospects". *FAO Nucis Newsletter* 5 (1996): 10-13.
6. Becquey J. "Les noyers a bois. Fore t prive e française". In: Les guides du sylviculteur. Troise me Edition (1997): 144.
7. Paris P., *et al*. "Primi risultati di prove comparative d'accessioni europee di noce da legno". Atti del III Congresso Nazionale SISEF" Alberi e Foreste per il Nuovo Millennio", Viterbo (2001): 15-18.
8. Aleta N., *et al*. "Characterization of the agroforestry performance of 12 walnut (Juglans sp.) genotypes grown in two locations of Catalonia". *Forest Systems* 12.1 (2003): 39-50.
9. Clark J and Gabriel H. "Walnut hybrids in the UK: fastgrowing quality hardwoods". *Quarterly Journal of Forestry* 104.1 (2010): 43-46.

10. Aleta N and Vilanova A. "El nogal hí brido". *Navarra Forestal* 13 (2006): 18-21.

11. Contreras A., *et al*. "New set of microsatellite markers for the walnut hybrid progeny Mj209xRa and assessment of its transferability into Juglans genus". *Forest Systems* 28.2 (2019): 009.

12. Kalinowski ST. "How many alleles per locus should be used to estimate genetic distances?" *Heredity* 88.1 (2002): 62.

13. Ross-Davis A and Woeste KE. "Microsatellite markers for Juglans cinerea L. and their utility in other Juglandaceae species". *Conservation Genetics* 9.2 (2008): 465-469.

14. Victory ER., *et al*. "Genetic homogeneity in Juglans nigra (Juglandaceae) at nuclear microsatellites". *American Journal of Botany* 93.1 (2006): 118-126.

15. Wagner HW and SEFC KM. "Identity 1.0". Centre for Applied Genetics, University of Agricultural Sciences, Vienna 500 (1999).

16. Woods JG., *et al*. "Genetic tagging of free-ranging black and brown bears". *Wildlife Society Bulletin* 27.3 (1999): 616-627.

17. Waits LP., *et al*. "Estimating the probability of identity among genotypes in natural populations: cautions and guidelines". *Molecular Ecology* 10.1 (2001): 249-256.

18. Perrier X and Jacquemoud-Collet JP. "DARwin software" (2006).

19. Woeste K., *et al*. "Thirty polymorphic nuclear microsatellite loci from black walnut". *Journal of Heredity* 93.1 (2002): 58-60.

20. Dangl GS., *et al*. "Characterization of 14 microsatellite markers for genetic analysis and cultivar identification of walnut". *Journal of the American Society for Horticultural Science* 130.3 (2005): 348-354.

21. Turchetto C., *et al*. "Genetic differentiation and hybrid identification using microsatellite markers in closely related wild species". *AoB Plants* 7 (2015).

22. Hu YH., *et al*. "De novo assembly and characterization of transcriptome using Illumina sequencing and development of twenty five microsatellite markers for an endemic tree Juglans hopeiensis Hu in China". *Biochemical Systematics and Ecology* 63 (2015): 201-211.

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