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Development of 185 polymorphic simple sequence repeat (SSR) markers from walnut (*Juglans regia* L.)

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ABSTRACT

Juglans regia L. is the only commercially grown species for nut consumption within the *Juglans* genus. Simple sequence repeats (SSRs) are the markers of choice, especially when constructing genetic linkage maps for plants. There are only a limited number of simple sequence repeat (SSR) markers in the literature for *J. regia* that can be used to construct a SSR based genetic linkage map. Therefore, genomic libraries enriched with CA, GA, AAC, and AAG repeats were constructed using genomic DNA from *J. regia* cv. 'Maraş-18' to develop SSR markers for walnut. A total of 624 clones were sequenced; 516 had repeats and 94 were duplicates. Two-hundred and seventy-six primer pairs were designed, and 246 generated PCR products and 185 polymorphic loci were obtained by characterizing 15 walnut cultivars. The number of alleles ranged from two to ten among the 185 polymorphic loci with an average of 4.3. The GA-enriched library was the best among the four libraries in terms of number of alleles, polymorphism, productivity, and information content. A higher number and frequency of SSRs were obtained from the dinucleotide-enriched libraries than from the trinucleotide libraries. The SSR markers developed in this study may help genetic studies on *J. regia* and related species, especially when constructing and integrating maps.

1. Introduction

Central Asia and its neighboring regions are believed to be the origin of a number of *Juglans* L. species (Browicz, 1976). In addition, North and South America are also known to be distribution centers for *Juglans* species (Aradhya et al., 2007). The only commonly cultivated species in the genus *Juglans* in Europe is *Juglans regia* L. Turkey is one of the origins of this species, and is the fourth largest walnut producing country in the world after China, USA, and Iran, and produces 194,298 t of nuts annually (Faostat, 2015). *J. regia* is a monoecious and a wind pollinated species.

Several molecular marker assays, such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), randomly amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), and single nucleotide polymorphism (SNP), have already been applied in walnut cultivar identification, genetic diversity studies, analyzing phylogenetic relationships, and germplasm characterization (Ciamielloi et al., 2011; Doğan et al., 2014; Fjellstrom et al., 1994; Kafkas et al., 2005; Nicese et al., 1998; Potter et al., 2002). However, most of them are dominant and have limited application in marker-assisted breeding, when integrating genetic linkage maps, especially with a heterozygous out-breeding perennial species like walnut.

Simple sequence repeat (SSR) markers have been widely used for genotyping and linkage map construction due to their good genome coverage, high polymorphism, multi-allelic nature, co-dominance, and reproducibility. These characteristics have made them one of the most useful molecular markers for detecting genetic diversity, association mapping, genetic linkage mapping, and population and evolution analysis (Jiao et al., 2012). Despite their importance, especially in genetic linkage mapping and comparative mapping studies, little attention has been paid to the development of polymorphic SSR markers in walnut. Therefore, the numbers of available SSR markers in the literature are inadequate, especially when attempting to construct a SSR-based genetic linkage map for walnut.

The first SSR development study on *Juglans* was performed by Woeste et al. (2002) who generated 30 SSR markers using *J. nigra* genomic DNA. Then Dangl et al. (2005) published 12 more SSRs, and Hoban et al. (2008) generated 13 polymorphic SSR loci from *J.*

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Table 1
The comparison of sequenced clones from CA-, GA-, AAC-, and AAG-enriched genomic libraries in walnut.

	Enriched libraries				Total/average
	CA	GA	AAC	AAG	
No. of sequenced clones	180	180	84	180	624
No. of clones with repeats	168 (93.3)[a]	146 (81.1)	67 (79.8)	135 (75.0)	516 (83.2)
No. of duplicate clones	39 (23.2)[a]	10 (6.8)	16 (23.9)	29 (21.5)	94 (18.2)
No. of remaining clones	129	136	51	106	422
No. of designed primer pairs	70	97	27	82	276
No. of amplified loci	61 (87.1)[a]	88 (90.7)	22 (81.5)	76 (92.7)	247 (89.5)
No. of monomorphic loci	15	8	8	31	62
No. of polymorphic loci	46 (75.4)[a]	80 (90.9%)	14 (63.6)	45 (59.2)	185 (74.9)
Productivity[b] (%)	25.5	44.4	16.7	25.0	29.8
No. of total allelles	219	397	42	146	804
Avg. no. of allelles (*Na*)	4.8	5.0	3.0	3.2	4.3
Avg. effective no. of allelles (*Ne*)	2.9	3.0	2.1	2.2	2.7
Expected heterozygosity (*He*)	0.58	0.60	0.47	0.47	0.55
Observed heterozygosity (*Ho*)	0.50	0.44	0.36	0.34	0.43
Polymorphism information content (PIC)	0.53	0.56	0.40	0.41	0.50

[a] Percentages.
[b] Percentage of amplified polymorphic loci in the number of sequenced clones.

cinerea. Several authors (Yi et al., 2011; Zhang et al., 2010, 2013) used the EST database of *Juglans* in the NCBI to develop 148 SSR markers. Chen et al. (2013) generated 20 SSR markers in *J. mandshurica*, and Najafi et al. (2014) and Chen et al. (2014) developed 12 and 13 SSR markers in *J. regia*, respectively. In total, there are approximately 250 published SSR markers for *Juglans* species, but this amount is still inadequate, especially when attempting to construct a SSR based genetic linkage map for walnut. Therefore, this study aimed to develop new SSR markers in *J. regia* by enriching genomic DNA with four different motifs.

2. Materials and methods

2.1. Plant materials and workflow

Genomic DNA from *J. regia* 'Maraş-18' was used to develop four microsatellite-enriched libraries. In this study, SSR primer development followed three consecutive steps: in the first step, enriched libraries with four motifs were constructed and sequenced, and the SSR primer pairs were designed. In the second step, amplification of the candidate SSR markers was tested by gradient PCR to determine their optimum annealing temperatures. In the third step, 15 diverse walnut cultivars (Chandler, Franquette, Pedro, Hartley, Fernor, Serr, Midland, Maraş-12, Kaplan-86, Şebin, Bilecik, Yalova-1, Kaman-1, Sütyemez-1, and Sütyemez-2) were used to examine allele sizes and polymorphism by capillary electrophoresis. All the plant materials in this study were obtained from the walnut germplasm collection at Sütçüimam University in Kahramanmaraş province, Turkey.

2.2. DNA extractions, construction of enriched libraries, sequencing, and primer designing

The total genomic DNA from the walnut cultivars was extracted from frozen leaves by the CTAB method of Doyle and Doyle (1987) with minor modifications (Kafkas et al., 2005). The DNA concentration was estimated visually by comparing the band intensity with known quantities of λ DNA marker after 0.8% agarose gel electrophoresis and ethidium bromide staining, or was measured by a Qubit fluorimeter (Invitrogen) according to the manufacturer's protocol. The DNAs were diluted to 10 ng/μl for the PCR reactions.

Walnut enriched genomic libraries were constructed for two dinucleotide repeats (CA and GA) and two trinucleotide repeats (AAC and AAG). The library construction and sequencing of the inserts were undertaken by Genetic Identification Services (GIS, Chatsworth, CA, USA) as described by Gürcan et al. (2010). The primers were designed using the BatchPrimer3 web-based software (You et al., 2008) with the standard parameters. Eighty-four clones were sequenced from the AAC library and 180 clones were sequenced from the other libraries (624 clones in total).

2.3. SSR-PCR reactions

Gradient PCR was first performed to determine the optimum annealing temperatures of the SSR primer pairs. It also indicated whether the primer pairs had amplifications. The gradient PCR products were run on 3% agarose gels and visualized after ethidium bromide staining.

All SSR-PCR reactions were carried out based on a three primer strategy used by Scheulke (2000), with minor modifications (Zaloğlu et al., 2015). The PCR reactions were performed in a total volume of 12.5 μL containing 10 ng DNA, 75 mM Tris–HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 2.0 mM $MgCl_2$, 0.01% Tween 20, 200 μM of each dNTP, 10 nM M13 tailed forward primer at the 5′ end, 200 nM reverse primer, 200 nM universal M13 tail primer (5′-TGTAAAACGACGGCCAGT-3′) labeled with FAM, VIC, NED or PET dye, and 0.6 U of *Taq* DNA polymerase.

PCR amplifications were conducted in two consecutive steps. The first step involved initial denaturation at 94 °C for 3 min, followed by 28 cycles of 94 °C for 30 s, 54–60 °C for 45 s, and 72 °C for 60 s. The second step consisted of 10 cycles of 94 °C for 30 s, 52 °C for 45 s, and 72 °C for 60 s, with a final extension at 72 °C for 5 min. When the PCRs were completed, the reactions were subjected to denaturation for capillary electrophoresis in an ABI 3130xl genetic analyzer [Applied Biosystems Inc., Foster City, CA, USA (ABI)] using a 36-cm capillary array with POP7 as the matrix (ABI). Denaturation of the samples was done by mixing 0.5 μl (in 6-FAM and VIC labeled primers) or 1.0 μl (in NED and PET labeled primers) of the amplified product, 0.25 μl of the size standard, and 9.75 μl of Hi-Di formamide. The fragments were resolved using the ABI data collection software 3.0, and SSR fragment analysis was performed using GeneScan Analysis Software 4.0 (ABI). The loci were given acronyms, which were 'CU' for Cukurova University, 'JR' for *J. regia* from which the SSRs were isolated, and 'A' (library enriched with the CA motif), 'B' (GA motif), 'C' (AAC motif), or 'D' (AAG motif) to indicate the library from which the sequence was isolated.