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Developing new microsatellite markers in walnut (*Juglans regia* L.) from *Juglans nigra* genomic GA enriched library

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ABSTRACT

We attempted to develop new polymorphic SSR primer pairs in walnut using sequences derived from *Juglans nigra* L. genomic enriched library with GA repeat. The designed 94 SSR primer pairs were subjected to gradient PCR in 12 walnut cultivars to determine their optimum annealing temperatures and to determine whether they produce bands. Then, the primer pairs which had amplification in agarose gel were analyzed in capillary electrophoresis to determine their allele sizes. According to the gradient PCR and capillary electrophoresis results, 60.6% of the SSR primer pairs did not amplify any bands in agarose gel. Rest of the 37 primer pairs produced bands and their annealing temperatures and allele sizes were determined. From the amplified primer pairs, 18 of them were monomorphic, while 19 of them were polymorphic. As a result, 20.2% polymorphism was obtained from 94 SSR primer pairs tested in this study which had lower ratio when compared to the literature.

Keywords: SSR, walnut, polymorphism, PCR

Introduction

There are 21 species in the genus *Juglans* of which *Juglans regia* L. is the species with major economical importance (Manning 1978). Turkey has an considerable quantity of walnut production in the world. The major producing countries in the world are China, USA, Turkey, Iran and Ukraine (FAOSTAT, 2014). Walnut has monoecious feature, so clusters of male and female flowers are on the same tree, but located in different places and is pollinated by wind (Şen 1986). The diversity within this species is low and the cultivars are mostly from natural selections. Walnut has a long juvenility period, therefore, its breeding takes a few years. It is very important to characterize the cultivars for breeding purposes. However, morphological, physiological and biochemical methods used in the characterization are time consuming and are influenced by the environment. Development of DNA-based marker may overcome these problems.

Among the PCR-based DNA molecular marker techniques, simple sequence repeat markers (SSRs) are a perfect polymorphism source for eukaryotic genomes. Because SSRs have more genetic information and are co-dominant, and it is preferred in the areas of genetic mapping and population genetics (Powell et al. 1996). In walnut, biochemical methods have been first used to identify the cultivars (Arulsekar et al. 1985, 1986;

Aleta et al. 1990, ; Germain et al. 1993; Malvolti et al. 1993; Solar et al. 1994; Fornari et al. 2001; Vyas et al. 2003). Then, RFLP (Fjellstrom et al. 1994), RAPD (Nicese et al. 1997; Malvolti et al. 1997, 2001; Woeste et al. 1996), ISSR (Potter et al. 2002), SSR (Woeste et al. 2002; Dangl et al. 2005; Foroni et al. 2005, 2007; Victory et al. 2006; Robichaud et al. 2006; Ross-Davis and Woeste 2008a,b), Pollegioni et al. 2008; Wang et al. 2008; Hoban et al. 2008, Zhang ve ark. 2010) have been used in the characterization of genetic resources, genetic mapping and population genetic studies.

By now 56 SSR primer pairs from *J. nigra* genomic DNA, 13 microsatellite primer pairs from *J. cinerae* species and 41 EST-SSR primer pairs were developed from *J. regia* species (Woeste et al. 2002; Dangl et al. 2005; Foroni et al. 2005,2007; Victory et al. 2006; Robichaud et al. 2006; Ross-Davis and Woeste 2008a,b; Pollegioni et al. 2008, Wang et al. 2008, Zhang ve ark. 2010). So, there are 110 SSR primer pairs reported so far that can be used in genetic studies of *Juglans* species.

Studies on the genetic linkage map construction in *Juglans* species are limited and SSRs are powerful tools in genetic mapping studies because of their co-dominant nature. SSRs are very useful markers especially for reference genetic map construction in a plant species. However, there have to be enough SSR markers developed to use in the construction of a reference genetic map in walnut. Because of the limited number of SSR markers in the literature, there is no reference map in walnut. Therefore, we conducted a study to develop new SSRs in walnut.

Materials and methods

Plant material and DNA isolation

As plant materials, 'Maraş-12', 'Kaplan-86', 'Chandler', 'Franquette', 'Serr', 'Pedro', 'Van-4', 'Yalova-1', 'Bilecik', 'Şebin', 'Karabodur' and 'Maraş-18' cultivars were used in this study. Ninety-four SSR primer pairs developed by Dr. Woeste (Department of Forestry and Natural Resources, Purdue University) were used.

Genomic DNA was extracted using the CTAB method of Doyle and Doyle (1987) with minor modifications (Kafkas et al. 2006). DNA concentration was determined by gel electrophoresis (0.8 % agarose gel) and adjusted to 5 ng/µl for SSR reactions (Figure 1).

SSR analysis

PCR reactions and cycling condition in SSR analysis were done according to Zaloglu (2008) by using M13 tailed primer in accordance with the method developed by Schuelke (2000). M13 universal primer 5'-TGTAAAACGACGGCCAGT-3' is attached to the forward primer at the 5' end and synthesized. Forward primers became 38-42 base-length. At the same time, 5' end of M13 primer of which base sequence is given are synthesized by labelling with 6-FAM, VIC, NED and PET fluorescent dyes. Therefore, SSR reaction included reverse primer, forward primer with M13 primer tailed at the 5' end and labelled M13 primer. The optimum annealing temperatures of SSR primer pair were determined by gradient PCR by applying six different temperatures.

12.5 µl PCR amplification reaction includes 75 mM Tris-HCl, pH = 8.8, 20 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.1% Tween 20, 0,2 mM dNTP, 10 nM forward primer with an M13 tail at the 5' end, 200 nM reverse primer, 200 nM universal M13 primer labelled with one of the following dyes (6-FAM,VIC, NED, PET), 0.6 unit Taq DNA Polimerase and 10 ng DNA. Denaturation was 3 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 45 s at 50-60 °C and 1 min at 72 °C, and then followed by 10 cycles of 30 s at 94 °C, 45 s at 52 °C and 1 min at 72 °C. Final extension was included a cycle of 5 min at 72 °C.

Electrophoresis of PCR products

The products of gradient PCR reactions were stained with etidium bromide in 3%-agarose gel, and their photos were taken under UV transsimilator. 50 base pair size standard was used to identify DNA band size in the gel. In order to identify exact allele size of SSR primer pairs, the electrophoresis of PCR reactions was done on automatic base sequencing device using the model of ABI 3130xl having 16 capillary array (capillary electrophoresis), and the allele sizes were defined using Genemapper 4.0 software.

Results

Gradient PCR analysis

Ninety-four SSR primer pairs were screened by using DNAs of 'Maraş-12' and 'Kaplan-86' cultivars. An example of the agarose gel image in gradient PCR is shown in Figure 2**.**

37 (39.4%) of 94 SSR primer pairs produced DNA band and, therefore, their annealing temperatures were determined (Table 1). In the conclusion of the gradient PCR, the annealing temperatures of the SSR primer pairs varied from 50°C to 60°C.

Determination of allele sizes and polymorphism level of the SSR primer pairs

The PCR reactions were done using 37 SSR

primer pairs which produced band in agarose gel in 12 *Juglans regia* cultivars and the results are given in Table 1. According to the results of capillary electrophoresis (Figure 3), 18 of 37 analyzed primer pairs produced monomorphic bands, while 19 of them (51.4%) were polymorphic. So, 60.6% (57 primers) of 94 SSR primer pairs were out of the evaluation because of non-amplification in the PCR, and 19.2% of them (18 primers) were monomorphic and 20.2% of them (19 primers) were polymorphic.

32 of the 37 primer combinations produced alleles in single locus, 3 of them had amplification in two loci and 2 of them amplified in three loci. Sixteen out of 32 SSR primer pairs amplified in single locus were identified as monomorphic, while the rest of them were polymorphic. Three SSR primer pairs amplified two loci and they produced 4 monomorphic and 2 polymorphic loci. Two SSR primer pairs amplified three loci and they produced 2 monomorphic and 4 polymorphic ones. From 37 evaluated SSR primer combinations, 44 loci were produced and 22 (50.0%) of them were monomorphic loci, while 22 (50.0%) of them produced polymorphic ones. Totally, 124 alleles were produced from 44 loci and there were an average of 2.8 alleles per locus.

Discussion

The low rate of amplification success which 37 (39.4%) of 94 SSR primer pairs produced DNA band can be attributed to transferability of the SSRs from *J. nigra* to *J. regia*. Similar results were also reported earlier (Woeste et al. 2002; Dirlewanger et al. 2002).

In SSR primer development studies in walnut as reported in scientific literature, the primers have been developed in *J. nigra* species as in this study. The highest number of primer pairs have been developed by Zhang et al. (2010), followed by this study and Woeste et al. (2002), respectively. The allele sizes varied between 100 and 362 base pairs in this study. The highest rate of average number of allele per primer was obtained by Victory et al. (2006), whereas the lowest rates were obtained by Zhang et al. (2010) and in this study (Table 2).

In conclusion, 94 SSR primer combinations were designed from genomic DNA library of *J. nigra* and tested in *J. regia* . Consequently, new SSR primers were developed in this study. They can be used in genetic characterization, genetic mapping and population genetic studies in walnut. Moreover, polymorphic SSR markers together with monomorphic ones should be tested in the other walnut species in the genus *Juglans*.

Figure 1. A concentration image of walnut DNA after agarose gel electrophoresis.

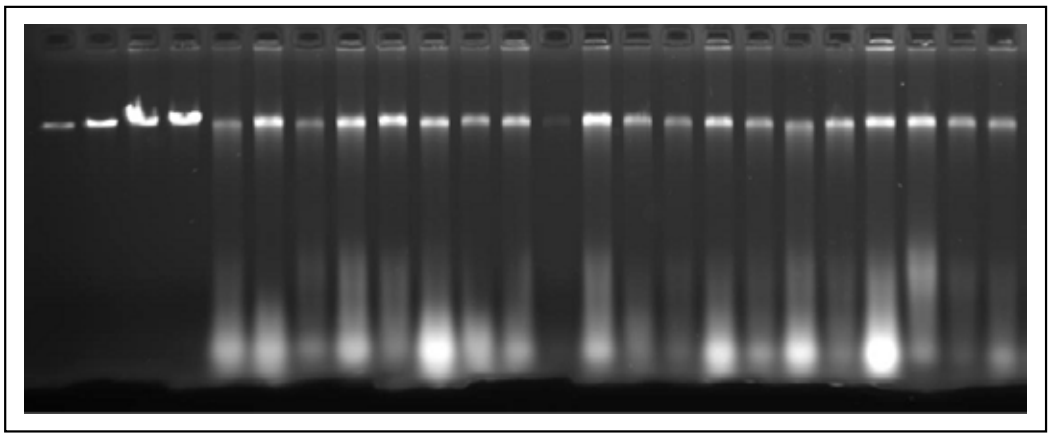
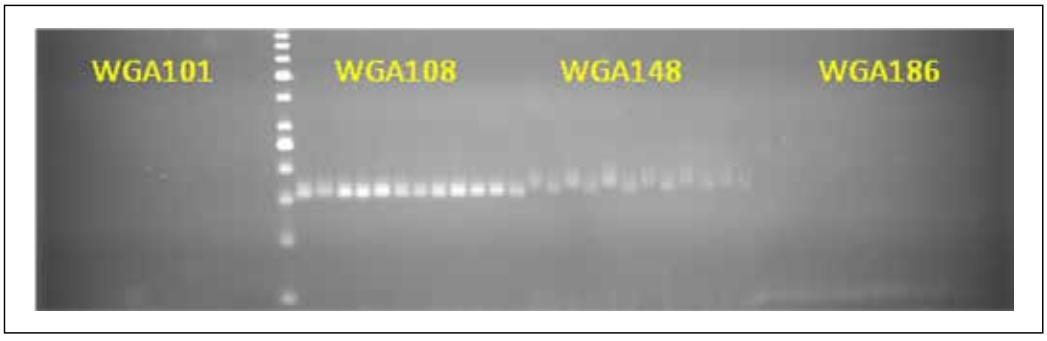


Figure 2. A gel image of three SSR primer pairs after gradient PCR.



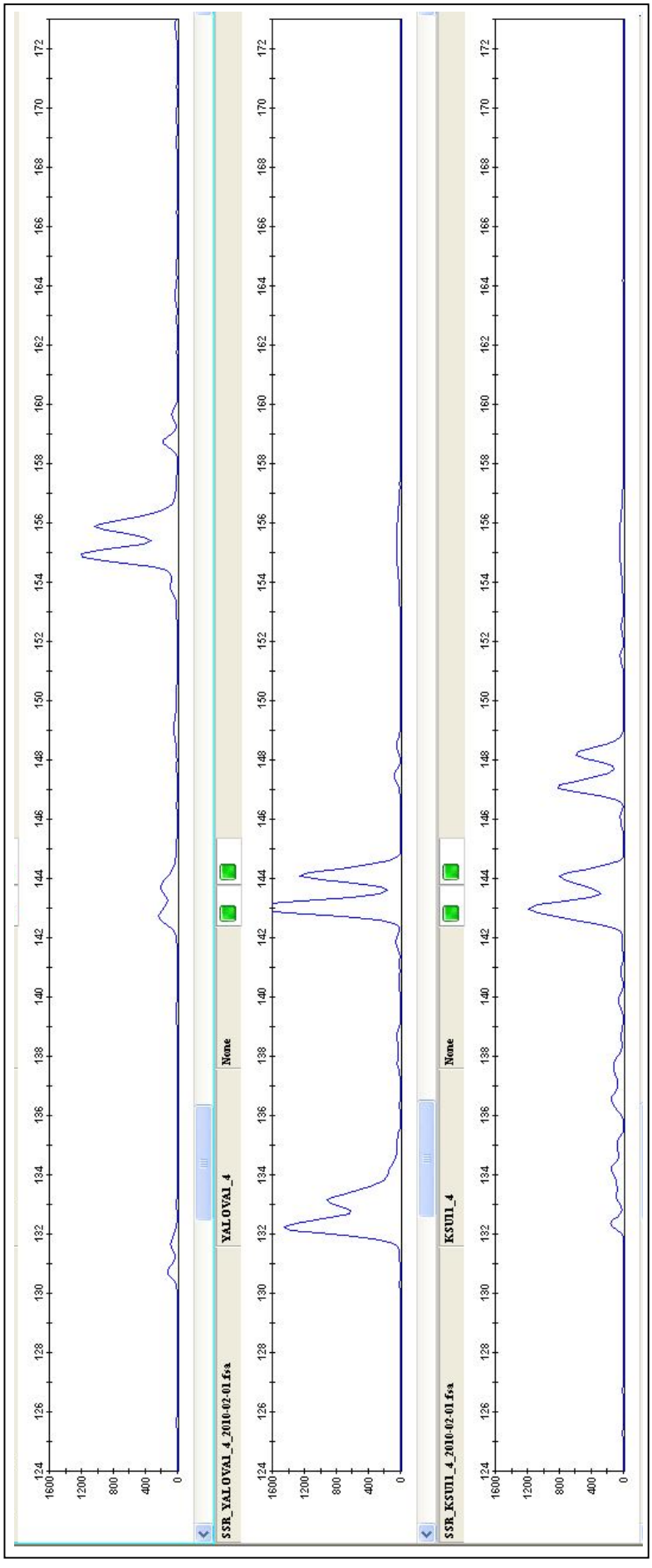


Figure 3. An electropherogram of WGA123 locus in *Juglans* cultivars obtained from capillary electrophoresis.

Table 1. Annealing temperatures, number of locus, allele sizes and allele numbers of the SSR primer combinations.

No	Primer Name	Annealing Temperature	Fluorescent Dye	Allele Size Range (bp)	No. of Alleles	Number of Locus	Type of marker
1	WGA101	58	PET	120	1	1	Monomorphic
2	WGA104	60	PET	121-139	6	1	Polymorphic
3	WGA108	58	VIC	145-157	2	1	Monomorphic
4	WGA110	54	VIC	103	1	1	Monomorphic
5	WGA111	60	NED	250	1	1	Monomorphic
6	WGA112	56	VIC	106	1	1	Monomorphic
7	WGA116	50	FAM	362	1	1	Monomorphic
8	WGA123	52	FAM	105-271	13	3	Polymorphic
9	WGA125	52	NED	173-253	2	2	Monomorphic
10	WGA126	52	NED	100-350	5	3	Polymorphic
11	WGA127	52	PET	238-281	6	1	Polymorphic
12	WGA131	56	FAM	179-298	5	2	Polymorphic
13	WGA133	50	PET	121-130	3	1	Polymorphic
14	WGA134	52	NED	362	1	1	Monomorphic
15	WGA135	58	NED	355	1	1	Monomorphic
16	WGA136	54	FAM	218-268	9	1	Polymorphic
17	WGA137	54	PET	227	1	1	Monomorphic
18	WGA139	56	NED	171-186	4	1	Polymorphic
19	WGA140	58	FAM	230	1	1	Monomorphic
20	WGA142	54	VIC	243-271	5	1	Polymorphic
21	WGA145	56	VIC	153-161	3	1	Polymorphic
22	WGA148	52	VIC	151-171	3	1	Polymorphic
23	WGA150	60	FAM	199-209	4	1	Polymorphic
24	WGA153	58	FAM	113	1	1	Monomorphic
25	WGA160	54	NED	245	1	1	Monomorphic
26	WGA167	52	FAM	241-253	4	1	Polymorphic
27	WGA168	54	NED	222-259	2	1	Monomorphic
28	WGA169	52	PET	175-188	6	1	Polymorphic
29	WGA171	58	VIC	131-147	5	1	Polymorphic
30	WGA182	60	PET	181-254	3	2	Monomorphic
31	WGA185	54	VIC	262-269	2	1	Polymorphic
32	WGA190	58	PET	135-143	3	1	Polymorphic
33	WGA193	56	FAM	228-267	8	1	Polymorphic
34	WGA195	54	NED	172-198	6	1	Polymorphic
35	WGA196	52	NED	260	1	1	Monomorphic
36	WGA198	52	PET	137-178	2	1	Monomorphic
37	WGA200	54	PET	181	1	1	Monomorphic

Table 2. Comparison of data in this study with the SSR primer development studies in the literature.

No.	Species	Reference	Number of primer pair	Allele Size range (bp)	Average number of alleles
1	*J. nigra*	Woeste et al. (2002)	30	150-242	7.3
2	*J. nigra*	Dangl et al. (2005)	12	143-275	5.2
3	*J. nigra*	Foroni et al. (2005;2007)	4	120-266	6.0
4	*J. nigra*	Victory et al. (2006)	4	162-236	23.8
5	*J. nigra*	Robichaud et al. (2006)	1	208-250	-
6	*J. nigra*	Ross-Davis and Woeste (2008a)	5	161-164	12.2
7	*J. cinerae*	Hoban et al. (2008)	13	103-358	13.6
8	*J. regia*	Zhang et al. (2010)	41	-	3.0
9	*J. nigra*	In this study	37	100-362	3.4

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