Journal of Nuts 5(1):51-55, 2014
ISSN: 2008-9937

RAPD Analysis for Sex Determination in *Pistacia vera* L.

*F. Kamiab**[1], *A. Ebadi,*[2] *B. Panahi*[3], *A. Tajabadi*[3]

[1] Department of Horticulture, Rafsanjan Branch, Islamic Azad University, Rafsanjan, Iran
[2] Department of Horticulture, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran
[3] Iran's Pistachio Research Institute, Rafsanjan, Iran

Received: 22 December 2013 Accepted: 5 March 2014

Abastract

Random Amplified Polymorphic DNA (RAPD) analysis of *Pistacia vera* L. was used to distinguishing sex type in progeny plants. Varied genotypes of the pistachio were analyzed by PCR amplification of a ten base primer previously found to be linked to the expression of a 945bp amplification band in female trees and its absence in males. Results revealed that genetic material from cultivar samples amplified well with relative primer, but some male progeny produced the 945bp band when amplified with the 10bp primer. Despite numerous subsequent tests for reamplification of the DNA, unfortunately, these male progeny failed to give reproducible results from its sample. None the less, presence of the 945bp amplification band in a male sample proved that an error rate of 15% is possible in sex determination of *P. vera* by RAPD analysis with the 10bp primer.

Keywords: Female, Male, PCR, *Pistacia vera*, RAPD, Sex.

Introduction

Pistachio is a dioecious species originated from the central-west region of Asia**.** *Pistacia vera* L., the cultivated species of pistachio in the middle East, Europe and California is a dioecious plant with male and female flowers on separate trees. Pollination is a critical for this crop. The marketable product of tree is seed. To assure a good fruit set, male trees should be interplanted in the proportion of one male to twenty females in Rafsanjan area conditions. Rafsanjan is the major plantation area of pistachio production in Iran. Since pollination is done by wind and *Pistacia vera* is a dioecious species, the main desirable character of a pollinator is an adequate production of viable pollen at a time when the female cultivars are in full bloom condition (Niklas and Buchmann, 1988). Many dioecious plants breeding schemes are influenced by gender (Alstrom-Rapaport et al. 1998).The sex of pistachio trees cannot be established until they reach a reproductive age (Hormaza *et al.,* 1994).

Pistachio seedlings, however, do not flower until they reach to 5 to 8 years old. Since identification of sex is done by analysis of the tree's flowers, currently it is infeasible to distinguish sex prior to maturity. Early diagnosis of sex type of seedlings would be assisted breeders and nursery managers in pistachio orchards. Current study, developed a method of sex identification in young *P. vera* plants. Among numerous, Random Amplified Polymorphic DNA (RAPD) analysis of potentially sex linked genetic markers, however, has shown to have promise. RAPD analysis, was first proposed by Williams *et al*. (1990), uses short, arbitrarily sequenced oligonucleotide primers to amplify polymorphisms in the DNA of related specimens. This technique, theoretically, is more convenient than standard restriction fragment length polymorphysm (RFLP) and polymerase chain reaction (PCR) since less work and sequence are required for amplification using the RAPD primers. Thus, RAPD analysis has been used to study polymorphisms in *P. vera* and other organisms. Using a decamer oligonucleotide primer with sequence (CCTCCAGTGT), Hormaza *et al.,* (1994) found a 945bp band in only female *P. vera* samples and proposed that the 945bp band be used as a marker in sex identification in *P. vera* progenies. This research was conducted on few samples with 20% error. Kafkas *et al.,* (2001) found, one primer, OPAK09, amplified a female – associated band (850bp), that was present in all 46 female individuals tested and absent in all 38 male trees tested. BC156 and BC360 used in order to determine sex in *P. eucarpa* that 1300bp band and 500bp band were amplified, respectively, with BC 150 and BC 360, were present in the most of females and were absent from all of the males. Tracy *et al*., (2004) found, Amplified fragment length polymorphism (AFLP) marker for distinguishing sex in fig and 246bp band was present in all of the males, but was absent in all of the females.

***Corresponding author:** E-mail: f.kamiab56@gmail.com

Several researchers have shown that random amplified polymorphic DNA (RAPD) banding patterns are linked to sex in plants such as Carica papaya (Urasaki *et al.,* 2002), Encephalartos natalensis (Prakash *et al.,* 2006) and Sequence characterized amplified regions (SCAR) markers have also been widely used to distinguish between the two sexes in *Asparagus officinalis* (Gao *et al.,* 2007), Carica papaya (Bedoya *et al.,* 2007), and *Rumex nivalis* (Stehlik and Blattner, 2004).

RAPD method performed for distinguishing sex about for many plants, i.e. *Salix viminalis, Piper longum and Trichosanthes dioica* Roxb. (Alstrom Ranagport *et al*., (1998), Banjeree *et al*., **(**1999) and Singh *et al.,* (2001). This article, describes the utility and reproducibility of RAPD analysis using this decamer primer for distinguishing sex in *Pistacia vera* seedlings.

Materials and Methods

Plant Material

The plant material used in this experiment was leaf tissue collected in the Spring season from a population of mature grafted pistachio trees located in the No.2 pistachio research station, Rafsanjan, Iran and garden of Karaj faculty of agriculture. The progeny samples, from 39 male and 4 female trees were collected and labeled in a laboratory of department of horticulture, faculty of agriculture, university of Tehran, Karaj, Iran. This research was conducted during years 2003-2005.

DNA Extraction and Isolation

Genomic DNA extraction with a high quantity and quality is one of the most important primary needs in genomic laboratories. In extraction of DNA from woody plants, such as fruit trees, besides the presence of polysaccharides, presence of phenolic compounds negatively affects on DNA quality. Therefore using methods for DNA extraction to enabling reduction these compounds to the lowest level is highly preferred. In order to use cetyltrimethylammonium bromide (CTAB) isolation protocol for DNA extraction (Table 1). Leaf tissue (0.5 g) was used for each sample. The leaves of each sample were then ground to a fine powder in liquid nitrogen, transferred to 5ml buffer extraction (Tris-Hcl pH8 2M, Na-EDTA 0.5M and NaCl 5M + CTAB 2%+ 2-mercaptoethanol 2%) and incubated at 60°C for 30 minutes. The samples were then mixed with 3.5 ml chloroform-isoamyl alcohol (24:1) and centrifuged at 10,000 rpm for 15 minutes. The aqueous layer was recovered and mixed with 0.6 volume of cold isopropanol to precipitate the DNA, then put them in laboratory condition for 10 minutes and centrifuged at 10000 rpm for 15 minutes. The isopropanol was decanted and the precipitated nucleic acid washed twice with ethanol 75%. The isolated DNA was dried for 30 minutes and resuspended in 500 µl water. Quantity and quality of extracted genomic DNA was determined by the spectrophotometer and agarose gel electrophoresis. The isolated DNA was diluted to approximately 5 ng/µL and subjected to polymerase chain reaction amplification.

Table 1. Mury & Thompson Extraction buffer (1980).

Autoclave	Chemical substrates	Final concentration	Stock contractions	For 100cc
Yes	Tris-HCl pH8	100 mM	2 M	5cc
Yes	Sodium EDTA	200 mM	0.5 M	4cc
Yes	NaCl	1.4 M	5 M	28cc
No	CTAB	-------	------	2g
No	B-mercaptoethanol	-------	-------	2cc

Polymerase chain reaction

PCR reactions were performed as described in Hormaza *et al*. (1994) with slightly modifications. The 25 µl reactions each contained 5 ng genomic DNA, 1.75 mM $MgCl_2$, PCR reaction Buffer lx, 0.2 mM dNTPs, 0.2 µm opo-08 primer, one unit Taq DNA polymerase and water. After gently mixing the samples, amplification reactions were performed in a MJ-Research Inc. Mini cycler, using the following amplification program: 1 cycle of 4 minutes and 94 °C followed by 35 cycles of one minutes at 92 °C, 1 minute at 37 °C and 2 minutes at 72 °C, then one cycle 5 minutes at 75°C. PCR reaction was performed in a thermal cycler-Bio-Rad Model (1-cycler) machine. After PCR reaction, 5 µl loading buffer added to each sample. The Samples were then stored at 4 °C.

Analysis of the PCR products was done by gel electrophoresis with 1.5% agarose and lx TBE Buffer. The samples were then tested by electrophoresis under electricity, 100 Volt during 2hours. The produced gels were then stained with ethidium bromide and visualized with a UV trans-illuminator by Gel document, UVP machine.

Results

The results of this experiment revealed opo-08 primer with sequence (CCTCCAGTGT) as having the capacity to differentiate between sex types (Fig. 1 and 2). This primer was found to be able to amplify even a weak 954-bp fragment in female

pistachio (*P. vera*). but this fragments was absent in most of the male samples. There was an error rate of 15% in the results. Six samples of thirty-nine samples of males (Lane No: 11, 13, 14 and 4, 17, 20) revealed 954-bp fragment similar to females. (Table 2 and Table 3)

Table 2. PCR results in different pistachio genotypes

Lane No.	Cultivar	Sex	945bp band	Lane No.	Cultivar	Sex	945bp band
	1kb	*ladder*		12	P2	Male	No
1	F3	Female	Yes	13	F4	Female	Yes
2	R29	Male	No	14	R28	Male	Yes
3	R21	Male	No	15	R30	Male	No
4	R20	Male	No	16	P2	Male	No
5	R25	Male	No	17	R19	Male	No
6	P9	Male	No	18	R27	Male	No
7	P3	Male	No	19	P4	Male	No
8	R23	Male	No	20	R30	Male	No
9	P10	Male	No	21	P6	Male	No
10	R22	Male	No		*1kb*	*ladder*	
11	R26	Male	Yes				

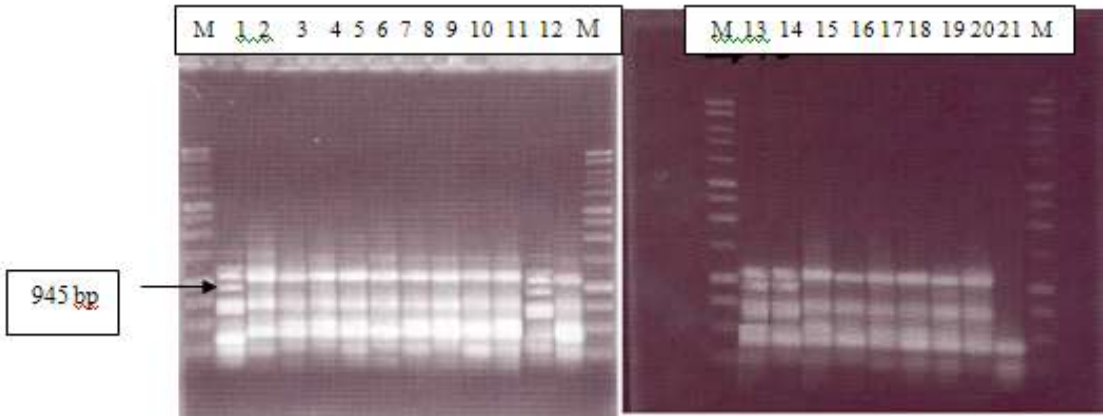

Fig. 1. Lanes labeled from 1 to 21 (left to right). The marker size (M: 1Kb) is loaded before the first genotype and after the last genotype.

Table 3. PCR results in different pistachio genotypes

Lane No.	Cultivar	Sex	945bp band	Lane No.	Cultivar	Sex	945bp band
1	1 kb ladder			13	K35	Male	NO
2	F1	Female	Yes	14	N14	Male	NO
3	N1	Male	NO	15	N7	Male	NO
4	N17	Male	Yes	16	N5	Male	NO
5	N19	Male	NO	17	K34	Male	Yes
6	O32	Male	NO	18	N11	Male	NO
7	K37	Male	NO	19	M1	Male	NO
8	N15	Male	NO	20	N17	Male	Yes
9	N13	Male	NO	21	K38	Male	NO
10	N16	Male	NO	22	M2	Male	NO
11	N2	Male	NO	23	F2	Female	Yes
12	N9	Male	NO	24	1kb ladder		

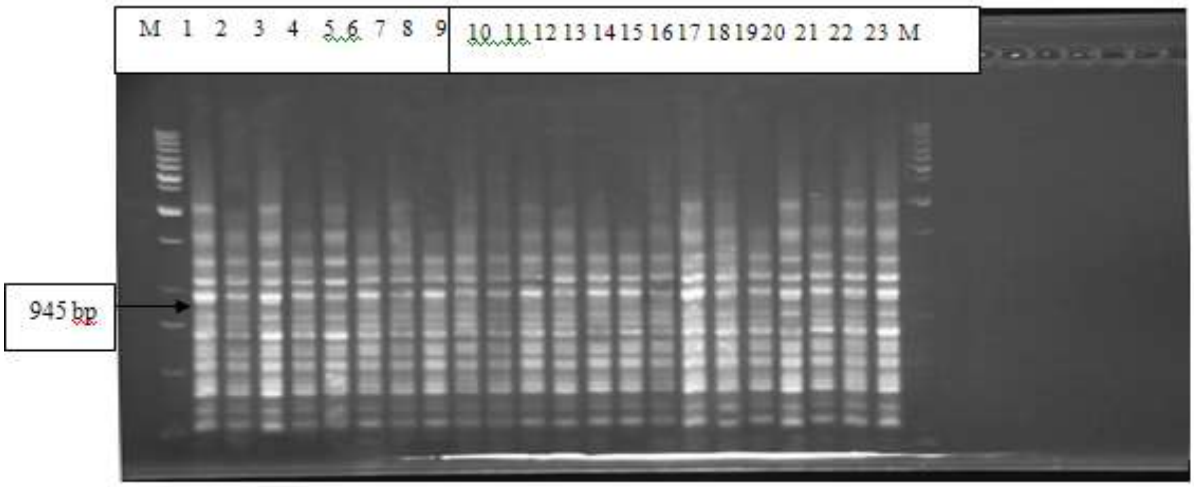

Fig. 2. Lanes labeled from 1 to 24 (left to right). The marker size (M: 1Kb) isloaded before the first genotype and after the last genotype.

Discussion

Amplification of cultivar DNA using the opo-08 primer was done and yielded results (Table 2 and 3, and Fig. l and 2). The data were in agreement with Hromaza *et al.*, (1994), the experimental female cultivars produced a 945bp band after PCR amplification while the male cultivars did not, but 15% of the male progeny samples (6 of 39 samples) produced the 945bp bands. Since it was proposed that only female samples amplified the 945bp band, and its presence in a male specimen throws in to a question: the reliability of the 945bp band in distinguishing sex in *P. vera*.

It was not revealed that the produced 945bp band, whether was due to contamination, to inherent errors of RAPD analysis, or to imperfect heredity of the 945bp band in *P. vera* female progeny? In any case, the presence of the 945bp band in a male sample supports the conclusion of an error rate of 15% in heredity of the band. According to the Staub *et al.* (1996), their results were without the amplification band. Further analysis, with appropriate changes is needed.

As genetic sex determining factors are highly conserved sequences in different species. we tentatively suggest that in these species with different chromosome numbers, the amplified DNA fragment is closely linked to the sex-determining genes.

In dioecious plants, it has been postulated that many genes are involved in the differentiation of male and female flowers but that sex differentiation could be controlled by a single locus acting as a trigger. In such a scenario, genes having the genetic information for carpel or stamen development would be present in both male and female plants, with one major gene being the only difference between the two sexes (Irish and Nelson1989). Pistachio may have a similar system, with a single major gene controlling sex determination. It is possible to identify this key gene using bulked segregant analysis via the crossing of two male and female genotypes of pistachio as performed by Hormaza *et al.*, (1994) and confirmed by Yakubov *et al.* (2004), showing that this reproducible sex marker can possibly be used for sex determination of other species of Pistacia.

References

Alstrom Ranagport C, Lascoux M, Wang YC, Roberts G, Tuskan GA (1998) Female sex associated RAPD marker in *Salix viminalis*. Journal of Heredity. 89, 44-49.

Banjeree NS, Manoj P, Das MR (1999) Female sex associated RAPD marker in *Piper longum*. Current Science. 7, 693-695.

Bedoya GC, Nunez V (2007) A SCAR marker for the sex types determination in Colombian genotypes of Carica papaya. Euphytica. 153, 215–220.

Bushra C, Afshan Y, Tayyab H, Riazuddin S (1999) Mini-Scale genomic DNA extraction from cotton. Plant Molecular Biology Reporter. 17, 1-7.

Gao WJ Li RLLi F, Deng CHL Li SP (2007) Identification of two markers linked to the sex locus in dioecious Asparagus officinalis plants. Russian Journal of Plant Physiology. 54, 816–821.

Hormaza JL, Dollo L, VS Polito (1994) Identification of a rapid marker linked to sex determination in *Pistacia vera* using bulked segregant analysis. Theoretic applied Genetics. 89, 9-13.

Irish E, Nelson T (1989) Sex determination in monoecious and dioecious plants. Plant Cell. 1,737–744

Kafkas S (2001) Development of sex associated RAPD markers in wild *pistacia* species.

Journal of Horticultural Science and Bitechnology. 18-22

Parrish T (2004) Identification of a male- specific AFLP marker in a functionally dioecious fig (Moraceae). Sex Plant. 17, 17-22.

Singh M, Kumar S (2001) Female sex associated RAPD marker in pointed gourd (*Trichosanthes dioica* Roxb). Current Science. 82,131-132.

Staub JK, Sergen FC, Gupta M (1996) Genetic markers map contraction and their application in plant breeding. Horticultural Science. 31, 729-741.

Staub J, Bacher J, Poetter K (1996) Sources of potential errors in the application of random amplified polymorphic DNAs in cucumber. Horticultural Science. 31, 262-266.

Stehlik I, Blattner FR (2004) Sex-specific SCAR markers in the dioecious plant Rumex nivalis (Polygonaceae) and implication for the evolution of sex chromosome. Theoretical and Applied Genetics. 108, 238–242

Williams JGK, Kubelic AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA Polymorphism amplified by abstrary primers are useful as genetic markers. Nucleic Acids Research. 18, 6531-6535.

Yakubov B, Barazani O, Golan-Goldhirsh A (2004) Combination of SCAR primers and Touchdown-PCR for sex identification in *Pistacia vera* L. Scientia Horticulturae. 103, 473–478.