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Sex determination in *Pistacia* species using molecular markers

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Abstract

Sex identification in *Pistacia* species are economically desirable. Regarding long juvenile stage in *Pistacia* species and lack of morphological method to identify sex in this stage, molecular marker could facilitate breeding program. Aim of the study was to identify a marker, closely linked to sex locus in *Pistacia atlantica* Desf *mutica*, *P. khinjuk* and *P. vera* var. Sarakhs. For this purpose, samples were collected from male and female individual trees from each species and their band patterns were analysed according to band specific presence or absence. Twenty Random Amplified Polymorphic DNA (RAPD) primers and a pair Sequence Characterized Amplified Regions (SCAR) primer were tested to determine sex in wild *Pistacia* species. Among RAPD primers, only BC1200 amplified a specific sex band which was present in female plant. The results indicated that all individual samples amplified an approximately 300 base pairs fragment in female trees which was absent in male samples. Although sex determination mechanism in *Pistacia* is unknown, it might be controlled by single locus acting as a trigger. However, SCAR technique is a reliable technique to identify gender genotypes in seedling stage of *Pistacia* species, that would help to save time and expanses in breeding program.

Key words: *Pistcia* spp, sex identification, SCAR- PCR, juvenile stage.

Introduction

Pistachio belongs to *Anacardiaceae* family which includes 11 species (Zohary, 1952). *Pistacia atlantica* Desf. *mutica*, *P. khinjuk* and *P. vera* var. Sarakhs are three main wild *Pistacia* species in Iran, which are applied as rootstock for *P. vera* and oil extraction in some countries (Kafkas *et al.,* 2002). Dioecious plants are thought to be the most evolved members of the plant kingdom in terms of sex differentiation. Therefore, it is impossible to determine sex during the pre-reproductive phase unless a genetic sex marker is available for this purpose (Korpelainen *et al.,* 2008). In many dioecious plants, their economic value and breeding schemes for commercial use of genetically transformed materials are influenced by sex (Alstrom-Rapaport *et al.,* 1998). The sex of pistachio cannot be known until it reaches reproductive age (Hormaza *et al.,* 1994).

With regard to long juvenile stage in dioecious trees, molecular markers have been extensively used in dioecious plant breeding to save time and economic source (Jiang and Sink, 1997). Several researchers showed that random amplified polymorphic DNA (RAPD) banding patterns have been linked to sex in *Hippophae rhamnoides* (Persson and Nybom, 1998), *Salix viminalis* (Alstrom-Rapaport *et al.,* 1998), *Piper longum* (Banerjee *et al.,* 1999), *Silene latifolia* (Zhang *et al.,* 1998), *Pistacia vera* (Hormaza *et al.,* 1994), *Encephalartos natalensis* (Prakash and Van Staden, 2006), *Actinidia chinensis* (Harvey *et al.,* 1997) and *Ginkgo biloba* (Longdou *et al.,* 2006). Sequence characterized amplified regions (SCAR) markers have been widely used to identify between two sexes in *Asparagus* (Gao *et al.,* 2007), *Carica papaya* (Bedoya and Nunez, 2007), *Rumex nivalis* (Stehlik and Blattner, 2004).

In many dioecious plants, even when sex determination is regulated genetically, no heteromorphic sex chromosomes are found. Sex chromosomes were indistinguishable in *Pistacia* species. Matsunaga (2006) has reported, in such cases, because sex chromosome cannot be identified by their size or shape, they are identified by trisomic analysis or analysis of genetic marker.

Sex-linked genetic markers based selection is an appropriate technique in breeding programs and is useful for understanding of genetic map and dioecism in *Pistacia* species. It was identified that RAPD marker is linked to sex locus in *P. vera* (Hormaza *et al.,* 1994), *P. eurycarpa* and *P. atlantica* (Kafkas *et al.,* 2001). Then by converting the RAPD primer to SCAR and Touchdown-PCR technique, sexual genotype identified in *P. vera* (Yakubov *et al.,* 2004).

To our knowledge, no sequence information on the aspect of *Pistcia* species genome is available. There are only few reports on molecular sex markers for wild *Pistacia*, so the present study is the first report on the use SCAR technique for sex determination in *P. atlantca* Desf. *mutica*, *P. khinjuk* and *P. vera* var. Sarakhs.

Materials and methods

Plant material and genomic DNA isolation: Fresh leaf samples from *P. khinjuk*, *P. atlantica* Desf. *mutica* and *P. vera* var. Sarakhs trees were collected from Iran Pistachio Research Institute (IPRI) in Rafsanjan. The samples were quickly transported to laboratory

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and kept at -80°C until use. Genomic DNA was extracted according to Doyle and Doyle (1987) with minor modification. About 0.3 g leaf tissue was ground to a fine powder in liquid nitrogen and mixed with 700 µL of CTAB (Cetyl Trimethyl Ammonium Bromide) extraction buffer (100 mM Tris-HCl (pH 8), 1.4 MNaCl, 20 mMEDTA (pH 8), 2% CTAB 1% β-mercaptoethanol, 1% PVP). The mixture was first incubated at 60°C for 90 min and then an equal volume of phenol: chloroform: isoamylalchol (25:24:1) was added, centrifuged at 12000 rpm for 20 min. The aqueous phase was decanted and transferred to a fresh microtube to reduce impurity between two phases. Extraction steps were repeated using phenol: chloroform: isoamylalcohol (25:24:1) mixture. The last aqueous phase was mixed with 2/3 of isopropanol and stored at -20°C for at least 2h to precipitate DNA and centrifuged at 12000 rpm for 15 min. The nucleic acid precipitate was washed with 70% ethanol, air-dried and suspended in 100 µL of ddH_2O. The extracted DNA was diluted in ddH_2O to 50 ng/µL and subjected to polymerase chain reaction (PCR).

DNA concentration was assayed with nano drop spectrophotometer (ND1000, USA) and quality was verified on 0.8 agarose gel electrophoresis.

RAPD-PCR amplification: RAPD-PCR was performed in a 25 µL volume using Gradiant Thermocycler (Eppendorf, Germany). Each reaction contained 25 ng template DNA, 10 pmol of primers, 300 mM dNTP, 25 mM $MgCl_2$, $(NH_4)_2SO_4$ buffer (1X) as PCR buffer and 0.5 unit Taq polymerase (Fermentase). Thermocycler conditions were 5 min at 94°C followed by 35 cycles of 94°C for 45 sec, 32°C for 30 sec and 72°C for 2 min.

SCAR-PCR amplification: PCR reaction for the SCAR marker were carried out with a final volume of 25 µL and 50 ng of genomic DNA, 10 pmol of each primer (PVF1 (Forward): 5´- GTCGTAGATGAAAACACC -3´, PVF2 (Reverse): 5´-TAATAGAAGCCATAGA -3´), 300 mM dNTPs, 25 mM $MgCl_2$, $(NH_4)_2SO_4$ buffer (1X) and 0.5 unit Taq polymerase (Fermentase). Amplification condition were: 1 cycle at 94°C for 2 min, 25 cycles at 94°C for 30 sec, 42°C for 15 sec, 72°C for 25 sec followed by 7 cycles increasing annealing temperatures in decrement of 0.4°C per cycle and finalized at 94°C for 30 sec, 45°C for 15 sec, 72°C for 15 sec and 72°C for 2 min to complete extension. Amplified products were then separated by electrophoresis in 1.2% agarose gel and stained with ethidium bromide. The expirement was replicated three times.

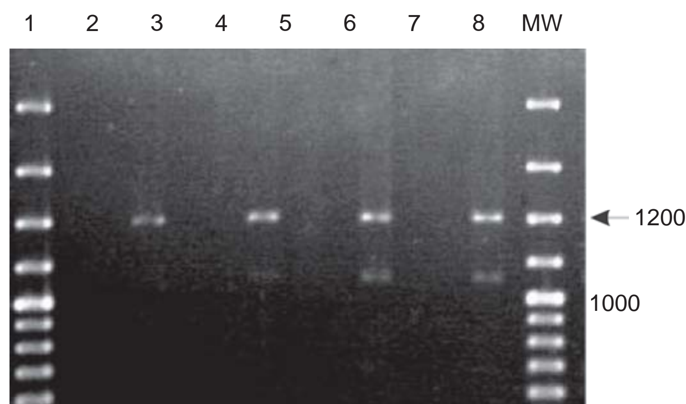

Fig. 1. A female-specific RAPD fragment in *P. vera* var Sarakhs amplified using primer OPO08. MW indicated size marker. The arrows denote the position of the 1200-bp fragment,

Results and discussion

After screening of 20 arbitrary 10-mer primers, we identified just BC1200 primer (5´GCCTGATTGC-3´) as being able to differentiate sex type (Fig. 1). This primer could amplify a weak 1200 bp fragment just in female *P. vera* var. Sarakhs. Amplification of genomic DNA from both male and female plants using SCAR primers showed that an approximately 300 bp fragment is present in all female samples of *Pistacia* species (Fig 2 and 3). By cloning and sequencing of RAPD marker (OPO08) and designing of the appropriate SCAR primers, a single specific band (297 bp segment) could be identified in female trees of *P. vera* (Yakubov *et al.,* 2004). Our study confirmed the results of Yakubov *et al.* (2004) in wild *Pistacia* species. In *Pistacia*, the genetic mechanism of sex identification is still unknown. The frequency of sex-linked markers depends on some factors such as the chromosome number, the total size of the genome and relative size of the chromosomal segments that determined sex. Mulcahy *et al.* (1992) reported four sex RAPD markers from screening of 64 random primers in *Silene latifolia*, which has a large Y chromosome; while Harvey *et al.* (1997) reported just two sex marker from testing of 500 RAPD primers. By screening of 1000 primers in *P. vera*, Hormaza *et al.* (1994) found just one sex marker. Kafkas *et al.* (2001) also found only two sex associated RAPD marker in screening of 472 primers. The low frequency of sex linked bands could indicate that loci gene which involved in sex determination is small and probably represents a single gene, or few genes. To learn about sex-inheritance and map distance measuring between a sex gene and the marker, segregating populations must be generated as test cross. Paran and Michelmore (1993) reported that sensitivity to reaction conditions and the requirement for high quality DNA, can hinder the use of RAPD markers, while SCAR primers amplify single band corresponding to genetically defined loci, are less sensitive to reaction conditions and have the capability of becoming co-dominant markers.

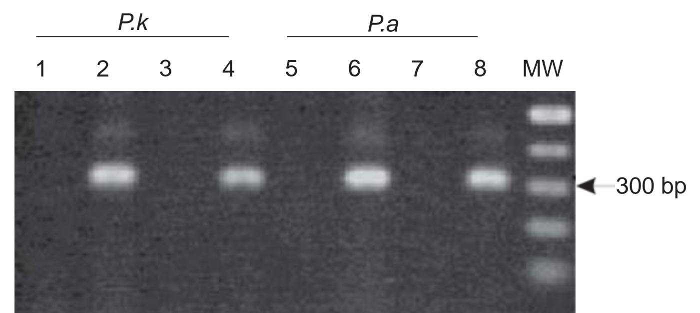

Fig. 2. SCAR pattern obtained from 8 genotypes of *P. khinjuk* (*P.k*) and *P. atlantica (P.a)* with PVF1 and PVF2 primers. Lanes 1-8 represent PCR-product of 8 trees. The arrow show the position of the 300 bp fragment which was present in female trees. The marker size (MW) is loaded before first genotype.

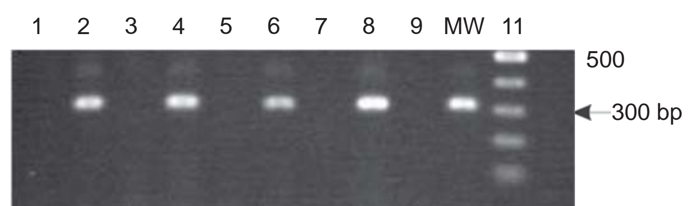

Fig. 3. Female - specific DNA fragments produced by PCR with PVF primers. Lanes 1-10 show band pattern of 10 genotypes of *P. vera* var. Sarakhs. Lane 11 show negative control.

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Recent studies of sex determing mechanisms have illuminated clearly that angiosperm have evolved a variety of sex determining mechanism that involve a number of different genetic and epigenetic parameters, from sex chromosomes in *Marchantia polymorpha* and *Silene latifolia* to hormonal regulation in *Zea mays* (Peng *et al.,* 1999) and *Cucumis sativus* (Perl-treves, 1999).

Based on the association of molecular markers and sex, the presence of an XX/XY sex chromosome system was proved for *Hippophae rhamnoides* (Persson and Nybom, 1998), *Dioscorea tokoro* (Terauchi and Kahl, 1999), *Carica papaya* (Parasnis *et al.,* 1999) and *Actinidia* (Harvey *et al.,* 1997). By contrast, similar studies in *Atriplex garettii* suggest that sex determination involves a single locus on a homologous pair of chromosomes (Ruas *et al.,* 1998).

Despite increasing research efforts on a number of different plant species, there is relatively little information available on the molecular basis of sex determination. It is also difficult to estimate the number of genes involved in sex determination (Ainsworth, 2000).

Durand and Durand (1990) also reported that a single major gene controls sex determination in *Asparagus* and *Vitis*. *Pistacia* species might have a similar system with a major gene controlling sex determination. According to the results in *Pistacia* species, sex determination might be controlled by single locus acting as a trigger.

Quick sex determination would help farmers while selecting the female seedlings and maintain an optimum sex ratio at plantation, therefore, this will save time, economic cost and increase efficiency of pistachio breeding programs. Our study demonstrated that sex determination mechanism in pistachio might be controlled by single locus acting as a trigger. However, SCAR-PCR is a useful and reliable method to detemined sex genotype in *Pistacia* genus.

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