Malays. Appl. Biol. (2008) 37(2): 25–28

SEX DETERMINATION OF *Pistacia vera* L. USING ISSR MARKERS

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ABSTRACT

Gender of four female and male individual *Pistacia vera* cultivars (Akbari, Ahmad Aghaii, Fandoghi, Kaleh Ghochi) were identified using nine Inter Simple Sequence Repeat (ISSR) primers. Two primers (AC)8CG and (AC)8TA were able to identify male and female plants by producing sex dependent DNA bands in female plants. PCR condition was reproducible and reliable for sex determination of Pistacia cultivars. It can be recommended for sex determination of Pistacia plants at early stage of seedling growth.

Key words: Pistacia vera, sex determination, ISSR

INTRODUCTION

Most angiosperms are hermaphrodites, producing male (stamen) and female (carpel or pistil) organs in the floral parts of the plants. About 10% of flowering species develop unisexual flowers. For example, *Zea mays* (maize) is a monoecious species in which male and female flowers develop on separate inflorescences. *Silene latifolia* (white campion), *Rumex acetosa* (sorrel) and the genus *Pistacia,* in the family Anacardiaceae are dioecious, bearing flowers of a single sex on one plant. On the other hand, when unisexual and bisexual flowers are produced on the same plant, it is called monoecy (Irish and Nelson, 1989; Wu and Tanksley, 1993).

Pistacia vera L. (pistachio) (2n=32) is cultivated widely in south west of Iran. Pistachio breeding programs have recently been initiated to develop new cultivars. *P. vera* shows perfect dioecy and maturity of pistachio seedlings takes between 5 and 8 years. Female flowers have no trace of stamens and mature male flowers lack any evidence of female structures (Wannan and Quinn, 1991). This clear differentiation of sexual phenotype, combined with its perennial nature, an increasing economic importance of the crop and recent interest in breeding improved cultivars, makes the species attractive for the study of different aspects of sex determination.

Currently there is no method for distinguishing between male and female pistachio seedlings prior to flowering. A method to determine the gender of plants before flowering would facilitate breeding as well as cultivation of female plants and selection by enabling screening for gender at the seedling stage, thereby simplifying the selection of male and female plants for different objectives, with a saving of time and economic resources (Zietkiewicz *et al*., 1994).

The first ISSR studies were published in 1994 and focused on cultivated species (Wolfe and Liston, 1998). These studies demonstrated the hypervariable nature of ISSR markers. Microsatellites, or Simple Sequence Repeats (SSRs), are polymorphic loci present in nuclear DNA and organellar DNA that consist of repeating units of 1-4 base pairs in length. They are typically neutral, co-dominant and are used as molecular markers which have wide-ranging applications in the field of genetics, including kinship and population studies. Microsatellites can also be used to study gene dosage (looking for duplications or deletions of a particular genetic region). They have been shown to be extremely useful as markers for the DNA fingerprinting of eukaryotes, including plants (Epplen, 1982; Ramakrishna, 1995). Sex-specific differences has been reported using micro- and minisatellites such as (GATA)4 and (GACA)4 in guppy fish (Nanda, 1992) and mice (Schafer, 1986). In recent years, the technique of ISSR due to simplicity,

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relatively low cost, and the requirement for only a small amount of material has been used in the detection of genetic diversity and sex determination of papaya (Parasnis et al., 1999), apple (Goulão, Lu'1s & Oliveira C.M., 2001), and rice (Navinder et al. 2004). However, no data in sex determination of Iranian cultivars of *Pistacia vera* has been reported using ISSR markers.

The aim of the present investigation is to introduce a reliable ISSR marker for identification of male and female individuals of cultivated *Pistacia* plants.

MATERIALS AND METHODS

Plant material and DNA extraction

Fresh leaf segments from 7 year old individual female *Pistacia* plant cultivars Kaleh Ghochi (K), Akbari (A), Akbar Aghaii (AA), and Fandighii (F), and male plants were harvested from Pistacia garden in Ardestan, Isfahan.

DNA was extracted according to the CTAB (hexadecyltrimethylammonium bromide) method of Doyle and Doyle (1987) with some modifications. Young leaf tissue (100mg) was ground to fine powder in liquid nitrogen in 1.5 centrifuge tubes and mixed with 0.5 ml of CTAB extraction buffer (100 mM TRIS-HC1, 1.4 M NaC1, 20 mM EDTA, 2% CTAB, 1% PVP, 0.2% mercaptoethanol, 0.1% NaHSO3). The sample was incubated at 65 ~ for 1 h, mixed with an equal volume of chloroform-isoamyl alcohol (24:1) and centrifuged at 13000 rpm for 5 min in a desktop centrifuge. The aqueous phase was recovered and mixed with equal volume of isopropanol to precipitate the DNA. The nucleic acid pellet was washed with 1 ml of 10 mM ammonium acetate in 76% ethanol, dried overnight and resuspended in 100 µl modified TE buffer (10 mM TRIS-HCI, 0. 1 mM EDTA). DNA was extracted separately from each individual plant. In all cases, extracted DNA (25 ng per 20 µl reaction mix) was subjected to polymerase chain reaction (PCR) amplification.

PCR Amplification:

PCR was performed using ISSR primers and amplification reactions were carried out in an Eppendorf Master cycler gradient (Eppendorf Netheler-Hinz, Hamburg, Germany). Conditions were 1 cycle at 94ºC for 5 min, 44 cycles at 94ºC for 1 min, 35ºC for 30 sec and 72ºC for 30 sec. PCR reactions were carried out in a volume of 25 µl with 30 ng of genomic DNA, 2 µM primer ISSR primers (AC)8CG, (CAG)5, (AG)8T, (ACTC)7, (GACA)4, (CAA)5, (AG)8C, (GATA)4, (AC)8TA, 1 U of Taq polymerase, 0.2 mM dNTPs, 10 mM Tris–HCl (pH 8.3), and 2.5 mM MgCl2. After amplification, the gels were stained with 0.5 µg/ ml ethidium bromide solution and visualized by illumination under UV light.

RESULTS AND DISCUSSION

Nine primers were initially tested using *Pistacia* DNA. Of the 9 ISSR primers screened, two produced clear and repeatable fragments and were selected for further analysis. Seven ISSR primers showed products in female and male but they could not reveal the difference between individual plants. However, two primers namely (AC)8CG and (AC)8TA generated excellent results while the other primers produced smears or fuzzy patterns that could not show acceptable differences. Primer (AC)8CG produced one specific band about 2400 bp in size present in female plants but was missing in male (Fig. 1). Primer (AC)8TA revealed 5 to 6 bands present in female plants but they were absent in male (Fig. 2). We tried adjusting annealing temperatures and changing amplifcation cycles, but no significant improvements were made for these seven primers. We believe that the poor results obtained were due either to characteristics of the primers or to the relative abundance of priming sites in the *Pistacia* genome.

ISSR markers offer a great potential for differentiating closely related Pistacia male and female plants. We have distinguished male and female individual plants with ISSR markers. To the best of our knowledge, ISSR is the first marker system that could be used for sex determination of *Pistacia*.

The use of molecular techniques in order to assess polymorphism among plant material at the DNA level and, to assess and study sex determination due to their genetic variation is widely used. Methods such as RAPDs (Random Amplification of Polymorphic DNA), AFLPs (Amplified Fragment Length Polymorphism), RFLPs (Restriction Fragment Length Polymorphism), SSRs (Simple Sequence Repeats) and ISSR (Inter-Simple Sequence Repeat) are used for such studies (Karp et al., 1996). Reliability of data derived from RAPDs for 'typing' is questionable, since their reproducibility is low, especially between different laboratories (Lanham, and Brennan, 1999).

Any system to be used for the comprehensive identification of sex in *Pistacia* cultivars should be reproducible and able to distinguish between male and female which are closely related genotypes. Band profiles generated by ISSR-PCR in the present study were unaffected by replicated PCRs or DNA extractions. Furthermore, independent

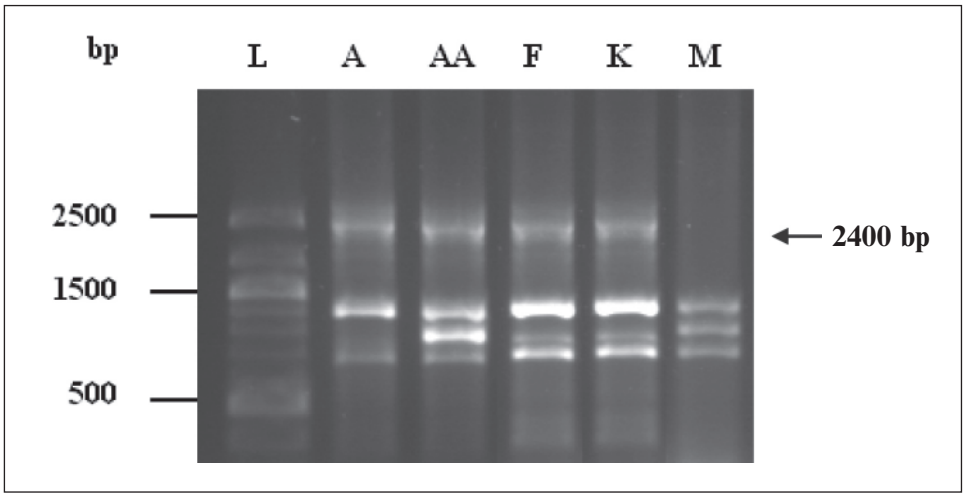

Fig. 1. PCR amplification of different *Pistacia vera* cultivars using ISSR primer (AC)8CG. L (DNA ladder), A, AA, F, K (female cultivar Akbari, Akbar Aghaii, Fandoghi, Kalehghochi), M (Male).

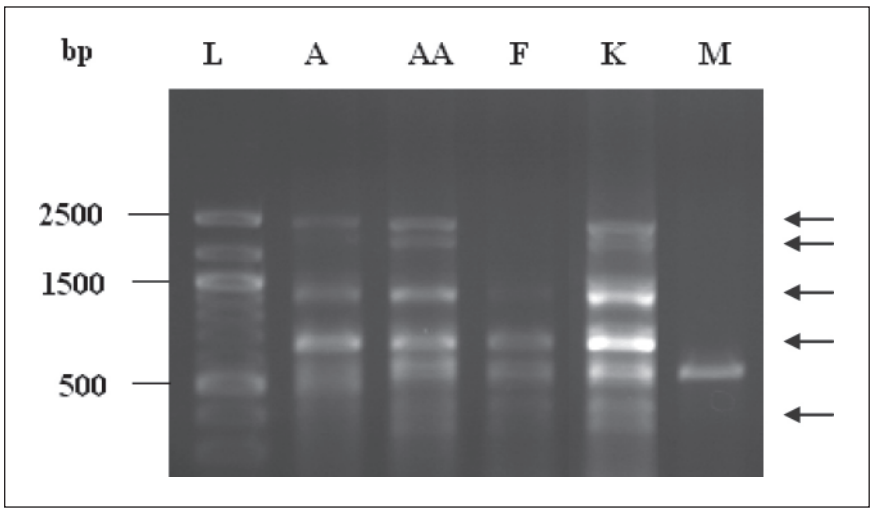

Fig. 2. PCR amplification of different *Pistacia vera* cultivars using ISSR primer (AC)8TA. L (DNA ladder), A, AA, F, K (female cultivar Akbari, Akbar Aghaii, Fandoghi, Kalehghochi), M (Male).

scorings of gels produced identical data sets. The protocol therefore appears to be sufficiently reproducible for sex determination purposes. This is in concordance with previous studies where the reliability of the protocol was demonstrated between PCRs, DNA extractions and even laboratories when applied to papaya (Parasnis et al., 1999). Each of the two ISSR-PCR primers used here generated polymorphisms. This was sufficient to allow all four female Pistacia cultivars to be distinguished. Thus, ISSR-PCR appears to produce reliable and highly polymorphic band profiles. A key additional feature of ISSR-PCR lies in its technical simplicity and speed. PCR products can be generated, fractionated and detected within 9 hours. It seems to have potential, therefore, for the large-scale of sex differentiation in Pistacia cultivars. *Fragaria vesca* (Albani et al., 2004), and Papaya (Parasnis et al., 1999) and genetic variation among plant cultivars or species, for example *Apostichopus japonicus* (YAO et al., 2007)

ACKNOWLEDGMENTS

Authors thank all members of Research Council of the University of Isfahan for their support from research project No. 860411.

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