Theor Appl Genet (1994) 89: 9–13

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Identification of a RAPD marker linked to sex determination in *Pistacia vera* using bulked segregant analysis

Received: 26 October 1993 / Accepted: 12 January 1994

Abstract The Random Amplified Polymorphic DNA (RAPD) technique was used to amplify DNA segments, with the objective of finding markers linked to sex determination in the dioecious species, *Pistacia vera*. Progenies from two female parents pollinated by a common male parent were studied. Two bulks of DNA were made in each cross, one from males and one from females, by pooling an equal weight of fresh leaves from each individual contributing to the bulk prior to DNA extraction. DNA was extracted from each bulked sample and from each of the contributing individuals. DNA was also extracted from 14 cultivars of *P. vera* and from 94 open-pollinated, few-weeks-old *P. vera* seedlings of unknown sex. Seven hundred different decamer oligonucleotide primers were used to perform DNA amplification, with 1 of these (OPO08) producing a 945 bp amplification band that was present only in the bulked female samples and absent in the bulked male samples of the two crosses. The relationship between band presence and female sex expression was conserved in every individual obtained from the two crosses and in the 14 cultivars unrelated to the crosses. We propose that this band is tightly linked to the gene(s) that control sex determination in pistachio. The $OPO08_{945}$ RAPD marker could be used in a breeding program to screen the gender of pistachio plants long before they reach reproductive maturity, resulting in considerable savings of time and economic resources. In order to verify that assumption we screened 94 additional seedlings with the OPO08 primer and obtained results consistent with a 1:1 male:female ratio.

Key words RAPD · Sex determination
Molecular markers · Pistachio · Dioecy

Communicated by G. Wenzel

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Introduction

The genus *Pistacia*, in the family Anacardiaceae, contains at least 12 tree and shrub species. *P. vera* L. (pistachio) is cultivated widely in the Mediterranean regions of Europe, the Middle East and California. The species is dioecious and in some areas where pistachio nuts are produced, a single female cultivar dominates local production. Two examples include 'Kerman' in California and 'Bronte' in Sicily. In California, pistachio growers depend almost entirely on one male cultivar, 'Peters', as a pollinizer.

Pistachio breeding programs have recently been initiated to develop new cultivars. Dioecy represents an inconvenience to pistachio breeding since the reproductive maturity of pistachio seedlings takes between 5 and 8 years. 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 and selection by enabling screening for gender at the seedling stage, thereby simplifying the breeding of male and female plants for different objectives, with a saving of time and economic resources.

Dioecy is the rule in most animals. In the plant kingdom, however, dioecy is found in only approximately 4% of the angiosperms (Yampolsky and Yampolsky 1922), although that frequency may be an underestimate because of incomplete information on many tropical species where the occurrence of dioecy appears to be higher than in temperate zones (Bawa 1980). Dioecism has arisen independently in different families and genera (Westergaard 1958), and several distinct genetic mechanisms regulating dioecy have been found in different plant species (Irish and Nelson 1989; Durand and Durand 1990). In contrast to the situation found in most animals, where highly differentiated sex chromosomes typically control dioecy, chromosomal heteromorphism is the exception rather than the rule in dioecious plant species. *Silene*, which has a well characterized male (XY) and female (XX) chromosome system, is one of the few genera where the system appears to be sim-

ilar to that of most animals. The presence of sex chromosomes has been claimed for other dioecious angiosperms, but in only a few cases has that claim been documented (Westergaard 1958; Lewis and John 1968; Parker and Clark 1991). Examples include the genera *Humulus*, *Rumex* and, perhaps, *Cannabis*, although the presence of heterochromosomes in the later is controversial (Westergaard 1958; Durand and Durand 1990). More often, the sex ratio in dioecious plant species is controlled by the expression of alleles at one to several loci (Irish and Nelson 1989). Well documented examples include species of the genera *Asparagus*, *Vitis*, *Spinacia* and *Mercurialis*. In most of the species examined in detail, the study of sex determination is complicated by the presence of additional alleles or factors that can modify the effect of the major sex determining genes (Durand and Durand 1990). *P. vera* shows perfect dioecy; mature 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.

Genetic marker systems based on direct analysis of the genomic DNA have been used widely for genetic mapping, disease diagnostics and evolutionary studies, and they could prove very useful in the study of sexual determination in dioecious plants such as pistachio. Michelmore et al. (1991) described an application of the random amplified polymorphic DNA (RAPD) technique termed bulked segregant analysis (BSA) to identify molecular markers linked to a trait of interest. They used RAPD analysis to compare two groups of pooled DNA from individuals of a segregating population originating from a single cross from parents that differed by only a single discrete character. BSA analysis uses a large number of segregating individuals in each pool, with the objective of minimizing or eliminating variation not associated with the specific trait of interest. Michelmore et al. (1991) used BSA to identify markers linked to a gene for resistance to downy mildew in lettuce making two bulks of 17 F_2 individuals each. Haley et al. (1993) used BSA methodology to identify two RAPD markers linked to a gene block for rust resistance in bean, and Barua et al. (1993) identified seven RAPD markers linked to a locus for resistance to leaf blotch in barley. Mulcahy et al. (1992), using the same approach, found several markers for sex determination in *Silene latifolia*, a dioecious species that possesses heteromorphic sex chromosomes. We followed a similar approach in pistachio with the goal of finding molecular markers present in one sex and absent in the other. In this paper we report the finding of a RAPD marker associated with females that is not present in males of pistachio. The importance of this finding in the early assessment of gender in pistachio as well as the possible implications in understanding the molecular basis of sex determination in dioecious species will be discussed.

Materials and methods

Plant material and genomic DNA isolation

The plant material used in this study were two populations of 17 year old seedlings obtained from the female *P. vera* cvs 'Kerman' and 'Lassen'. The seeds were collected from open-pollinated trees growing in a commercial orchard in which 'Peters' was the only male cultivar present (J. Allen, personal communication). Fourteen males and 10 females from the 'Lassen'×'Peters' cross and 9 males and 12 females from the 'Kerman'×'Peters' cross were available for this study.

For each cross two bulked samples of DNA were made; one for males and one for females. Bulked samples were obtained by pooling the same weight of fresh leaves from each individual. DNA was extracted according to the CTAB (hexadecyltrimethylammonium bromide) method of Doyle and Doyle (1987) with minor modifications. Young leaf tissue (5.0 g) was ground to fine powder in liquid nitrogen and mixed with 20 ml of CTAB extraction buffer (100 m*M* TRIS-HCl, 1.4 *M* NaCl, 20 m*M* EDTA, 2% CTAB, 1% PVP, 0.2% β-mercaptoethanol, 0.1% $NaHSO_3$). The sample was incubated at 65 °C for 1 h, mixed with an equal volume of chloroform-isoamyl alcohol (24:1) and centrifuged at 2,000 *g* for 15 min in a desktop centrifuge. The aqueous phase was recovered and mixed with 2/3 volume of isopropanol to precipitate the DNA. The nucleic acid precipitate was recovered with a glass hook, washed with 10 ml 10 m*M* ammonium acetate in 76% ethanol, dried overnight and resuspended in 1 ml modified TE buffer (10 m*M* TRIS-HCl, 0.1 m*M* EDTA).

DNA was extracted separately from each individual of the progeny used to make the bulks, from 7 male and 7 female *P. vera* cultivars unrelated to the plants comprising the pools (Table 1), and from 94 3-to-5-week-old seedlings originating from open pollinated seeds from a single 'Kerman' tree but unknown male parentage from a *Pistacia* collection that includes several male individuals. In all cases extracted DNA was diluted to 10 ng/ µl and subjected to polymerase chain reaction (PCR) amplification.

Polymerase chain reaction conditions

PCR amplification reactions were carried out as described in Williams et al. (1990) with minor modifications. The 25-ml reaction mixtures contained 50 ng genomic DNA, 10 m*M* TRIS-HCl, pH 8.3, 50 m*M* KCl, 1.9 m*M* $MgCl_2$, 0.001% gelatin, 100 µ*M* each of dATP, dGTP dCTP, and dTTP (Promega, Madison, Wis.), 0.4 mM primer (obtained from Operon Technologies, Alameda, Calif. and the University of British Columbia, Vancouver, B.C.) and 0.75 Units of AmpliTaq DNA polymerase (Perkin-Elmer-Cetus, Norwalk, Conn.). The samples were overlaid with 25 ml of mineral oil to prevent evaporation and briefly centrifuged prior to amplification. DNA amplification reactions were performed in a MJ-Research Inc MiniCycler (Watertown, Mass.) using the following cycling parameters: 1 cycle of 2 min at 94 °C followed by 45 cycles of 45 s at 94 °C, 1 min at 36 °C,

Table 1 *Pistacia vera* cultivars examined for specificity of the RAPD marker OPO08 for sex identification

Number	Cultivar	Sex
1	Peters	Male
2	Kastel	Female
3	Ask	Male
4	Red Aleppo	Female
5	Nazareth	Male
6	Bronte	Female
7	Gazvin	Male
8	Rashti	Female
9	O2–18	Male
10	Kerman	Female
11	O2–16	Male
12	Damghan	Female
13	Eil	Male
14	Trabonella	Female

and 2 min at 72 °C. After the final cycle, the samples were incubated for 5 min. at 72 °C and then held at 4 °C prior to analysis. Amplification products were analyzed by gel electrophoresis in 2% Sea-Kem agarose (FMC, Rockland, Me.) in 1× TBE buffer. Gels were stained with ethidium bromide and visualized on a UV transilluminator. A total of 700 RAPD primers were screened, and each amplification reaction was performed using a single primer and repeated at least once in order to verify the reproducibility of the results obtained.

Results and discussion

All but 1 of the 700 decamer primers used to amplify the pooled DNA obtained from the two crosses produced amplification patterns showing no differences between the male and female pooled samples. All markers were scored for the presence or absence of the amplification products. The primers used produced an average of approximately 7 scorable bands per primer. One primer (OPO08, with the sequence CCTCCAGTGT) produced an amplification pattern that was different in the pooled male and pooled female samples of the two crosses: a 945-bp amplification band was obtained from the pooled female DNA and not from the pooled male DNA (Figs. 1, 2). When amplification reactions were carried out with DNA from the single individuals comprising the pools, the pattern was consistent; i.e. the 945 bp band appeared in every amplification reaction made with DNA from females and was absent in amplifications with DNA from males (Figs. 1, 2). Consequently, we conclude that such a band is linked to the gene(s) that control sex determination in pistachio and can be considered to be a reliable genetic marker ($OPO08_{945}$) for sex determination in this species.

In order to determine if the marker can be broadly applied as an indicator of sex in *P. vera*, the OPO08 primer was used to screen individuals from 14 cultivars (7 males and 7 females). The pattern obtained was consistent with that of the pooled samples: $OPO08_{945}$ was present in all of the females and absent in all of the males (Fig 3). When a binomial model for distribution of the $OPO08_{945}$ marker among these 7 males and 7 females is used, the probability of the null hypothesis that the marker is not linked to sex is less than 10^{-4}. Thus, the marker is present in all females obtained from two different crosses as well as in each of the unrelated females we examined, and it is absent in all of the males studied. These observations indicate that the marker is closely linked to the gene(s) of interest and that recombination events do not occur very often between the marker and the gene(s) controlling sex determination. This is a finding of potentially great importance in pistachio breeding since the marker can be used to analyze a broad range of genetic material.

In a further step to demonstrate the usefulness of the marker as a determinant of sex in pistachio, 94 seedlings of unknown sex were screened with OPO08. The segregation obtained (54 negative and 40 positive) does not differ significantly from a 1:1 male:female sex ratio (χ^2, $P<0.05$, 1 *df*). This 1:1 ratio is expected at the zygote level in dioecious species if we assume a Mendelian inheritance of a monogenic sex determining factor (Fisher 1930, Charnov 1982).

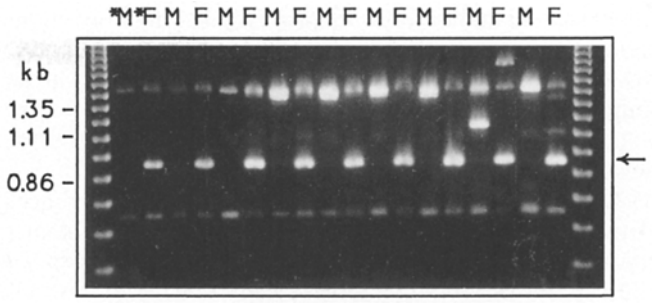

Fig. 1 RAPD banding patterns from pooled DNA from male and female 'Kerman' progeny (**M*, **F*, respectively) and DNA extracted from 7 each of the male (M) and female (F) individuals comprising the pools using primer OPO08. $OPO08_{945}$ is indicated with an *arrow*

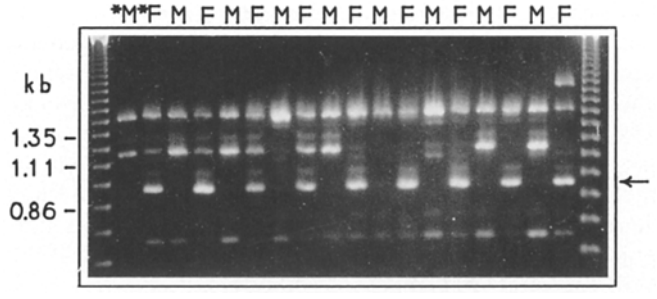

Fig. 2 RAPD banding patterns from pooled DNA from male and female 'Lassen' progeny (**M*, **F*, respectively) and DNA extracted from 7 each of the male (M) and female (F) individuals comprising the pools using primer OPO08. $OPO08_{945}$ is indicated with an *arrow*

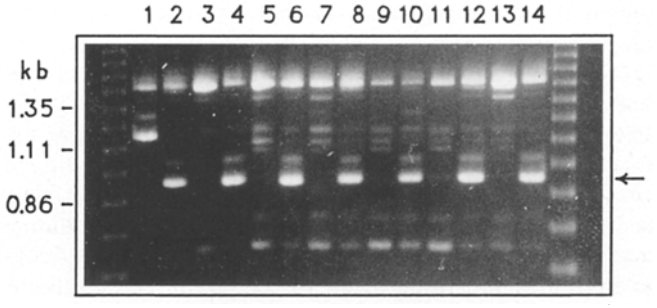

Fig. 3 RAPD banding patterns from DNA from 7 male and 7 female *P. vera* cultivars using primer OPO08. $OPO08_{945}$ is indicated with an *arrow*. Cultivars are numbered as in Table 1

We conclude, therefore, that we have identified a marker closely linked to a sex-determining chromosome segment in *P. vera* and that this marker can be used to screen satisfactorily the sex of pistachio seedlings well before they attain reproductive maturity.

A band of 1,050 bp is occasionally present that is associated with the $OPO08_{945}$ marker in some females of pistachio, as seen in Figs. 2 and 3. The band is not conspicu-

ously present in the 'Kerman' progeny shown in Fig. 1, although sometimes it has been observed in those individuals as well, which suggest the possibility that it may be an amplification artifact. Consequently, at this point we cannot consider the 1,050 bp band to be a reliable marker for sex in pistachio.

Different attempts to find molecular differences between male and female plants, such as immunochemistry, differences in proteins and enzymes, comparison of mRNA and tRNA populations or RNA hybridization kinetics, have been made in several dioecious species (see Durand and Durand 1990 for a review). Those studies deal with gene expression and could just show a differential expression of shared genes between males and females. A better approach to understand how sexual determination operates in dioecious species is to study differences at the DNA level. The discovery of markers linked to sex determining genes could eventually allow us to clone the gene(s) involved in this process. Although promising results have been obtained with *Asparagus* using RFLPs (Bracale et al. 1991), the RAPD technique appears to offer better prospects for rapid progress. Its advantages relative to RFLPs include technical simplicity, lower cost per data unit, small amount of DNA required and the higher level of polymorphism obtained with RAPDs (Waugh and Powell 1992; Williams et al. 1993).

Paran and Michelmore (1993) described the development of sequence characterized amplified regions (SCAR) markers that could be advantageous over standard RAPD markers because the amplification is less sensitive to reaction conditions. Currently, we are investigating this approach. We are also continuing to screen our pooled samples with additional primers with the aim of finding more markers linked to sex in this species and of developing more specific screening strategies using a multiple primer approach. The fact that a large number of 10-base primers have been tried (700 primers, giving approximately 4,900 loci) and that so far only 1 has produced a marker linked to sex determination leads us to conclude that the DNA segment(s) involved in sex determination is not very large and probably involves a single or a very few genes. It has been proposed (Truong et al. 1991) that the basic scheme of sex determination in animals involves a key gene that activates a cascade of regulatory genes. In plants, the system could be similar (Frankel and Galun 1977). If that were the case, of several to many genes could be involved in the differentiation of male and female flowers in dioecious plants, but sex determination could be controlled by a single locus acting as a trigger. In such a scenario, genes having the genetic information for carpels or stamens development would be present in both male and female plants with one major gene being the only difference between the two sexes. In their review of the subject, Durand and Durand (1990) observed that a single major gene controls sex determination in some plant genera (eg. *Asparagus* and *Vitis*), although there are exceptions like *Mercurialis* where the system is more complex with three genes involved in sex determination. Pistachio may have a similar system with a single major gene controlling sex determination. Another possibility is that the gene(s) controlling sex determination are present in a genomic region not subject to frequent recombination events, perhaps as an early step in the differentiation of sexual chromosomes (Lewis and John 1968).

Further research is underway studying other species within the genus *Pistacia* as well as other dioecious genera in the Anacardiaceae. Although much additional work is needed, the approach we have taken opens new avenues of research in the molecular characterization of sexual determination in dioecious species as well as in the practical breeding of pistachio.

Acknowledegments We gratefully acknowledge the assistance and advice of Drs. S. Arulsekar, F. Bliss, T. Gradziel and Ms. K. Pinney, and thank Mr. J. Allen for providing some of the plant material used in this study. JIH was supported by an INIA Fellowship from the Spanish Ministry of Agriculture. Support for this research was provided by a grant from the California Pistachio Board.

References

Barua UM, Chalmers KJ, Hackett CA, Thomas WTB, Powell W, Waugh R (1993) Identification of RAPD markers linked to a *Rhynchosporium secalis* resistance locus in barley using near-isogenic lines and bulked segregant analysis. Heredity 71: 177–184

Bawa KS (1980) Evolution of dioecy in flowering plants. Annu Rev Ecol Syst 11:15–39

Bracale M, Caporali E, Galli MG, Longo C, Marziani-Longo G, Rossi G, Spada A, Soave C, Falavigna A, Rafaldi F, Maestri E, Restivo FM, Tassi F (1991) Sex determination and differentiation in *Asparagus officinalis* L. Plant Sci 80:67–77

Charnov EL (1982) The theory of sex allocation. Princeton University Press, Princeton, N.J.

Doyle JJ, Doyle JL (1987) A rapid isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15

Durand R, Durand B (1990) Sexual determination and sexual differentiation. Crit Rev Plant Sci 9:295–316

Fisher RA (1930) The genetical theory of natural selection. Oxford University Press. Oxford, UK

Frankel R, Galun E (1977) Pollination mechanisms, reproduction, and plant breeding. Springer, Berlin Heidelberg New York

Haley SD, Miklas PN, Stavely JR, Byrum J, Kelly JD (1993) Identification of RAPD markers linked to a major rust resistance gene block in common bean. Theor Appl Genet 86:505–512

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

Lewis KR, John B (1968) The chromosomal basis of sex determination. Int Rev Cytol 23:277–379

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci USA 88:9828–9832

Mulcahy DL, Weeden NF, Kesseli R, Carroll SB (1992) DNA probes for the Y-chromosome of *Silene latifolia*, a dioecious angiosperm. Sex Plant Reprod 5:86–88

Paran I, Michelmore RW (1993) Development of PCR-based markers linked to downy mildew resistance in lettuce. Theor Appl Genet 85(8):985-993.

Parker JS, Clark MS (1991) Dosage chromosome systems in plants. Plant Sci 80:79–92

Truong AT, Heinderyckx M, Installé P, Negrutiu I (1991) Sex determination and differentiation in organisms other than higher plants. Plant Sci 80:201–211

Wannan BS, Quinn CJ (1991) Floral structure and evolution in the Anacardiaceae. Bot J Linn Soc 107:349–385
Waugh R, Powell W (1992) Using RAPD markers for crop improvement. TiBtech 10:186–191
Westergaard M (1958) The mechanism of sex determination in dioecious flowering plants. Adv Genet 9:217–281
Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535
Williams JGK, Hanafey MK, Rafalski JA, Tingey SV (1993) Genetic analysis using random amplified polymorphic DNA markers. Method Enzymol 218:704–740
Yampolsky C, Yampolsky H (1922) Distribution of sex forms in the phanerogamic flora. Bibl Genet 3:1–62