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RAPD markers linked to sex in the genus *Pistacia*

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SUMMARY – Molecular markers have been used in breeding to diagnose and select a genotype based on a linked DNA marker, long before the phenotype is apparent. Marker-assisted selection would be very important in pistachio rootstock breeding programs, as well as in cultivar improvement, because of their long juvenility period. We aim to find Random Amplified Polymorphic DNA (RAPD) markers linked to sex in *P. atlantica*, *P. terebinthus* and *P. eurocarpa*, the major wild species in Turkey used as rootstocks for *P. vera*. Knowing the sex of a seedling may provide early selection in the nursery and help to establish and manage germplasm collections. Leaf samples were collected from ten male and ten female trees from each species. Sex-pooled DNA samples were prepared by mixing the DNA of ten male and ten female individuals, respectively. Among all the 312 primers screened so far, three bands, amplified by primers OPL11 and BC152 appeared to be sex correlated in *P. terebinthus* and three bands amplified by primers BC156 and BC360, appeared to be sex correlated in *P. eurocarpa*. The results showed that in each of the putative sex-related bands, the correlation with sex is only partial, and some individuals have the marker-phenotype of the opposite sex. It is possible that the marker is linked to a sex-determining locus but not very tightly. We also observed that many of the RAPD bands screened in this experiment can be identified as species-specific. Additional male female individuals were sampled and their testing is underway.

Key words: RAPD, *Pistacia*, sex determination, dioecy.

RESUME – "Marqueurs RAPD liés au sexe chez le genre *Pistacia*". Des marqueurs moléculaires ont été utilisés pour l'amélioration, pour diagnostiquer et sélectionner un génotype basé sur un marqueur lié à l'ADN, bien avant que le phénotype ne soit apparent. La sélection assistée par marqueurs serait très importante pour les programmes de sélection de porte-greffes de pistachier, ainsi que pour l'amélioration des cultivars, en raison de leur longue période de juvénilité. Notre but était de chercher des marqueurs RAPD liés au sexe pour *P. atlantica*, *P. terebinthus* et *P. eurocarpa*, qui sont les principales espèces sauvages en Turquie utilisées comme porte-greffes pour *P. vera*. Connaissant le sexe d'un plant, on peut faire une sélection précoce en pépinière et aider à établir et gérer les collections de germoplasme. Des échantillons de feuilles ont été prélevés sur 10 arbres mâles et 10 arbres femelles de chaque espèce. Les échantillons d'ADN tous sexes confondus ont été préparés en mélangeant l'ADN des 10 individus mâles et 10 individus femelles, respectivement. Parmi les 312 amorces criblées jusqu'à ce jour, trois bandes, amplifiées par amorces OPL11 et BC152, paraissent être corrélées au sexe chez *P. terebinthus*, et trois bandes amplifiées par amorces BC156 et BC360, semblent être corrélées au sexe chez *P. eurocarpa*. Les résultats ont montré que pour chacune des bandes censées être liées au sexe, la corrélation au sexe n'est que partielle, et certains individus ont le marqueur-phénotype du sexe opposé. Il est possible que le marqueur soit lié à un locus de détermination du sexe mais pas de façon très étroite. Nous avons également observé qu'un grand nombre de bandes RAPD objet de crible dans cette expérience peuvent être identifiées comme spécifiques à l'espèce. Des individus supplémentaires mâles et femelles ont été échantillonnés, et sont actuellement sous testage.

Mots-clés : RAPD, *Pistacia*, détermination du sexe, dioécie.

Introduction

The genus *Pistacia* is a member of the Anacardiaceae family and consists of at least eleven species (Zohary, 1952; Whitehouse, 1957). There are six *Pistacia* species in Turkey (Yaltirik, 1967a), five of which (*P. vera*, *P. khinjuk*, *P. atlantica*, *P. terebinthus* and *P. lentiscus*) were described by Zohary (1952) and one (*P. eurocarpa*) by Yaltirik (1967b,c). *P. vera* has edible nuts and is commercially important. The other species grow in the wild and are used as rootstock for *P. vera*. They are dioecious, although several exceptions were described (Ozbek and Ayfer, 1958; Crane, 1974; Kafkas *et al.*, this volume).

Molecular markers have been used in breeding to diagnose and select a genotype based on a linked DNA marker, long before the phenotype is apparent. The Random Amplified Polymorphic

DNA (RAPD) technique is technically simple and easily automated. It requires small quantities of DNA and no previous sequence information on the target genome; the level of polymorphism obtained is usually high (Williams *et al.*, 1990, 1993). Marker-assisted selection would be very important in pistachio rootstock breeding programs, as well as in cultivar improvement, because of their long juvenility period, which is a major handicap for breeders. Reproductive maturity of *Pistacia* seedlings takes four to eight years under optimum growing conditions. A RAPD-PCR based method was reported recently to distinguish between male and female *Pistacia vera* seedlings (Hormaza *et al.*, 1994). A RAPD band (OPO08₉₄₅) was shown to be effective in distinguishing male and female seedlings before flowering. However, this RAPD marker has been ineffective for gender determination in wild *Pistacia* species (Hormaza, 1994).

A marker that determines the gender of pistachio rootstocks at the seedling stage may be useful in the case that rootstock sex will affect scion performance (effects of rootstock sex are under study in our laboratory). Such marker may also help pistachio breeders to establish and manage germplasm collections. We aim to find RAPD markers linked to sex in *P. atlantica*, *P. terebinthus* and *P. khinjuk* (or *P. eurocarpa*, see Kafkas *et al.*, this volume), that are the major wild species used as rootstock for *P. vera* in Turkey.

Materials and methods

The plant material for this study was collected in Turkey, in the Adana, Manisa and Aydin provinces (*P. atlantica* and *P. terebinthus* samples) and Siirt province (*P. eurocarpa* samples). Leaf samples were collected from ten male and ten female trees from each species. DNA extraction was done according to Doyle and Doyle (1987) with some modifications. Sex-pooled DNA samples were prepared by mixing the DNA of ten male and ten female individuals. RAPD reactions were performed using random decamers from Operon (Sets A, B, C, D, L and R) and British Columbia (Sets of 101-200 and 301-400) according to Williams *et al.* (1990, 1993) using a PTC-100 thermocycler (MJ-Research Inc., MA, USA). The reaction products were subjected to electrophoresis on 1.8% TBE-agarose gels, stained with ethidium bromide and visualized under UV light. RAPD primers were first screened in pooled DNA samples. Primers that detected polymorphism between the two sex pools were tested in the twenty individual plants. When polymorphism correlated with sex in the 20 individuals, additional male and female individuals were sampled and their testing is underway.

Results and discussion

Screen for sex-specific markers

Among all the 312 primers screened so far, three bands amplified by primers OPL11 and BC152 appeared to be sex correlated in *P. terebinthus* and three bands amplified by primers BC156 and BC360 appeared to be sex correlated in *P. eurocarpa*. The observed segregation of these bands among 20 individuals is shown in Table 1.

Table 1. Segregation of sex-related RAPD bands

	Sex-related bands	Females		Males	
		Present	Absent	Present	Absent
<i>P. terebinthus</i>	OPL11 ₆₅₀	6	4	1	9
	OPL11 ₇₅₀	7	3	1	9
	BC152 ₁₂₅₀	8	2	1	9
<i>P. eurocarpa</i>	BC156 ₁₃₀₀	1	9	10	0
	BC360 ₅₀₀	8	2	0	10
	BC360 ₁₂₀₀	1	9	8	2

For example, we observed two female-correlated bands with the OPL11 primer in *P. terebinthus* (Fig. 1). We see that 750 bp band was amplified in 7 females and 1 male whereas

650 bp band was amplified in 6 females and 1 male out of 10 individuals for each sex. In *P. eurocarpa*, we observed one band of approximately 1300 bp produced by primer BC156 (Fig. 2), in the 10 male individuals, and only in one female individual. We can see that in each of the putative sex-related bands, the correlation with sex is only partial, and some individuals have the marker-phenotype of the opposite sex. It is possible that the marker is linked to a sex determining locus but not very tightly, and there are recombinant individuals, that have the alleles of the "opposite sex".

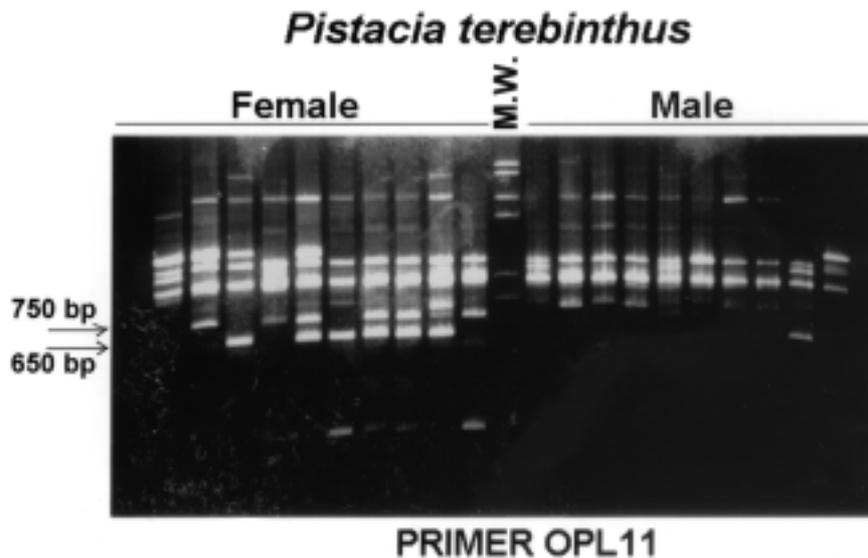


Fig. 1. RAPD banding patterns from 10 female and 10 male *P. terebinthus* individuals using primer OPL11. The 650 bp and 750 bp bands are indicated with an arrow.



Fig. 2. RAPD banding patterns from 10 female and 10 male *P. eurocarpa* individuals using primer BC156. The 1300 bp band is indicated with an arrow.

Testing a larger number of individuals is important in order to estimate more correctly the sex-specificity of our markers. Screening a larger number of markers may be required to find a more tightly linked one. Hormaza (1994) screened 1000 primers in *P. vera* for sex determination and found only one marker which is absent in males and present in females. He suggested that the low frequency of sex linked bands may indicate that the DNA segment(s) involved in sex

determination is small and probably involves a single gene, or very few genes. In order to measure the map-distance between a putative sex gene and the marker, a segregating population from a single cross should be tested. Such populations were prepared but they are still in the juvenility stage.

Sex determination mechanisms in plant are diverse, and may involve sex chromosomes as in *Silene*, *Actinidia*, *Vitis* and *Asparagus* or individual sex genes as in *Mercurialis*, *Cucumis*, *Spinacia*. In *Pistacia*, the genetic mechanism of sex determination is still unknown. The sex-specific markers may represent, therefore, amplified DNA from a sex chromosome. Alternatively, they may represent DNA polymorphisms (e.g., point mutations) that are linked to individual sex genes.

If sex specific RAPD markers will be verified with more individuals, we plan to convert them to SCAR markers (Sequence Characterized Amplified Regions; Paran and Michelmore, 1993) to increase the reliability and speed of the testing.

Species-specific markers

During the screen for sex-specific markers we that many of the primers produced species-specific bands, i.e. they are present in 20 individuals of the one species and not in the 20 individuals of the other two species. One of the primers, BC135, tested in four *Pistacia* species for species-specificity is shown in Fig. 3. It was tested in ten individual plants of each species and produced a species-specific band in each of the four species.

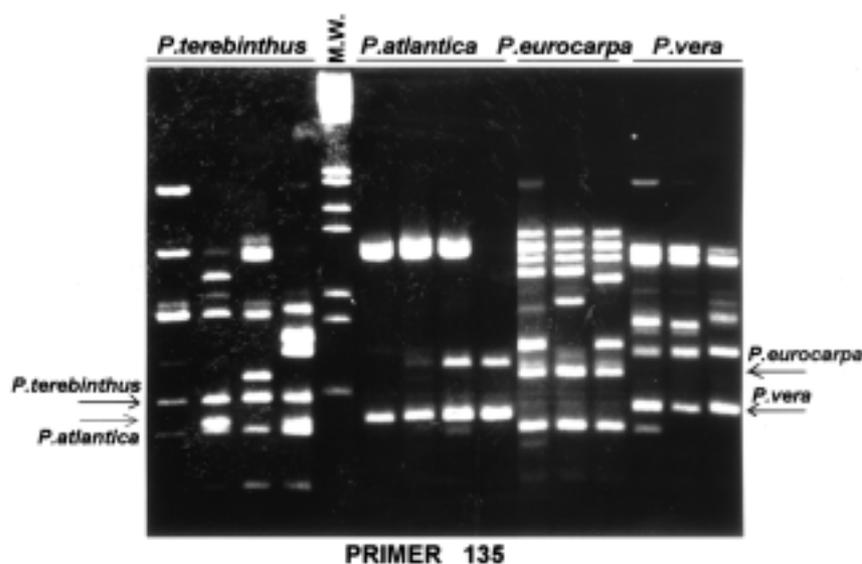


Fig. 3. RAPD banding patterns from DNA from 4 *P. terebinthus* and *P. atlantica* individuals, and 3 *P. eurocarpa* and 3 *P. vera* individuals using primer BC135. The species-specific bands are indicated for each species with an arrow.

The domestication of *P. vera* and spread of pistachio cultivation far beyond the natural range of its wild progenitor brought the crop in contact with several southwest Asian and Mediterranean *Pistacia* species. In traditional areas of pistachio cultivation, contacts between the cultivated clones and the wild species *P. terebinthus*, *P. palaestina*, *P. khinjuk* and *P. atlantica* are quite common. Moreover, many of these contacts have existed for hundreds or even thousands of years (Zohary, 1996). Introgression of *P. vera* onto indigenous rootstock species is occurring, and in some areas wild rootstocks have been extensively grafted (Maggs, 1973). Also in Turkey top-working of wild *Pistacia* species has been done for many years, and it may prove very difficult to obtain uncontaminated wild material of these species and correctly identify a *Pistacia* tree based on its morphology. Species-specific DNA markers may therefore provide an accurate tool to identify *Pistacia* germplasm of unknown origin.

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