

Molecular variation between and within wild *Pistacia* species in Turkey

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SUMMARY – The most common wild *Pistacia* species in Turkey are *P. terebinthus*, *P. khinjuk* and *P. atlantica*. They have been used as rootstocks for *P. vera* by top-working for many years. Their phenotypic appearance and productivity are highly variable. The objective of this study was to characterize, *in situ*, *Pistacia* germplasm of Turkey at the molecular and phenotypic level. This will enable the preservation of biodiversity of *Pistacia* species of Turkey before losing these trees by cutting or top-working, and may also clarify taxonomic relationships in the genus *Pistacia*. A total of 42 genotypes from different parts of Turkey were sampled for this study: (i) 10 *P. khinjuk* genotypes from Siirt and Gaziantep provinces; (ii) 20 *P. atlantica* and 10 *P. terebinthus* genotypes from Adana, Aydin and Manisa provinces; and (iii) 2 *P. vera* varieties (Kirmizi and Siirt) from the Pistachio Research Institute, Gaziantep. Leaf samples of these trees were used for DNA fingerprinting by the Randomly Amplified Polymorphic DNA (RAPD) technique. From a preliminary screen of RAPD primers, the most polymorphic 10 primers were selected and used for fingerprinting and characterization of 42 *Pistacia* genotypes. A total of 138 fragments were generated and 128 were polymorphic at the intra- or inter-specific level. The four *Pistacia* species are clearly separated from each other. *P. terebinthus* appears to be the most diverged species. The samples identified as *P. khinjuk* should be probably classified as *P. eurocarpa* based on their morphology and molecular data. Samples 27-K-01 and 27-K-02 are probably hybrids between *P. vera* and *P. terebinthus*, while tree 56-K-08 may be a hybrid between *P. vera* and *P. eurocarpa*.

Key words: *Pistacia*, genetic variation, RAPD, *P. eurocarpa*, fingerprinting.

RESUME – "Variation moléculaire inter et intra espèces sauvages de *Pistacia* en Turquie". Les espèces sauvages les plus courantes de *Pistacia* en Turquie sont *P. terebinthus*, *P. khinjuk* et *P. atlantica*. On les a utilisées comme porte-greffes pour *P. vera* par "topworking" pendant plusieurs années. Leur apparence phénotypique et leur productivité sont fortement variables. L'objectif de cette étude était de caractériser, *in situ*, le germoplasme des *Pistacia* de Turquie au niveau moléculaire et phénotypique. Ceci permettra la préservation de la biodiversité des espèces de *Pistacia* de Turquie avant de perdre ces arbres par abattage ou topworking, et pourrait également éclaircir les relations taxonomiques du genre *Pistacia*. Un total de 42 génotypes de différentes parties de la Turquie a été échantillonné pour cette étude : (i) 10 génotypes de *P. khinjuk* des provinces de Siirt et Gaziantep ; (ii) 20 génotypes de *P. atlantica* et 10 de *P. terebinthus* des provinces d'Adana, Aydin et Manisa ; et (iii) 2 variétés de *P. vera* (Kirmizi et Siirt) de l'Institut de Recherches sur le Pistachier, Gaziantep. Des échantillons de feuilles de ces arbres ont été utilisés pour des empreintes d'ADN par technique RAPD. D'après un criblage préliminaire des amorces RAPD, les 10 amorces les plus polymorphes ont été sélectionnées et utilisées pour les empreintes et la caractérisation des 42 génotypes de *Pistacia*. Un total de 138 fragments ont été obtenus dont 128 étaient polymorphes à niveau intra ou inter espèces. Les quatre espèces de *Pistacia* sont clairement séparées entre elles. *P. terebinthus* semble être l'espèce la plus divergente. Les échantillons identifiés comme *P. khinjuk* devraient probablement être classifiés comme *P. eurocarpa* en se basant sur leur morphologie et données moléculaires. Les échantillons 27-K-01 et 27-K-02 sont probablement des hybrides entre *P. vera* et *P. terebinthus*, tandis que l'arbre 56-K-08 pourrait être un hybride entre *P. vera* et *P. eurocarpa*.

Mots-clés : *Pistacia*, variation génétique, RAPD, *P. eurocarpa*, empreinte.

Introduction

The genus *Pistacia* is a member of Anacardiaceae family and consists of 11 species (Zohary, 1952). *P. vera* has edible nuts and commercial importance. In Turkey, the most common wild *Pistacia*

species are *P. terebinthus*, *P. khinjuk* and *P. atlantica*. They have been used as rootstock for *P. vera* by top-working for many years (Bilgen, 1968, 1973; Kaska and Bilgen, 1988). There are approximately 66 millions wild *Pistacia* trees in Turkey (Kuru and Ozsabuncuoglu, 1990) and mixed populations of *P. vera*, *P. atlantica*, *P. terebinthus* and *P. khinjuk* grow in many places for hundred of years. Yaltirik (1967a,b,c) described *Pistacia* species in Turkey and added a new species, *P. eurocarpa*, which grows in southeast Turkey. *P. eurocarpa* is probably a hybrid between *P. khinjuk* and *P. atlantica* (Yaltirik, 1967c).

P. atlantica trees grow wild in Marmara, Aegean, Mediterranean, Black Sea and Middle Anatolia regions. *P. khinjuk* trees are found in southeast Anatolia, especially in Siirt, Hakkari, Bitlis, Sirnak, Batman and Tunceli provinces. *P. terebinthus* trees grow naturally all over Turkey except for the too cold (east Anatolia) and rainy areas (Black Sea). *P. eurocarpa* trees grow in Bitlis, Mardin and Hakkari provinces (Ayfer, 1963; Yaltirik, 1967a,b,c; Bilgen 1968, 1973; Kaska and Bilgen, 1988).

A few studies have been conducted in *P. vera* concerning inter- and intra-specific genetic relationships, inheritance and breeding, based on morphological, physiological and biochemical data (Zohary, 1952, 1972; Yaltirik, 1967a,b,c; Grundwag and Werker, 1976; Lin *et al.*, 1984; Dong and Baas, 1993; El-Oqlah, 1996). Few studies have used isozyme and DNA markers to distinguish between *Pistacia* species and between *P. vera* varieties (Louskas and Pontikis, 1979; Hormaza *et al.*, 1994a,b, 1998; Dollo *et al.*, 1995; Rovira *et al.*, 1995, 1998; Vezvaei, 1995; Barone *et al.*, 1996; Dollo, 1996; Parfitt and Badenes, 1997; Caruso *et al.*, 1998). However, there is no study on intra-specific variation in wild *Pistacia* species at the DNA level.

Several marker techniques are available to study genetic diversity of plant taxa. The lack of sufficient polymorphism and isozyme systems are the major limitations of isozyme markers (Tanksley, 1983), and DNA markers overcame these problems. The Restriction Fragment Length Polymorphisms (RFLPs) technique was developed (Tanksley *et al.*, 1989) and used widely for species or cultivar characterization and for genetic mapping. Williams *et al.* (1990) developed the Random Amplified Polymorphic DNA (RAPD) technique, in which random DNA segments are amplified using decamer oligonucleotide primers of arbitrary sequence.

The objective of this study is to characterize, *in situ*, wild *Pistacia* germplasm of Turkey at the molecular level. This will help the preservation of biodiversity of *Pistacia* species in Turkey before losing the trees by cutting or top-working, and may also clarify the taxonomic relationships in the genus *Pistacia*.

Materials and methods

A total of 42 genotypes from different parts of Turkey were sampled for this study: (i) 10 *P. khinjuk* genotypes from Siirt and Gaziantep provinces; (ii) 20 *P. atlantica* and 10 *P. terebinthus* genotypes from Adana, Aydin and Manisa provinces; and (iii) 2 *P. vera* varieties (Kirmizi and Siirt) from the Pistachio Research Institute, Gaziantep. The wild trees were described with respect to tree, leaf and nut morphology (Kafkas *et al.*, unpublished results), and their seedling progeny is being tested as potential rootstock for *P. vera*. Leaf samples taken from these trees were used for DNA fingerprinting by the RAPD technique. DNA was extracted according to Doyle and Doyle (1987) with some modifications. RAPD reactions were performed using random decamers (University of British Columbia Primers 147, 165, 189, 302, 304, 322, 348, 353, 354, 356) 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.

From a preliminary screen of RAPD primers, the most polymorphic 10 primers were selected and used for fingerprinting and characterization of the 42 *Pistacia* genotypes. Parsimony analysis was performed using the PAUP 3.1 program (Swofford, 1993) with different Heuristic search-options, and "majority rule" consensus trees were constructed from sets of shortest trees, and from bootstrap replicates of the data.

Results and discussion

Separation between species; mis-identification of *P. khinjuk*?

A total of 138 fragments were generated from 10 arbitrary primers. The number of amplified fragments generated varied from 8 to 23 per primer (average of 13.8) and 128 were polymorphic at the intra- or inter-specific level. RAPD fingerprinting patterns of *Pistacia* genotypes using primer BC322 is shown in Fig. 1. A total of 169 bands were scored as present (1) and absent (0) for cluster analysis. Only the most clear and strong bands were used. Reproducibility of the patterns was tested by running part of the reactions in duplicates. Fig. 2A shows a typical dendrogram resulting from parsimony analysis of the data, using the PAUP 3.1 software. Fig. 2B shows a consensus-dendrogram from bootstrap analysis of our data with 25 replicates. Genetic distances between all pairwise combinations of species were calculated by the same program.

Figure 2 shows that the four *Pistacia* species are clearly separated from each other. *P. terebinthus* appears to be the most diverged species. A branch of 35 steps appearing in 100% of the bootstrap replicates separates all *P. terebinthus* genotypes from the rest of the trees. The average genetic distances between *P. terebinthus* and each of the other three species (*P. atlantica*-0.505 units, *P. khinjuk*-0.519, *P. vera*-0.471) were similar. The average distance between *P. vera* and *P. khinjuk* (0.330), and between *P. vera* and *P. atlantica* (0.346) were smaller. A branch of 26 steps appearing in 100% of the bootstrap replicates separates the two *P. vera* varieties from all the *P. khinjuk* and *P. atlantica* genotypes. The two species that appear closest are *P. khinjuk* and *P. atlantica* (genetic distance 0.233): a branch of 16 steps appearing in 91% of the bootstrap replicates separates all *P. khinjuk* genotypes from all the *P. atlantica* ones. According to the distance matrix, the closest relative of *P. vera* is *P. khinjuk*, while *P. atlantica* is a little more distant, and *P. terebinthus* is the most distant species from *P. vera*. The tree derived by parsimony analysis reflects this relationship, although *P. atlantica* appears significantly more distant from *P. vera*, as compared to *P. khinjuk*. These results were somewhat unexpected. According to previous studies (Zohary, 1952; Parfitt and Badenes, 1997) *P. vera* and *P. khinjuk* were the two closest species, while in our study *P. khinjuk* and *P. atlantica* species are the closest pair, with rather similar distance to *P. vera*. We believe that the reason for this discrepancy is a mis-identification of the trees classified as *P. khinjuk* in our study. In our study, the leaf rachis of *P. khinjuk* samples had narrow wings, especially in the terminal leaflet petiole, or between terminal leaflet petiole and the former leaflets, and the nuts were 6-7 x 8-10 mm, while according to the literature (Zohary, 1952; Yaltirik, 1967a), *P. khinjuk* should not have leaf rachis-wings and the nuts should be smaller, 4-6 x 4-5 mm. From both genetic and morphological considerations, the plants identified as *P. khinjuk* based on information from local growers are *P. eurocarpa* trees described by Yaltirik (1967b,c) except for trees 27-K-01 and 27-K-02.

In Siirt province *Pistacia* species grow in the wild either as forests or individual trees, and we collected the seeds from several villages close to the cities. According to local growers and agricultural engineers, this wild species is called *P. khinjuk*, but wild trees were apparently top-worked many years ago with a *P. eurocarpa* variety called 'large buttum' or 'fatty buttum', valued for oil and soap production and salty consumption. The trees that we sampled are probably progeny of this variety. Zohary (1952) described this species a variety of *P. atlantica* (var. *kurdica*) because of the presence of a leaf rachis wing. Yaltirik (1967a,b,c), on the other hand, treated this plant as a different species because their leaves are light green on both sides (instead of being dark green above and pale below), and the nuts are depressed and bigger. Furthermore, the leaflets are usually thicker and never as numerous or narrow as in *P. atlantica*, and the rachis wing is much narrower or even absent. Based on growers information, 56-K-07 was a top-worked tree and the others may be its progeny.

Figure 3 shows a schematic relationship of these *Pistacia* species according to the literature, as compared to our results, and our hypothesis regarding the re-identification of our samples as *P. eurocarpa*. Our hypothesis would explain the phylogenetic position of the *P. khinjuk* samples, closer to *P. atlantica* than to *P. vera*. To prove this hypothesis, we should go back and sample true *P. khinjuk* trees from Siirt, and see whether they would cluster further away from *P. atlantica* and closer to *P. vera*.

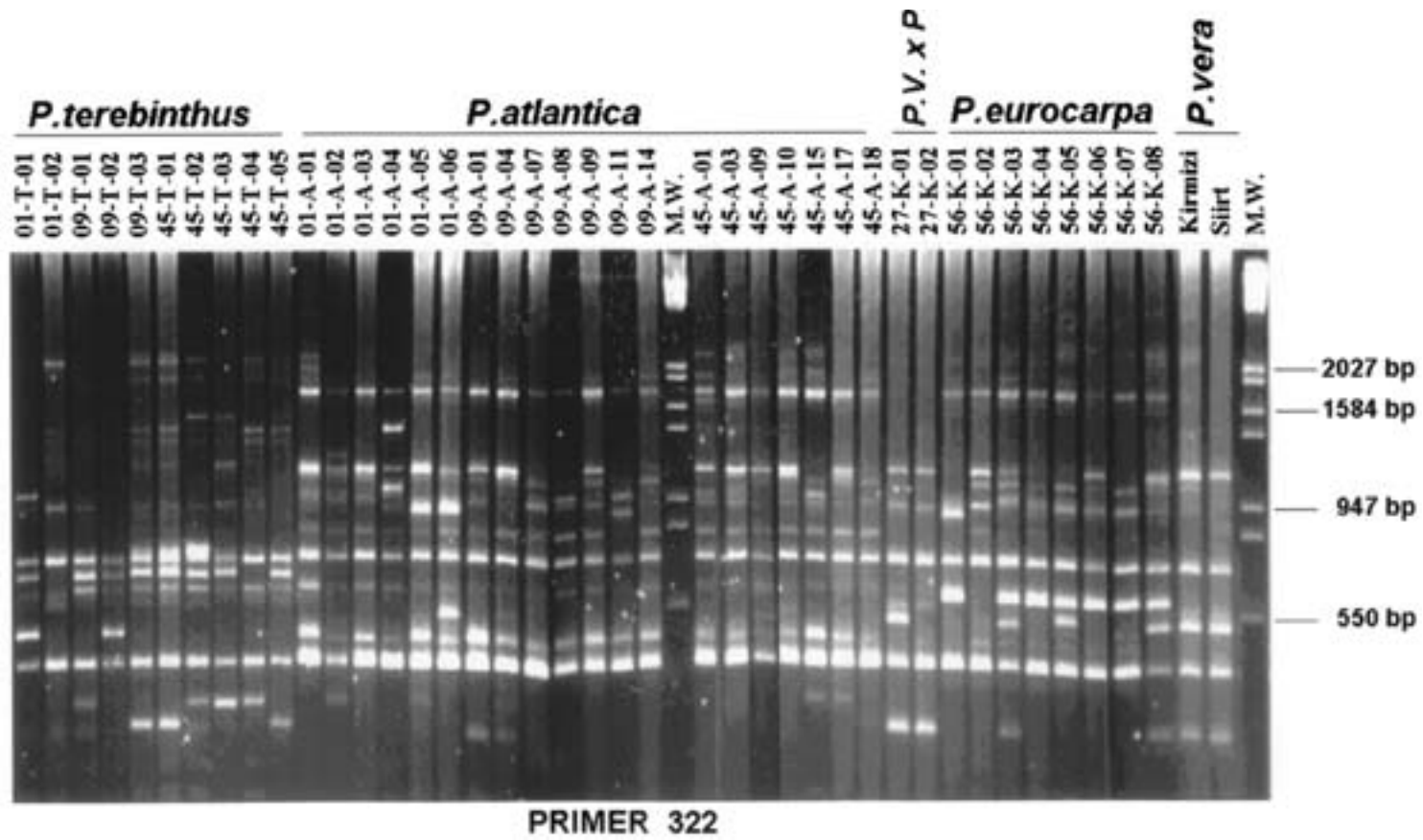


Fig. 1. RAPD fingerprinting *Pistacia* genotypes using BC Primer 322.

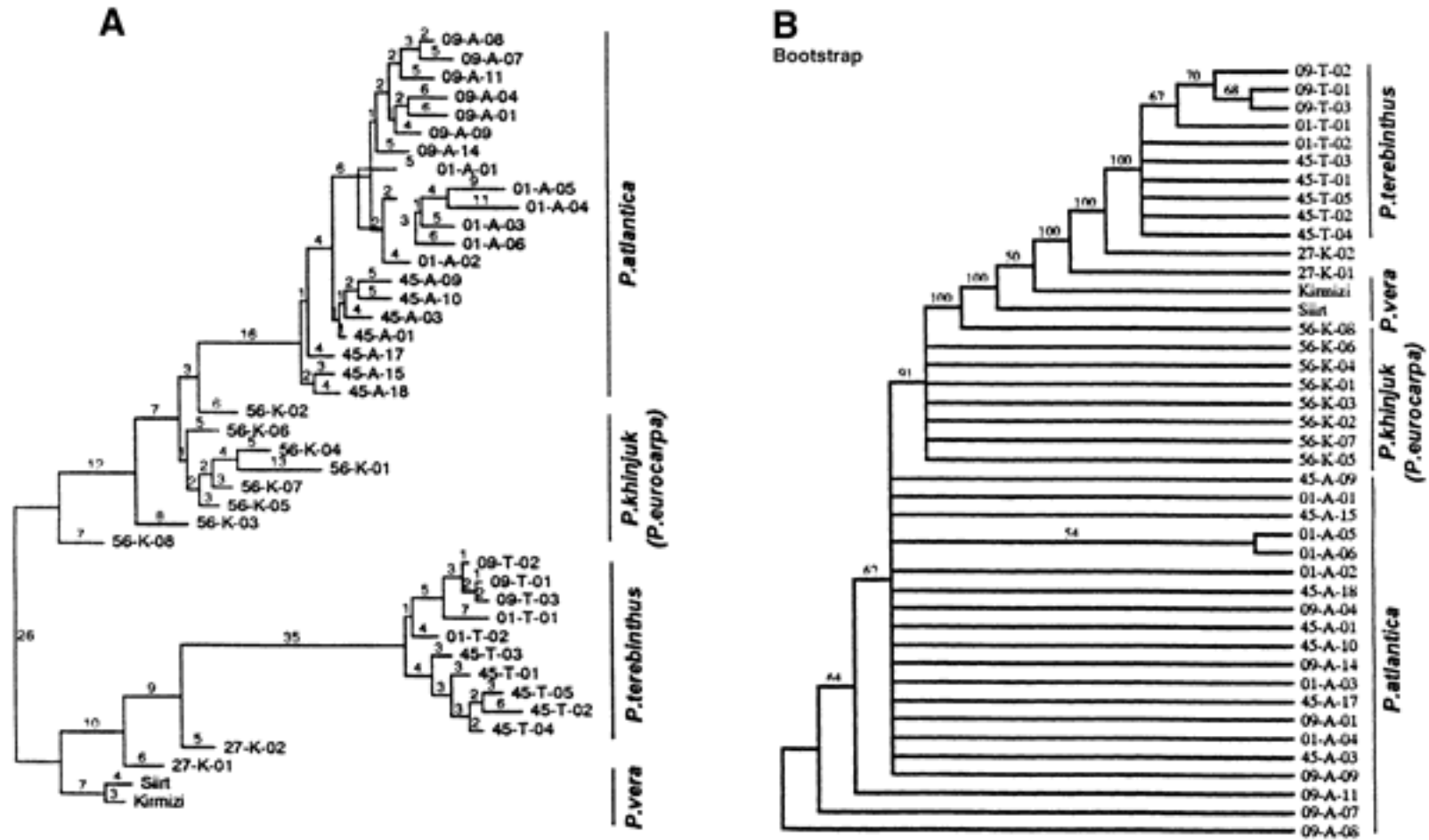


Fig. 2. Cluster analysis of molecular fingerprinting data from 42 *Pistacia* genotypes. Data base included 138 RAPD bands. (A) A heuristic search was conducted by the PAUP software using TBR optimization option, resulting in 3304 trees of 384 steps. One of these trees is depicted. Numbers indicate the length (no. of steps) of each branch. (B) Consensus tree obtained from 25 Bootstrap replicates of same data. Tree codes indicate the province, species, and individual tree number. Province codes were: 01-Adana, 09-Aydin, 27-Gaziantep, 45-Manisa, and 56-Siirt. Species codes were: A-*P. atlantica*, K-*P. khinjuk*, T-*P. terebinthus*.

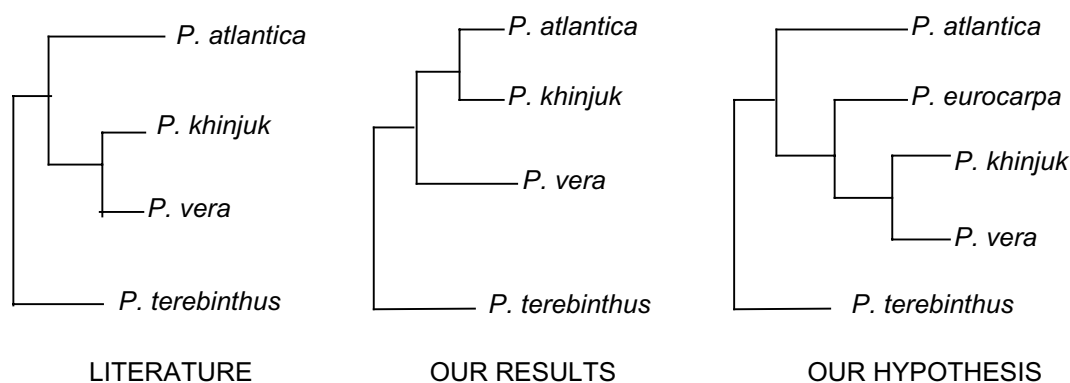


Fig. 3. A schematic phylogenetic trees of five *Pistacia* species according to the literature, our study and our hypothesis.

Separation between trees from different locations

P. atlantica and *P. terebinthus* genotypes from different regions were separated according to their geographical distribution. The *P. atlantica* genotypes from Aydin and Adana provinces were more similar to each other and the genotypes from Manisa were more distant: a branch of 6 steps separates the genotypes from Adana and Aydin from Manisa ones (Fig. 2). However, the *P. atlantica* genotypes from the different locations appear inter-mixed in the bootstrap dendrogram (Fig. 2B), indicating that the separation between geographical locations is weakly supported by the data. Also, when we compared the genotypes morphologically, we did not observe traits that can identify the Manisa group as a separate variety or subspecies. The *P. terebinthus* individuals from Aydin are separated from Manisa and Adana samples by a branch of 3 steps appearing in 70% of the bootstrap replicates.

Putative inter-specific hybrids

Trees 27-K-01 and 27-K-02 occupied an intermediate position between *P. vera* and *P. terebinthus*, and they may be hybrids between these two species. However, they were closer to *P. vera* than *P. terebinthus*. The genetic distance of 27-K-01 and 27-K-02 to *P. vera* is 0.149 and 0.214 units, respectively, and the distance to *P. terebinthus* is 0.390 and 0.346, respectively. Also from a morphological point of view, they were closer to *P. vera*, having large leaflets and nuts. Tree 56-K-08 occupied an intermediate position between *P. vera* and *P. eurocarpa*. It had a similar genetic distance to *P. vera* (0.200) and to *P. eurocarpa* (0.228). A branch of 12 steps appearing in 100% of the bootstrap replicates separates 56-K-08 from the rest of the *P. eurocarpa* trees. Also the sizes of the nuts and the leaflets were intermediate between the two species. The growers in Siirt province have recently brought this sample named Mardin Buttum, from Mardin province as a budstick because of its large nuts.

Conclusions

(i) RAPD appears to be an efficient technique to classify *Pistacia* species at the intra- and inter-specific levels, and to determine the genetic diversity present in germplasm collections.

(ii) The four *Pistacia* species are clearly separated from each other and *P. terebinthus* appears to be the most diverged species.

(iii) The samples identified as *P. khinjuk* are probably *P. eurocarpa*.

(iv) Samples 27-K-01 and 27-K-02 are probably hybrids between *P. vera* and *P. terebinthus*, while tree 56-K-08 may be a hybrid between *P. vera* and *P. eurocarpa*.

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