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Morphological and molecular phylogeny of *Pistacia* species in Turkey

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Abstract This study addresses the taxonomic relationships and genetic variation of wild *Pistacia* germplasm in Turkey using morphological data and RAPD analysis. *P. atlantica*, *P. terebinthus* and *P. eurycarpa* are common wild species in the flora of Turkey, and their phenotypic appearance and productivity are highly variable. Understanding such variation would facilitate their use in rootstock breeding programs as rootstock for edible pistachio. We have sampled and characterized a total of 40 wild *Pistacia* genotypes from different parts of Turkey for this study. These included 10 *P. eurycarpa* (locally identified as *P. khinjuk*) genotypes from Siirt and Gaziantep provinces and 20 *P. atlantica* and 10 *P. terebinthus* genotypes from Adana, Aydin and Manisa provinces. In addition, two local *P. vera* varieties, cvs. Kirmizi and Siirt, were added for comparison. Cluster analysis based on morphological data revealed that the closest species to *P. vera* is *P. eurycarpa,* followed by *P. atlantica* and *P. terebinthus*. Ten polymorphic RAPD primers, yielding a total of 138 scorable bands, were selected and used for DNA fingerprinting of these genotypes. In the resulting molecular phylogeny, the four *Pistacia* species are clearly separated from each other. *P. terebinthus* appears to be the most diverged species, and the closest pair of species was found to be *P. atlantica* and *P. eurycarpa*. This supported the classification of trees that had been identified by local growers as *P. khinjuk*, as *P. eurycarpa*. Comparison of these samples with a reference *P. khinjuk*, obtained from a germplasm collection in the USA, also supported such identification. Several wild genotypes were found to be inter-specific hybrids, and the RAPD patterns revealed their probable origin. Species-specific markers were identified for each of the four species, and these may aid in future classification of new germplasm materials.

Keywords *Pistacia* · *P. eurycarpa* · *P. khinjuk* · Phylogeny · RAPD

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Introduction

The genus *Pistacia* L. is a member of the *Anacardiaceae* family and consists of at least eleven species (Zohary 1952; Whitehouse 1957; Yaltirik 1967a; Kokwaro and Gillett 1980). *Pistacia vera* L., the pistachio, has edible nuts and considerable commercial importance. There are approximately 66 million wild *Pistacia* trees in Turkey (Kuru and Ozsabuncuoglu 1990), mostly *P. terebinthus* L., *P. khinjuk* Stocks, *P. atlantica* Desf. and *P. eurycarpa* Yalt.. Mixed populations of *P. vera* and these wild species have been growing in many locations for hundreds of years. The sex habit is dioecious (with few exceptions; Ozbek and Ayfer 1958; Crane 1974; Kafkas et al. 2000), and the trees bear apetalous flowers, which are wind-pollinated. They have been used in Turkey for many years as rootstock for *P. vera* by top-working (i.e. grafting of mature trees; Bilgen 1968).

The first classification of the genus *Pistacia* was done by Zohary (1952). In his monographic study, the genus was subdivided into four sections. The main diagnostic traits used to distinguish between the various species were, and remain, leaf characteristics and nut morphology. Zohary (1972) classified *Pistacia* species in Israel, and Yaltirik (1967a) classified *Pistacia* species in Turkey, identifying *P. atlantica* var. kurdica Zoh. as a different species, which he named *P. eurycarpa*. Al-Yafi (1978) described a few *P. atlantica* subspecies on the basis of their leaf morphology and retained *P. eurycarpa* as a variety of *P. atlantica*. Kokwaro and Gillet (1980) described a new *Pistacia* species in East Africa and named it *P. aethiopica* Kokwaro. Lin et al. (1984) compared the

leaf morphology, photosynthesis and leaf conductance of nine *Pistacia* species. El-Oqlah (1996) described the *Pistacia* species in Jordan morphologically, anatomically and palynologically. Recently, Parfitt and Badenes (1997) classified *Pistacia* species based on chloroplast DNA profiles and subdivided the genus into two sections, *Terebinthus* and *Lentiscus*. A few other studies addressed intra-specific genetic relationships in *P. vera*, based on molecular data. Isozyme and DNA markers were used to distinguish *P. vera* varieties (Louskas and Pontikis 1979; Hormaza et al. 1994, 1998; Dollo et al. 1995; Vezvaei 1995; Barone et al. 1996; Dollo 1996; Rovira et al. 1998; Caruso et al. 1998). However, none of the studies analyzed the inter- and intra-specific molecular variation of wild *Pistacia* species using genomic DNA profiles. The objective of the study reported here was to clarify the taxonomic relationships among *Pistacia* germplasm surveyed in Turkey using morphological data and random amplified polymorphic DNA (RAPD) analysis. An analysis of such variation may help to preserve the biodiversity of *Pistacia* in Turkey, which is under threat by extensive forest cutting and top-working with *P. vera*, and will provide a framework for future efforts to incorporate wild germplasm into rootstock breeding programs.

Materials and methods

Plant material

A total of 40 wild *Pistacia* female genotypes from different parts of Turkey were sampled for this study: 10 *P. eurycarpa* (locally identified as *P. khinjuk*) genotypes from Siirt and Gaziantep provinces, 20 *P. atlantica* and 10 *P. terebinthus* genotypes from Adana, Aydin and Manisa provinces. Sampling and evaluation were performed in the wild, and the seeds of the wild genotypes were collected and germinated for rootstock selection in Cukurova University, Adana. An additional nine female *P. vera* varieties (Bademi, Degirmi, Halebi, Keten Gomlegi, Kirmizi, Ohadi, Siirt, Sultani and Uzun) and three male varieties (Atli, Kaska and Uygur) from the Pistachio Research Institute, Gaziantep, were sampled to test the species-specific RAPD markers, and two of them (Kirmizi and Siirt) were used as *P. vera* "standards" for the phylogenetic study.

Scoring of morphological traits

A total of 30 traits (19 leaf, 4 tree and 7 nut traits) were scored in the 40 wild *Pistacia* genotypes and used for cluster analysis. The list of morphological characters and the assigned character states for cluster analysis are given in Table 2. *P. vera* descriptors were taken from the literature (Zohary 1952, 1972; Yaltirik 1967a; Anonymous 1993). Twenty of the characters were qualitative ones, and the remaining ten were quantitative ones; for cluster analysis (see below), these were divided into discrete classes ('states'; Table 1). The scoring of each individual tree was based on a sample of ten leaves and 100 nuts. A more detailed morphological description of our germplasm will be provided elsewhere (Kafkas and Perl-Treves, unpublished results).

RAPD analysis

Leaf samples were collected, frozen in liquid nitrogen and stored at −70°C until use. Genomic DNA was extracted from leaf tissue by the CTAB method of Doyle and Doyle (1987) with minor modifications. RAPD analysis was performed according to Williams et al. (1990) with minor modifications. Amplification reactions were carried out in a 25-µl volumes each containing 10 m*M* TRIS-HCl, pH 9.0, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.1% Triton X-100, 0.2 µ*M* primer, 100 µ*M* each of dATP, dGTP, dCTP and dTTP, 1 U *Taq* DNA polymerase and 10 ng of genomic DNA. DNA reactions were performed in a PTC-100 thermal cycler (MJ-Research, Mass.). The program included 1 cycle of 2 min at 94°C, followed by 35 cycles of 45 s at 94°C, 1 min at 36°C, and 2 min at 72°C and concluded with a final incubation for 5 min at 72°C. Amplification products were analyzed by gel electrophoresis in 1.8% agarose in 1× TBE buffer, stained with ethidium bromide and photographed under UV light. From a preliminary screen of a large number of RAPD primers, the most polymorphic ten primers (from University of British Columbia laboratories) were selected and used for fingerprinting and characterization of the 40 wild, and two cultivated *Pistacia* genotypes. These primers are listed in Table 1.

Table 1 Polymorphic RAPD markers used in this study. Decamer primers (from University of British Columbia) selected to perform the phylogenetic analysis of *Pistacia* are shown, along with their sequences

Primer	Sequence
BC147	5′ GTG CGT CCT C 3′
BC165	5′ GAA GGC ACT G 3′
BC189	5′ TGC TAG CCT C 3′
BC302	5′ CGG CCC ACG T 3′
BC304	5′ AGT CCT CGC C 3′
BC322	5′ GCC GCT ACT A 3′
BC348	5′ CAC GGC TGC G 3′
BC353	5′ TGG GCT CGC T 3′
BC354	5′ CTA GAG GCC G 3′
BC356	5′ GCG GCC CTC T 3′

Band scoring and cluster analysis

Only the clearest and strongest bands were used for cluster analysis. Reproducibility of the patterns was tested by running part of the reactions in duplicates. For both morphological and molecular data, parsimony analysis was performed using the PAUP 3.1 program (Swofford 1993) with different Heuristic search-options. A consensus tree was constructed from bootstrap replicates of the same data. Genetic distances between all pair-wise combinations of accessions were calculated by the same program. The distance values are based on the proportion of different bands between all possible pairs of genotypes. The same data was also analyzed by distance matrix methods using the PHYLIP package software (Felsenstein 1993): Kimura's genetic distances were computed between all pairs of genotypes using the RESTDIST program. These values served as input to the NEIGHBOR program that constructed a tree using the UPGMA option.

Results and discussion

Inter- and intra-specific relationships of *Pistacia* genotypes based on morphological data

To investigate the inter- and intra-specific relationships between the *Pistacia* genotypes on the basis of morphological data, we coded a total of 30 phenotypic characters as discrete states. Out of the 30 characters surveyed, 24 were polymorphic at the intra- or inter-specific level.

Table 2 Morphological characters and their discrete character states used for cluster analysis

Characters	Character states				
	0	1	2	3	4
Tree vigor	Low	Intermediate	High		
Growth habit	Shrub	Tree branched at the base	Single trunk tree		
Branching habit	Sparse	Intermediate	Dense		
Leaf persistency	Evergreen	Deciduous			
Leaf length (cm)	10.0–14.0	14.1–17.0	17.1–20.0		
Leaf width (cm)	7.0–9.0	9.1–11.5	11.6–14.0		
Leaf color	Light green	Green	Dark green		
Leaf indumentum	Glabrous	Puberulent			
Leaf waxiness	Absent	Present			
Resin smell of leaf	Weak	Intermediate	Strong		
Leaf rachis wing	Absent	Present			
Number of leaflet pairs	1.5–2.4	2.5–3.9	4.0–5.0		
Terminal leaflet	Absent	Present			
Relative size of terminal leaflet to basal ones	Smaller	Similar	Bigger		
Terminal leaflet length	2.0–3.5	3.6–5.0	5.1–6.5		
Terminal leaflet width	0.5–1.2	1.3–2.5	2.6–3.5		
Leaf petiole	Absent	Present			
Leaflet petiole	Present	Absent			
Leaf petiole length	1.5–3.0	3.1–4.0	4.1–5.0	5.1–6.0	6.1–7.0
Leaf petiole shape	Rounded	Angled	Flattened		
Leaflet shape	Lanceolate	Elliptical or narrow elliptical	Ovate, oblong or ovate-oblong		
Terminal leaflet shape	Lanceolate	Elliptical or narrow elliptical	Ovate, oblong or ovate-oblong		
Terminal leaflet apex shape	Mucronulate Acute, or obtuse	Acumunate or mucronate	Retuse or Emarginate		
Hull tip	Absent	Present			
Hull texture	Fleshy-juicy	Fleshy-dry			
Nut weight per 100 nuts (g)	5.0–11.0	11.1–20.0	20.1–40.0	40.1–70.0	
Nut thickness (mm)	3.0–4.0	4.1–5.4	5.5–7.0	7.1–8.0	
Nut width (mm)	4.0–5.4	5.5–7.4	7.5–11.0		
Nut length (mm)	4.0–7.4	7.5–9.5	9.6–13.0		
Nut shape	Obovoid	Obovoid-globular	Globular	Globular-compressed	

Leaf persistency, leaf waxiness, presence of terminal leaflet, hull texture, presence of leaf petiole and leaflet petiole were the non-polymorphic traits (Table 2). We applied parsimony analysis using the PAUP 3.1 program (Swofford 1993) to obtain a dendrogram that depicts the relationship between the genotypes. Figure 1A shows a typical dendrogram resulting from such analysis, one of the 4700 short trees that were obtained. Figure 1B shows a consensus-dendrogram from a bootstrap analysis of the same data with 100 replicates. Bootstrap analysis was used to test the consistency of the results by analyzing random samples of half of the data in each replicate and then constructing a consensus dendrogram that included only the nodes that appeared in the majority of the replicates.

Figure 1A and B shows that the four *Pistacia* species are clearly separated from each other on the basis of their morphology. A branch of 11 steps appearing in 95% of the bootstrap replicates separates all *P. terebinthus* genotypes from the rest of the tree. A branch of 13 steps appearing in 92% of the replicates separates all *P. eurycarpa* genotypes from *P. atlantica*. Branches appearing in 65% and 58% of the bootstrap replicates separate *P. vera* from all the *P. eurycarpa* genotypes (except for 27-K-01, 27-K-02 and 56-K-08, see below). Genetic distances between all pair-wise combinations of accessions were calculated by the same program. According to the average distances between genotypes from different species, *P. atlantica* was closer to *P. eurycarpa* (0.60 genetic distance units) than to *P. vera* (0.71). The average distance between *P. vera* and *P. eurycarpa* (0.46) was the smallest. The closest relative of *P. vera* is, therefore, *P. eurycarpa*, whereas *P. terebinthus* is the most distant species from *P. vera*.

Zohary (1952) classified *P. eurycarpa* trees as a variety of *P. atlantica* (var. kurdica) because of the presence of a leaf rachis wing. Yaltirik (1967b), on the other hand, treated this plant as a different species because the leaves are light-green on both sides (instead of being dark-green above and pale below as in *P. atlantica*) and the nuts are depressed and bigger. Furthermore, the leaflets are usually wider and thicker and never as numerous as in *P. atlantica*, and the rachis wing is narrower or even absent. Our phenotypic cluster analysis supports

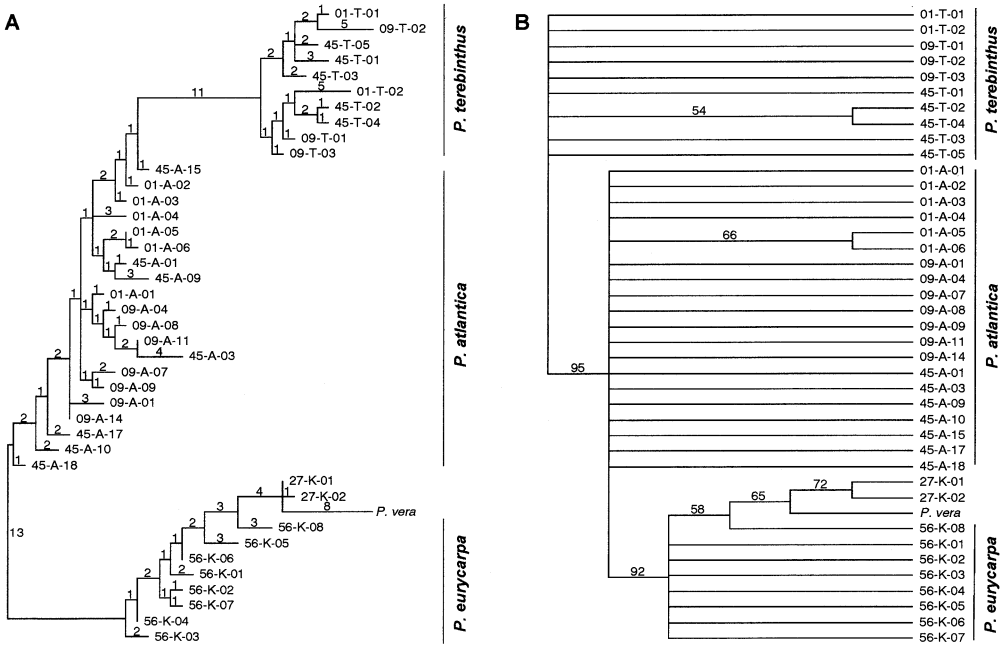

Fig. 1A, B Cluster analysis of morphological data from 41 *Pistacia* genotypes. The database included 30 morphological characters. **A** Heuristic search was conducted by the PAUP software using the TBR optimization option, resulting in 4700 trees of 341 steps. One of the trees is depicted. *Numbers* indicate the length (no. of steps) of each branch. **B** Consensus tree obtained from 100 Bootstrap replicates of the same morphological data. *Numbers* indicate the percentage of replicates that included a given node

Yaltirik's classification of *P. eurycarpa* as a separate species (Fig. 1) and not as a variety of *P. atlantica* as suggested by Zohary (1952). Growers in Siirt province, where we sampled the trees, called them *P. khinjuk*. We concluded, however, that these trees could not represent *P. khinjuk* because they had a leaf rachis wing, mostly in the terminal leaflet petiole or between the terminal leaflet petiole and the former pair of leaflets. Moreover, their nuts measured 6–7×8–10 mm, while according to the literature (Zohary 1952; Yaltirik 1967a), *P. khinjuk* should not have a leaf rachis-wing, and the nuts should be smaller, 4–6×4–5 mm. We therefore concluded that most of the plants identified as *P. khinjuk* by local growers are *P. eurycarpa* trees as described by Yaltirik (1967b).

After collecting more data from local growers, we have learned that the wild trees named *P. khinjuk* in this region were apparently top-worked many years ago with a *P. eurycarpa* variety called 'Large Buttum' or 'Fatty Buttum', valued for oil and soap production and salty consumption. Based on growers' information, tree 56-K-07 had been top-worked, and the other trees that we sampled may be its sexual progeny. Original, true-to type *P. khinjuk* trees may be difficult to locate near these villages and were inaccessible to us in this survey.

Phylogenetic analysis of *Pistacia* genotypes based on RAPD data

The same 40 wild *Pistacia* genotypes and two additional *P. vera* varieties (cvs. Kirmizi and Siirt) were analyzed using the RAPD technique. A total of 138 scorable, i.e. strong and clear fragments, were generated by ten arbitrary-sequence primers (Table 1) and scored as present (1) or absent (0) for cluster analysis. The number of such fragments varied from 8 to 23 per primer (average 13.8),

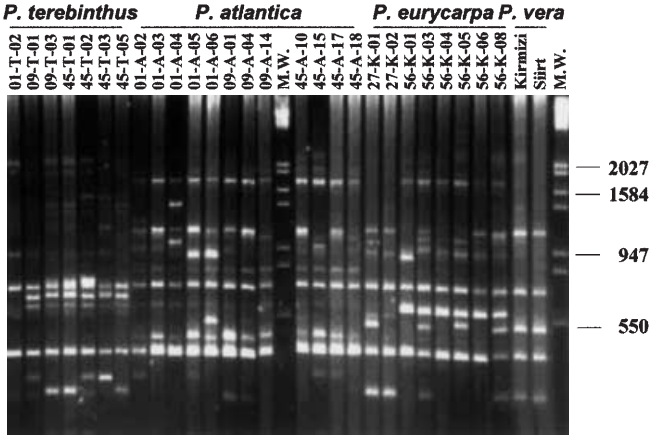

Fig. 2 RAPD fingerprinting patterns of *Pistacia* genotypes using primer BC322. *M.W.* Molecular-weight standards. Size of fragments are shown in base pairs

and 128 bands were polymorphic at the intra- or interspecific level. RAPD fingerprinting patterns of *Pistacia* genotypes using primer BC322 are shown in Fig. 2. Data were analyzed using two different computational methods to obtain dendrograms that depict the relationship among the genotypes. The first method was parsimony analysis (using the PAUP program), in which only phylogenetically informative characters are used to construct the shortest tree by the stepwise addition of taxa and characters. Subjecting our data to PAUP yielded 3304 shortest trees, 384 steps long; Fig. 3A depicts one of them. Figure 3B shows a consensus-dendrogram from bootstrap analysis of the same data with 100 replicates.

Figure 3A and B shows that the four *Pistacia* species are clearly separated from each other on the basis of RAPD fingerprinting. *P. terebinthus* appears to be the

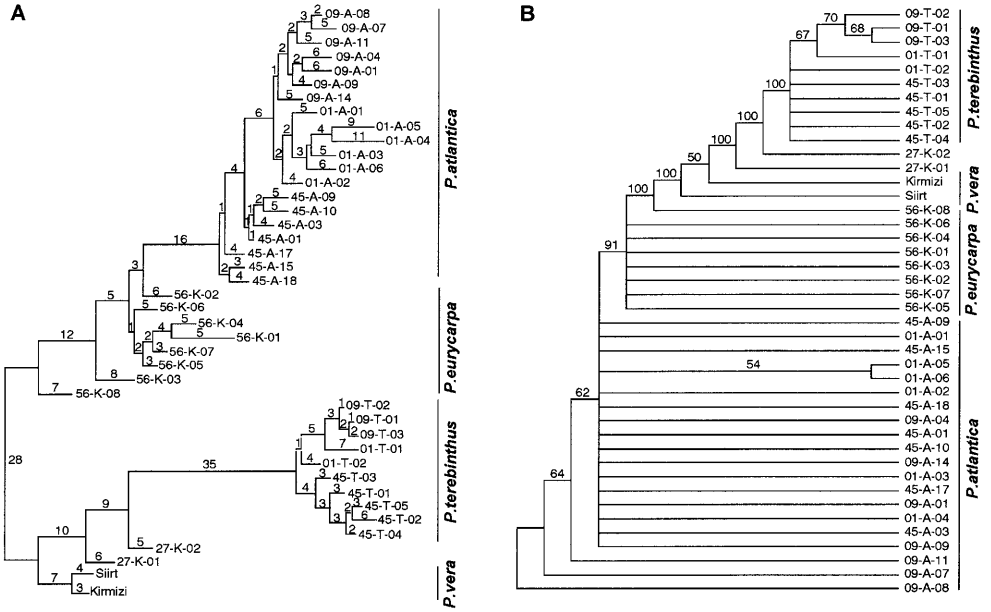

Fig. 3A, B Cluster analysis of molecular fingerprinting data from 42 *Pistacia* genotypes. The data base included 138 RAPD bands. **A** Heuristic search was conducted by the PAUP software using the TBR optimization option, resulting in 3304 trees of 384 steps. One of the trees is depicted. *Numbers* indicate the length (no. of steps) of each branch. **B** Consensus tree obtained from 100 Bootstrap replicates of the same molecular fingerprinting data. *Numbers* indicate the percentage of replicates that included a given node

most diverged species, separated from the rest of the tree by a branch of 35 steps appearing in 100% of the bootstrap replicates. The average genetic distances calculated by PAUP between *P. terebinthus* and each of the other three species were similar (*P. atlantica-P. terebinthus* 0.50 units; *P. eurycarpa-P. terebinthus* 0.52, *P. vera-P. terebinthus* 0.47). Smaller average distances were observed between *P. vera* and *P. eurycarpa* (0.33 units) and between *P. vera* and *P. atlantica* (0.35). A branch of 28 steps appearing in 100% of the bootstrap replicates separated all the *P. eurycarpa* and *P. atlantica* genotypes (except for 27-K-01, 27-K-02) from the rest of the tree. The two species that appear closest are *P. eurycarpa* and *P. atlantica* (average genetic distance 0.23); these two are separated by a branch of 16 steps appearing in 91% of the bootstrap replicates. According to the distance matrix and to parsimony analysis, the closest relative of *P. vera* is *P. eurycarpa*, while *P. terebinthus* is the most distant one.

In order to confirm the validity of this phylogeny, we generated a second set of RAPD data. This set was obtained by fingerprinting bulks of individuals, 20 individuals from each of the three wild species surveyed. A total of 518 scorable fragments were generated from 50 arbitrary primers, and 415 bands were polymorphic at the inter-specific level. Parsimony analysis was performed using the PAUP program. The tree obtained (not shown) exhibited the same relationships between the three species as depicted in Fig. 3, with *P. terebinthus* being the most distant species. Moreover, the genetic distances computed from the second data set between *P. terebinthus* and *P. atlantica*, between *P. terebinthus* and *P. eurycarpa* and between *P. atlantica* and *P. eurycarpa* were 0.50, 0.53 and 0.23, respectively, whereas the genetic distances in the detailed molecular survey of the 42 individual genotypes were 0.50, 0.52 and 0.23, respectively. The near-identical values obtained from the two data sets show that the phylogenetic relationship revealed by a sample of ten primers was probably accurate, since it is so similar to that obtained with 50 primers.

We applied a second, independent computational approach to analyze the same data based on a distant matrix method. For this purpose, we applied the RESTDIST and NEIGHBOR programs using the UPGMA algorithm (Fig. 4). The tree that we obtained was similar, in its salient features, to the one obtained by PAUP: the four species were separated from each other and *P. terebinthus* appeared to be the most diverged species. *P. eurycarpa* and *P. atlantica* appeared to be the closest pair, similarly distant from *P. vera*.

Our identification of the samples locally named *P. khinjuk* as *P. eurycarpa* explains their phylogenetic position – closer to *P. atlantica* than to *P. vera* (Figs. 3, 4). On the other hand, true *P. khinjuk* samples would be expected to cluster closer to *P. vera*. To prove this hypothesis, we obtained a reference *P. khinjuk* sample from Dr. D.E. Parfitt (University of California, Davis). This sample was used in the study by Parfitt and Badenes (1997), and we wished to know whether such a sample would cluster further away from *P. atlantica*, and from the trees that we identified as *P. eurycarpa*, and closer to *P. vera*.

The 'standard' *P. khinjuk* was subjected to RAPD analysis along with one individual sample from each species using the same ten primers. Parsimony analysis was performed using the PAUP program. Figure 5A depicts one of the shortest trees, while Fig. 5B shows a consensus-dendrogram from bootstrap analysis of the data with 100 replicates. A branch of 61 steps appearing in 100% of the bootstrap replicates separates *P. terebinthus* from all the other species, representing genetic distances of about 0.55 units between this species and each of the other four species. A branch of six steps appearing in 53% of the bootstrap replicates separates *P. vera* from *P. khinjuk*, *P. atlantica* and *P. eurycarpa*. The species clos-

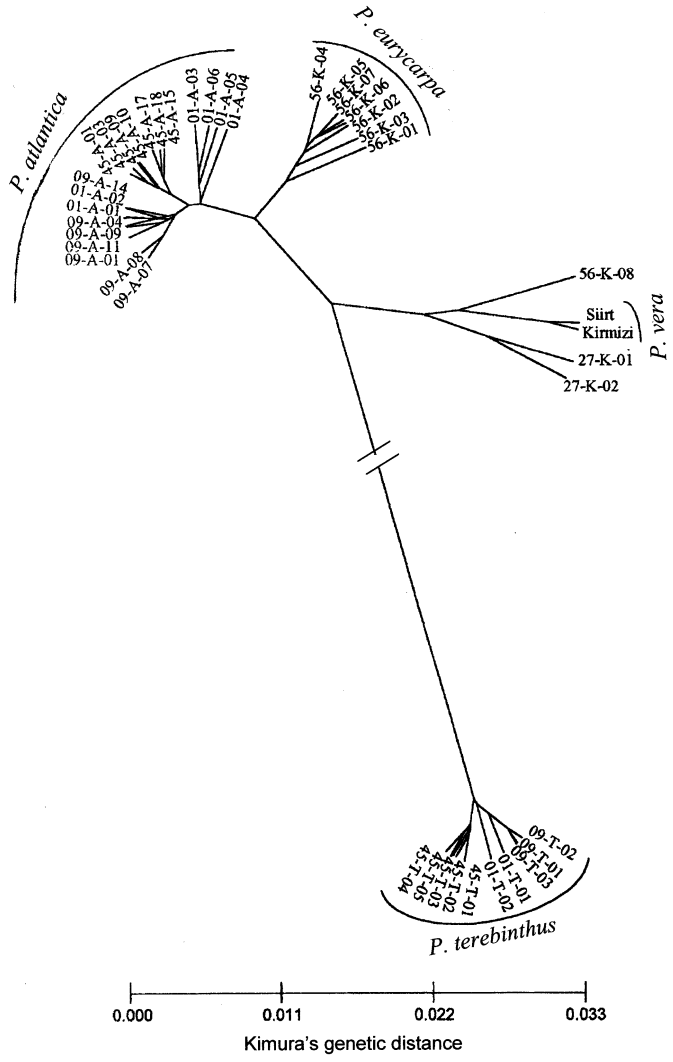

Fig. 4 UPGMA analysis of the same RAPD data as in **Fig. 3**, performed by the PHYLIP package software. Kimura's genetic distances were computed between all pairs of genotypes using the RESTDIST program. These served as input to the NEIGHBOR program that constructed a dendrogram using the UPGMA option

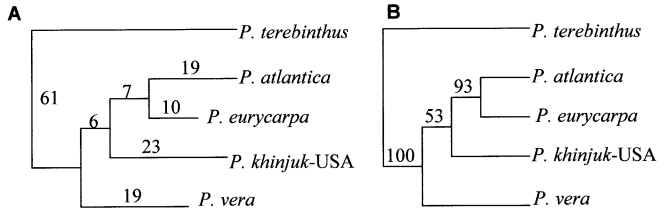

Fig. 5A, B Cluster analysis of molecular fingerprinting data from five *Pistacia* species. The data base included 137 RAPD bands. **A** Heuristic search was conducted by the PAUP software using the TBR optimization option, resulting in two trees of 139 steps. One of the trees is depicted. *Numbers* indicate the length (no. of steps) of each branch. **B** Consensus tree obtained from 100 Bootstrap replicates of the same data. *Numbers* indicate the percentage of replicate that included a given node

est to *P. vera* was indeed *P. khinjuk* (0.29 units), followed by *P. eurycarpa* (0.31 units) and *P. atlantica* (0.34 units), respectively. A significant node defining a branch of seven steps appears in 93% of the bootstrap replicates and separates *P. atlantica* and *P. eurycarpa* from the other species; these two form the closest pair of species within the tree (0.21 units).

This experiment supports, therefore, our idea that the trees identified as *P. khinjuk* by local growers and villagers in our study do not correspond to the *P. khinjuk* sample in the study of Parfitt and Badenes (1997). In view of the peculiar morphology of the former and the history of grafting in the Siirt province, they most likely represent *P. eurycarpa*, as described by Yaltirik (1967b). Our molecular data (Figs. 3, 4), in agreement with the morphological data (Fig. 1), also seems to support the designation of *P. eurycarpa* as a separate species, as suggested by Yaltirik (1967b) rather than as a variety of *P. atlantica* as suggested by Zohary (1952) since it is well-separated from *P. atlantica*.

Because of the leaf rachis wing character, Zohary (1952) grouped *P. vera* and *P. terebinthus* in the same, *Eu-terebinthus*, group, and *P. atlantica* was placed separately in the *Butmela* group. On the other hand, Parfitt and Badenes (1997) placed all three species in the same, *Terebinthus*, group by molecular analysis. According to our dendrograms, *P. eurycarpa*, *P. vera* and *P. atlantica* are rather close to each other and may be placed in a single group, while *P. terebinthus* is more distant.

Possible evolution of *Pistacia* species

According to the hypothesis of Zohary (1952) regarding the evolution of the genus *Pistacia*, *P. vera* is the most primitive species in the genus based on morphological characters (simple, imparipinnate leaves, small number of leaflets per leaf, symmetrical leaflets, rounded leaflet apex, wingless petiole, highly branched panicles, deciduous character). *P. khinjuk* may be a descendent of *P. vera*, and *P. atlantica* var. *kurdica* (syn *P. eurycarpa*) may have originated either directly from *P. vera* or through *P. khinjuk*. Zohary noted that var. *kurdica* resembles certain forms of *P. vera* due to the large nuts and large, ovate leaves bearing only a few pairs of leaflets. Moreover, he found samples of this variety in places where *P. vera* and *P. khinjuk* grow naturally. Based on the leaf rachis wing, Yaltirik (1967b) placed *P. eurycarpa* between the Eu-terebinthus group (whose leaves have a wingless rachis) and the *Butmela* group (with a winged rachis, including only *P. atlantica*) and concluded that *P. eurycarpa* may be a hybrid between *P. atlantica* and *P. khinjuk* since *P. eurycarpa* is a widespread species in southeastern Turkey, northern Iraq, Iran and Afghanistan, and part of its range overlaps with *P. khinjuk* and *P. atlantica*.

According to our molecular cluster analysis (Fig. 5) and morphological data, *P. eurycarpa* may indeed be a hybrid between *P. khinjuk* and *P. atlantica*, and *P.*

khinjuk may have originated from *P. vera*. It is also possible that *P. eurycarpa* is a primary descendant from *P. vera*, *P. khinjuk* is a hybrid between *P. eurycarpa* and *P. vera*, while *P. atlantica* originated from *P. eurycarpa*.

In a recent study, Parfitt and Badenes (1997) performed a phylogenetic study of ten *Pistacia* species based on an analysis of the chloroplast genome. They found that the closest species to *P. vera* is *P. khinjuk*, followed by *P. atlantica* and *P. terebinthus*. They could not discriminate *P. khinjuk* from *P. vera* because the chloroplast genome is very conserved within genera, and they suggested that *P. khinjuk* and *P. vera* may be considered one species, despite the difference in morphology. Our study was based on the more sensitive RAPD technique that mostly labels the nuclear genome. The present study largely confirmed the relationships described by Parfitt and Badenes (1997) but, in addition, it was able separate *P. khinjuk*, *P. vera* and *P. eurycarpa* from each other by nodes that were significant according to the bootstrap analysis.

Inter-specific hybrids

Several *Pistacia* species grow together in several regions of Turkey, and introgression of *P. vera* into wild species occurs naturally because of the allogamous character of the genus. Inter-specific hybrids between *Pistacia* species are, therefore, rather common in nature, and RAPD analysis may provide a tool to identify such hybrids. For example, genotypes 27-K-01, 27-K-02 and 56-K-08 may represent inter-specific hybrids.

Genotype 56-K-08 (a *P. eurycarpa* variety named Mardin Buttum used to graft trees) seemed to occupy an intermediate position between *P. vera* and *P. eurycarpa* (Fig. 1). Nut and leaf sizes of this genotype were intermediate, and it may represent a hybrid between *P. eurycarpa* and *P. vera*. Molecular analysis also placed tree 56-K-08 at an intermediate position between *P. eurycarpa* and *P. vera* (Fig. 3).

As for genotypes 27-K-01 and 27-K-02, our morphological analysis (Fig. 1) separated them from the other *P. eurycarpa* samples. Leaf and nut characters of these two genotypes were more similar to those of *P. vera* as compared to the other *P. eurycarpa* genotypes, and also the distance matrix places them closer to *P. vera* (0.29 and 0.32 distance units, respectively) than to the other *P. eurycarpa* genotypes (0.34 and 0.37 units, respectively). The genotypes may, therefore, represent hybrids between *P. vera* and *P. eurycarpa*. According to the molecular data, however, they occupied an intermediate position between *P. vera* and *P. terebinthus* (closer to *P. vera*). In Gaziantep province, where these two samples were collected, *P. terebinthus* and *P. vera* are the common species, and it is possible that they originated from an interspecific hybrid between *P. vera* and *P. terebinthus*.

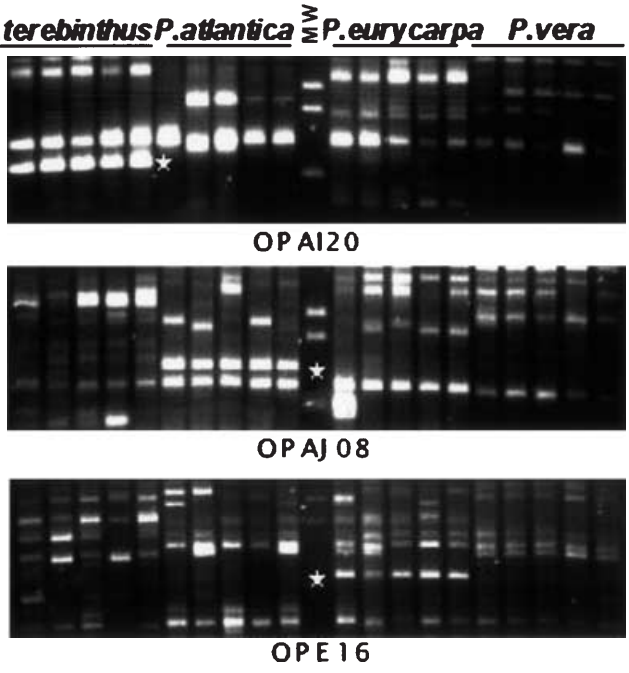

Fig. 6 RAPD banding patterns of OPAI20, OPAJ08 and OPE16 primers in five individual DNAs of each *Pistacia* species. Species-specific markers are indicated with *asterisks*. *MW* Molecular-weight standard

Identification of species-specific RAPD markers

During the screen for polymorphic primers, we noted that a few of the primers produced species-specific bands that appeared in all of the individuals of a species but not in the other species. We have found a total of 16 species-specific bands out of 138 bands analyzed: 10 were specific to *P. terebinthus*, 2 for *P. vera*, 2 for *P. atlantica* and 2 *for P. eurycarpa*. To confirm their specificity, we tested these in a larger sample of 50 individuals of each of the three wild species *P. terebinthus*, *P. atlantica* and *P. eurycarpa*, and in 12 varieties of *P. vera*. Examples of such markers are shown in Fig. 6, where primers OPAI20, OPAJ08 and OPE16 produced a species-specific band in *P. terebinthus*, *P. atlantica* and *P. eurycarpa*, respectively.

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, contact between the cultivated clones and the wild species *P. terebinthus*, *P. palaestina*, *P. khinjuk* and *P. atlantica* is quite common and has 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). In Turkey, top-working of wild *Pistacia* species has been done for many years, and it may sometimes prove difficult to obtain uncontaminated wild material of these species and correctly identify a *Pistacia* tree based on its

morphology. Our problems in identifying correctly the '*P. khinjuk*' samples exemplify this problem. Species-specific DNA markers may therefore provide an additional tool for the identification of *Pistacia* germplasm from an unknown origin.

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