



ORIGINAL ARTICLE

Molecular polymorphism in *Pistacia vera* L. using non-coding regions of chloroplast DNA



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Abstract The present study describes plastid DNA polymorphism and reports a comparative analysis of two non-coding cpDNA regions (*trnC-trnD* and *atpB-rbcL*) in pistachio. Seventeen different genotypes of domestic and wild pistachio from Iran, Syria, Turkey and America were sampled. Total genomic DNA was extracted and amplified with *trnC-trnD* and *atpB-rbcL* specific primers and then were sequenced. Phylogenetic relationships and depiction of phylogenetic trees were conducted. Cultivated genotypes of *Pistacia vera* were classified in a group regardless of their geographic location. *P. vera* was isolated from Sarakhs but they placed in the two close groups. Among cultivated genotypes, Jalab was separated from other cultivated genotypes. *Pistacia Khinjuk* was classified with *Pistacia atlantica* subsp. *mutica*. The findings confirm the common splitting hypothesis for commercial pistachio genotypes of the *P. vera* wild-type and also indicated the direct impact of Iranian genotypes in the evolutionary process of cultivated pistachios in other parts of the world. In conclusion it can be inferred that cultivated varieties of pistachio and *P. vera* var. *sarakhs* have the same origin, moreover genomic chloroplast could appropriately identify the interspecies relationships of pistachios.

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1. Introduction

The pistachio, *Pistacia vera* L. ($2n = 30$), is a deciduous, dioecious and wind-pollinated tree species member of the Anacardiaceae family [1,2]. It is referred to as the “green gold tree” due to its high economic value [3,4]. Its origin is still

indeterminate, but most experts come to an agreement that it probably originated in the Middle East [5–7].

Iran is the world’s major producer and has the greatest cultivation area of pistachio in the world. According to the number of pistachio cultivars and genotypes, Iran is one of the rich resources of pistachio in the world [6,8]. Nevertheless, the number of varieties constituting the species *P. vera* L. is considerable, there is some ambiguity about the exact location of some Iranian pistachio nut cultivars.

Evidence on the genetic and evolutionary relationships among species within the genus *Pistacia* is not adequate and

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is chiefly based on morphological, biochemical, and molecular markers [9,10]. Investigation on genetic diversity and relationships within different pistachio genotypes is necessary for breeding program decisions. Over the last decade, DNA sequence data have contributed prominently to improve our knowledge of the phylogenetic relationships of flowering plants [11,12].

Chloroplast DNA (cpDNA) sequence comparisons have been used commonly in studies of plant phylogenetics and genome evolution [13]. Accessibility of numerous universal chloroplast primers to amplify non-coding regions has simplified analyses of phylogenetic relationships at the generic and inter-specific levels [10]. Actually, the non-coding regions provide the most practical source of data for phylogenetic inference at lower taxonomic levels [14]. Among several non-coding chloroplast spacers the *trnC-trnD* (the *trnC-petN* intergenic spacer, the partial *petN* gene, the *petN-psbM* intergenic spacer, the partial *psbM* gene, the *psbM-trnD* intergenic spacer, and the partial *trnD* gene) region has been more satisfactory to be used in declaring phylogenetic relationships [15–19]. The *atpB-rbcL* spacer separating the plastid genes, *atpB* and *rbcL*, is one of the first non-coding regions used for phylogeny interpretation [20]. The molecular evolution of *atpB-rbcL* spacer was studied in depth [21] because it contained promoter elements for genes encoding subunits of the chloroplast ATP synthase and the large subunit of ribulose-1,5-biphosphate carboxylase [22,23].

In the present study, we describe plastid DNA polymorphism and report a comparative analysis of two non-coding cpDNA regions (*trnC-trnD* and *atpB-rbcL*) in pistachio.

2. Materials and methods

2.1. Plant material

Seventeen different genotypes of domestic and wild pistachio from Iran, Syria, Turkey and America were sampled in this study. Iranian pistachio genotypes were collected from the Rafsanjan Pistachio Germplasm collection situated in Rafsanjan, Iran. Syrian samples were obtained from the

Department of Horticulture, University of Amer Ibrahim Basha, Aleppo, Syria. American and Turkish pistachios (out of shell) were obtained from the market (Table 1).

2.2. DNA extraction and PCR amplification

Total genomic DNA was extracted according to the Hormaza et al. method [24]. The quality and quantity of DNA samples were determined using agarose gel electrophoresis and verified by spectrophotometry. DNA samples were diluted to 10 ng/μl and stored at –20 °C.

DNA amplifications were performed in 15 μL reactions containing approximately 10 ng genomic DNA, 1.5 μl of 10× PCR buffer, 1.5 mM MgCl₂, 0.2 mM of dNTPs, 0.3 μM of each primer, and 1.0 unit of *Taq* DNA polymerase. PCR amplification of the two regions, *trnC-trnD* and *atpB-rbcL*, was carried out in Techne (Burlington, NJ, USA) thermal cycler (Fig 1) using specific primers (Table 2). The *trnC-trnD* region was amplified using three pairs of primers, *trnC-petN2R*, *petN1-psbM2R*, and *psbM2-trnD*, as described by Lee and Wen [25]. The conditions of the PCR were as follows: an initial step of 95 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 3 min with a final extension step at 72 °C for 5 min. Amplified products were detected by electrophoresis on a 0.7% agarose gel followed by staining with ethidium bromide and under a UV transilluminator.

2.3. DNA cloning and sequencing

For sequencing the PCR amplified fragments, we need to clone these fragments into a cloning vector. So, for sequencing process, the amplified fragments were ligated into the pTZ57R/T cloning vector and transferred to *Escherichia coli* MC1061 using InsTAclone™ PCR Cloning KIT (Fermentas #K1213, K#1214) following the manufacturer's instructions. Plasmids were purified by Bioneer plasmid extraction kit (AccuPrep® Plasmid Mini Extraction Kit, Daejeon, South Korea) according to manufacturer's instructions and colonies containing the fragments were selected by colony PCR and digested with

Table 1 Seventeen different *Pistacia* genotypes used in this study.

No.	Species name	Genotype name	Code	Origin
1	<i>P. vera</i>	Ohadi	OH	Iran
2	<i>P. vera</i>	Khanjaridamghan	KD	Iran
3	<i>P. vera</i>	Kaleghochi	C3	Iran
4	<i>P. vera</i>	Sarakhs	S15	Iran
5	<i>P. atlantica</i> Subsp. <i>mutica</i>	Baneh	BO	Iran
6	<i>P. atlantica</i> Subsp. <i>mutica</i>	Baneh	B19	Iran
7	<i>P. khinjuk</i>	Kasur	Pke5	Iran
8	<i>P. atlantica</i> (Male)	Atlantica	P. Atla	Syria
9	<i>P. palaestina</i> (Male)	Palaestina	P. Pala	Syria
10	<i>P. vera</i>	Oleimy	P.V.OI	Syria
11	<i>P. vera</i>	Ashoury	P.V.ASHO	Syria
12	<i>P. vera</i>	Jalab	P.V.J	Syria
13	<i>P. lentiscus</i> (Male)	Lentiscus	P. Lent	Syria
14	<i>P. vera</i>	Siirt Anatolia	Sirrt	Turkey
15	<i>P. terebintus</i>	Terebintus	P.T	Turkey
16	<i>P. integerrima</i>	Integerrima	P.I	Turkey
17	<i>P. vera</i>	Eley	Kerman 2	USA

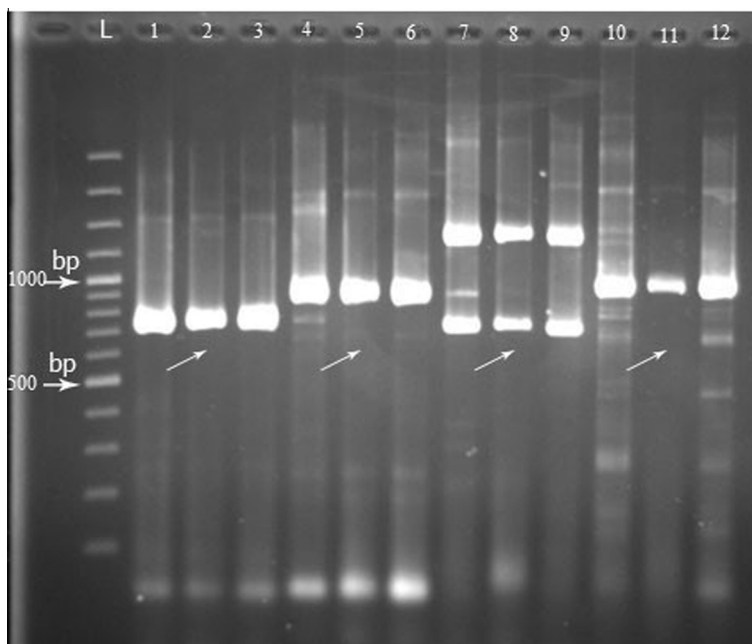


Figure 1 Wells 1, 2 and 3: 750 bp fragments amplified with *trnC*–*petN2R* primers. Wells 4, 5 and 6: 900 bp fragments amplified with *petN1*–*psbM2R* primers. Wells 7, 8 and 9: two band amplification with *psbM1*–*trnD* primers. Wells 10, 11 and 12: 900 bp fragments amplified by *atpB*–*rbcL* primers. In wells that have been marked with arrows, the annealing temperature is 58 °C. The first line of each binding row indicates the annealing temperature of 56 °C and the third line refers to 60 °C.

Table 2 Primer sequences used for analysis of *Pistacia vera* L. with optimal annealing temperatures (T_a).

Primer name	Sequence	Optimal T_a (°C)	Length (bp)
<i>trnC</i>	5'-CCAGTTCAAATCTGGGTGTC-3'	58	750
<i>petN2R</i>	5'-CCATTAAAGCAGCCCAAGCAAGAC-3'		
<i>petN1</i>	5'- GGATATAGTAAGTCTTGCTTGGG-3'	58	900
<i>psbM2R</i>	5'-TTCTTGCATTTATTGCTACTGC-3'		
<i>psbM2</i>	5'- GCAGTAGCAATAAATGCAAGAA-3'	58	1300
<i>trnD</i>	5'-GGGATTGTAGTTC AATTGGT-3'		
<i>atpB</i>	5'-ACATCKARTACKGGACCAATAA-3'	58	900
<i>rbcL</i>	5'-AACACCAGCTTTRAATCCAA-3'		

EcoRI and *PstI* (Fermentase Co. Germany). These steps were done in our lab and confirmed colonies were sent to Korea for sequencing process (MacrogenInc Company, Seoul, South Korea).

2.4. Data analysis

The sequences were assembled and edited by Chromas software version 2.13 [26] and the confirmed ones were compared to sequences within the NCBI database (National Center for Biotechnology Information) using BLASTn. Multiple sequence alignment was carried out by ClustalW, and phylogenetic tree was constructed by neighbor-joining method using MEGA 5.0 software [27].

3. Results and discussion

The processes of evolutions such as gene replication, deletion, and pseudogene formation, in chloroplast genomes are very

tardy, whereas, such processes are common among the nuclear genome and changes their DNA sequence [28]. It can be inferred that the application of genomic chloroplast is an appropriate technique to identify the interspecies relationships. To investigate the ability of each locus for elucidating the phylogenetic relationships, relative dendrogram of each region was constructed separately. In the present investigation the *atpB*–*rbcL* region could not segregate the pistachio species and illustrate the relationships among different pistachio species (Fig. 2). It may be because of its high conservative sequences in pistachio varieties. Also in the other reports this area has been applied along with other chloroplasts or nuclear parts for better representing the evolutionary relationships [29]. The *trnC*–*trnD* region consists of the distances among the four genes (*trnC*, *petN*, *psbM* and *trnD*). Following the study of dendrogram related to each part, *psbM*–*trnD* region was the only part that to some extent could show the relationships between the pistachio species. In this dendrogram, *P. vera*, *P. khinjuk* and *P. atlantica* subsp. *mutica* had a close relationship; however, other wild species were well

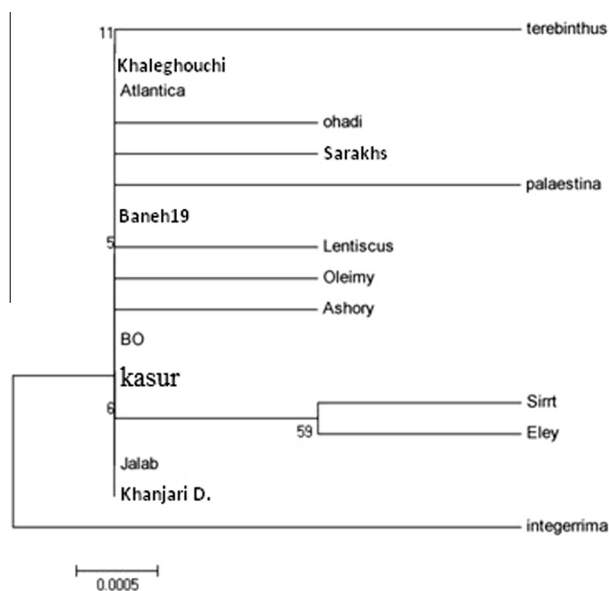


Figure 2 Neighbor-joining tree of genetic relationships among pistachio species based on the sequences of *atpB-rbcL* spacer according to the Kimura-2 method.

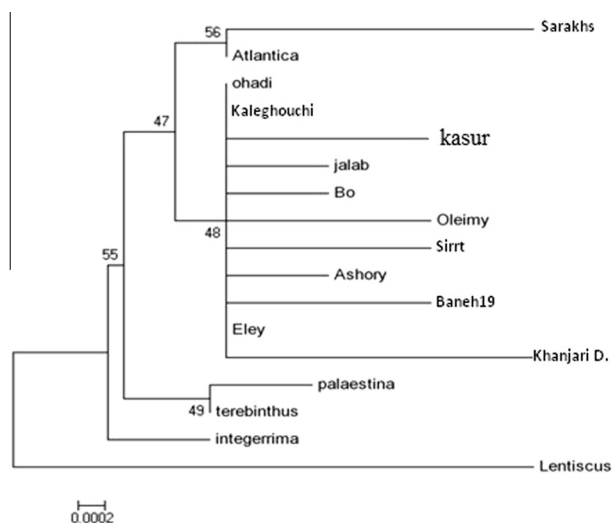


Figure 3 Neighbor-joining tree of genetic relationships among pistachio species based on the sequences of *psbM-trnD* spacer according to the Kimura-2 method.

discriminated from these three species (Fig. 3). *Pistacia lentiscus* was classified as a separate category and the two species of *Pistacia palaestina* and *Pistacia terebinthus* were classified in a similar group. In the phylogenetic tree derived from *trnC-petN* region, cultivated and wild species were separated (Fig. 4). Cultivated species had a close relationship with each other while the relationship between the wild species was not in accordance with other studies. In the classification obtained from *petN-psbM* region; cultivated genotypes were placed among the wild species which seems the sequences of this region are solitary, and not useful for pistachio phylogenetic determination; but from the other point of view, it could reveal the fact that both the wild and cultivated genotypes share this region and might

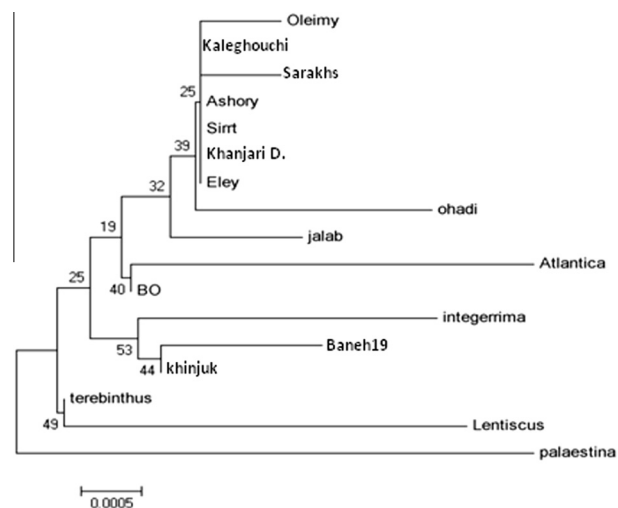


Figure 4 Neighbor-joining tree of genetic relationships among pistachio species based on the sequences of *trnC-petN* spacer according to the Kimura-2 method.

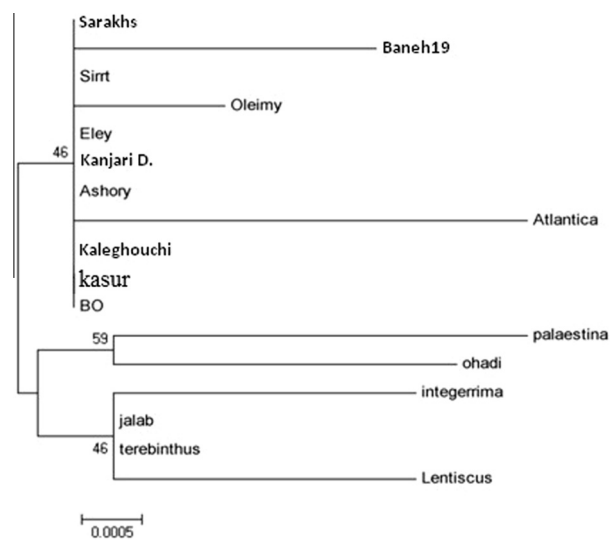


Figure 5 Neighbor-joining tree of genetic relationships among pistachio species based on the sequences of *petN-psbM* spacer according to the Kimura-2 method.

have a common ancestor (Fig. 5). In combining the three parts of *trnC-trnD* in different ways, the *trnC-psbM* region could separate domestic species from wild species (Fig. 6). The results of *petN-trnD* showed a close relationship of *P. vera*, *P. khinjuk* and *P. atlantica* and also two species of *P. palaestina* and *P. terebinthus* and in addition, classified *P. lentiscus* into a separate category (Fig. 7). The total data obtained from the genetic distance between the *trnC-petN* and *psbM-trnD*, approximately could provide a dendrogram similar to the dendrogram obtained from the overall data (Fig. 8).

The combination dendrogram of three regions of *trnC-petN*, *petN-psbM* and *psbM-trnD* (which are generally known as *trnC-trnD*), plotted the overall process showed by the total collection of data (Fig. 9); thus, it seems that *trnC-trnD* could better determine the pistachio phylogenetic relationships, as it

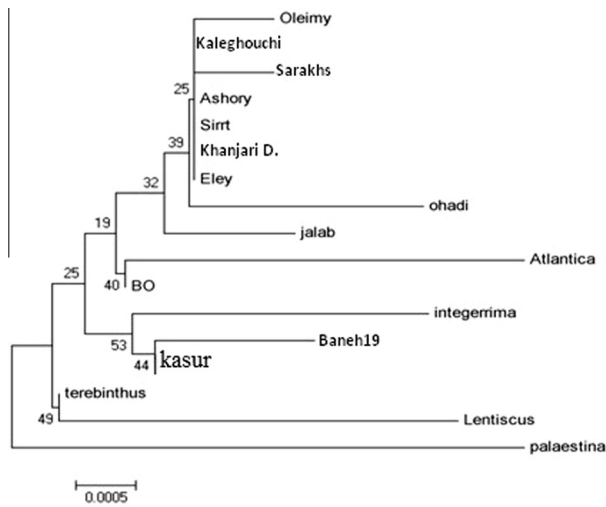


Figure 6 Neighbor-joining tree of genetic relationships among pistachio species based on the sequences of *trnC-psbM* spacer according to the Kimura-2 method.

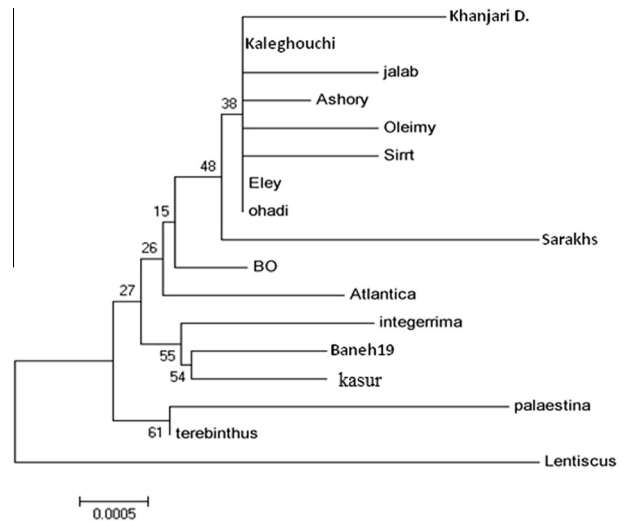


Figure 8 Neighbor-joining tree of genetic relationships among pistachio species based on combining the sequences of *trnC-petN* and *psbM-trnD* spacer according to the Kimura-2 method.

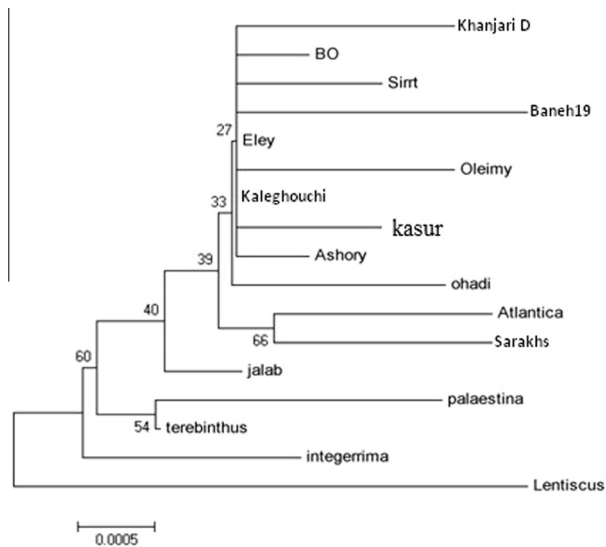


Figure 7 Neighbor-joining tree of genetic relationships among pistachio species based on the sequences of *petN-trnD* spacer according to the Kimura-2 method.

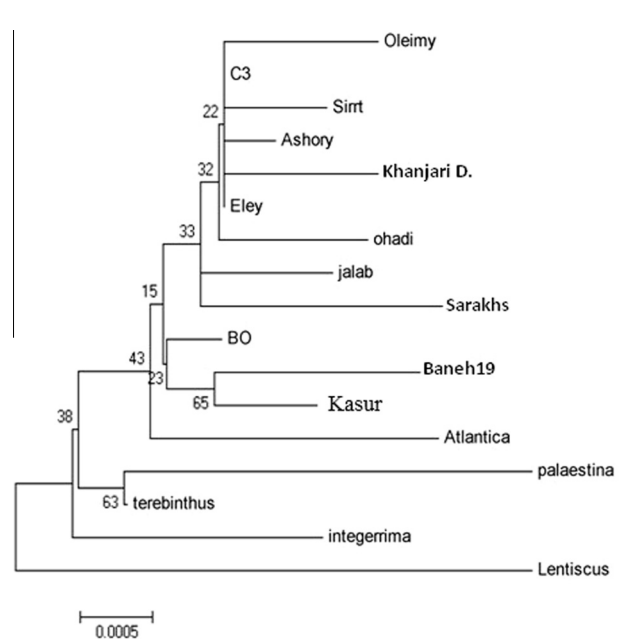


Figure 9 Neighbor-joining tree of genetic relationships among pistachio species based on the sequences of *trnC-trnD* spacer according to the Kimura-2 method.

could well express other interspecies relationships of the plants [19,25]. According to the plotted dendrograms, increasing the number of regions for plotting the dendrograms and accordingly increasing the number of single-nucleotide differences (i.e. the number of phylogenetic characters) could help to plot a better interspecies relationship. The combination of *trnC-trnD* parts in different forms, could isolate the cultivated and wild species.

According to the single nucleotide difference between sequences in chloroplast *trnC-trnD* and *atpB-rbcL* regions, the cultivated genotypes of *P. vera* were classified in a group regardless of their geographic location (Fig. 10). The cultivated genotypes of *P. vera* were separated from Sarakhs but they placed in the two close groups. There was a considerable similarity between the cultivated genotypes. Among these

genotypes, Jalab had a distinct banding pattern and was separated from other cultivated genotypes. *P. Khinjuk* was classified with *P. atlantica* subsp. *mutica* (Baneh). *P. lentiscus* given to its highly different sequence was discriminated from other species. This species is an evergreen shrub and for this reason is different from other studied species. *P. lentiscus* has also been discriminated from deciduous species and was classified with other evergreen species in a previous report. There was a close genetic relationship between three species of *P. atlantica*, *P. khinjuk* and *P. vera* and also between *P. terebinthus* and *P. palaestina* [18]. The study of genetic

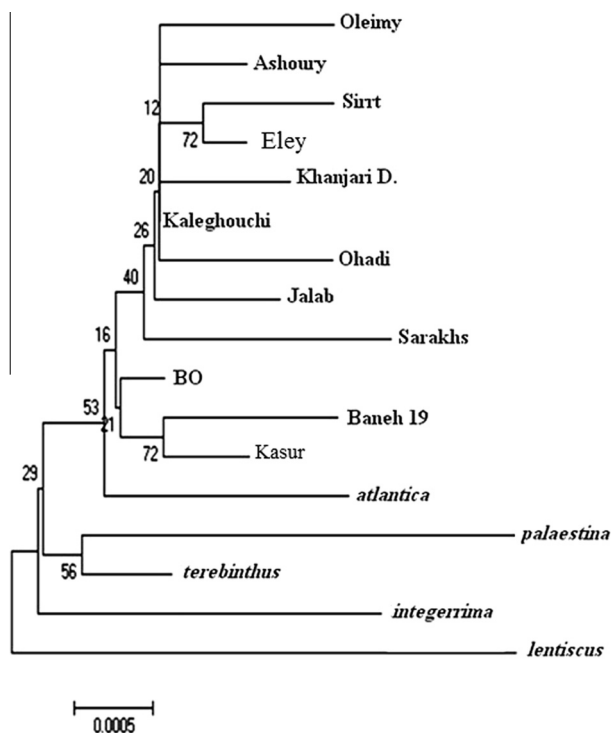


Figure 10 Neighbor-joining tree showing the genetic relationships among different pistachio species based on the combined sequences of *trnC-trnD* and *atpB-rbcL* spacers.

diversity among and within pistachio species using AFLP, ISSR, and RAPD markers showed the discrimination among accessions according to its geographical origin (i.e. Mediterranean regions, North Africa, Middle East, and Eastern Zagros Mountains). In addition, the Iranian accessions of *P. vera* classified in separate groups of Mediterranean cultivars [30] unlike, in the present study, the cultivated genotypes of *P. vera* were classified in one group regardless of their geographic location. Similar results were obtained based on SSR marker, which classified the cultivated pistachio genotypes of Syria, Turkey, and the U.S. among the Iranian genotypes. This finding could confirm the common splitting hypothesis of commercial pistachio genotypes from the wild-type *P. vera* and also indicates the direct impact of Iranian genotypes in evolutionary process of cultivated pistachios in other parts of the world [31]. According to the plotted dendrogram, the cultivated *P. vera* genotypes were separated from its wild-type counterparts (Sarakhs), while were placed in their close cluster. It can be inferred that cultivated varieties of pistachio and *P. vera* var. *sarakhs* have the same origin. Given that wildling woods in northern parts of Iran, is covered by *P. vera* var. *sarakhs*, so it could be possibly the area that was the common ancestor of cultivated varieties and *sarakhs* and the primary origin of cultivated varieties can be attributed to these regions. Our finding is keeping with the results of Ahmad et al. [32] and classified the Jalab separated from other cultivated genotypes. In some former studies using genomic markers such as RAPD, AFLP, ISSR and SSR the *P. khinjuk* has been reported as the most similar species to *P. vera* [9,33–35].

Unlike, in this study, *P. khinjuk* was classified with *P. atlantica* subsp. *mutica* (Baneh). Similar to our result in a classification based on morphological characteristics,

P. khinjuk has been shown to have closer relationship with cultivated *P. atlantica* genotypes than cultivated *P. vera* [36]. However, the genetic distance between 1500-year-old *P. atlantica* subsp. *mutica* (Baneh, BO) and *P. vera* species is lower than *P. khinjuk* which might be due to the high antiquity of this pistachio probably not being involved in the evolutionary process. In a previous pistachio evolutionary study using chloroplast DNA [37,38], *P. atlantica* subsp. *mutica* (Baneh) was not among the studied species; hence, the present study could be more reliable because of covering more species of pistachio. Two species of *P. palaestina* and *P. terebinthus* were classified in one cluster; it is worth noting that these two species are morphologically and ecologically alike [39]. Also the AFLP and RAPD results by Katsiotis et al. [33], classified the *P. palaestina* along with *P. terebinthus*. Furthermore, in the study of Golan Goldhirsh et al. [34], using AFLP and RAPD techniques, a similar classification for these two species was observed. In keeping with our findings, in the morphological and molecular literatures investigating the relationship of different pistachio species, the *P. vera*, *P. khinjuk* and *P. atlantica* had a closer relationship than other species which is in accordance with their geographical distribution that is central Asia [18,35,40]. In the plotted dendrogram, *P. lentiscus* was placed in a separate group given its very different sequence than other species. Similar results have also been obtained in previous studies using morphological and molecular markers [33,34,37,39]. This species is an evergreen shrub and is different from other studied plants. In a morphological classification of pistachio genera by Zohary [39], *P. atlantica* was classified in a separate group from the other species, while in the present study, *P. atlantica* was grouped with *P. vera* and *P. khinjuk*, and confirmed the report of Parfitt and Badenes [37] regarding the elimination of Butmela part (including *P. atlantica*) and classifying *P. atlantica* in Eu-Terebinthus. There are some consistencies between the results obtained here and the classification obtained from nuclear, chloroplast and ITS [18]. There was a close genetic relationship among three species of *P. atlantica*, *P. khinjuk* and *P. vera* and also between *P. terebinthus* and *P. palaestina*. Integration of Eu-Terebinthus and Butmela parts was also supported by this study. According to the results obtained here, and due to the fact that in the chloroplast genome evolutionary processes such as replication, gene deletion and pseudo-gene formation are happening slowly, while this process is common among nuclear genes and can change the evolutionary process of DNA sequence it can be inferred that the chloroplast genome is an appropriate method for determining the relationships between species.

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