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***Full Length Article***

# Retrotransposon Marker Systems as a Tool to Analyze Molecular Diversity of Mediterranean *Pistacia* Species

**Emrah Kırdök and Yelda Özden Çiftçi***
*Gebze Technical University, Department of Molecular Biology and Genetics, 41400 Çayırova Kocaeli, Turkey*
*For correspondence: yelda75@yahoo.com

## Abstract

Retrotransposon movements could lead to major genome rearrangements because of their special transposition mechanism and may be used to analyze differences between species as a marker system. In this context inter primer binding site (iPBS), inter-retrotransposon amplified polymorphism (IRAP) and retrotransposon-microsatellite amplified polymorphism (REMAP) marker systems were used to resolve genetic differences in 7 Mediterranean *Pistacia* species (*P. vera* L. cv. Siirt, *P. khinjuk* Stocks, *P. mutica* Fischer, *P. atlantica* Desf., *P. palaestina* Boiss., *P. terebinthus* L. and *P. lentiscus* L.). High level of polymorphism ratio observed in all tested marker systems. Except REMAP, iPBS and IRAP marker systems showed strong mantel correlations. Phylogenetic tree with neighbor joining (NJ) method depicted that Mediterranean *Pistacia* species may be divided into three clades: (i) *P. vera – P. khinjuk*, (ii) *P. mutica – P. atlantica* and (iii) *P. palaestina – P. terebinthus* with *P. lentiscus*. Principal component analysis (PCA) plots showed 4 distinct clusters. In conclusion, retrotransposon based marker systems could be useful for analyzing molecular diversity in *Pistacia* genus due to their highly polymorphic nature and ease of use. © 2016 Friends Science Publishers

**Keywords:** *Pistacia*; Retrotransposon marker system; Characterization; iPBS; IRAP; REMAP

## Introduction

Transposons are the main genetic properties of higher eukaryotes as well as plants and consist of two different major groups (DNA and RNA transposons or retrotransposons) according to their transposition mechanism. Upon discovery of DNA transposons by Barbara McClintock (McClintock, 1951), it was found that they are one of the major genome reshaping elements via transcriptional activation and transposition under environmental stress conditions (Grandbastien, 2015). Among other types of transposons, retrotransposons (especially long terminal repeat, LTR retrotransposons) provides a very big contribution to this phenomenon because of their special type of transposition mechanism with their capability to integrate various copies through the genome while preserving the original copy (Schulman, 2013). Having this retrotransposition ability, retrotransposons can induce massive chromosomal rearrangements thus influence an increase, change and functionality of genome shape which have been documented extensively in different type of plant species (Grandbastien, 2015). Currently these genetic elements drive too much attention for using as a DNA marker system due to their structural characteristics including conserved PBS (primer binding site, initiation site for reverse transcription of retrotransposon mRNA) region and LTRs at both ends. Moreover their abundance in plant genomes, dispersity, demonstration of Mendelian inheritance, detection of big chromosomal rearrangements and ease of use make them suitable for genetic markers (Fig. 1a; Kalendar *et al*., 2011; Kalendar and Schulman, 2014). In this context Kalendar *et al.* (1999; 2010) developed three retrotransposon based marker systems (Fig. 1b, c and d): IRAP (primers designed to amplify between LTR regions), REMAP (primers designed for both LTR and adjacent SSR region) and iPBS (primers designed to amplify between PBS regions).

*Pistacia* genus belongs to the *Anacardiaceae* family consists of at least 11 species (Kafkas and Perl-Treves, 2002) that are mostly dioecious and evergreen or deciduous trees which was extensively studied using both morphological and molecular aspects. Two different diversity centers are available for *Pistacia*: (i) Mediterranean region of South Europe, Northern Africa, Middle East (ii) West and Central Asia (Al-Saghir and Porter, 2012). The most complete characterization study was conducted by Zohary (1952) who divided the genus into 4 main sections with using morphological markers: Lenticella (*P. mexicana* and *P. texana*), Eu-Lentiscus (*P. lentiscus*, *P. saportae* and *P. weinmannifolia*), Butmela (*P. atlantica*), Eu-Terebinthus (*P. chinensis*, *P. khinjuk*, *P.*

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*palaestina*, *P. terebinthus* and *P. vera*). Lately with 19 different morphological markers, Al-Saghir and Porter (2012) revised the status of the genus and clustered the species in 2 main sections. In addition various molecular characterization studies were also carried on different species, cultivars and accessions of *Pistacia* including RAPD (Kafkas and Perl-Treves, 2001; Kafkas and Perl-Treves, 2002; Katsiotis *et al.,* 2003; Golan-Goldhirsh *et al.*, 2004), AFLP (Katsiotis *et al*., 2003; Golan-Goldhirsh *et al.*, 2004; Kafkas, 2006; Karimi *et al.*, 2009), ISSR (Kafkas, 2006), SSR, SRAP (Talebi *et al.*, 2012), IRAP (Ghaemmaghami *et al.*, 2013) and restriction analysis of chloroplast fragments (Parfitt and Badenes, 1997) in order to resolve the phylogenetic relationships. These studies were mostly dealt with classification of *Pistacia* species, which were originated from Middle East region and tried to classify either 4 (as Lenticella, Eu-Lentiscus, Butmela and Eu-Terebinthus) or 2 (as Terebinthus and Lentiscus) groups. Moreover numerous plastid (*trnL-F*, *ndhF*, *psA-ycf3*, *rps16*, *rpl16*, *atpB-rbcL*, *psbA-trnH*) and nuclear (ITS, ETS) markers were also sequenced for molecular characterization (Yi *et al.*, 2008; Xie *et al.*, 2014) and it was found that these two studies support neither 2 nor 4 sections but showed a reticulate evolution in *Pistacia* genus. Those studies showed strong similarity between *P. vera* - *P. khinjuk*, *P. atlantica* - *P. mutica* and *P. terebinthus* - *P. palaestina* in *Pistacia* species for both morphological and molecular aspects. However, previous classifications did not seem to have strong supports (Xie *et al.*, 2014). Besides presence of a high ratio of interspecific hybridizations in *Pistacia* genus makes very hard to resolve phylogenetic relationships (Al-Saghir and Porter, 2012).

As retrotransposon marker systems were successfully used for molecular characterization of *Diospyros kaki* (Guo *et al.*, 2006), wheat (Carvalho *et al.*, 2010), *Citrus* (Biswas *et al*., 2010), grapevine species and cultivars (D'Onofrio *et al*., 2010; Guo *et al.*, 2014), flax (Smykal *et al.*, 2011), alfalfa (Abdollahi Mandoulakani *et al*., 2011), cocoyam (Doungous *et al.*, 2015), taro (Doungous *et al.*, 2015), lilium (Lee *et al.*, 2015) and howtron (Rahmani *et al*., 2015). The aim of the study was to use three iPBS, IRAP and REMAP retrotransposon based marker systems for the first time to analyze the genetic diversity, to compare three different marker systems in order to reveal their diversity indices, to construct phylogenetic trees in order to resolve the relationships in Mediterranean *Pistacia* species.

## Material and Methods

### Plant Materials

Seeds which were obtained under controlled pollination from 6 different *Pistacia* species (*P vera* cv. Siirt, *P. mutica*, *P. khinjuk*, *P. terebinthus*, *P. palaestina*, *P. lentiscus* L.) kindly obtained from Pistachio Research Institute (Gaziantep) by Msc Veysel Süzerer. *P. atlantica* seeds were kindly obtained from Algeria by Dr. Limane Abdulkerim (Mouloud Mammeri University of Tizi Ouzou) and *P. lentiscus in vitro* samples which were originated from Çeşme, İzmir were provided by İbrahim Koç.

### Sterilization, *in Vitro* Germination and DNA Isolation and PCR Reactions

*Pistacia* seeds were sterilized as described in Ozden-Tokatli *et al.* (2005). For *in vitro* germination, sterilized seeds were cultured on MS (Murashige and Skoog, 1962) medium devoid of growth regulators for 2–4 weeks. DNA isolation of 5 germinated plantlets from each species based on CTAB (cetyltrimethylammonium bromide) protocol was carried out according to Lodhi *et al.* (1994). Isolated genomic DNAs were visualized on 1.5% agarose gel electrophoresis along with 1 kb + 100 bp DNA Ladders (Invitrogen) and quantified with Shimadzu Biotech, Biospec Nano spectrometer. PCR protocol for all tested marker systems was based on Kalendar *et al.* (2010): 95°C for 3 min pre-denaturation; 30 cycle of; 15 sec 95°C denaturation, 1 min for annealing temperature (30' for IRAP and REMAP PCR, annealing temperatures were presented in Table 1), 1 min for 68°C extension (for IRAP and REMAP PCR, extension time is 3 min) and 72°C 10 min for final extension step. PCR final concentrations were: 1 X PCR buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTP, 1 µM primer (for REMAP PCR, 0.5 µM for both primers indicated in Table 2), 50 ng gDNA and 2 U Taq polymerase (iTaq, IntronBio CAT. 25022) in 25 µL final volume. PCR products were visualized on 1.5% agarose gel electrophoresis that run at 80 volt for 6 h and gel images were recorded with ChemiDoc XRS+ (Biorad) imaging system.

### Statistical Approach

Gel images were analyzed and each band treated as a di allelic locus, 1 for presence and 0 for absence. Then discrimination capacity of the marker systems calculated as described in Table 3 (Roldan-Ruiz *et al.*, 2000; Belaj *et al.*, 2003; Campbell *et al.*, 2010). Similarity matrices between species calculated by Jaccard coefficient (Jaccard, 1908) with MVSP (Multivariate Statistics Package, Kovacs computing) software. Neigbour-joining (NJ) phylogenetic trees were constructed using Darwin (Dissimilarity Analysis and Representation for Windows), with Jaccard distance, and 900 bootstrap replicates. Mantel permutation test was conducted using R statistical package (64 bit version) with vegan library installed (implementation based on Legendre and Legendre, 1998). Principal component analysis (PCA) was conducted for individual and all marker systems using Mat Lab (64 bit, version 2013a).

**Table 1:** List of primer sequences used in this study

| Primer Name | Sequence | Annealing temperature (°C) |
|---|---|---|
| iPBS Marker System (Kalendar et al. 2010) | | |
| iPBS - 1 | GCTCTGATACCA | 53 |
| iPBS - 2 | CTCATGATGCCA | 53 |
| iPBS - 4 | CTCACGATGCCA | 54.3 |
| iPBS - 5 | GCGGAGTCGCCA | 62.9 |
| iPBS - 6 | AGGTGGGCGCCA | 62.6 |
| iPBS - 7 | GCTCGGATACCA | 55 |
| iPBS - 8 | CCCAGCAAACCA | 54.3 |
| iPBS - 9 | TCGCATCAACCA | 53 |
| iPBS - 10 | TAGATGGCACCA | 51.4 |
| IRAP Marker System (Smykal et al. 2011) | | |
| LTR - 2 | CTTGCTGGAAAGTGTGTGAGAGG | 55 |
| LTR - 3 | TGTTAATCGCGCGCTCGGGTGGGAGCA | 55 |
| LTR - 4 | AGCCTGAAAGTGTTGGGTTGTCG | 55 |
| LTR - 5 | CTGGCATTTCCATTGTCGTCGATGC | 55 |
| LTR - 6 | GCATCAGCCTGGACCAGTCCTCGTCC | 55 |
| LTR - 7 | CACTTCAAATTTTGGCAGCAGCGGATC | 55 |
| LTR - 10 | TGAGTTGCAAGGTCCAGGCATCA | 55 |
| ISSR Primers | | |
| ISSR-10 | GAAGAAGAAGAAGAAGG | 55 |
| ISSR-13 | AGAGAGAGAGAGAGAGT | 55 |

**Table 2:** Combinations for REMAP marker system used in this study

| Primer Set | Combination | | Primer Set | Combination | |
|---|---|---|---|---|---|
| | LTR | ISSR | | LTR | ISSR |
| REMAP 1 | 2 | 10 | REMAP 8 | 2 | 13 |
| REMAP 2 | 3 | 10 | REMAP 9 | 3 | 13 |
| REMAP 3 | 4 | 10 | REMAP 10 | 4 | 13 |
| REMAP 4 | 5 | 10 | REMAP 11 | 5 | 13 |
| REMAP 5 | 6 | 10 | REMAP 12 | 6 | 13 |
| REMAP 6 | 7 | 10 | REMAP 13 | 7 | 13 |
| REMAP 7 | 10 | 10 | REMAP 14 | 10 | 13 |

## Results

### Diversity Indices and Mantel Correlation of Marker Systems

Among primer sets, iPBS marker system supplied the most locus information (319 total loci, 35.44 loci per assay and 35.44 polymorphic loci per assay) and discrimination capacity in comparison with the other markers (as it resulted in high total number of alleles, efficiency index and resolving power). However, polymorphism proportions (0.94, 0.93 and 0.93), expected heterozygosity (0.35, 0.32 and 0.3) and PIC values (0.35, 0.35 and 0.32) were nearly similar for all tested markers (Table 4). Mantel correlations with 1000 permutations showed that the correlation between iPBS and IRAP markers were quite strong (0.79) (Table 5). Moreover, mantel correlation values were quite high in between iPBS (0.96), IRAP (0.91) but very low in REMAP (0.47) in comparison with combined marker data.

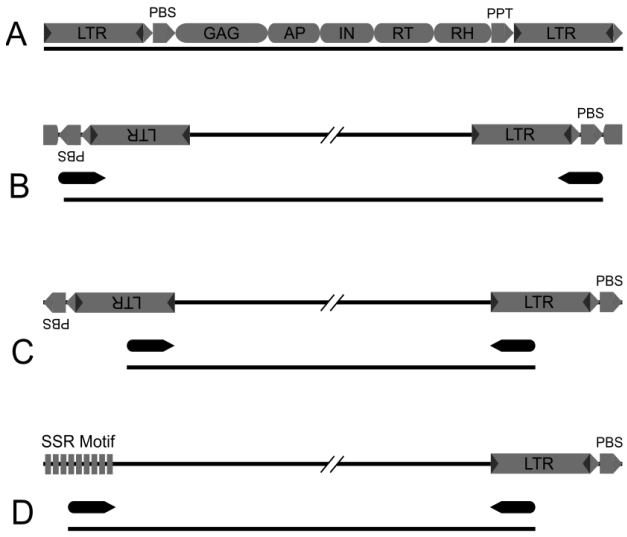


**Fig. 1:** Schematic representation of a LTR retrotransposon structure and molecular markers used in this study (Kalendar *et al.*, 2010; Kalendar *et al.*, 2011): (A) Two LTR regions for transcriptional initiation and termination sites forms the head and tail structure. Inverted repeats represented as inward black triangles. PBS and PPT (polypurine tract) sites are for initiation of reverse transcription from transcribed RNA. Coding region contains GAG (capsid protein, packaging the mRNA), AP (Aspartic protease, cleaving the polyprotein complex), IN (Integrase, inserting the DNA form of the retrotransposon), RT (Reverse transcriptase, reverse transcription of retrotransposon mRNA), RH (Rnase H, digesting mRNA while reverse transcription). (B) For using iPBS marker system, specific iPBS primers used for amplifying inter retrotransposonal are where for head to head oriented retrotransposons. (B) IRAP marker system aims to amplify inter retrotransposonal are like iPBS but primers designed for LTR regions and again head to head oriented retrotransposons (C) In REMAP marker system one IRAP primer and one SSR primer used in order to amplify between LTR and the adjacent SSR region

### Analysis of Genetic Similarities and Phylogenetic Relationships in Pistacia Species

Using all the marker information; the closest species pairs were found to be *P. vera* - P. *khinjuk* (0.587), *P. mutica* - *P. atlantica* (0.540) and *P. khinjuk* - P. *terebinthus* (0.564) (Table 6). Construction of a phylogenetic tree by using combined marker dataset resolved 3 clades: (Clade-I) *P. vera – P. khinjuk* cluster has the most bootstrap support (98%) followed by (Clade-II) *P. mutica – P. atlantica* (59%) and (Clade-III) *P. lentiscus – P. palaestina – P. terebinthus* (23%) cluster (Fig. 2).

**Table 3:** Description of diversity indices for analyzing marker systems

| | Diversity indices |
|---|---|
| Total number of primers | $U$ |
| Number of loci | $L$ |
| Number of polymorphic loci | $n_p$ |
| Number of monomorphic loci | $n_{np}$ |
| Proportion of polymorphic loci | $P_p$: |
| Number of loci per primer | $n_u=L/U$ |
| Expected heterozygosity | $H_e=1-\Sigma p_i^2$ |
| Effective number of alleles per locus ($n_e$): | $n_e=1/\Sigma p_i^2$ |
| Total number of effective alleles ($N_e$): | $N_e=\Sigma n_e$ |
| Assay efficiency index ($A_i$): | $A_i= N_e/U$ |
| Effective multiplex ratio ($E$): | $E=n_u \times P_p$ |
| Marker index ($M$): | $M=H_e \times L$ |
| Resolving power ($R_p$): | $R_p= \Sigma(1-(2 \times \|0.5 - pi\|))$ |
| Polymorphism information content | $PIC= (\Sigma 2fi(1-fi))/L$ |

*pi* is the frequency for $i^{th}$ allele

**Table 4:** Diversity indices of 3 different retrotransposonal marker systems

| Diversity Indices | iPBS | IRAP | REMAP |
|---|---|---|---|
| Number of Assays | 9 | 7 | 8 |
| Number of Polymorphic Bands | 319 | 145 | 116 |
| Number of Monomorphic Bands | 21 | 9 | 9 |
| Total Number of Loci | 340 | 154 | 125 |
| Proportion of Polymorphic Bands | 0.94 | 0.94 | 0.93 |
| Proportion of Polymorphic Bands per Assay | 35.44 | 20.71 | 14.5 |
| Proportion of Loci per Assay | 37.78 | 22 | 15.62 |
| Expected Heterozygosity | 0.35 | 0.32 | 0.3 |
| Effective Number of Alleles per Locus | 1.61 | 1.53 | 1.5 |
| Total Number of Effective Alleles | 548.29 | 235.94 | 188.01 |
| Efficiency Index | 60.92 | 33.71 | 23.5 |
| Effective Multiplex Ratio | 35.51 | 20.68 | 14.53 |
| Resolving Power | 180.57 | 79.89 | 59.37 |
| PIC | 0.35 | 0.35 | 0.32 |

### Principal Component Analysis of *Pistacia* Species

After calculation of eigenvalues and eigenvectors; the first three principal coordinates covered 50.26% of the total variability for combined marker data (Table 7) and species were clustered in 4 different groups in PCA plot: *P. vera* – *P. khinjuk*, *P. terebinthus* - *P. palaestina* and *P. mutica* - *P. atlantica* formed three clusters, while *P. lentiscus* remained as individual (Fig. 3).

## Discussion

In this research, 3 different retrotransposon based marker systems were used for the analysis of genetic diversity and phylogenetic relationships in 7 Mediterranean *Pistacia* species. There was just one report dealing with using retrotransposon based marker systems for characterization of *Pistacia* genus from Iranian *Pistacia* species (*P. vera*, *P. khinjuk*, *P. atlantica*) at the time manuscript was written and resolved 89.6% polymorphism in IRAP marker system (Ghaemmaghami *et al.*, 2013). Retrotransposon based marker systems showed a high proportion of polymorphism ratio similar with RAPD

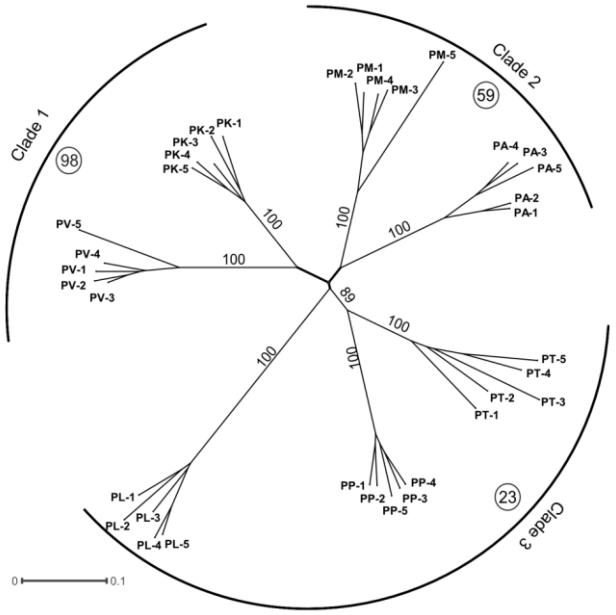


**Fig. 2:** Unrooted NJ phylogenetic tree using Jaccard coefficient with combined marker systems. Bootstrapping ratios for clades were showed in the circles under respective clades and on the arms. (PV: *P. vera*, PK: *P. khinjuk*, PM: *P. mutica*, PA: *P. atlantica*, PP: *P. palaestina*, PT: *P. terebinthus*, PL: *P. lentiscus*)

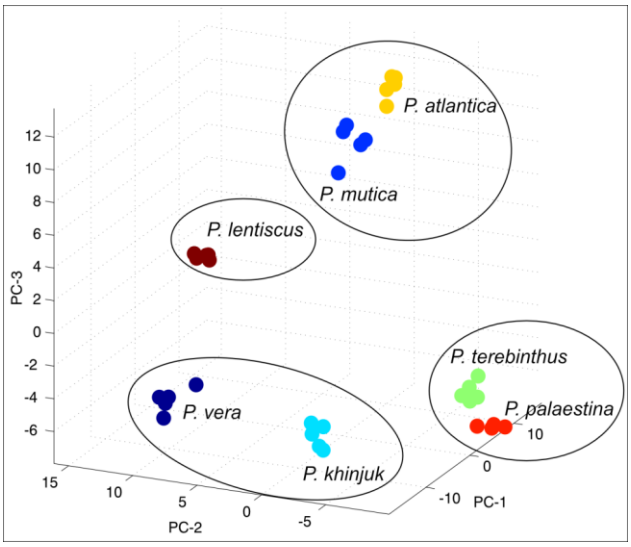


**Fig. 3:** Principal component analysis of Mediterranean *Pistacia* species. Resolved 4 groups were shown in circles

(Golan-Goldhirsh *et al.*, 2004; Kafkas, 2006) and AFLP (Golan-Goldhirsh *et al.*, 2004) while revealed out relatively greater heterozygosity and Rp than AFLP (Shanjani *et al.*, 2009; Kafkas, 2006; Karimi *et al.*, 2009) marker systems. However, PIC values were lower than the ones reported in the literature (Kafkas, 2006; Karimi *et al.*, 2009).

**Table 5:** Jaccard similarity matrices both (iPBS + IRAP + REMAP) marker systems

| Species | *P. vera* | *P. mutica* | *P. khinjuk* | *P. terebinthus* | *P. atlantica* | *P. palaestina* | *P. lentiscus* |
|---|---|---|---|---|---|---|---|
| *P. vera* | 0.855 | | | | | | |
| *P. mutica* | 0.512 | 0.797 | | | | | |
| *P. khinjuk* | 0.587 | 0.538 | 0.860 | | | | |
| *P. terebinthus* | 0.439 | 0.491 | 0.564 | 0.767 | | | |
| *P. atlantica* | 0.449 | 0.540 | 0.503 | 0.490 | 0.846 | | |
| *P. palaestina* | 0.452 | 0.476 | 0.532 | 0.532 | 0.497 | 0.878 | |
| *P. lentiscus* | 0.368 | 0.370 | 0.414 | 0.414 | 0.415 | 0.414 | 0.841 |

**Table 6:** Pairwise Mantel correlations of the 3 marker systems

| | iPBS | IRAP | REMAP | ALL |
|---|---|---|---|---|
| iPBS | 1 | | | |
| IRAP | 0.79 | 1 | | |
| REMAP | 0.39 | 0.51 | 1 | |
| ALL | 0.96 | 0.90 | 0.47 | 1 |

*All correlations are significant under p<0.001

**Table 7:** Relative explained variance and cumulative variability of all primer sets

| Principal Coordinates | | Relative Explained Variance | Cumulative Variability (%) |
|---|---|---|---|
| All Primer Sets | 1 | 0.1883 | 18.83 |
| | 2 | 0.1653 | 35.36 |
| | 3 | 0.1490 | 50,26 |

Our mantel correlation test results between marker systems were not high in comparison with the literature (Katsiotis *et al.*, 2003; Golan-Goldhirsh *et al.*, 2004), however this could be due to the different mobile retrotransposon regions targeted in each marker system.

Recently, Xie *et al.* (2014) pointed out that the evolution in *Pistacia* genus was not likely to be previous classification but to be a reticulated pattern based on geographical locations as (i) North and Central America, (ii) Mediterranean and adjacent areas and (iii) Asia. Here, reconstruction of phylogeny with combined marker data showed that *Pistacia* genus is likely to be a reticulated pattern. PCA plot was able to clearly separate the species except *P. lentiscus*, which was remained individual. Our results showed a good agreement between other marker systems which were used previously to analyze molecular relationships between *Pistacia* species. We found that *P. vera* and *P. khinjuk* were closely related with each other as consistent with the previous studies by using RAPD (Kafkas and Perl-Treves, 2002; Golan-Goldhirsh *et al.*, 2004) and AFLP (Golan-Goldhirsh *et al.*, 2004) marker systems also showing a high bootstrapping clustering ratio (Kafkas and Perl-Treves, 2002). Restriction analyses of chloroplast genome (Parfitt and Badenes, 1997) together with *NIA-i3*, *ITS* and plastid genes (Yi *et al.*, 2008) demonstrated the closer relationship between these two species. It is known that *P. khinjuk* not only shares the dispersion locations (Afghanistan, Iran, Iraq, Syria, Palestine and Egypt) but also it shows similar morphological variation patterns with *P. vera* (Zohary, 1952). Previously, *P. atlantica* and *P. mutica* species pair also clustered together in numerous marker studies (Kafkas, 2006; Karimi *et al.*, 2009). In our phylogenetic reconstruction and PCA plot the closer relationship between *P. atlantica* and *P. mutica* could be easily observed. However, there was an ongoing debate about classification of *P atlantica* in a separate section Butmela which was classified by Zohary (1952), because of rachis wing character. Parfitt and Badenes (1997) suggested that the Butmela section could be eliminated and merged with section Terebinthus. On the contrary, Golan-Goldhirsh *et al.* (2004) found that *P. atlantica* clustered in a separate section and suggested that Butmela section could not be eliminated. Third clade was formed as *P. terebinthus* - *P. palaestina* species pair and *P. lentiscus*. Previous studies showed that *P. terebinthus* and *P. palaestina* were clustered together with high similarity values like RAPD (Kafkas and Perl-Treves, 2002; Katsiotis *et al.*, 2003; Golan-Goldhirsh *et al.*, 2004), AFLP (Katsiotis *et al*., 2003; Golan-Goldhirsh *et al.*, 2004; Kafkas, 2006). Sequencing of *ITS4*, *NIA-3* and plastid marker data showed that these two species could not be separated at sequence level (Yi *et al.*, 2008). While *P. lentiscus* clustered in Clade 3 with a low bootstrapping support, it remained individual in PCA plot. Previous molecular marker studies showed that clustering patterns of *P. lentiscus* varied as RAPD and AFLP (Kafkas and Perl-Treves, 2002; Kafkas, 2006) analyses suggested *P. lentiscus* clustered with *P. palaestina* and *P. terebinthus* while some other studies concluded *P. lentiscus* is separated from other species and formed an individual cluster (Katsiotis *et al.*, 2003; Golan-Goldhirsh *et al*., 2004). In a morphological view, *P. lentiscus* was the most divergent species in our study in comparison with other specimens that we used (Katsiotis *et al.*, 2003).

## Conclusion

Results showed a high similarity between *P. vera – P. khinjuk*, *P. atlantica – P. mutica* and *P. terebinthus* - *P palaestina* species pairs. Among other tested markers, iPBS was supplied the most polymorphism ratio and correlated with IRAP. Phylogenic reconstructions depicted that Mediterranean *Pistacia* species may be divided in 3 different clades, which was supported with previous molecular marker studies. Thus retrotransposon based molecular markers may be used for molecular characterization and molecular breeding studies for other *Pistacia* species.

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