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DISTINCTNESS AND INTER RELATIONSHIPS OF PISTACIA L. SPECIES IN IRAN AS EVIDENCED BY RETROELEMENT INSERTIONAL POLYMORPHISMS (IRAP METHOD)

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This study concerns taxonomic status and the distinctness of *Pistacia* taxa occurring in Iran. Regarding different taxonomic treatments based only on the leaf morphological traits in this study IRAP marker was sued in order to evaluate relationships and distinctness among the putative *Pistacia* taxa in Iran. A number of 16 geographical and morphological representative OTUs out of 383 collected and herbarium samples were subjected both to IRAP study and root-tip chromosome counting. The resulted data were analyzed using both phonetics and cladistics methods. Based on the results of this study and also literature review we concluded and suggested that the genus *Pistacia* in this country includes three cytodemes resulted from aneuploidy. These are to be justified based on the taxonomic concept of species.

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Key words. Pistacia, IRAP, Chromosome number, Taxonomy and Iran.

جدائي و خويشاوندي گونه‌هاي جنس پسته در ايران بر پايه روش **IRAP**

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اين مطالعه به بررسی موقعيت حدود تاکسون‌های مفروض جنس پسته در ايران می‌پردازد. با توجه به تفاوت نظرات تاکسونوميک ارايه شده و از صفات ريخت‌شناختی مبتنی بر شکل برگ ار مارکر آی‌رَپ استفاده شد. همينطور عدد کروموزومی انتهای ريشه شمارش شد. پس از مطالعه ريخت‌شناسی و جغرافيايی از ميان ۳۸۳ نمونه تعداد ۱٦ واحدعمل‌کردی (اوتی‌يو) به عنوان نماينده انتخاب و بررسی شد. داده‌های حاصل به صورت فنتيک و کلاديستيک تحليل گرديد و بر پايه مشاهدات و بررسی منابع اين نتيجه‌گيری و پيشنهاد حاصل شد که در نتيجه آنيوپلوييدی اين جنس در ايران ۳ سيتوديم دارد و گونه‌های تاکسونوميک را بايد بر اين اساس توجيه نمود.

Introduction

P. vera L., the source of pistachio cultivars is an important element in the woodlands of its natural distributional range including NE of Iran (Khanazarov et al. 2009; Khatamsaz, 1989). The genus *Pistacia* L. contains about eleven species of dioeciously, wind-pollinated shrubs or small trees extended in Mediterranean, Irano-Turanian, mountainous East African, Sino-Japanese and Mexican regions which were arranged in four sections by Zohary (1952).

The dioeciously and anemophilous nature of *Pistacia* taxa resulted in the simplicity and uniformity of the reproductive characteristics in this genus and made the *Pistacia* workers to over emphasizing the foliage features in the taxonomic treatments of this taxon (e.g., Engler 1896; Zohary 1952).

Taxonomically, the genus was treated in different ways in the flora of Iran. In his monograph, Zohary (1952) recognized three *Pistacia* (*P. vera*, *P. khinjuk* Stocks and *P. atlantica* Desf.) species for the flora of

Table1: A summarized taxonomic background of the genus *Pistacia* in Iran.

Author	Sect.	Species	Subspecies	Variety
Boissier (1872-1873)		*P. vera* L.		
		P. khinjuk Stocks		
		P. mutica Fisch. & Mey.		
		P. cabulica Stocks		
Parsa (1948)		*P. vera*		
		P. khinjuk		
		P. mutica		
		P. cabulica		
Zohary (1952)	*Eu-terebinthus*	*P. khinjuk*		*latifolia* DC.*
	Butmela	*P. vera*		*kurdica*
		P. atlantica		
Rechinger (1969)		*P. vera*	*cabulica*	
		P. khinjuk	*mutica*	
		P. atlantica	*kurdica*	
Khatamsaz (1989)		*P. vera*	*cabulica* (Stocks) Rech. f.	
		P. khinjuk	*mutica* (Fisch. & Mey.) Rech. f.	
		P. atlantica	*kurdica* (Zohary) Rech. f.	

Table 2: Summarizing the chromosome number reported for three *Pistacia* species occurring in Iran.

Species name	2n =	References
P. vera	30	Zohary (1952)
		Bocnatseva (1972)
		Harandi & Ghafari (2001)
		Ghaffari & Harandi (2002)
		Ila et al. (2003)
		Ayaz & Namli (2008)
P. atlantica s.l.		Zohary (1952)
	28	Ghaffari & Harandi (2002)
	30	Vogt & Aparicio (1999)
		Ila et al. (2003)
P. khinjuk s.l.	24	
		Ghaffari & Harandi (2002)

Iran. These are the main *Pistacia* species occurring in Iran; although there are some other taxa mentioned for this country, but their designation to specific or infra-specific levels is controversial (Boissier 1872; Parsa 1948; Rechinger 1969) (Table 1). Reviewing the chromosomal reports of the genus summarized in Table 2 implies the prevalence of aneuploidy among this group of species.

Many biosystematic aspects of *Pistacia* taxa including genetic variability, inter-specific relationships, geographical movements, phylogeny and reticulate evolution were surveyed using a vast range of molecular data (Hormozan et al. 1994, RAPD; Parfitt & Badenes 1997, Chloroplast genome; Hormozan et al. 1998, RAPD; Kafkas & Perl-Treves 2000, RAPD; Werner et al. 2001, RAPD; Barazani et al. 2003a & b, RAPD; Golan-Goldhirsh et al. 2004, RAPD; Kafkas 2006, AFLP; Yi et al. 2008, nrDNA ITS and *NIA-i3ndh*F, *trn*L-F & *trn*C-*trn*D regions; Salehi Shanjani et al. 2009, AFLP; Pazouki et al. 2009, SSRs).

Among the informative and currently used plant genomic segments as molecular markers are repetitive sequences dispersed in the DNA. During the recent years, PCR methods revolutionized the application of DNA markers including repetitive sequences to analyze the genetic diversity, intra- and inter-population and specific relationships of both wild and cultivated plant gene pool. Among the repetitive DNA markers, retrotransposons (Flavell et al., 1992; Voytas et al., 1992; Heslop-Harrison et al., 1997; Saiedi et al. 2008) are often the most frequent ones which through reverse transcription can amplify their sequences in many copies and insert them into the nuclear genomes (Boyko et al., 2002). These elements can be used as

Table 3: List of the accessions of *Pistacia* used for IRAP study.

P. atlantica sensu lato: 25 at: Chaharmahal-e-Bakhtiari: after Guerdeh-Bisheh, Shamsabad, Alt. 1700-2000, HUI Rahiminejad and Saeidi 14794; L.L 5-7 70 at: Hormozgan:Bekhoon mountain area, Alt. 1600, HUI Zaeifi 14786; L.L 5 107 at: Kerman: Sarcheshme ,near Shahid-e-Gomnam cave, Alt. 2100, HUI Rahiminejad and Dehghan14782; L.L 5-7; ch No 28 144 at: Kordestan: Baneh to Sardasht, Poorsheh village, Alt. 1300, HUI Rahiminejad 14789; L.L 5-7; ch. No. 28 149 at: Kordestan:Maryvan, HUI Ranjbar 14793; L.L 5; ch. No 28 178 at: Sistan-va-Baloochestan: Zahedan toward Khash, HUT 5102; L.L 3-5; ch. No 28 180 at: Tehran: Chitgar TARI 2204, 1450
Taxonomically between *P. atlantica* and *P. khinjuk* 4 atkh: Azerbaijan: Arasbaran, HUI Rahiminejad 14827; No. L.L 5-7; ch. No 28
P. khinjuk 210 kh: Chaharmahal-Bakhtyari: between Lordegan & Semirom, Alt. 2000; Malkhalifeh, HUI Rahiminejad, Saeidi and Dehghan 14765; L.L 1-2 (-3); ch No 24 224 kh: Fars: Between 50 km to Darab from Fasa, Alt. 1300, HUI Rahiminejad and Mirtazhedini 14750; L.L 1-3 (-4); ch. No 24 243 kh: Hormozgan: Roodan, HUI Modarres 14773; L.L 1-3-5; ch. No 24 318 kh: Kuhkiluyeh- Bouyerahmad: Gachsaran, Kolak, HUI Modarres 14770; L.L 3-7; ch. No 24 356kh: Sistan-Baloochestan: Khash, HUI Modarres 14752; L.L 1-(2); ch. No 24
P. vera 370 vc: Kerman: Baghin, Talashojaie garden (cv Fandoghi), HUI Ghaemmaghami and Rahiminejad 14813; L.L (1)-2-3-4-5; ch. No 30 374 vc: Kerman: Rafsanjan, Tavakolabad (cv Akbari), HUI Ghaemmaghami and Rahiminejad 14811; L.L 3-5; ch. No 30 377 vs: Khorasan: Sarakhs, HUI Modarres 14810; L.L 3-5; ch. No. 30

HUI = Herbarium of the University of Isfahan; L.L = leaflet number; ch. No = chromosome number.

multi copy and polymorphic elements to examine many aspects of speciation and evolutionary processes (Shimamura et al., 1997). The polymorphism of retrotransposon inserted into the DNA can be detected using a PCR technique named inter-retrotransposon amplified polymorphisms (IRAPs). In retroelement insertional polymorphisms (IRAP) outward-facing primers designed for conserved sequences such as long terminal repeats (LTRs) within the retrotransposon elements are used (Waugh et al., 1997; Flavell et al., 1998; Kalendar et al., 1999; Boyko et al., 2002). The revealed polymorphisms appeared as bands on the agarose gel can be used to evaluate the amount and model of insertion events in order to establish hypotheses about the genetic variability, speciation and phylogeny encountered in a group of taxa. This marker was first used successfully in Iran by Saeidi et al. (2008) in surveying the diversity and Phylogeography within diploid, D-genome *Aegilops tauschii* (Triticeae, Poaceae) Sub-taxa. Considering the IRAP method as a powerful tool to detect the genomic variability and lack of any study using it in *Pistacia*, this marker was selected to be tested and also for analyzing the distinctness and inter-specific relationships in this genus in Iran.

Materials and methods

From a total of 383 specimens examined (both herbarium sheaths and our field collections), a number of 16 accessions as the representatives of the geographical and morphological variability of three species (*P. atlantica* sensu lato 7, *P. khinjuk* sensu lato 6 and *P. vera* 3) were selected for IRAP studies (Table 3). As a generally accepted monograph the taxonomic identifications were based on the Zohary's (Zohary 1952).

From any of the 16 accessions, about 30 drupes were soaked in water for about a week in late winter, after removal of the fleshy exocarp they were grown in pots consisting loam and moss peat in an open experimental field (University of Isfahan, Iran). Only a small portion of seeds (5-7) germinated after about two months, which were used for both DNA extraction and chromosome counting. The bulk DNA was isolated from 1.5-2 g fresh leaves from at least five plants of each accession following Gawel and Jarret (1991). In order to increase the validity of identifications, diploid chromosome numbers were counted in all the accessions studied. Root tips were fixed, hydrolyzed, squashed and stained with aceto-orcein following |

Table 4: List of IRAP pair primers, annealing temperature, and the PCR resultant of any pair primer used among the Iranian *Pistacia* materials used in this study.

IRAP pair primers and (T_a °C)	Number of bands:				Size of Bands	No of Com. & Spec. bands in:		
	Tot.	Poly.	Mono.	Spec.		*P. at. sl*	*p. kh. sl*	*p. ver.*
LTR 6150 + LTR 1 (57)	23	15	4	4	300 - 4000 bp	18 & 2	14 & 1	19 & 1
LTR + Nikita (52)	15	14	1	-	100 – 1000 bp	10 & -	15 & -	14 & -
LTR + LTR 6149 (54)	12	10	2	-	200 – 2000 bp	8 & -	11 & -	12 & -
LTR + LTR 6150 (54)	17	13	3	1	100 – 1200 bp	15 & -	15 & 1	14 & -
LTR + LTR 1 (50)	23	21	1	1	100 – 900 bp	17 & 1	19 & -	18 & -
Nikita + LTR 6150 (54)	26	22	1	3	100 – 1500 bp	18 & 1	16 & -	16 & 2
Total	116	95	12	9	-	86 & 4	90 & 2	93 & 3

Abbreviations are as: T_a = annealing temperature; Tot. = total; Poly. = polymorph; Mono. = monomorph; Spc. = specific; Com. = Common; *p.at. sl = P. atlantica sensu lato*; *P. kh. Sl = P. khinjuk sensu lato*; *P. ver. = P. vera*. For the sequence of primers see: Kalender et al. 1999; Vicient et al. 2001; Boyko et al. 2002.

(Fukui and Nakayama1996).

IRAP analysis used 18 pair combinations of 9 LTR primers (Kalender et al. 1999; Vicient et al. 2001; Boyko et al. 2002) from which 6 pair combinations resulted in resolvable bands (see Table 4). For PCR reactions, genomic DNA samples were diluted with sterile de-ionized water to 50 ng ml^{-1}. The IRAP PCR was performed in a 20 ml reaction mixture containing 50 ng DNA, 1× PCR buffer, 2 mM $MgCl_2$, 5 pmol of each primer, 200 mM dNTP mix and 1 U *Taq* polymerase. The annealing temperature was optimized using gradient PCR. The PCR reaction parameters consisted of: 95 ºC, 2 min; 30 cycles of 95 ºC, 60 s, annealing at the T_a specified in Table 2 for 60 s, ramp +0.5 ºCs^{-1} to 72 ºC, and 72 ºC for 2 min adding 3 s per cycle, with a final extension at 72 ºC for 10 min. PCR products were analyzed by electrophoresis on 2% (w/v) agarose gels and detected by Ethidium bromide staining.

Data analysis was carried out using presence (1) or absence (0) of clear and distinct bands of particular mobility scored from the gels for each accession. In order to visualize the closeness the accessions examined a phenetic analysis was performed among the accessions by constructing a UPGMA cluster based on Jaccard's similarity coefficient using NTSYSpc software (Rohlf 1998). The relationship of the OTUs examined was evaluated by constructing the most parsimonious un-rooted cladogram using heuristic branch swapping method by a bootstrap of 1000 rounds in PAUP*4.0beta10.

Results and discussion

In this study we could not prepare qualified chromosome spreads suitable enough for karyotyping and chromosomal measurements and merely chromosome counting was possible. Based on this study all individuals from the 16 populations surveyed, showed a sporophytic chromosome number of 2n = 24, 28 and 30 for *P. khinjuk* sensu lato, *P. atlantica* sensu lato and *P. vera* respectively.

This study showed that the IRAP primers designed for the other species (Kalender et al. 1999; Vicient et al. 2001; Boyko et al. 2002) are applicable for this genus. Six IRAP pair primers resulted in multiple DNA fragments from genomic DNA of all 16 accessions of three *Pistacia* species (Table 3). Of the 116 bands resolved from *Pistacia* species, 104 (89.6%) were polymorphic from which 9 bands (i.e., 8.66% of the polymorphic bands) were species specific (see table 4). Comparing IRAPs with the other dominant markers used in this genus: RAPD (Hormozan et al. 1994; Hormozan et al. 1998; Kafkas & Perl-Treves 2000; Werner et al. 2001; Barazani et al. 2003a & b) and AFLP (Kafkas 2006; Golan-Goldhirsh et al. 2004) showed their relative advantage.

Despite the presence of a number of species-specific bands (9 bands), finding 12 monomorphic, against 95 polymorphic IRAP bands (Table 4) can be taken at the same time as evidence for both a relative isolation of and an ongoing gene flow after divergence from a common ancestor between *Pistacia* accessions studied.

The similarity visualized in the UPGMA dendrogram (Fig. 1) showed a non-distinctive and

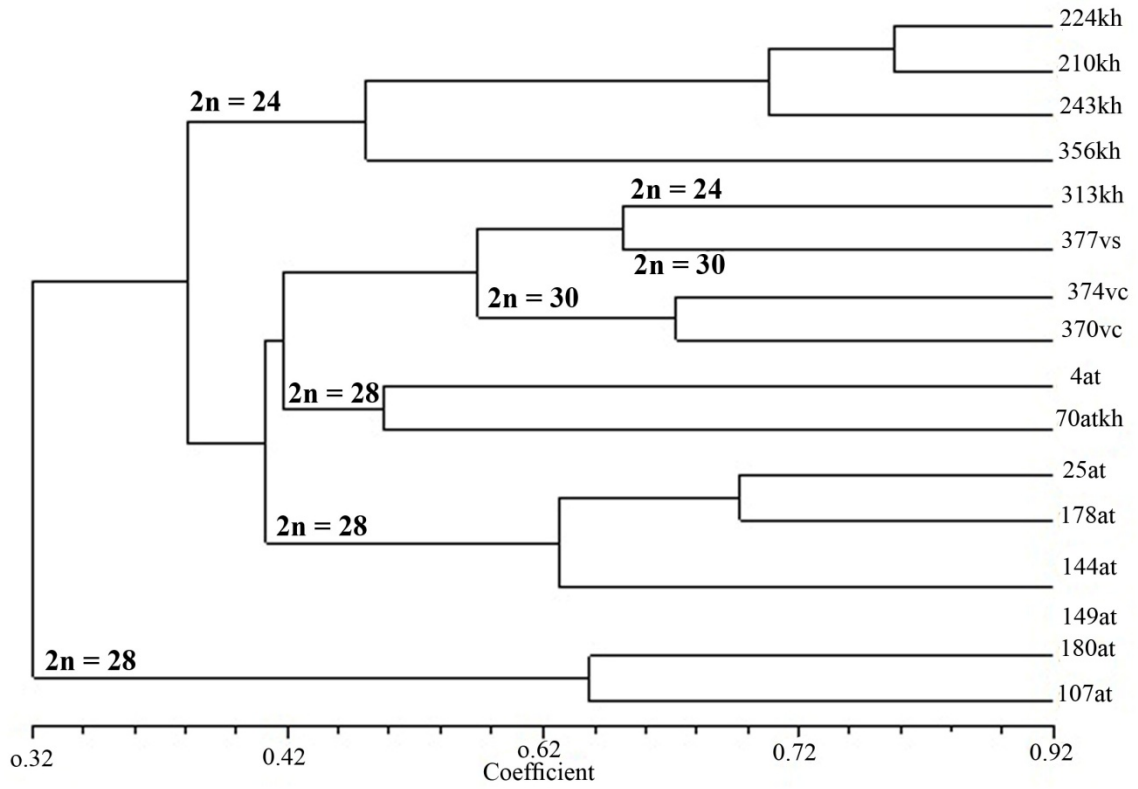

Fig. 1: UPGMA dendrogram showing the similarity relationship among 16 OUTs belonging to three *Pistacia* species occurring in Iran. For the abbreviations (accessions number) see Table 3; the diploid chromosome number of lineages are presented on the dendrogram.

discriminative pattern between the OTUs. While OTUs 107at and 180at belonging to *P. atlantica* sensu lato stands apart at a similarity of 0.32 from the core of the dendrogram, their relatives i. e., accessions 144at and 149at are tied at a similarity of 0.92 and nested in a sub-cluster with two other *P. atlantica* accessions. The OUT 70at-kh which its leaves and fruits resemble those of *P. atlantica* and *P. khinjuk* respectively is jointed to the last *P. atlantica* element in this study. Based on Fig. 1 and also considering OTU 70atkh, *P. atlantica* is of high diversity and occupied an intermediate place between the two other species. As Fig. 1 shows *P. vera*'s OTUs are clustered with an OTU of *P. khinjuk* and the other four *P. khinjuk*'s have their own group. This dendrogram implies that *P. khinjuk* is more similar to each of the two other taxa than they are to each other.

As the un-rooted cladogram presented in Fig. 2 shows generally clade bootstrap support among the *Pistacia* OTUs studied is low. This can be interpreted based on the randomized distribution of IRAP loci among the OUTs (EUs) examined. The highest bootstrap support belongs to 107at-1701at clade, a pair-group of two *P. atlantica* elements geographically separated in two mountainous and isolated habitats in Sirjan-Sanandadje and Elborz formations respectively (see Table 3). Also the two latter with a similarity of 0.65 formed their own sub-cluster in the dendrogram of Fig.1. It can be inferred that these two OTUs are isolated relicts remained from an extended *Pistacia* gene-pool once occurring all around Iran plateau; the existence of scattered trees in the local high lands of this area can be taken as evidence for this suggestion. Clade comprising of the OTUs 210kh-224kh-243kh showed a bootstrap support of 81 (Fig. 2); *P. khinjuk*'s OTUs that were collected along a transect line from the middle Zagros Mountains to the most southern point of this Mountains (see Table 3). Noticeably, the presence of OTU 350kh with 1(-2) leaflet leaves in the root of the above 3 OTUs clade which all possess one-leaflet leaves among the others implies the primitiveness of this clade. Two cultivated accessions belonging to *P. vera* form their own clade with a relatively weak bootstrap support (63) while the third *P. vera* OTU

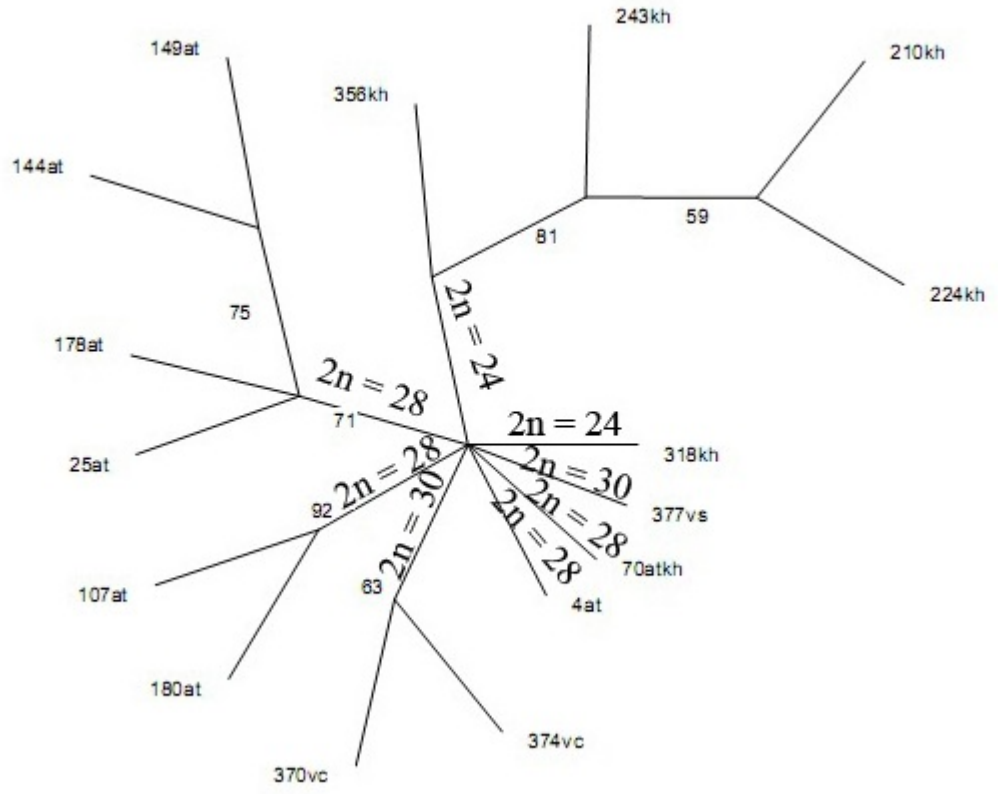

Fig. 2: The IRAP based most parsimonious unrooted cladogram using heuristic branch swapping method by a bootstrap of 1000 rounds of 16 OUTs (EUs) belonging to three *Pistacia* species occurring in Iran. For the abbreviations see Table 3; the diploid chromosome number of lineages are presented on the dendrogram.

(377vs) which belongs to its wild gene-pool is tied to an unsolved polytomic node including 318kh, 4at and 70atkh in which the latter is morphologically intermediate between *P. atlantica* and *P. khinjuk* (see Table 3). This aggregate can be taken as evidence for the gene-pool integration of all *Pistacia* taxa in Iran. As Fig. 1 and Fig. 2 show the general congruency between IRAP segregation, taxonomic species and diploid chromosome number is broken by the conflicts encountered in this aggregate.

Conclusions

Based on the literatures reviewed in this study it is obvious that taxonomically this genus was continuously the matter of intensive controversies among *Pistacia* workers (Linnaus 1753, Willdenow 1806, Desfontaines 1799, De Candoll 1825, Stocks 1852, Marchand 1869, Engler 1883, also see Table 1). This is mainly caused by the nature of breeding system (anemophilous) and growing habit of *Pistacia* which facilitate both inter- and intra-populational gen flow and long rang establishment of the occasional inter- and intra-specific hybrids created among the *Pistacia* species. Zohary (1952) believed that despite the immense importance of floral characteristics as diagnostic traits in most plant taxa, the anemophilous nature of the genus resulted in simplicity and uniformity of this system in *Pistacia*. Therefore, *Pistacia* taxonomists have over emphasized on the foliage features in their taxonomic treatments of the genus and consequently added to the taxonomic confusion encountered.

The results of this study showed that in the lack of good diagnostic floral characters, not only are leaf features (number of leaflets, shape of leaflets and the presence/absence of petiole wing) of low taxonomic values but also can complicate inter- and intra-specific taxonomic treatments. In terms of fruits' traits, despite are being good and diagnostic between *P. vera* and the rest of the species, however not always clear cut and sharp between *P. atlantica* sensu lato and *P. khinjuk* sensu lato (Ghaemmaghami et al. 2010; Ghaemmagahmi 2010).

It can be concluded that the taxonomic species concept in *Pistacia* is to be adopted with the

Ecogenetic Species Concept (Levin 2000). Regarding the geographical distribution of *Pistacia* in Iran it can be suggested that the local populations are adapted to their habitat conditions and the main partially isolating barrier between them is caused due to aneuploidy. The results of this study which are mostly in accordance with the previous studies (see Table 2), suggested that aneuploidy phenomenon created three cytodemes of 2n = 30, 28 and 24 in *Pistacia* gene-pool in Iran. This phenomenon prevents *Pistacia* taxa in Iran from an easy and ongoing gene flow that otherwise would be resulted in only one species. However, the close relationships between these three cytodemes and their natural tendency (genetic and ecological) to amalgamate made a suitable platform to create a great number of long-lived single trees. This can in turn provide an endless array of "Jordanons" for a hair-splitting plant taxonomist to add a long list of new subspecies, varieties, subvarieties, forms and subforms to the previous one including 55 names under *Pistacia* (see http://www.ipni.org). Therefore, it is suggested here that based on three cytodemes three species: *P. vera* (2n = 30), *p. atlantica* sensu lato (2n = 28) and *P. khinjuk* sensu lato (2n = 24) are to be recognized in this country which consists with Zohary's treatment (1952). In addition based on the results of this study and ICBN rules for *Pistacia* taxa in Iran only one section i. e., section *Terebithus* is recognized.

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