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MORPHOMETRIC ANALYSIS AND GENETIC DIVERSITY IN *Pistacia* SPECIES POPULATIONS USING SEQUENCE RELATED AMPLIFIED POLYMORPHISM

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The Anacardiaceae family includes 11 or more species, including the genus *Pistacia. Pistacia vera* L. is the most commercially developed of them all, thanks to its tasty nut seeds. *Pistacia khinjuk* Stocks, *Pistacia atlantica* Dsef, *Pistacia vera*, *Pistacia eurycarpa* Yalt. (*Pistacia atlantica* subsp. *Kurdica* Zoh.) and all have their origins in Iran. The present study aimed to investigate the SRAP (Sequence-related amplified polymorphism) markers in 13 wild pistachio accessions, which comprised three different species: *Pistacia khinjuk*, *Pistacia vera*, and *Pistacia atlantica*. Through polymerase chain reaction amplifications (PCR) of three *Pistacia* species, a total of 170 (Number of total loci) (NTL) DNA bands were obtained. Ten different selective primers were combined to generate these bands. The number of amplified pieces ranged from nine to twenty-six. The projected impartial gene diversity (UHe) ranged from 0.053 (*Pistacia khinjuk*) to 0.417 (*Pistacia khinjuk*) (*Pistacia atlantica* subsp. *Kurdica*). The genetic similarity of three species is estimated to be between 0.61 to 0.90. Two significant groupings emerged from the clustering findings: *Pistacia khinjuk* and *Pistacia atlantica* subsp. *Kurdica*

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exhibited the least similarity in the SRAP markers study. Our findings revealed excellent molecular recognition of all genotypes tested, indicating that a significant amount of genetic variety exists among pistachio accessions. This discovery might be useful in breeding management techniques for genetic preservation and cultivar improvement.

Key words: Gene Flow, Genetic Diversity, *Pistacia,* Sequence-related amplified polymorphism

INTRODUCTION

People are split owing to genetic or geographical barriers, resulting in dispersed populations, hence genetic variety and diversity are crucial for species survival. Because these people have restricted gene flow, population number is more likely to shrink (ZHENG *et al*., 2021; ZHU *et al.,* 2021). Considering the importance of genetic variety in conservation methods, disentangling genetic diversity in species of plants, especially vulnerable and uncommon species, is critical (ESFANDANI-BOZCHALOYI *et al.,* 2018a; 2018b; 2018c; 2018d).

There are at least 11 varieties in the *Pistacia* genus, which is thought to be around 80 million years old (PARFITT and BADENES, 1997). *Pistacia vera* is the sole commercially viable species in this genus. *Pistachio* trees are dioecious (2n=30) and diploid (2n=30), which means they have distinct male and female trees (ZOHARY, 1996).

Although the genus *Pistacia* is thought to have originated in the European and North African zones, the majority of scientists now agree that it most likely originated in Central Asia. As the early sources revealed, two main ideas exist about the spread of *Pistacia* species: one concentrates on the Mediterranean region of Europe, Northern Africa, and the Middle East, and the other on the rest of the world. The eastern part of the Zagros Mountains (Iran) and the Caucasus regions from Crimea to the Caspian Sea are further options (ZOHARY, 1952; JI *et al* 2020). *Pistacia eurycarpa* Yalt. *Pistacia khinjuk* Stocks, *Pistacia vera,* (*Pistacia atlantica* subsp. *Kurdica* Zoh.), and *Pistacia atlantica* Dsef. all have their origins in Iran (HORMAZA *et al*.1994) Iran is home to three important wild *Pistacia* species: *Pistacia atlantica*, *Pistacia khinjuk*, and *Pistacia vera.* Although wild *P. vera* has grown to an area of over 75,000 hectares in focal Asia, which encompasses Turkmenistan, Afghanistan, and Northeast Iran, where *P. vera* thrives in the Sarakhs region and covers around 17,500 ha (BEHBOODI, 2003). Iran is the world's biggest pistachio supplier, having the world's largest pistachio production area (AHMAD *et al.,* 2003a), although its output has been poor in recent years when compared to nations such as the United States and Turkey.

Pistachio plants have a lengthy life span, with a juvenile phase of 5–10 years. Wild *Pistacia* species also have edible seeds and are utilized as rootstock seed sources for farmed *Pistacia vera,* as well as for fruit eating, oil extraction, soap manufacture, and as forest trees (KATSIOTIS *et al*., 2003).

Pistacia genetic diversity has been investigated in a number of research based on morphological, physiological, and biochemical properties (ZOHARY, 1952; BARONE *et al.,* 1993) Experts are now studying genetic variety in crops using molecular technologies, such as genomic editing at the DNA level. Many genetic research on *Pistacia* are based on similar approaches in this regard (STRUSS *et al* 2003; AL-SOUSLI *et al*., 2014). While prior research have partly

documented pistachio variety in Iran, they did not undertake a comprehensive examination of wild *Pistacia* discrimination, breeding potential, and conservation implications.

Pistacia species induction of diversity is based on morphological traits, which are commonly done by blooming or grafting chosen scions onto seedling rootstocks with same species or different *Pistacia* species. Because of their dioecious nature and pollination technique, *Pistacia* species exhibit a significant genetic diversity. Because of these variables, rootstock breeding requires a high level of selectivity, and understanding the genetic links across *Pistaci*a species would be extremely beneficial in pistachio rootstock development.

Even though the exact amount of diversification and connections among and within the genus *Pistacia* are unknown, new understanding and techniques that can be used to comprehend these interactions are required before they can be used in plant growth (PARFITT and BADENES, 1997). Genetic markers, in particularly, are valuable and dependable because they stay constant in a variety of environments (MA *et al*., 2021; PENG *et al*., 2021; YIN *et al.*, 2021).

SRAP stands for sequence-related amplified polymorphism, which is a system marker based on PCR. It is among the most efficient and straightforward marker systems for studying gene mapping and gene tagging in plant species (LI and QUIROS, 2001), and SRAP might be used to evaluate plant taxonomic classification and genetic variety investigations. Furthermore, WU *et al*. (2010) used SRAP markers to investigate genetic diversity and population structure in *Pogostemon cablin*. The study's goals were as follows: a) assessing genetic diversity; and b) using NJ methods to analyze population linkages. The current findings have ramifications for breeding and conservation efforts. The current work is the first one to use SRAP markers to investigate genetic variation and phylogenetic connections across and within wild *Pistacia* populations in Iran.

MATERIALS AND METHODS

Plants collection

During July-August 2018-2020, three wild *Pistacia* species (*Pistacia atlantica*, *Pistacia khinjuk*, and *Pistacia vera*) were chosen and sampled in East Azerbaijan, Kurdistan, Esfahan, Semnan, Khorasan, Kerman, Arak, and Lorestan Provinces of Iran (Table 1). On 13 plant accessions, morphometric and SRAP studies were performed. Based on additional eco-geographic criteria, five to twelve samples from each population of three distinct species were chosen. The samples were kept at - 20°C until they were needed. There is a lot of detail on the sites of the specimens and the geographical distribution of the species (Table 1).

Morphological studies

Each species had twelve specimens processed and was submitted to morphometric analysis. The researchers looked at qualitative (3) and quantitative (4) morphological characteristics (Appendix). Before calculating, the data was converted. Flowers, leaves, and seeds were researched for their morphological characteristics. The Euclidean distance was used to conduct the ordination analysis (PODANI, 2000).

Table 1. List of the investigated the populations of Iranian pistachio species including origin of voucher specimens

No	Sp.	Locality	Latitude	Longitude	Altitude (m)
Sp1	*Pistacia vera* L.	Khorasan, Koppeh Dagh	38˚ 52'37″	47 ˚ 23' 92	1144
Sp1	*Pistacia vera* L.	Semnan, Damghan	32°50′03″	51°24′28″	1990
Sp1	*Pistacia vera* L.	Khorasan, Mashhad	29◦20′07″	51° 52′08″	1610
Sp1	*Pistacia vera* L.	Kerman, Rafsanjan	38˚ 52'373	47 ˚ 23' 92"	1144
Sp1	*Pistacia vera* L.	Arak, Saveh	33° 57′12″	47° 57′32″	2500
Sp2	*Pistacia khinjuk* Stocks	Lorestan, Oshtorankuh, above Tihun village	34˚ 52'373	48 ˚ 23' 92"	2200
Sp2	*Pistacia khinjuk* Stocks	Esfahan, Semirom	38˚ 52'373	47 ˚ 23' 92"	1144
Sp2	*Pistacia khinjuk* Stocks	Kurdistan, Sanandaj	35°50′03″	51°24′28″	1700
Sp2	*Pistacia khinjuk* Stocks	Lorestan, Khoramabad	36°14′14″	51°18′07″	1807
Sp2	*Pistacia khinjuk* Stocks	Esfahan:Ghameshlou, Sanjab	32◦36′93″	51°27′90″	2500
Sp3	*Pistacia atlantica* subsp. *Kurdica* Zoh	East Azerbaijan, Arasbaran	37°07′02″	49°44′32″	48
Sp3	*Pistacia atlantica* subsp. *Kurdica* Zoh	East Azerbaijan, Urmia	28◦57′22″	51°28′31″	430
Sp3	*Pistacia atlantica* subsp. *Kurdica* Zoh	Lorestan, Azna	30◦07′24″	53° 59′06″	2178

Sequence-related amplified polymorphism method:

Young leaves from one to twelve plants were utilized at random. Silica gel powder was used to dry them. Following the prior process, genomic DNA was isolated (ESFANDANI-BOZCHALOYI *et al.*, 2019). The SRAP test was carried out as previously reported (LI and QUIROS, 2001). Ten SRAP were employed, each with a distinct primer combination (Table 2). PCR reactions were carried out in a 25l volume containing 10 mM Tris-HCl buffer at pH 8, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM of each dNTP (Bioron, Germany), 0.2 mM of single primer, 20 ng of genomic DNA, and 3 U of Taq DNA polymerase (Bioron, Germany). The total volume of the reaction was 25 l.

Table 2. SRAP primer information and results

Primer name	NTL[a]	NPL[b]	P[c]	PIC[d]	RP[e]
Em1-Me1	15	14	93.31%	0.44	43.77
Em2-Me2	17	17	100.00%	0.56	46.77
Em1-Me4	18	17	95.4%	0.59	30.46
Em2-Me4	13	13	100.00%	0.49	43.76
Em2-Me5	9	9	100.00%	0.44	40.99
Em3-Me4	11	11	100.00%	0.37	42.24
Em3-Me1	26	20	76.00%	0.39	36.55
Em4-Me1	11	11	100.00%	0.44	44.23
Em5-Me1	16	16	100.00%	0.47	38.55
Em5-Me2	26	24	95.00%	0.38	39.65
Mean	17	16	92.40%	0.55	44.99
Total	170	150			

a) Number of total loci (NTL), b) Number of polymorphic loci (NPL), c) Polymorphic ratio(P %), d) Polymorphic information content (PIC), e) Resolving power (Rp)

Data Analyses

To evaluate morphological characteristics, the UPGMA (Unweighted paired group using average) ordination approach was used. To analyze morphological differences across species, an ANOVA (analysis of variance) was used. To find variable morphological features in *Pistacia* species, principal component analysis (PCA) was used. PAST software version 2.17 was used to perform multivariate statistical studies, often known as PC analysis (HAMMER *et al.*, 2001).

RESULTS

Mophometery

In terms of quantitative morphological traits, the ANOVA analyses revealed significant differences (p0.01) between the species. The results of principal component analysis explained 62 percent of the total variance. The first PCA axis was responsible for 46% of the overall variance. Morphological characters such as width of leaves, fruit thickness, thickness of multiple buds, number of leaflets, fruit width, length of leaves, fruit length, number of fruit per inflorescence, length of petioles, kernel infestation, width of terminal leaf, length of terminal leaf, and length of inflorescence showed the highest correlation (> 0.7). In a PCoA plot, the morphological features of three wild *Pistacia* species are displayed (Figure 1). Based on physical characteristics, each species was divided into several groups. The morphometric study revealed distinct differences among *Pistacia* species, allowing each group to be distinguished.

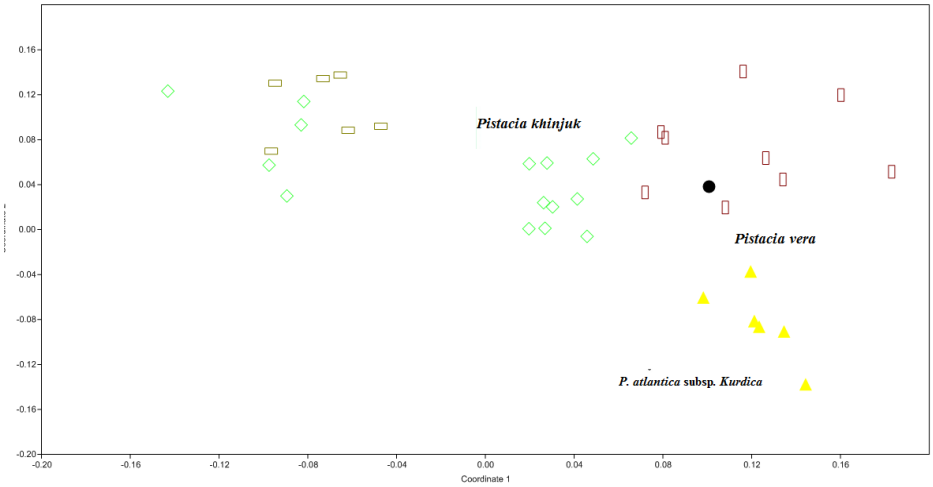

Fig 1. Morphological characters analysis of the *pistachio* species by PCoA plot.

Species identification and genetic diversity

The present research looked at ten (10) acceptable primer combinations (PCs) out of a total of twenty-five (25). The SRAP marker profile shows the banding pattern of Em3-Me4, Em4-Me1, Em5-Me2, and Em2-Me2 primers in Figure 2. A total of 150 amplified polymorphic bands (number of polymorphic loci) were generated. These bands (fragments) ranged in size from 100 to 3000 bytes. For Em5-Me2 and Em2-Me5, the maximum and least number of polymorphism bands were 24 and 9, respectively. On average, each primer produced 16 polymorphic bands. The PIC for the 10 SRAP primers ranged from 0.37 (Em3-Me4) to 0.59 (Em1-Me4), with a mean of 0.55 per primer. The mean RP of the primers was 44.99, ranging from 30.46 (Em1-Me4) to 46.77 (Em2-Me2) (Table 2). The genetic variables of *Pistacia* species that have been computed are displayed (Table 3). With a mean of 0.20, the unbiased heterozygosity (H) ranged from 0.053 *(Pistacia khinjuk*) to 0.417 (*Pistacia atlantica* subsp. *Kurdica).* Shannon's information index (I) was highest in *Pistacia atlantica* subsp. *Kurdica* (0.670), whereas Shannon's information index was lowest in *Pistacia khinjuk* (0.083). The number of alleles (Na) found in *Pistacia khinjuk* varied from 1.167 to 2.333 in *Pistacia atlantica* subsp. *Kurdica.* The number of significant alleles (Ne) varied from 1.078 (*Pistacia vera*) to 1.922 (*Pistacia ulmaria*) (*Pistacia atlantica* subsp. *Kurdica*).

Pistacia species have considerable genetic differences (p = 0.01), according to analysis of molecular variation. The bulk of genetic diversity was found within species. According to the findings of AMOVA, 59 percent of total variation is found between species, whereas genetic variation is found at a lower level at the species level. Genetic statistics (Nei's GST) revealed genetic differences across *Pistacia* species, as evidenced by substantial p values, i.e. Nei's GST (0.567, p = 0.01) and D est values (0.987, p = 0.01).

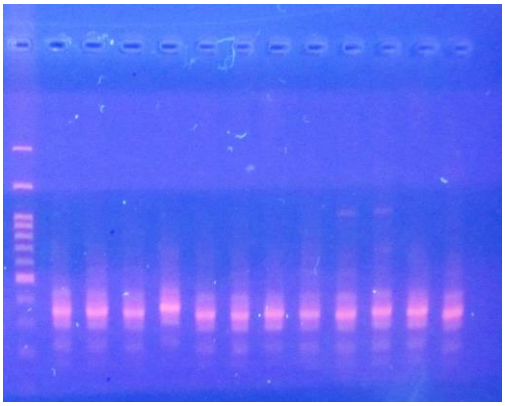

Fig 2. Electrophoresis gel of studied ecotypes from DNA fragments produced by SRAP profile; 1,4,7,10: *Pistacia vera*; 2, 5,8,11: *Pistacia khinjuk* ; 3,6,9,12*: Pistacia atlantica.* L = Ladder 100 bp

Table 3. Genetic diversity parameters in the different pistachio populations, species, and cultivars

Population	%P	N	Na	Ne	I	He	UHe
Pistacia vera L.	50.00%	5.000	1.500	1.462	0.337	0.200	0.240
Pistacia vera L.	16.67%	5.000	1.333	1.196	0.150	0.133	0.090
Pistacia vera L.	33.33%	5.000	1.500	1.366	0.271	0.067	0.173
Pistacia vera L.	50.00%	5.000	1.500	1.078	0.337	0.000	0.240
Pistacia vera L.	33.33%	5.000	1.500	1.366	0.271	0.000	0.173
Pistacia khinjuk Stocks	16.67%	5.000	1.167	1.366	0.083	0.000	0.053
Pistacia khinjuk Stocks	66.67%	6.000	1.667	1.491	0.407	0.083	0.280
Pistacia khinjuk Stocks	35.33%	2.000	1.333	1.333	0.231	0.333	0.167
Pistacia khinjuk Stocks	33.33%	8.000	1.333	1.267	0.209	0.000	0.146
Pistacia khinjuk Stocks	50.00%	5.000	1.500	1.311	0.279	0.067	0.187
Pistacia atlantica subsp. *Kurdica* Zoh	83.33%	5.000	2.333	1.922	0.670	0.133	0.417
Pistacia atlantica subsp. *Kurdica* Zoh	50.00%	5.000	1.500	1.441	0.330	0.133	0.233
Pistacia atlantica subsp. *Kurdica* Zoh	33.33%	5.000	1.500	1.366	0.271	0.000	0.173

Abbreviations: (N = number of samples, Na= number of different alleles; Ne = number of effective alleles, I= Shannon's information index, He = gene diversity, UHe = unbiased gene diversity, P%= percentage of polymorphism, populations).

Because the findings of the NJ tree and the UPGMA clustering were comparable, only the NJ tree is shown and analyzed (Figure. 3). This finding indicates that the molecular features investigated can separate *Pistacia* genotypes into two distinct groups or clusters. In general, two large clusters developed in the NJ tree (Fig. 3), with 10 genotypes of *Pistacia khinjuk* cultivars forming one cluster. Cluster II was divided into two sub-clusters, with cluster II containing the majority of genotypes of *Pistacia atlantica* and *Pistacia vera*. This cluster has a total of 21 people in it.

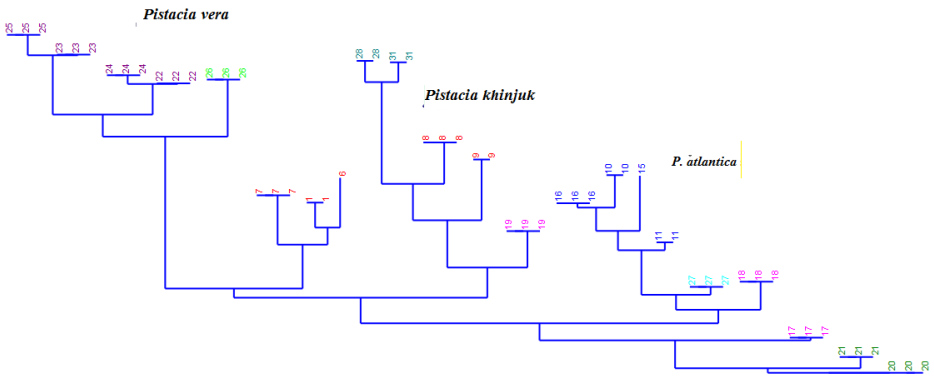

Fig. 3. Neigbor-Joingin tree of populations in *Pistacia* species based on SRAP molecular markers.

We discovered a substantial connection (r = 0.56, p=0.0002) between geographical and genetic distances, as well as a gene flow (Nm) score of 0.456 among species. The genetic distances and genetic identity (Nei's) are discussed in detail (Table not included). *Pistacia khinjuk* and *Pistacia vera* had the highest degree of genetic similarity (0.90), according to the data. *Pistacia khinjuk* and *Pistacia atlantica* subsp. *Kurdica* (0.61), on the other hand, exhibited the least genetic similarity.

DISCUSSION

We employed morphological and molecular (SRAP) data to assess species relationships in *Pistacia* species in this work. *Pistacia* species morphological investigations revealed that quantitative markers (ANOVA test results) and qualitative traits are well separated. According to PCA analysis, morphological features like the pistachio kernel to testa ratio, the length of the pistachio kernel, the width of the pistachio kernel, and the thickness of the pistachio kernel have the ability to identify and delimitate *Pistacia* species. The use of morphological features to identify and delimit *Pistacia* species is suggested by the findings of principal component analysis. Fruit length, fruit breadth, fruit thickness, number of fruits per inflorescence, and kernel infection are all morphological traits that play a role in plant systematics and taxonomy. Our

findings also demonstrated the importance of morphological and molecular data in identifying and studying species genetic diversity. In general, morphometric measurements and genetic correlations acquired from SRAP data agree. This is consistent with AMOVA parameters and genetic diversity results. The use of SRAP molecular markers revealed significant genetic differences across species. These findings suggest that SRAP might be used to investigate plant systematics and taxonomy in *Pistacia* species.

Considering the harmful effects of biodiversity challenges and overexploitation of *Pistacia* plant species in Iran, genetic variety research on *Pistacia* species are required. Studies on genetic variety help us learn how to build conservation efforts (ESFANDANI-BOZCHALOYI *et al.*, 2017a, b, c, d). Polymorphic information content (PIC) and marker index (MI) are significant measures to comprehend genetic variation in species and are used in genetic diversity research (MIRZAEI *et al*., 2005). According to common reasoning, various manufacturers have varying skills to assess genetic variety, and genetic diversity is frequently associated with polymorphism (MIRZAEI *et al*., 2005).

In Iran's dry and semi-arid agricultural regions, pistachio has significant socio-economic and ecological implications (KAFKAS *et al.*, 2006). Furthermore, Iran has a large genetic variety of *Pistacia* spp., with over 300 pistachio genotypes gathered around the nation. As a result, Iran has significant germplasm for pistachio development and conservation. It is crucial to assess genetic diversity and linkages among Iranian *Pistachio* cultivars using discriminative and robust markers (BI *et al*, 2021; JIA *et al*, 2020; CHENG *et al.*, 2021).

In this study, 10 SRAP markers were used to describe 13 *Pistacia* cultivars. The findings back up the effectiveness of microsatellite markers in fingerprinting. Our findings revealed that the PIC for the 10 SRAP primers ranged from 0.37 (Em3-Me4) to 0.59 (Em1-Me4), with an average of 0.55 per primer. The average RP of the primers was 44.99, ranging from 30.46 (Em1-Me4) to 46.77 (Em2-Me2). Such principles were higher than those reported by ARABNEJAD *et al*. (2008), who calculated the mean of 3.69 alleles per primer pair and an average PIC of 0.46 in 20 commercial Iranian *Pistachio* cultivars; and also higher than those reported by BAGHIZADEH *et al.* (2010) (an average of 2.75 alleles per primer pair and an average of 0.44 in 31 Iranian *Pistachio* cultivars) and AHMAD *et al year* (an average of 3.30 alleles per locus in 17 *Pistachio* cultivars). KOLAHI-ZONOOZI (2014) used 12 nSSR markers to assess genetic diversity of 45 commercially available Iranian cultivars and found that PIC ranged from 0.19 to 0.56 with an average of 0.33, while Ho and He mean values were 0.49 and 0.35, respectively. Among 22 Iranian *Pistachio* cultivars and wild *Pistachio* species, MIRZAEI *et al*. (2005) found 80.00 percent polymorphism. In a research published by GOLAN-GOLDHIRSH *et al.* (2004), 27 primers were used to analyze polymorphisms in 28 Mediterranean *Pistachio* accessions, resulting in 259 total bands (an average of 9.59).

Many cultivars have similar name and physical identity in different areas, yet molecular analyses revealed discrepancies. Badami-Zarand, for example, was distinguished from Badami-Kaj and Badami-Zoodras cultivars. In addition, Ghazvini-Zodras differed from Ghazvini. These differences might be attributable to the inherent nature of nSSRs, as it's quite improbable that the microsatellites amplified correspond to the altered DNA region when they're separated at random from the entire genome. Due to the species' dioecious and cross-pollination characteristics, the

findings of this study revealed that the investigated cultivars had a substantial genetic variation (AHMAD *et al.*, 2005; SI *et al*, 2020, SUN *et al.*, 2021).

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MORFOMETRIJSKA ANALIZA I GENETIČKA RAZNOVRSNOST U POPULACIJAMA VRSTE *Pistacia* KORIŠĆENJEM SRAP (*Sequence-related amplified polymorphism*)

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Izvod

Rod *Pistacia* je član porodice Anacardiaceae, koja obuhvata 11 ili više vrsta. Među njima, *Pistacia vera* L. je glavna ekonomski razvijena vrsta, zbog ukusnog semena orašastih plodova. Iran je centar porekla četiri važne vrste *Pistacia*: *P. vera*, *P. khinjuk* Stocks, *P. eurycarpa* Yalt. (*P. atlantica* subsp. *Kurdica* Zoh.), i *P. atlantica* Dsef. Cilj ovog istraživanja bio je da se analiziraju markeri SRAP (*Sequence-related amplified polymorphism*) kod ukupno 13 uzoraka divljih vrsta pistaća, među kojima su tri vrste *Pistacia vera*, *Pistacia khinjuk*, *Pistacia atlantica*. Ukupno 170 (Broj ukupnih lokusa) (NTL) DNK traka je proizvedeno PCR-om za tri vrste *Pistacia*. Ove trake su proizvedene kombinacijama 10 selektivnih prajmera. Ukupan broj amplifikovanih fragmenata kretao se od 9 do 26. Predviđena nepristrasna genska raznovrsnost (UHe) varirala je između 0,053 (*Pistacia khinjuk*) i 0,417 (*Pistacia atlantica* subsp. *Kurdica*). Genetske sličnosti između tri vrste se procenjuju od 0,61 do 0,90. Rezultati grupisanja su pokazali dva glavna klastera. Prema SRAP (*Sequence-related amplified polymorphism*) analizi markera, *Pistacia khinjuk* i *Pistacia atlantica* subsp. *Kurdica* su imale najmanju sličnost. Naši rezultati su dali odličnu molekularnu identifikaciju svih ispitivanih genotipova, koji su pokazali da postoji velika količina genetske raznovrsnosti među uzorcima pistacija. Rezultati bi mogli da pruže upečatljive informacije u strategijama upravljanja oplemenjivanjem za konzerviranje genetičkih resursa i razvoj novih sorata.

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