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Evaluation of genetic diversity and Gene-Pool of *Pistacia khinjuk* Stocks Based On Retrotransposon-Based Markers

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Abstract. *Pistachio* genetic variety includes a wide range of female variations and male genotypes, and Iran is regarded as one of the critical sites for this diversity in the world. The genus *Pistacia* consists of eleven species that only have edible nuts and are commercially important. Four important species of pistachios include *Pistacia vera, P. khinjuk* Stocks, *P. eurycarpa* Yalt. (*P. atlantica* subsp. *Kurdica* Zoh.), and *P. atlantica* Dsef are found in Iran. Genetic diversity is one aspect of biological diversity that is extremely important for conservation strategies, especially in rare and narrowly endemic species. In Iran, there is no knowledge concerning the genomic organization of the population, genetic diversity, or phenotypic variations of the species. *Pistacia khinjuk* has eight distinct regional populations, all of which were studied for genetic variation and demographic organization because of the species' therapeutic value. For this reason, we employed six inter-retrotransposon amplified polymorphism (IRAP) indicators and 15 mixed IRAP indicators to highlight genomic variation in this plant both within and across populations in this study. It was discovered that 73% of overall genomic variability was related to within-population variety and 27% was attributable to inter-population genomic divergence using the AMOVA test among the examined populations (PhiPT = 0.49, P = 0.010). It was discovered by the Mantel analysis that there was a substantial positive association between genomic isolation and geographic distance among the tested populations. STRUCTURE analyses and population assignment tests revealed some degree of gene flow among these populations. There was consistency between the MDS plots of communities and the NJ grouping of molecular information. Based on (IRAP) indicators, these findings demonstrated that regional communities of the plant *Pistacia khinjuk* are well distinct.

Keywords: gene flow, IRAP, *Pistacia khinjuk,* population differentiation.

INTRODUCTION

According to current estimates, the *Pistacia* genus has at least twelve species and has existed for around eighty million years (Karimi et al. 2009). *Pistacia vera* is the sole commercially viable species throughout this genus

(Fares et al. 2009). According to previous theories, the *Pistacia* genus originated in Europe and North Africa, but recent research suggests that it probably originated in Central Asia. *Pistacia* species have been reported to have spread over the world, based on initial research. One theory concentrates on the Mediterranean region of Europe, Northern Africa, and the Middle East. The eastern portion of the Zagros Mountains (Iran) and the Caucasus regions stretching from Crimea to the Caspian Sea are further options (Zohary 1952). Four important species of pistachios include *P. vera, P. khinjuk* Stocks, *P. eurycarpa* Yalt. (*P. atlantica* subsp. *Kurdica* Zoh.), and *P. atlantica* Dsef are found in Iran (Karimi et al. 2009). *Pistacia vera, P. khinjuk*, and *P. atlantica* are three of the most important wild *Pistacia* species that thrive in Iran. In Central Asia, which includes Turkmenistan, Afghanistan, and Northeast Iran, wild *P. vera* has grown in an area of approximately 75,000 hectares. In the Sarakhs region, *P. vera* grows in an area of approximately 17,500 hectares (Behboodi 2003). With the biggest area under cultivation, Iran is the world's leading pistachio exporter, although recent years have seen poor yields relative to other nations, notably the United States and Turkey (Ahmad et al. 2003a).

Pistachio plants are long-living with a juvenile period of approximately 5–10 years. In addition, wild *Pistacia* species have edible seeds. They are used as rootstock seed sources for cultivated *P. vera*, and sometimes, fruit consumption, oil extraction, soap production, and as forest trees (Katsiotis et al. 2003).

Pistacia genetic diversity has been the subject of several investigations that have been conducted on the basis of examination of morphological, physiological, and metabolic properties (Tayefeh Aliakbarkhany et al. 2013). A number of these methods have been employed to characterize pistachio cultivars across time, with RAPD (Williams et al., 1990) being the most extensively utilized (Kafkas et al., 2002; Katsiotis et al., 2003). To examine the evolutionary connection between *Pistacia* species and cultivars, AFLP and SSR approaches have also been utilized on *pistachio* (Katsiotis et al., 2003; Ibrahim Basha et al., 2007; Ahmad et al., 2003; Ahmad et al., 2005; Ahmadi Afzadi et al., 2007). *Pistachio* pollination difficulties may be solved by identifying the genetic variety of male cultivars and genotypes in Iran because there is not enough data surrounding their genetic characteristics (Ahmad et al., 2005). Most of the taxonomic and nomenclatural ambiguity in European species has been cleared up thanks to the later research. To examine the genetic diversity and connections among *Pistacia khinjuk* cultivars and landraces, randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR), simple sequence repeat (SSR), and inter-retrotransposon amplified polymorphism (IRAP) were some molecular marker techniques employed during recent years.

There is also the potential that this species might have infra-specific taxonomic variants owing to the wide range of morphological variation throughout the nation. As a result, we conducted the first-ever nationwide demographic genetic evaluation and morphometric examination of eight distinct regional groups. Through amplifying the segments of DNA between two retrotransposons for genomic analysis, we employed the inter-retrotransposon amplified polymorphism (IRAP) approach to detect insertional polymorphisms. It has been employed in various investigations on genomic variation (Smykal *et al*., 2011). The objectives of this research were to study genetic diversity among *Pistacia khinjuk* cultivars/populations with a different geographical origin by inter-retrotransposon amplified polymorphism (IRAP) method to determine genetic variation among and within materials using IRAP markers.

MATERIALS AND METHODS

Plant materials

During the months of July and August of 2019-2020, a number of 40 participants from eight natural communities of *Pistacia khinjuk* were collected in the Iranian provinces of Fars, Kerman, Sistan and Baluchestan, and Hormozgan (Table 1). Fresh leaves of 3-6 individuals from each population were collected and immediately dried in Silica Gel (Table 1). The accurate recognition of species was achieved through the utilization of numerous sources (*Pistacia khinjuk*) (Kafkas et al., 2002; Katsiotis et al., 2003). Table 1 list the locations where samples were taken.

DNA extraction and IRAP examination

Three to six plants from each group were randomly selected to collect fresh leaves. The silica gel powder was used to dry them. Genomic DNA was extracted using a CTAB stimulated charcoal technique (Esfandani-Bozchaloyi et al., 2019). By passing the isolated DNA across a 0.8% agarose gel, the purity of the DNA was determined. The IRAP assessment was conducted using a collection of six outward-facing LTR primers (Smykal et al., 2011;

Table 1. Populations studied their locality and ecological features.

Pop.no	Locality
1	Fars, Shiraz
2	Fars, 60 km south of Shiraz at the vicinity to Shiraz-Bushehr
3	Fars, Arjan Lake
4	Hormozgan, Bandar Lengeh
5	Hormozgan, Bandar Abbas
6	Hormozgan, Bandar Abbas, Genow
7	Kerman, Hamun-e Jaz Murian
8	Sistan and Baluchestan, Iranshahr

Table 2). Outward-facing LTR paired primers were additionally utilized in 15 distinct mixtures. PCR reactions were carried in a 25µl 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 µM of a single primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). An initial denaturation during 1 minute at 94°C was continued by 40 rounds divided into three sections, including 35 s at 95°C, the 40s at 47°C, and the 55s at 72°C, which comprised the thermal schedule. The final extension was performed at 72°C for 5 min. In order to see the amplification results, the gels were first to run on a 1 percent agarose solution and then stained with ethidium bromide. A molecular size ladder with a step size of 100 bp was used to determine the fragment size (Fermentas, Germany).

Data analyses

The IRAP profiles obtained for each samples were scored as binary characters. For grouping of the plant specimens, Ordination methods such as MDS (Multidimensional scaling) analysis were also performed (Podani 2000). Multivariate and all the necessary calculations were done in the PAST software, 2.17 (Hammer et al. 2012). Parameter like Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism were determined (Freeland *et al*., 2011).

Nei's genetic distance among populations was used for Neighbor Joining (NJ) clustering and Neighbor-Net networking (Freeland *et al*., 2011). Mantel test checked the correlation between geographical and genetic distance of the studied populations (Podani, 2000). These analyses were done by PAST ver. 2.17 (Hammer *et al*., 2012), DARwin ver. 5 (2012) and SplitsTree4 V4.13.1 (2013) software.

AMOVA (Analysis of molecular variance) test (with 1000 permutations) as implemented in GenAlex 6.4 (Peakall and Smouse, 2006), and Nei's Gst analysis as implemented in GenoDive ver.2 (2013) were used to show genetic difference of the populations. Moreover, populations' genetic differentiation was studied by G'ST est = standardized measure of genetic differentiation, and D_est = Jost measure of differentiation.

The genetic structure of populations was studied by Bayesian based model STRUCTURE analysis (Pritchard *et al*. 2000), and maximum likelihood-based method of K-Means clustering of GenoDive ver. 2. (2013). For STRUCTURE analysis, data were scored as dominant markers (Falush *et al*. 2007). The Evanno test was performed on STRUCTURE result to determine proper number of *K* by using delta *K* value. In K-Means clustering, two summary statistics, pseudo-F, and Bayesian Information Criterion (BIC), provide the best fit for k.

Gene flow was determined by (i) Calculating Nm an estimate of gene flow from Gst by PopGene ver. 1.32 (1997) as: Nm = 0.5(1 - Gst)/Gst. This approach considers equal amount of gene flow among all populations. (ii) Population assignment test based on maximum likelihood as performed in Genodive ver. in GenoDive ver. 2. (2013). The presence of shared alleles was determined by drawing the reticulogram network based on the least square method by DARwin ver 5. (2012).

Table 2. IRAP primers based on SMYKAL et al. (2011) study.

IRAP	Sequence (5′-3′)
GU735096	ACCCCTTGAGCTAACTTTTGGGGTAAG
GU980589	AGCCTGAAAGTGTTGGGTTGTCG
GU929878	GCATCAGCCTGGACCAGTCCTCGTCC
GU735096	CACTTCAAATTTTGGCAGCAGCGGATC
GU929877	TCGAGGTACACCTCGACTCAGG
GU980590	ATTCTCGTCCGCTGCGCCCCTACA

RESULTS

Genetic variation across communities.

Table 3 displays the genetic variation characteristics of *Pistacia khinjuk* collected from eight different geographic locations. Fars, Shiraz (population No. 1) exhibited the largest polymorphism percentage (53.75 percent) and the maximum scores for gene variation (0.39) and Shanon data indicator (0.40). Hormozgan, Bandar Abbas, and Genow (No.6) populations had the minimum polymorphism rate (17.15%) and the minimum values for Shanon, data score (0.15), and He (0.18).

Population genetic differentiation

AMOVA (PhiPT = 0.49, P = 0.010), and Gst analysis (0.844, p = 0.001) revealed significant difference among the studied populations (Table 4). Within-population variation accounted for 27% of overall genomic variation, whereas among-population genomic divergence accounted for 73% of variations. There were substantial variations in the communities analyzed using pairwise AMOVA analysis. Moreover, we got high values for Hedrick standardized fixation index after 999 permutation (G'st = 0.844, P = 0.001) and Jost, differentiation index (D-est = 0.116, P = 0.001). *Pistacia khinjuk* has been shown to be genetically distinct across its geographical communities, according to these findings.

Populations genetic affinity

There were different clusters of plants from each population in the NJ tree. No transitional stages were found throughout the samples that we examined. These results showed that IRAP data could differentiate the populations of *Pistacia khinjuk* in three different major clusters or groups (Figure 1). The first significant cluster supported with significant bootstrapping values of 94% so that plants of Fars, Shiraz (No.1) comprised the first cluster due to morphological similarity. In contrast, the plants of Hormozgan, Bandar Abbas pop 5 (B=94%), formed the second cluster and finally, the population 2 (Fars, 60 km south of Shiraz at the vicinity to Shiraz-Bushehr) with 97% of support. While plants of Hormozgan, Bandar Lengeh (pop 4), Hormozgan, Bandar Abbas, Genow (pop6), Kerman, Hamun-e Jaz Murian (pop7), Sistan and Baluchestan, Iranshahr (Pop 8) showed genetic affinity and intermixture.

Table 3. Genetic diversity parameters in the studied populations *Pistacia khinjuk* (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).

Pop	N	Na	Ne	I	He	UHe	%P
Pop1	5	0.241	1.158	0.40	0.36	0.39	53.75%
Pop2	6	0.355	1.077	0.377	0.34	0.32	35.05%
Pop3	4	0.449	1.167	0.24	0.23	0.24	19.26%
Pop4	4	0.535	1.020	0.22	0.25	0.28	43.13%
Pop5	4	0.231	1.088	0.30	0.22	0.25	31.63%
Pop6	3	0.355	1.121	0.15	0.18	0.12	17.15%
Pop7	6	0.538	1.091	0.207	0.23	0.280	23.93%
Pop8	5	0.291	1.333	0.231	0.333	0.167	21.59%

Table 4. Analysis of molecular variance (AMOVA) of the studied species.

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	55	116.596	22.329	17.077	73%	73%
Within Pops	14	33.757	29.580	33.590	27%	
Total	69	150.342		51.773	100%	

df: degree of freedom; SS: sum of squared observations; MS: mean of squared observations; EV: estimated variance; ΦPT: proportion of the total genetic variance among individuals within an accession, (P < 0.001).

Genetic divergence and separation of populations Fars, 60 km South of Shiraz at the vicinity to Shiraz-Bushehr (No.2) as well as Hormozgan, Bandar Abbas (No.5) and Hormozgan, Bandar Abbas, Genow (No.6) from the other communities is obvious in MDS design of IRAP information following 900 permutations (Figure.2). The other groups were genetically related to each

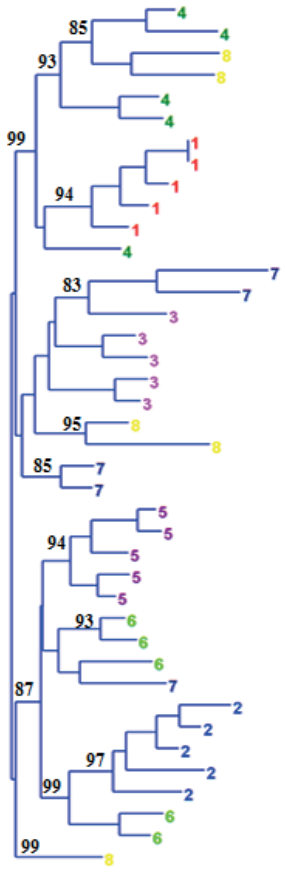

Figure 1. NJ tree of populations in *Pistacia khinjuk* based on IRAP data. Bootstrap value from1000 replicates are indicated above branches (Population numbers are according to Table 1).

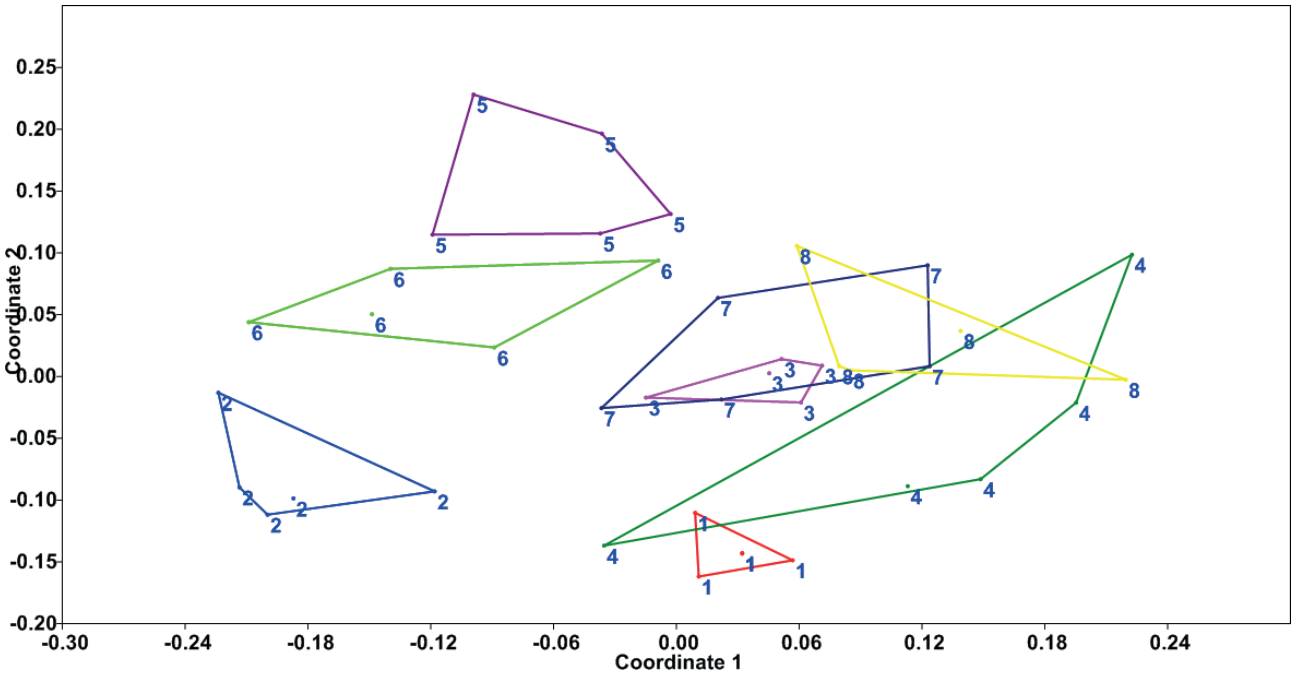

Figure 2. MDS plot of populations in *Pistacia khinjuk* based on IRAP data. (Population numbers are according to Table 1).

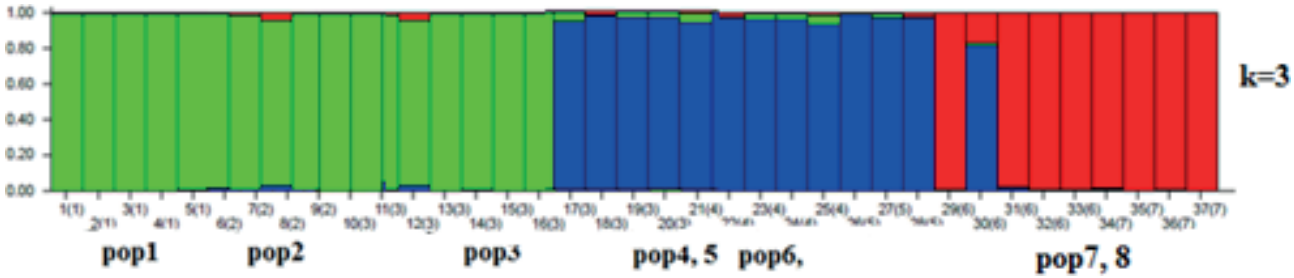

Figure 3. STRUCTURE plot of *Pistacia khinjuk* populations based on k = 3 of IRAP data. (Population numbers are according to Table 1).

other. A substantial association between genetic isolation and geographic separation was found in these communities after a Mantel analysis with 5000 permutations (r = 0.55, P = 0.001). We possess isolation by distance (IBD) in the *Pistacia khinjuk* species because communities that are spatially separated exhibit less genetic exchange.

Populations genetic structure

There are three genetic subgroups present when K = 3. When the Evanno examination was run on the STRUCTURE evaluation, it yielded a comparable outcome, with a large peak appearing at k=3. Both studies found genetic differentiation in *Pistacia khinjuk* groups.

STRUCTURE plot based on k = 3 revealed a genetic difference of populations 1-3 (differently colored), as well as 4-6 (Figure.3). But it showed genetic affinity between populations 7, 8 (similarly colored). The mean Nm = 0.29 was obtained for all IRAP loci, which indicates a low amount of gene flow among the populations and supports genetic stratification as indicated by K-Means and STRUCTURE analyses. It was also found that there was no substantial genetic exchange between these groups when the demographic allocation experiment was performed. It was found that populations 1 and 5, as well as populations 3 and 6, also 2 and 5 shared certain alleles, according to a reticulogram created using the least square approach (Figure not shown). Due to the proximity of both communities, our MDS map resulted in the same classification. Genetic differentiation among *Pistacia khinjuk* communities is clearly evident from the STRUCTURE plot, which shows that the common

genetic alleles throughout these communities represent only a small percentage of the genomes. It was possible to collect 75 IRAP bands totally; 15 of them were considered exclusive. Two to four unique bands were found in communities 3 and 6, and 8.

DISCUSSION

Genetic and breeding investigations benefit greatly from population genetics analysis. Data on the degrees of genomic diversity, genetic diversity distribution within and across communities, inbreeding and outcrossing, the efficient community size and bottleneck are presented by these studies (Ellis and Burke, 2007). Demographic genomic research has significantly advanced with the introduction of molecular biomarkers. Among the various *Pistacia* accessions, such indicators have been utilized to detect possibly unique genotypes (Martin *et al*.,1997). To examine the genetic diversity and connections among *Pistacia khinjuk* cultivars and landraces, randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR), simple sequence repeat (SSR), and inter-retrotransposon amplified polymorphism (IRAP) were some molecular marker techniques employed during recent years (Wiesnerova and Wiesner, 2004; REN and KHAYATNEZHAD 2021; KHAYATNEZHAD and NASEHI 2021, I *et al*., 2021; JIA *et al*, 2021). The majority of plant genomes are made up of transposable elements, especially retrotransposons. Genomic variety is generated through their replication, rendering them an ideal repository of molecular indicators (Smykal *et al*., 2011; GHOLAMIN and KHAYATNEZHAD, 2020a; 2020b, 2020c). Through replicating the sections of DNA between two retrotransposons, the inter-retrotransposon amplified polymorphism (IRAP) approach reveals insertional polymorphisms. Several genomic investigations have relied on this technique (Smykal *et al*., 2011).

Iranian *Pistacia khinjuk*'s genomic variation was evaluated during this research in order to help in the preservation of its germplasm. In order to formulate suitable conservation approaches, the data gathered on genetic diversity between and within various groups will be used to establish a solid foundation for future research. Iranian *Pistacia khinjuk* is very diverse, according to the results of the current study, which is likely owing to differences in genetic backgrounds across different geographical areas, breeding pressure, and/or restricted exchange of genomic information. Our findings demonstrate the distinct character of the Iranian *Pistacia khinjuk* germplasm, hence bolstering the rationale for deploying more intensive characterization, preservation, and reproduction techniques. It was possible to determine the genomic variation of the Iranian population employing IRAP indicators. The results of this molecular assay in fingerprinting the 8 *Pistacia khinjuk* population are presented in Table 3. A total of 75 bands were amplified by the six primers, an average of 8 bands per primer, of which 62 (84%) were polymorphic. The total number of amplified fragments was between 6 to 10, and the number of polymorphic fragments ranged from 5 to 9. NJ clustering and MDS plot (Figs 1–2), of the studied populations did not entirely delimit the studied populations and revealed that some plants in these populations are intermixed. In MDS plot, a higher degree of intermixture occurred between populations of 7, 8 and seem to be an area that populations of 1,4 and 7, 8 together with the gene exchange (Fig. 2). These results indicate that the geographical populations of *Pistacia khinjuk* are not genetically differentiated from each other. Evanno test performed on STRUCTURE analysis produced the best number of $k = 3$. This genetic grouping is in agreement with NJ clustering result presented before.

Throughout the semi-arid and dry farming areas of Iran, pistachio has significant socioeconomic and environmental implications (Kafkas et al., 2006). More than 300 *pistachio* genotypes have been identified in Iran, which is home to a diverse range of *Pistacia* species. *Pistachio* development and preservation efforts may thus benefit from Iran's *Pistachio* germplasm. It is consequently vital to evaluate genomic variation and interactions among cultivars of Iranian *Pistachio* employing discriminative and reliable indicators.

Genetic diversity is of fundamental importance to the survival of a species (Sun and Khayatnezhad 2021; Tao *et al*, 2021; Wang *et al*, 2021; Xu *et al*., 2021; Yin *et al*., 2021; Zhang *et al,* 2021).

There were three primers ultimately chosen for further testing out of the original six employed during ISSR following initial screening (Kafkas et al., 2006; Zheng, et al., 2021; Zhu *et al*, 2021), in accordance with the stated findings. The three primers replicated a maximum of 28 bands, with each primer amplifying an average of 9.3 bands among 13 types (or 46 percent), which were polymorphic. Approximately seven to 12 pieces of DNA were replicated, and three to five segments of DNA were polymorphic.

Between 22 Iranian cultivars and wild *Pistachio* varieties, Mirzaei et al. (2005) found 80% polymorphism. Because of the changes in genotypes and primers between the present research and the previous study, it is possible that variations in polymorphism are observed.

82.41%% polymorphism was discovered by Katsiotis and colleagues (2003); there were 18.2 polymorphic bands out of a total number of 22.11. In a study reported by Golan-Goldhirsh et al. (2004) in assessing polymorphisms among 28 Mediterranean *Pistacia* accessions, twenty-seven selected primers produced 259 total bands (average 9.59), and 86.1 of them were polymorphic. The genotypes investigated by Khadivi (2018) showed a significant degree of polymorphism. 18 alleles were produced by seven SSR primer pairs, thirteen among them were polymorphic across the genotypes. Averaging 2.57, the polymorphic alleles ranged from one for Ptms9, Ptms40, Ptms41, and Ptms42 loci to five for the Ptms7 locus. Allele lengths ranging from 120 to 250 bp were replicated. The coefficients of genomic homology between two individuals ranged from 0.20 to 0.75. To summarize, it can be concluded that one of two significant hubs of *Pistacia* variety is Iran. Genomic variation among several *Pistacia khinjuk* communities employing IRAP indicators was studied during the current research, offering useful data in the effort to conserve the species' germplasm. Because Iranian *Pistacia khinjuk* possesses limited genetic variety, its preservation and prospective reproduction initiatives are very vital. Preserving, core collecting, and reproducing the *Pistacia khinjuk* will be made easier thanks to the outcomes of this research.

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