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GENETIC VARIABILITY ANALYSIS IN *Peganum harmala* L. BY SCOT AND SRAP MOLECULAR MARKERS

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Peganum harmala L. is a perennial herbaceous plant and it has long been used for medicinal purposes as herbicide due to the presence of harmine. *P. harmala* is a famous medicinal plant used in the Iranian traditional medicine, due to the antimicrobial compounds found in its seeds and roots. Population genetic study is an essential scientific approach for studying medicinal plants; as it produces data on genetic variability, genetic structure, and gene flow versus genetic fragmentation of these plants. We have no detailed information on genetic structure of this plant species in the country. Therefore, a through population genetic study was conducted in four geographical populations of these valuable plants by using start codon targeted (SCoT), and sequence related amplified polymorphic (SRAP) molecular markers. Both these molecular markers are highly reproducible and polymorphic molecular markers and are very efficient in genetic variation studies in plants. In present study, both multidimensional scaling (MDS) plot and analysis of molecular variance (AMOVA) based on both SCOT and SRAP molecular markers revealed genetic differentiation of the studied populations. This indicates that local populations may be genetically isolated and due to that gained specific genetic

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content. This is good news for genetic conservation of these medicinally important plants. We should continue for hunting new *P. harmala* wild populations in the country and study their genetic structure; in this way we can broaden *P. harmala* gene pools which can be utilized for future breeding studies.

Keywords: Genetic variability, *Peganum harmala,* SCoT, SRAP

INTRODUCTION

Peganum harmala L. (The genus *Peganum* L., family Peganaceae) is a perennial herbaceous plant and it has long been used for medicinal purposes as herbicide due to the presence of harmine (ASTULLA *et al.,* 2008; TURMUKHAMBETOV *et al.,* 2009). It is one of the most frequently used medicinal plants for the relief of pain and as an antiseptic agent to treat hypertension, cardiac disease, some nervous system disorders such as Parkinson's disease, Lumbago asthma, colic, jaundice and as a stimulant emmenagogue (EL-BAKATOUSHI and ASEEL AHMED, 2018). It is also considered as a medicinal plant that all parts used as anti-inflammatory, sudorific, sedative and narcotic (ADNAN and HÖLSCHER, 2012).

Peganum harmala is a famous medicinal plant used in the Iranian traditional medicine, due to the antimicrobial compounds found in its seeds and roots (GOUDARZI and AZIMI, 2016). It has antimicrobial activity against methicillin-resistant *Staphylococcus* aureus Strains (GOUDARZI and AZIMI, 2016). It contains certain chemical compounds such as: alkaloids, flavonoids, saponins, reducing compounds, tannins, volatile oils, triterpenes or sterols and anthraquinone (ARSHAD *et al.,* 2008; MOLOUDIZARGARI *et al.,* 2013).

Peganum harmala is a perennial herb that grows in the landscape of steppe, semidesert, and desert territories in southern Europe, northern Africa, and southwestern Asia and has somatic chromosome number (2n = 24), and the 2C values, ranging from 0.61 to 0.67 pg (HAJJI *et al.,* 2017).

Genetic diversity analysis and identification of the gene pools in medicinal plants are very important for the conservation purpose and breeding programs. For this reason several studies were performed to investigate genetic variability of *Peganum harmala.* For example, EL-BAKATOUSHI and ASEEL AHMED (2018), investigated the genetic diversity of twelve *P. harmala* genotypes by different molecular markers and reported that the ITS (Internal transcribed spacer DNA sequences) and ISSR (Inter-simple sequence repeat) markers were more informative than the other used markers in the assessment of genetic diversity of *P. harmala*. AL RAWASHDEH et al. (2017), studied *P. harmala* wild populations in Jordan by RAPD molecular markers.

Population genetic study is an essential scientific approach for studying medicinal plants, as it produces data on genetic variability, genetic structure, gene flow versus genetic fragmentation of these plants (SHEIDAI *et al.,* 2016). It also can identify the molecular markers and genetic loci that can differentiate geographical populations and also suggest potential gene pools available (SHEIDAI *et al.,* 2018).

In spite of medicinal importance of *Peganum harmala* in Iran, we have no detailed information on genetic structure of this plant species in the country. Therefore, a through population genetic study was conducted in four geographical populations of these valuable plants by using start codon targeted (SCoT), and sequence related amplified polymorphic (SRAP) molecular markers. Both these molecular markers are highly reproducible and polymorphic molecular markers and are very efficient in genetic variation studies in plants (COLLARD and MACKILL, 2009; LI and QUIROS, 2001).

SCoT markers were developed by COLLARD and MACKILL (2009). These markers are highly reproducible due to the use of longer primers. The primers are designed according to the short conserved region surrounding the ATG translation start (or initiation) codon (or translational start site, TSS). It is a type of targeted molecular marker technique with the ATG context as one part of a functional gene, markers generated from SCoT marker technique may be mostly correlated to functional genes and their corresponding traits (COLLARD and MACKILL, 2009). Similarly, SRAP markers were developed by LI and QUIROS (2001). It is a simple marker technique aimed for the amplification of open reading frames (ORFs). It is based on two-primer amplification. The primers are 17 or 18 nucleotides long and consist of the following elements. Core sequences, which are 13 to 14 bases long, where the first 10 or 11 bases starting at the 5′ end, are sequences of no specific constitution ("filler" sequences), followed by the sequence CCGG in the forward primer and AATT in the reverse primer. The core is followed by three selective nucleotides at the 3′ end. The filler sequences of the forward and reverse primers must be different from each other and can be 10 or 11 bases long. SRAP technique combines simplicity, reliability, moderate throughput ratio and facile sequencing of selected bands. Further, it targets coding sequences in the genome and results in a moderate number of co-dominant markers (LI and QUIROS, 2001).

The experiment aimed to study the population genetic variability in four geographical populations of *Peganum harmala* by using Start codon targeted (SCoT), and sequence related amplified polymorphic (SRAP) molecular markers.

MATERIALS AND METHODS

Plant materials

In total 40 plants were collected randomly from 4 populations in Khorasan provinces (Table 1). We used these plants for both sequence related amplified polymorphism (SRAP) and start codon targeted (SCoT) analyses.

Table1. Populations studied their locality and ecological features in Peganum harmala.

NO	Province	Locality	Altitude (m)	Longitude	Latitude
1	Salehabad	Khorasan	739	351653	663200
2	Fariman	Khorasan	1066	361626	599977
3	Hosseinabad	Khorasan	950	363535	597640
4	Ferdosi	Khorasan	1025	364906	595326

DNA extraction and PCR

Fresh leaves were used randomly from 5-10 plants in each of the studied populations. These were dried by silica gel powder. CTAB activated charcoal protocol was used to extract genomic DNA. The quality of extracted DNA was examined by running on 0.8% agarose gel.

SRAP assay

Five sequences related amplified polymorphism (SRAP) primer pairs including forward primers: Me1, Me2, Me3, Me4, Me5 and reverse primers: Em1, Em2, Em3, Em4, Em5 were used (Table2). 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 1 U of *Taq* DNA polymerase (Bioron, Germany). The amplifications, reactions were performed in Techne thermocycler (Germany) with the following program: 5 min initial denaturation step at 94°C, followed by five cycles of 94°C for 1min, 35°C for 1min, and 72°C for 1 min; followed by 35 cycles of 94°C for 1min, 54°C for 1min, and 72 for 2 min; followed by 10 min at 72°C. The amplification products were observed by running on 1% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Table2. Primers name for SRAP sequences.

Primer name	5-3 seq	scale nml	Method	Purification	Chemistry	Length
Me1	TGAGTCCAAACCGGATA	40	ECONOMY	2XPAGE	DNA	17
Me2	TGAGTCCAAACCGGAGC	40	ECONOMY	2XPAGE	DNA	17
Me3	TGAGTCCAAACCGGAAT	40	ECONOMY	2XPAGE	DNA	17
Me4	TGAGTCCAAACCGGACC	40	ECONOMY	2XPAGE	DNA	17
Me5	TGAGTCCAAACCGGAAG	40	ECONOMY	2XPAGE	DNA	17
Em1	GACTGCGTACGAATTAAT	40	ECONOMY	2XPAGE	DNA	18
Em2	GACTGCGTACGAATTTGC	40	ECONOMY	2XPAGE	DNA	18
Em3	GACTGCGTACGAATTGAC	40	ECONOMY	2XPAGE	DNA	18
Em4	GACTGCGTACGAATTTGA	40	ECONOMY	2XPAGE	DNA	18
Em5	GACTGCGTACGAATTAAC	40	ECONOMY	2XPAGE	DNA	18

SCoT assay

For SCoT study, 10 SCoT primers were used (Table3). 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 1 U of *Taq* DNA polymerase (Bioron, Germany). The amplifications, reactions were performed in Techne thermocycler (Germany) with the following program: 5Min initial denaturation step 94°C, followed by 40 cycles of 1 min at 94°C; 1 min at 56°C and 2 min at 72°C. The reaction was completed by final extension step of 10 min at 72°C. The amplification products were observed by running on 1% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Table3. Primers name for SCoT sequences.

Primer name	5-3 seq	Length
SCOT1	CAACAATGGCTACCACCA	
S2	CAACAATGGCTACCACCC	17
S3	CAACAATGGCTACCACCG	17
S7	CAACAATGGCTACCACGG	17
S10	CAACAATGGCTACCAGCC	17
S11	AAGCAATGGCTACCACCA	17
S14	- ACGACATGGCGACCACGC	17
S17	ACCATGGCTACCACCGAG	17
S18	ACCATGGCTACCACCGCC	17
S20	ACCATGGCTACCACCGCG	17

Data analyses

Dentrented correspondance analysis (DCA) was performed to investigate suitability of the molecular markers for genetic diversity studies. If the loci obtained are scattered throughout the genome and are not closely tight to each other, they are considered as suitable molecular markers for genetic diversity analysis (PODANI, 2000). Discriminating power of SCOT and SRAP loci obtained was determined by Gst analysis as implemented in POPGENE 1.32 (YEH *et al.,* 1999).

Correlation between SCOT and SRAP loci was determined by Mantel test (PODANI, 2000). Nei genetic distance was estimated between the studied populations separately for both SCOT and SRAP markers and used for grouping of the plants by NJ (Neighbor Joining) clustering and MDS (Multidimentional Scaling) methods (PODANI, 2000).

RESULTS

Dentrented correspondance analysis of both SCOT and SRAP loci (Fig.1) revealed that these loci are well distributed in the genome and represent many genetic regions of the genome. Therefore, they can be utilized in germ plasm evaluation and genetic finger printing of *Peganum harmala* populations.

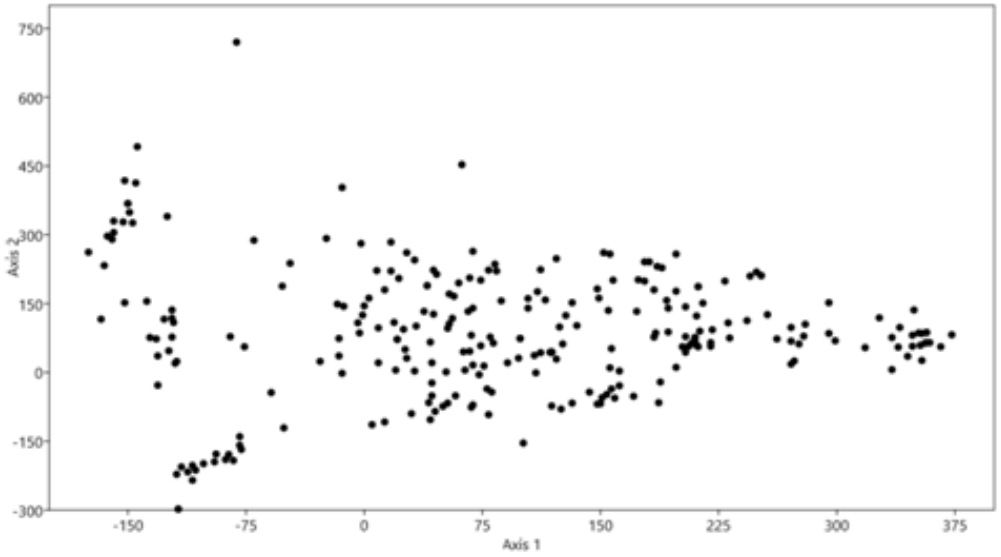

Fig.1. DCA plot of SCOT and SRAP loci utilized in *Peganum harmala* genetic finger printing.

Scot primers produced 214 bands/ loci. Some bands were private for the studied populations, while few bands were common and shared among these populations. Discriminating power of SCOT loci with >0.50 Gst value, determined by Gst analysis is given in Table 4. The result reveals that about 26 out of 214 loci, have discriminating power to differentiate *Peganum harmala* populations.

Table 4. Discriminating power of SCOT loci among Peganum harmala populations (Only loci with >0.5 Gst have been given).

Locus	Sample Size	Ht	Hs	Gst	Nm*
Locus2	40	0.2662	0.1162	0.5634	0.3874
Locus4	40	0.2662	0.1162	0.5634	0.3874
Locus6	40	0.2662	0.1162	0.5634	0.3874
Locus7	40	0.2662	0.1162	0.5634	0.3874
Locus8	40	0.2662	0.1162	0.5634	0.3874
Locus14	40	0.3875	0.0243	0.9372	0.0335
Locus23	40	0.4608	0.1800	0.6094	0.3204
Locus30	40	0.4649	0.1649	0.6453	0.2749
Locus95	40	0.4500	0.2162	0.5195	0.4625
Locus101	40	0.4500	0.2162	0.5195	0.4625
Locus102	40	0.4971	0.1764	0.6450	0.2751
Locus104	40	0.4968	0.1797	0.6384	0.2832
Locus113	40	0.3168	0.1553	0.5097	0.4810
Locus115	40	0.4416	0.1081	0.7552	0.1621
Locus134	40	0.4500	0.2162	0.5195	0.4625
Locus135	40	0.4875	0.1081	0.7782	0.1425
Locus137	40	0.4750	0.1236	0.7398	0.1759
Locus161	40	0.4939	0.2109	0.5729	0.3727
Locus162	40	0.4740	0.1719	0.6374	0.2845
Locus164	40	0.4744	0.1239	0.7389	0.1767
Locus166	40	0.4854	0.1708	0.6481	0.2715
Locus169	40	0.4618	0.1236	0.7323	0.1827
Locus187	40	0.3875	0.0243	0.9372	0.0335
Locus188	40	0.3750	0.0000	1.0000	0.0000
Locus190	40	0.3750	0.0000	1.0000	0.0000
Locus191	40	0.4752	0.1725	0.6369	0.2850
Mean	40	0.3005	0.2090	0.3046	1.1415

* Nm = estimate of gene flow from Gst or Gcs. E.g., Nm = 0.5(1 - Gst)/Gst;

SRAP primers produced 136 bands/ loci. Some bands were private for the studied populations, while few bands were common and shared among these populations.

Discriminating power of SRAP loci with Gst value >0.50, is given in Table 5. The result reveals that about 7 out of 136 loci, have discriminating power to differentiate *Peganum harmala* populations.

AMOVA produced significant difference (P = 0.001) among the studied populations which indicates these are genetically differentiated. It also revealed that about 40% of total genetic variability is due to among population difference, while 60% is due to within population genetic variability. This may indicate open pollination of these plants within each population.

Genetic differentiations of the studied populations were also shown by NJ tree (Fig.2) and MDS plot (Fig.3).

Table5. Discriminating power of SRAP loci among Peganum harmala populations (Only loci with >0.5 Gst have been given).

Locus	Sample Size	Ht	Hs	Gst	Nm*
Locus13	40	0.3875	0.0243	0.9372	0.0335
Locus14	40	0.3750	0.0000	1.0000	0.0000
Locus16	40	0.3750	0.0000	1.0000	0.0000
Locus17	40	0.4752	0.1725	0.6369	0.2850
Locus45	40	0.3750	0.0000	1.0000	0.0000
Locus47	40	0.4625	0.1239	0.7322	0.1829
Locus88	40	0.4500	0.1162	0.7417	0.1741
Mean	40	0.2985	0.2109	0.2934	1.2043
St. Dev	0.0140	0.0140			

* Nm = estimate of gene flow from Gst or Gcs. E.g., Nm = 0.5(1 - Gst)/Gst;

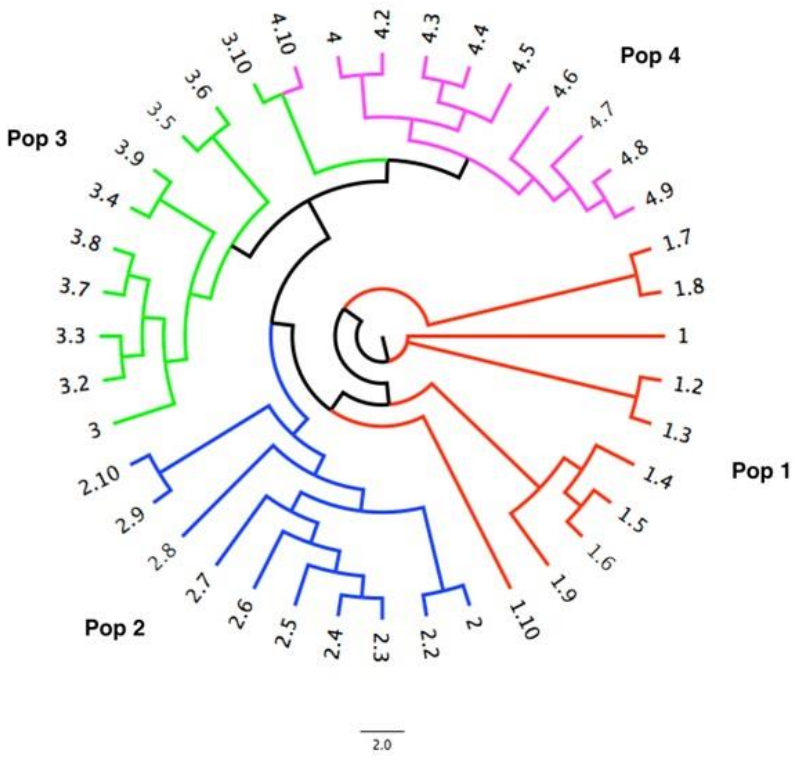

Fig.2. NJ tree of *Peganum harmala* populations based on combined SCOT and SRAP data (Populations 1-4 are according to Table 1).

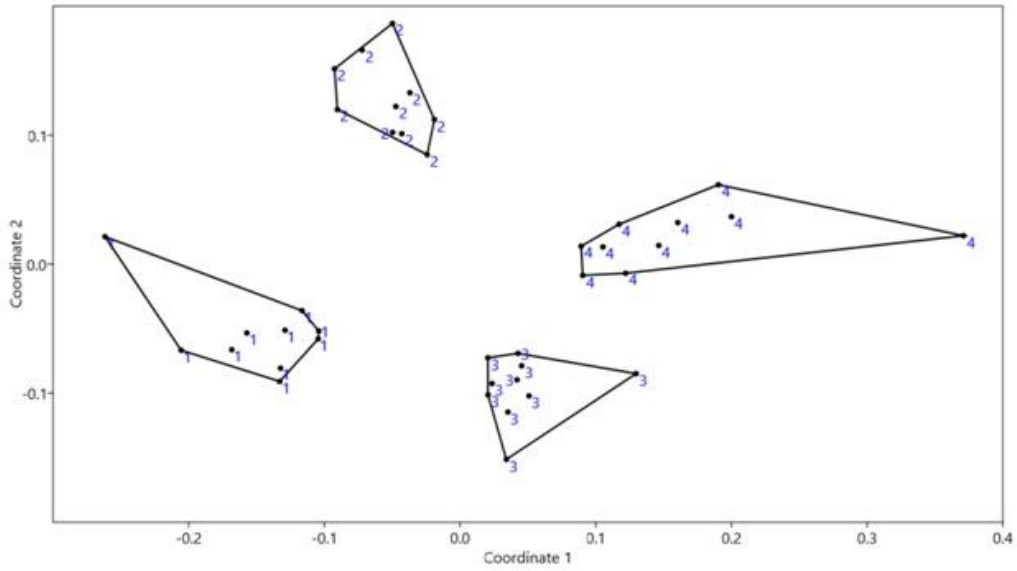

Fig.3.MDS plot of the studied *Peganum harmala* populations based on combined SCOT and SRAP data, showing population genetic distinctness (Populations 1-4 are according to Table 1).

DISCUSSION

Population genetic study of medicinal plants like *P. harmala* can produce valuable information on genetic diversity within these plants which can be used in conservation and future breeding programs. *P. harmala* is widely distributed in Iran and is consumed by many local peoples. Extensive utilization of the medicinal plants reduces the number of plants and creates a bottle-neck for them. Therefore, genetic conservation of medicinal plants is considered an important task. Similarly, continuous cultivation and artificial selection of these plants can bring about genetic erosion, therefore carrying out detailed population genetic study is an immediate and important job to do with regard to *P. harmala.* We could produce some detailed information on genetic structure and genetic variability in four *P. harmala* populations that are mainly located in north-east of the country. We observed a good level of genetic diversity both within and among the studied populations by using Scot and SRAP molecular markers. However, ZEBARJADI *et al.* (2016), studied the genetic diversity of few Iranian populations of *Peganum harmala* by application of ISSR markers. Accordingly, highest and lowest similarities among ecotypes were measured as 94% and 55%, respectively. Cluster analysis categorized the studied accessions into three groups that did not completely match to their geographic pattern place of collection.

In a similar investigation, EL-BAKATOUSHI and ASEEL AHMED (2018), studied the genetic diversity of twelve *P. harmala* genotypes by using a combination of inter-simple sequence repeats (ISSRs), PCR-RFLP of rDNA-ITS, PCR-SSCP of rDNA-ITS and simple sequence repeats (SSRs) markers. They reported the lowest level of polymorphism in ITS-SSCP among

molecular markers used. This was followed by ITS-RFLP then ISSR and the highest polymorphism level was reported for SSR marker. They also reported a higher within population genetic variability compared to that of among population variation. They considered partial out-crossing as the possible explanation for that.

AL RAWASHDEH *et al*. (2017), studied *P. harmala* wild populations in Jordan by RAPD molecular markers and reported a low level of genetic variation in them. These authors considered self-pollination along with environmental pressure for this low genetic diversity.

In present study, both MDS plot and AMOVA based on both SCOT and SRAP molecular markers revealed genetic differentiation of the studied populations. This indicates that local populations may be genetically isolated and due to that gained specific genetic content. This is good news for genetic conservation of these medicinally important plants. We should continue for hunting new *P. harmala* wild populations in the country and study their genetic structure; in this way we can broaden *P. harmala* gene pools which can be utilized for future breeding studies.

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ANALIZA GENETIČKE VARIJABILNOSTI *Peganum harmala* L. POMOĆU SCOT I SRAP MOLEKULARNIH MARKERA

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Izvod

Peganum harmala L. je višegodišnja zeljasta biljka i dugo se koristi u lekovite svrhe kao herbicid zbog prisustva harmina. *P. harmala* je poznata lekovita biljka koja se koristi u iranskoj tradicionalnoj medicini, zbog antimikrobnih jedinjenja koja se nalaze u njenom semenu i korenu. Populaciona genetika je suštinski naučni pristup za proučavanje lekovitih biljaka, jer proizvodi podatke o genetskoj varijabilnosti, genetskoj strukturi i protoku gena nasuprot genetskoj fragmentaciji ovih biljaka. Nemamo detaljne informacije o genetskoj strukturi ove biljne vrste u zemlji. Zbog toga je sprovedena pročavanje populaciione genetike na četiri geografske populacije ovih dragocenih biljaka korišćenjem ciljanih start kodona (SCoT) i SRAP molekularnih markera. Obe vrste molekularnih markera su visoko reproduktivni i polimorfni i veoma su efikasni u proučavanjima genetičke varijacije kod biljaka. U ovoj studiji, i multidimenzionalno skaliranje (MDS) i analiza molekularne varijance (AMOVA) zasnovana na SCOT i SRAP molekularnim markerima otkrili su genetsku diferencijaciju proučavane populacije. Ovo ukazuje da lokalna populacija može biti genetski izolovana i zbog toga je dobila specifičan genetski sadržaj. Ovo je dobra vest za genetsku zaštitu tih medicinski važnih biljaka. Potrebno je nastaviti traženje nove divlje populacije P. harmala u zemlji i proučavati njihovu genetsku strukturu; na ovaj način možemo proširiti genetičke grupe P. harmala koje se mogu koristiti za potrebe oplemenjivanja.

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