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Original scientific article

GENETIC DIVERSITY OF *Cordia myxa* L. ASSESSED BY ISSR MARKERS

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A large number of species of Cordia (Boraginaceae) are found across the tropical and subtropical parts of the planet. *Cordia* species are mentioned in popular medicine as being used to cure a variety of ailments affecting a variety of human organs. Population genetic structure, genetic diversity, and phenotypic variation are unknown for this species in Iran. For this reason, we collected morphological as well as molecular data on this plant species, which is significant in its own right. We employed 70 plants that were randomly picked from seven geographical communities spread throughout four provinces to conduct our research. These populations' genetic diversity variables were analyzed. The results of the structure and K-Means grouping analyses indicated the existence of five distinct gene pools in the nation, which were isolated from one another geographically. Genetic and geographical separation were shown to be correlated using the Mantel analysis. Among communities, AMOVA found considerable genetic differences, with within-population variance accounting for around 55% of the overall genetic variability. Upcoming breeding and conservation efforts for this vital medicinal plant in the nation may benefit from these findings.

Keywords: Cordia myxa L., Gene flow, Genetic admixture, ISSR, STRUCTURE analysis.

INTRODUCTION

A total of 300 species of the *Cordia* Boraginaceae group may be found, the majority of which are small trees or shrubs endemic to the Americas, ranging from Central America down to Argentina's central area (BARROSO and OLIVEIRA, 2009). *Cordia* species are mentioned in popular medicine as being used to cure various ailments affecting a variety of human organs. Numerous ethno pharmacological uses include antibacterial, anti-inflammatory, anthelmintic,

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analgesic, and diuretic properties, as well as the treatment of digestive, respiratory, urogenital, cardiovascular, and blood diseases (JIA *et al.*, 2020).

Botanical and reproductive properties of the *Boraginaceae* genus *Cordia* were covered in the initial publications. As a result of their inability to self-fertilize, the plants were classified as fertile, a key characteristic for identifying the species pertaining to the genus. There are several species of the genus *Cordia* that are farmed for ornamental plants, timber, and medicinal purposes, all of which are widely used by conventional societies across the world. Research on *Cordia* species has increased dramatically over the past several decades, reflecting the widespread enthusiasm in phytochemical, biological, and pharmaceutical research of this plant family. The Indian cherry (*Cordia myxa* L.), also known as Lehsua, Gonda, is an underappreciated fruit that grows across India in dry and semi-arid environments. In addition to India, it may also be discovered in Myanmar, Egypt, Sri Lanka, and the tropical Australian continent (). Originally from India, Lehsua belongs to the Boraginaceae family. Tolerant of mild shade and dryness, the plant is a medium-sized tree. However, it is not planted in orchards and instead thrives in a wild condition in wastelands along agricultural borders or roadside. Dispersed single trees of the species may be found on farmland as well. Both RAPD and SSR markers may be used effectively with the ISSR. As a result, it is capable of producing more polymorphism than RAPD while also being more responsive, stable, and repeatable in its reaction mechanism (BI *et al*, 2021; CHENG *et al*., 2021). Investigations on germplasm resource recognition, species phylogeny, plant taxonomy, evolution, and genetic variation have all relied on this molecular indicator (). A significant degree of morphological variety may be seen in *Cordia myxa*, which occurs in several regional communities across Iran. Various eco-geographical characteristics define these geographical communities, with some clustered together while others are dispersed over vast distances. In the case of *Cordia myxa* communities, we have no data regarding genetic diversity, gene flow, or genetic organization. It's possible that this species might have infra-specific taxonomic forms owing to the wide range of morphological variation throughout the country. For the first time in the nation, we conducted demographic genetic evaluation research of seven regional communities. We employed ISSR (Inter-simple sequence repeats) as a genetic analysis tool. Reproducibility, ease of use, and cost-effectiveness make these molecular biomarkers ideal for demographic genetic investigations and species or infra-specific taxonomic characterization (ESFANDANI- BOZCHALOYI *et al*., 2018a, 2018b).

MATERIALS AND METHODS

In July-August 2018-2020, 70 *Cordia myxa* specimens from nine natural communities were collected in Hormozgan, Kerman, Khuzestan, and Sistan-Baluchestan in Iran (Table 1). 70 plant accessions (four to eleven samples from each population) from seven distinct populations with varied eco-geographic features were collected and preserved in -20 °C until subsequent usage for ISSR evaluation. Table 1 and Figure 1 provide more details on the regional dispersion of accessions. *Cordia myxa* was correctly identified using a variety of references (RIEDL, 1967, DAVIS, 1978). Herbarium of the Science and Research Branch of Islamic Azad University, Tehran, Iran (IAUH), received certificates.

Table 1. Location and herbarium accession numbers of the studied populations of Cordia myxa in Iran

Pop No.	Locality	No. of collected accessions	Voucher No.	Elevation (m)	Latitude (N)	Longitude (E)	Mean Max. Temperature(C)	Mean Min. Temperature(C)	Annual rainfall (mm)	No. of frost days
1	Sistan and Baluchestan , Iranshahr	10	IAUH-822	239	274636.4	605869.6	40.12	-18.12	325	77
2	Khuzestan, Ahvaz	8	IAUH-446	229	315514	481172.8	35.55	-20.34	378	75
3	Khuzestan, Dezful	12	IAUH - 949	150	328309	481855.9	41.34	-10.34	377	96
4	Hormozgan, Bandar Abbas	9	IAUH - 676	11	272229	562628.2	39.14	-17.55	390	73
5	Kerman, Jiroft	11	IAUH - 323	650	305775	572954.5	36.88	-11.23	320	76
6	Khuzestan, Shush	6	IAUH - 780	87	324349	471384.8	32.55	-22.45	334	88
7	Sistan and Baluchestan Chabahar	14	IAUH - 365	222	253107	605655.9	30.44	-18.66	229	120

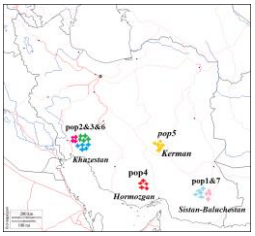

Fig. 1. Map of distribtion of populations of *Cordia myxa*

Extraction of DNA and an ISSR test

Five to ten leaves from each analyzed group were randomly selected and utilized in the experiment. Silica gel powder was used to dry them. Genomic DNA was extracted using a CTAB activated charcoal technique (ESFANDANI- BOZCHALOYI *et al*., 2019). A 0.8 percent agarose gel was used to test the isolated DNA's purity. The following ISSR primers have been employed: (AGC) 5GT, (CA) 7GT, (AGC) 5GG, UBC 810, (CA) 7AT, (GA) 9C, UBC 807, UBC 811, (GA) 9T, and (GT) 7CA commercially available from UBC (the University of British Columbia). 25-µl of 10 mM Tris/HCl buffer pH 8, 50 mM KCl, 1.5 mM MgCl2, 0.02 mM of each dNTP (Bioron, Germany), 0.02 micromoles of a single primer, 20 nanograms of genomic DNA, and three units of Taq DNA polymerase were used in the PCR procedures (Bioron, Germany). The following program was used on a Techne thermocycler (Germany) to accomplish the amplification and reactions: First denaturation phase of 5 minutes at 94°C, followed by 40 cycles of 1 minute at 94°C, 1 minute at 52-57°C, and 2 minutes at 72°C. The last extension step of 7 to 10 minutes at 72°C finished the reaction. In order to visualize the amplification products, the gels were first to run on a 1 percent agarose solution and then stained with ethidium bromide. A 100-bp molecular size ladder was used to assess the fragment size (Fermentas, Germany).

Data analyses

The UPGMA (Unweighted paired group using average) and Ward (Minimum spherical characteristics), as well as the MDS ordination techniques (Multidimensional scaling) and PCoA

(Principal coordinate analysis), were employed to categorize the plant samples (PODANI, 2000). Multivariate statistical analyses of morphological data were performed using PAST version 2.17 (HAMMER *et al*., 2012).

Molecular analyses

A genetic diversity study was performed using the ISSR bands that were acquired. The bands were recorded as binary characters (presence = 1, absence = 0). A variety of parameters, including Nei's gene diversity (H), Shannon information index (I), the number of effective alleles, and the percentage of polymorphism, were calculated in this study (FREELAND *et al*., 2011). Neighbor-Joining (NJ) grouping and Neighbor-Net networking were based on Nei's genetic distance between communities (FREELAND *et al*., 2011). The Mantel analysis was used to determine the relationship between geographical and genetic separation between the two communities under consideration (PODANI, 2000). PAST ver. 2.17 (HAMMER *et al*., 2012), DARwin ver. 5 (2012), and SplitsTree4 V4.13.1 (2013) software were used to conduct these studies. Analyzing genetic differences between populations was done using the AMOVA (Analysis of Molecular Variance) test, which was implemented in GenAlex 6.4 (PEAKALL and SMOUSE, 2006). G'ST est = standardized measure of genetic differentiation (HEDRICK, 2005) and D est = Jost measure of differentiation were used to study population genetic differentiation. The PopGene ver. 1.32 (1997) software used to calculate Nm, an estimation of gene flow (i) from Gst, where Nm = 0.5(1 - Gst)/Gst. It was assumed that all communities had an equivalent quantity of gene flow when using this methodology. Drawing the reticulogram network based on the least square approach using DARwin ver. 5 was used to identify the existence of common alleles (HAMMER *et al*., 2012).

RESULTS

The genetic diversity of populations

Table 2 summarizes the genetic diversity characteristics of *Cordia myxa* collected from seven different geographic locations. Considering gene diversity (0.23) and Shanon, information index (0.35), Sistan and Baluchestan, Iranshahr (population No. 1) had the greatest rate of polymorphism (79.59%). Khuzestan's Dezful (No. 6) community had the minimum proportion of polymorphism (23.33%), as well as the minimum values for Shanon, information index (0.111), and He (0.055).

Table 2. Genetic diversity parameters in the studied populations of Cordia myxa

Pop	N	Na	Ne	I	He	uHe	%P
pop1	9.000	0.517	1.143	0.355	0.233	0.084	79.59%
pop2	10.000	0.943	1.239	0.209	0.139	0.146	52.53%
pop3	11.000	1.092	1.256	0.237	0.155	0.162	61.72%
pop4	11.000	1.379	1.359	0.329	0.217	0.227	75.52%
pop5	10.000	1.115	1.295	0.257	0.171	0.180	55.57%
pop6	9.000	0.805	1.166	0.111	0.055	0.104	23.33%
pop7	10.000	0.724	1.167	0.143	0.095	0.100	38.74%

N = number of samples, Na = No. of Different Alleles, Ne = No. of Effective Alleles = 1 / (p^2 + q^2), I = Shannon's Information Index = -1* (p * Ln (p) + q * Ln(q)), He = Expected Heterozygosity = 2 * p * q, UHe = Unbiased Expected Heterozygosity = (2N / (2N-1)) * He, Where for Diploid Binary data and assuming Hardy-Weinberg Equilibrium, q = (1 - Band Freq.)^0.5 and p = 1 - q. *P%= percentage of polymorphism, populations*

Differentiation of demographic genetic

There was a statistically significant difference between the analyzed groups in AMOVA (PhiPT = 0.77, 0.001) and Gst analysis (0.899, 0.002), according to the findings. Within-population genetic variety accounts for 55% of overall genetic variation, while variation across populations accounts for just 45%. (Table 3). Significant differences between the analyzed groups were found by using pairwise AMOVA. After 999 permutations, we found high values of Hedrick's standardised fixation index (G'st = 0.555, P = 0.001) and Jost, differentiation indicator (D-est = 0.355, P = 0.001). Using these findings, it can be concluded that *Cordia myxa*'s regional communities are genetically distinct.

Table 3. Analysis of molecular variance (AMOVA) of the studied species.

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	12	396.576	28.327	4.082	45%	
Within Pops	58	494.767	8.530	8.530	55%	45%
Total	70	891.342		12.613	100%	

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

Genetic diversity within and across populations was seen using non-metric MDS plots of ISSR data (see Figure 2). Genetic diversity variables acquired from the community number 5 Kerman, Jiroft (Kerman province) supported the findings of the plots, which revealed more genetic variation within the population (Table 2).

The MDS plot of ISSR data following 900 permutations clearly demonstrated the genetic divergence and isolation of communities 5 and 4 from the others (Fig. 2). The genetic affinity between the other communities, such as populations 2 and 3, and 6, was evident. These communities' genetic and geographic distance were significantly correlated by the Mantel analysis following 5000 permutations (r = 0.81, P = 0.0001). Consequently, there was less gene flow between remote communities of *Cordia myxa*, and we observed isolation by distance (IBD) in this species.

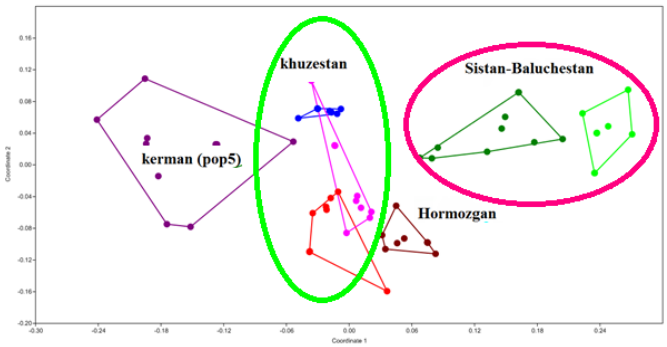

Fig. 2. MDS plot of *Cordia myxa* populations based on ISSR data. Note: Population numbers are according to Table 1.

Population classification based on genetic information

Dezful and Shush communities from Khuzestan province (no. 2 and 3) have a genetic resemblance of 0.93%. In contrast, groups from Sistan and Baluchestan, Iranshahr, and Kerman, Jiroft (Kerman province) have the minimum genetic similarity (0.711). (Pop. nos. 1 and 5).

The genetic affinity between populations

Fig. 3 shows the clustering of the communities analyzed by the NJ tree. In general, there were four primary groups. Our MDS plot showed that the Dezful and Shush communities (pop. nos. 2 and 3) were near one another (Fig. 2). As a result of isolation, drift, founder effects, and local selection, it was predicted that phenotypic features and allelic variation would vary across populations.

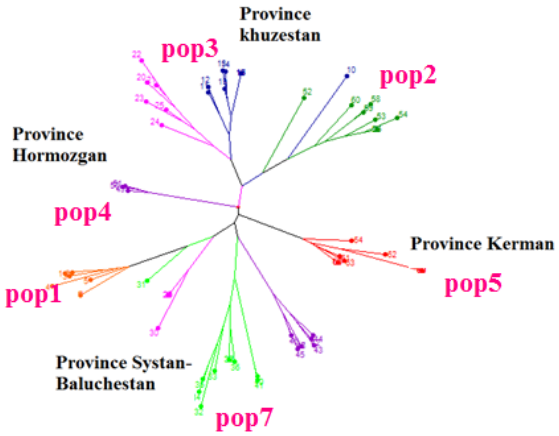

Fig. 3. NJ tree of ISSR data in *Cordia myxa* populations studied.

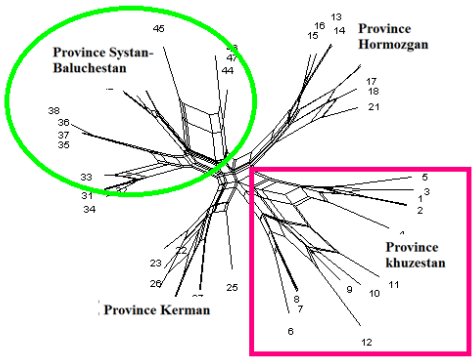

Fig.4. Neighbor Net diagram of ISSR data.

The AMOVA results were supported by the fact that we had practically full network isolation of the analyzed community. A significant distance separates the inhabitants of Bandar Abbas from Hormozgan province and Jiroft from Kerman province, which were different and stood out from the others. A closer genetic affinity and proximity to each other may be seen among the inhabitants of Dezful from Khuzestan province, Iranshahr, Chabahar (Sistan and Baluchestan Province) (Fig. 4).

The genetic organization of a population

In the STRUCTURE assessment, Evanno's test yielded k = 5 (Fig. 5). Results previously showed that this genetic classification was consistent with the NJ tree and NeighborNet graph. The presence of a high degree of within-population genetic differences demonstrated that the genetic content of the plants taken from different locations within each of the four analyzed provinces varied, with community 5 exhibited the minimum extent of within-population genetic diversity.

The mean Nm value was 2.55, and the Gst value was 0.9, based on the Nm assessment of genetic data from all communities. There was a lot of gene flow among the groups investigated, according to these statistics. Allele combinations in the examined populations were extremely comparable to those found in other populations that have been genetically admixed, as shown by the STRUCTURE plot created using the genetic admixture model (similarly colored segments). These prevalent similar alleles were either ancestral ones or the result of continuous gene flow between these groups. A considerable degree of genetic mixing was found among the examined groups, as evidenced by the Nm and STRUCTURE analyses. For all 80 ISSR loci, the Nm values ranged from 1 to 75, indicating a substantial extent of gene flow between them.

Alleles between communities 2 and 4 and between populations 1 and 4 were shared between groups 7 and 1, 3-5, according to a least-squares reticulogram (Fig. 6). Since these communities were so near to one another, this finding was consistent with the Neighbor-Net categorization. Genetic differentiation among *Cordia myxa* communities was clearly shown by the STRUCTURE plot based on the admixture model, showing that the common alleles make up a relatively small portion of the genomes throughout these communities.

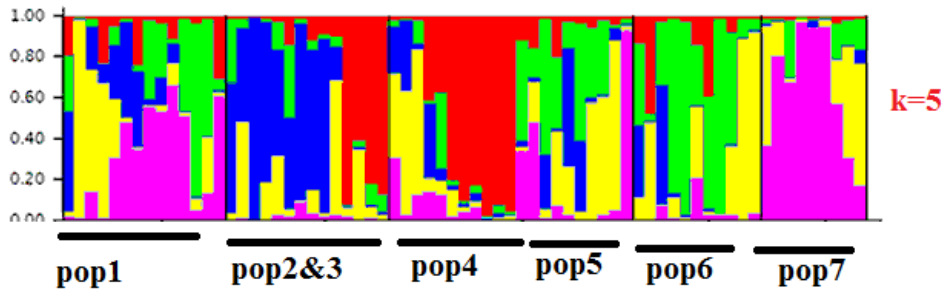

Fig. 5. STRUCTURE plot of *Cordia myxa populations* based on k = 5 of ISSR data.

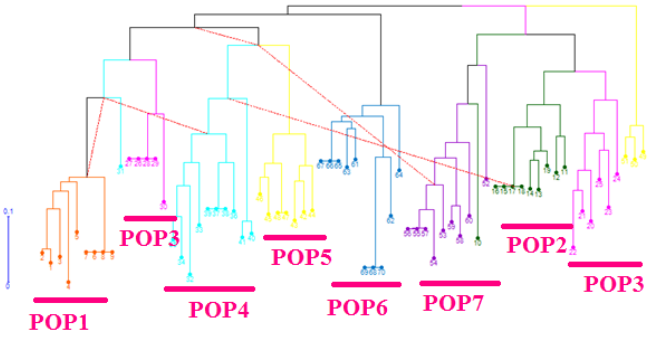

Fig. 6. Reticulogram of *Cordia myxa* populations based on least square method analysis of ISSR data. (Numbers below branches are population number according to Table 1).

DISCUSSION

Flora of Iran includes *Cordia myxa*, an uncommon species growing in the provinces of Hormozgan, Kerman, Khuzestan, and Sistan-Baluchistan (RIEDL, 1967, DAVIS, 1978). The genetic organization and comprehensive taxonomic data, on the other hand, were not known to us. The findings of the current research provided intriguing information on genetic diversity and genetic differentiation in the southern region of Iran.

The genetic diversity of the communities tested was low to moderate. As previously said, genetic variety is critical to the long-term survival of a species because it allows for the development of required adaptations to deal with alterations in the ecosystem. There is a strong correlation between the extent of genetic variation within a species and the manner of reproduction; the more open pollination/cross-breeding occurs, the more genetic variability is seen in the taxon under study (FREELAND *et al*., 2011; YIN *et al*., 2021). Given that *Cordia myxa* is mostly a self-pollinating species (HEDGE & LAMOND 1972), it is possible that the minimal degree of genetic diversity among communities is due to the intimate pattern of reproduction in this taxon.

High genetic and morphological diversity across populations is another well-known characteristic of self-pollinating species. The lack of gene flow or its scarcity across geographical populations in a single species is the cause of this phenomenon (FREELAND *et al*., 2011;). In line with the previously stated hypothesis, the results of this investigation also indicated considerable morphological and genetic variation across groups. The STRUCTURE plot, which found five distinct genetic subgroups in this population, as well as the consensual tree derived from morphological and genetic information, strongly corroborate this claim. Allele and phenotypic differences across communities may result from various factors, such as separation or drift, founder effects, or local selection. That said, there were quantifiable morphological differences across the investigated groups, and we don't determine how much of the morphological variation is influenced by genetics; it's possible that ecological factors are at play as well. To avoid introducing new taxonomic groupings below the species level, we only acknowledge these ecotypes to be distinct from each other.

In the Thar desert of Rajasthan, India, researchers NAGAR *et al.* (2013) studied the genetic diversity in Lehsua (*Cordia myxa*). The physicochemical characteristics of Lehsua's 15 provenances were examined for their variable features. Fruits per cluster, fruit diameter, pulp-to-seed ratio, fruit weight, and total soluble solids (TSS) showed genetic diversity. The majority of the variables, particularly fruits per cluster, fruit weight, fruit diameter, and TSS, exhibited a high degree of heritability and genetic progress as a mean percentage, suggesting that they are more genetically controlled. The high degree of variety identified in them may be used to enhance the genetics of this species.

Cordia dichotoma Frost F. accessions from the Nanded region in Maharashtra, India, show morphological and molecular differences in various places, according to NANDEDKAR and MULANI (2016). Leaf, fruit characteristics, and the pulp: stone proportion were shown to have a strong positive association. RAPD markers were used for the molecular evaluation. A maximum of 45 polymorphic scorable markers were obtained from the five primers that were evaluated. These markers were able to determine 67.86 percent of the polymorphism in these samples. There was a range of genetic variation shown by Jaccard's co-efficient, from 0.48 to 0.86. According to their findings, these accessions have a wide range of genetic variations. SIVLINGAM *et al* (2012) studied the genetic diversity of *C. dichotoma* from the arid area of Rajesthan and found a wide range of fruit morphological characteristics and molecular findings. The significance of the Randomly Amplified Polymorphic DNA (RAPD) marker has expanded in recent years since it provides valuable information regarding ecological and morphological changes throughout plant species. Across the span of dispersion of the analyzed groups, it is possible that demographic divergence is being influenced by isolation-by-distance. Distance may impede the spread of these groups, and gene flow is most probably to proceed between surrounding communities. Consequently, a higher degree of genetic similarity is observed between communities geographically near each other (HUTCHISON and TEMPLETON, 1999;).

Regional adaptation may accompany demographic divergence. SSR, AFLP, RAPD, ISSR, and other multilocus molecular markers are not immediately functioning as adaptive genes. Still, they may be connected to a gene or a genomic area with adaptive value. This is why we employ these markers for demographic genetic investigations.

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GENETIČKI DIVERZITET *Cordia myxa* L. ODREĐENO ISSR MARKERIMA

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Izvod

Veliki broj vrsta Cordia (Boraginaceae) su nađeni u tropskim i subtropskim delovima planete. *Cordia* vrste se spominju u narodnoj medicine ua lečenje brojnih obolenja različitih organa. Populaciono genetičke struktur, genetički diverzitet, i fenotipsko variranje su nepoznati za ovu vrstu u Iranu. Iz tog razloga smo sakupili morfološke i molekularne podatke za ovu biljnu vrstu. Sedamdeset biljaka su nasumično sakupljeni iz sedam geografskih oblasti rasprostranjenih nasumično kroz četiri provencije. Genetička varijabilnost populacija je analizirana. Rezultati strukture i K-prosek grupna analiza ukazuje na postojanje pet razdvojenih pulova gena u naciji , koje su bili izolovani jedni od drugih geografski, Genetičko i geografsko razdvajanje su pokazali da su korelisani sa Mantel analizom. Između oblasti, AMOVA pokazala značajnu genetičku razlike, sa unutra-populacijsko variranje od oko 55% ukupne varijabilnosti. Nastupajućei oplemenjivački i konzervativni napori za ovu biljku će imati koristi od ovih istraživanja.

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