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ARTICLE

Genetic diversity of *Salvia tomentosa* Miller (Lamiaceae) species using Touch-down Directed Amplification of Minisatellite DNA (Td-DAMD) molecular markers

Basel Saleh*

Department of Molecular Biology and Biotechnology, Atomic Energy Commission of Syria, Damascus, Syria.

ABSTRACT *Salvia tomentosa* Miller (Lamiaceae) a Mediterranean species has an important role in various pharmacological applications. To reveal genetic relationships among *S. tomentosa* natural populations, 35 samples were collected from different regions of Syria. Touch-down Directed Amplification of Minisatellite DNA (Td-DAMD) markers have been investigated for this goal. Td-DAMD assay produced 158 total bands of which 131 (82.911%) were polymorphic with a mean polymorphic information content (PIC) value of 0.264 and a mean marker index (MI) value of 2.269. Clustering profile based on Td-DAMD data showed that samples were grouped into two main clusters; the first cluster included Lattakia samples which split into two subclusters regardless their altitudes over the sea level. Whereas, the second cluster included Tartous and Hama samples. Td-DAMD assay successfully discriminate among the tested 35 samples belonged to the *S. tomentosa* natural population. **Acta Biol Szeged 63(2):135-141 (2019)**

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***Corresponding author**
E-mail: ascientific3@aec.org.sy

Introduction

Salvia tomentosa Miller [(*S. grandiflora* Etling) (incl. *S. nusairiensis* Post)] is a perennial flowering plant belonging to the Lamiaceae family. This species with a common name of balsamic sage known as tomentose sage is endemic in Lebanon, Syria and Palestine or wholly or partially restricted species to East Mediterranean area (Mouterde 1983; Ramadan-Jaradi and Matar 2012). Its native range is South-Eastern Europe to Transcaucasia, including Albania, Bulgaria, East Aegean Islands, Greece, Crimea, Lebanon-Syria, Transcaucasia, Turkey and former Yugoslavia (Govaerts 2003).

It worth to mention that plant population genetic structure reflects various interaction processes involving various phenomena [long-term evolutionary history of the species (shift in distribution, habitat fragmentation, and population isolation), mutations, genetic drifts, mating system, gene flow, and selection]. Genetic diversity background of wild resources is considered as backgrounds for establishing plant breeding programs: these result improvement and sustainable production that accumulate various desirable traits together (Liber et al. 2014). Phylogeographic survey of *S. tomentosa* as an endemic plant in East Mediterranean area is important for its conservation implications.

Various *Salvia* species have been described as sources of extracts and metabolites with wide range of biological (e.g., antibacterial, antiviral, antifungal, antioxidant, and cytotoxic) activities (Georgiev et al. 2011; Yilar et al. 2018). Of which, *S. tomentosa* is displayed antibacterial and antioxidant (Hanlidou et al. 2014; Bayan and Genc 2017) and antifungal (Bayan and Genc 2017; Yilar et al. 2018) effects. Furthermore, it is used as condiment and tea around the world (Georgiev et al. 2011).

Molecular characterization of a given species could consider as a potent tool in its conservation and also in breeding programs. Previous studies investigated the phylogenetic relationships of some *Salvia* species within populations of a given genus. Such examples are when, e.g., in *S. hispanica* L. Random Amplified Polymorphic DNA (RAPD) markers (Cahill 2004), in *S. lachnostachys* Inter Simple Sequence Repeat (ISSR) markers (Erbano et al. 2015), in *S. lutescens* var. *intermedia* nuclear ribosomal DNA and plastid DNA sequences (Takano 2017), in *S. divinorum* Chloroplast Simple Sequence Repeats (cpSSR's) (Casselman 2016), in *S. japonica* chloroplast and nuclear ribosomal DNA sequences and allozyme polymorphisms (Sudarmono and Okada 2008) and in *Salvia* sp. Directed Amplification of Minisatellite Region DNA (DAMD) markers (Karaca et al. 2008) have been investigated. Several such studies targeted *S. miltiorrhiza* Bge, e.g., RAPD (Guo et al. 2002), Amplified Fragment Length Polymorphism (AFLP) (Wen et al. 2007), Sequence-related Amplified Polymorphism (SRAP) and ISSR (Song et al.

2010), nrDNA Internal Transcribed Spacer (nrDNA ITS) sequences (Zhang et al. 2012) and ISSR (Zhang et al. 2013) markers have been investigated. Recently, Fabriki-Ourang and Yousefi-Azarkhanian (2018) applied Target Region Amplification Polymorphism (TRAP) and Conserved Region Amplification Polymorphism (CoRAP) markers in *Salvia* sp.

Previously, the Touch-down Directed Amplification of Minisatellite DNA Polymerase Chain Reaction (Td-DAMD-PCR) approach has been successfully employed in phylogenetic studies in *Salvia* species (Ince and Karaca 2012), in common bean landraces (Ince and Karaca 2011), in *Allium* sp. (Deniz et al. 2013), in carnation cultivars (Ince and Karaca 2015) as well as in commercial cotton (Gocer and Karaca 2016).

Despite of the importance of *S. tomentosa* species in various pharmaceutical and industrial applications, until now limited attention has been paid to its genetic variation. Thereby, this study has been focused on its genetic variability among different samples belonged to the *S. tomentosa* natural population.

Materials and methods

Plant materials

Leaf samples of *S. tomentosa* natural population included 35 samples (29 samples from Lattakia, 4 samples from Tartous and 2 samples from Hama); these represented different geographical regions in Syria which varied in term of altitude and annual rainfall (Table 1). Sample collection has been carried out during blooming stage. Wild *Stachys nivea* L. (Lamiaceae; collected from Damascus) was included as a outside reference.

Table 1. Original sites in term of their altitude (m) and annual rainfall (mm) in which the *S. tomentosa* natural population was collected.

Collection site	Code	Altitude (m)	Annual rainfall (mm)
Lattakia	L1	157	800
	L2	250	1000
	L3	346	1000
	L4	400	1100
	L5	420	1100
	L6	400	1100
	L7	500	1100
	L8	520	1100
	L9	482	1100
	L10	680	1250
	L11	134	800
	L12	153	800
	L13	198	800
	L14	123	800
	L15	150	800
	L16	114	800
	L17	347	800
	L18	194	850
	L19	350	1400
	L20	594	1000
	L21	540	1100
	L22	395	1000
	L23	465	850
	L24	212	1400
	L25	195	1400
	L26	200	1400
	L27	854	1500
	L28	830	1350
Tartous	L29	1100	1300
	T30	377	1500
	T31	240	1350
	T32	511	1500
	T33	346	1400
Hama	H34	300	1400
	H35	400	1500
Damascus	SN	970	240

DNA extraction

Total genomic DNA of was extracted according to CTAB (cetyltrimethylammonium bromide) protocol as previously reported by Doyle and Doyle (1987). DNA concentration was determined by DNA fluorimeter. Extracted DNA was kept at -80 °C until further use.

Td-DAMD-PCR test and data analysis

Sixteen DAMD primers (Table 2) were tested to investigate DNA genetic relationships among the studied samples. Td-DAMD amplification was performed according to Ince and Karaca (2012) in a total volume of 25 µl. PCR amplification was carried out in a T-gradient Thermal Cycler (Bio-Rad, USA) programmed as following: 1 cycle for 4 min at 94 °C, followed by ten cycles of pre-PCR involving of 30 s at 94 °C for denaturation, 45 s at 60 °C for annealing, and 3 min at 72 °C for extension. Annealing temperature was reduced 0.5 °C/cycle for the first 10 cycles. Then 30 cycles at a constant 55 °C as annealing temperature; followed by final extension at 72 °C for 10 min. Final PCR products were separated on a 2% ethidium bromide-stained agarose gel (Bio-Rad) in 0.5× Tris-borate-EDTA (TBE) buffer. Electrophoresis was carried out at 85 V for 2.5 h and visualized with a UV transilluminator. A VC 100bp Plus DNA Ladder (Vivantis) standard was used to estimate molecular weight of Td-DAMD-PCR amplification products.

The presence or absence of each band size was manually scored as 1 or 0, respectively. The Unweighted Pair

Table 2. Selected DAMD primers used in the current study.

Primer Nr.	Primer name	Primer sequence 5'-3'
1	URP1F	ATCCAAGGTCCGAGACAACC
2	URP2R	CCCAGCAACTGATCGCACAC
3	URP4R	AGGACTCGATAACAGGCTCC
4	URP9F	ATGTGTGCGATCAGTTGCTG
5	URP38F	AAGAGGCATTCTACCACCAC
6	HBV3	GGTGAAGCACAGGTG
7	14C2	GGCAGGATTGAAGC
8	6.2H(+)	AGGAGGAGGGGAAGG
9	M13	GAGGGTGGCGGCTCT
10	HBVb	GGTGTAGAGAGAGGGGT
11	URP2F	GTGTGCGATCAGTTGCTGGG
12	URP6R	GGCAAGCTGGTGGGAGGTAC
13	URP17R	AATGTGGGCAAGCTGGTGGT
14	M13	GAGGGTGGCGGTTCCT
15	HVA	AGGATGGAAAGGAGGC
16	HVV	GGTGTAGAGAGGGGT

Group Mean Arithmetic average (UPGMA) was constructed based on percent disagreement values (PDV) using Statistica program (Statistica 2003). Whereas, polymorphic information content (PIC) was determined according to the following formula:

$$PIC = 1 - \Sigma(Pij)^2$$

where Pij is the frequency of the i-th pattern revealed by the j-th primer summed across all patterns revealed by the primers (Botestein et al. 1980).

Moreover, marker index (MI) was determined as reported by Powell et al. (1996) according to the following formula:

$$MI = PIC \times \eta\beta$$

where PIC is the mean PIC value, η the number of bands, and β is the proportion of polymorphic bands.

Results

Td-DAMD amplification using sixteen DAMD primers produced 158 total bands of which 131 bands (82.911%) were polymorphic (Table 3). PCR products sizes yielded by Td-DAMD assay ranged between 150-3000 bp. Td-DAMD polymorphism profiles yielded by HBVb (a), URP17R (b) and HVA (c) DAMD primers for natural *S. tomentosa* population are presented in Fig. 1. Total band numbers ranged between 5 (URP2F) and 15 (M13 & HVA) bands with an average of 9.875 bands/primer (Table 3).

Table 3. Td-DAMD amplified fragments scored in term of Total Bands (TB), Polymorphic Bands (PB), Polymorphic % (P%), Polymorphic Information Content (PIC) and Marker Index (MI).

Primer name	TB	PB	P%	PIC	MI
URP1F	13	10	76.923	0.189	1.890
URP2R	11	10	90.909	0.386	3.860
URP4R	6	6	100.000	0.408	2.448
URP9F	7	6	85.714	0.296	1.776
URP38F	10	10	100.000	0.365	3.650
HBV3	7	6	85.714	0.216	1.296
14C2	10	8	80.000	0.197	1.576
6.2H(+)	14	9	64.286	0.225	2.025
M13	9	7	77.778	0.280	1.960
HBVb	10	9	90.000	0.390	3.510
URP2F	5	5	100.000	0.244	1.220
URP6R	8	4	50.000	0.093	0.372
URP17R	10	6	60.000	0.119	0.714
M13	15	15	100.000	0.319	4.785
HVA	15	13	86.667	0.290	3.770
HVV	8	7	87.500	0.207	1.449
Sum	158	131			
Average	9.875	8.188	83.468	0.264	2.269

The number of polymorphic bands ranged between 4 (URP6R) and 15 (M13) with an average of 8.188 polymorphic bands/primer (Table 3).

PIC value ranged between 0.093 (URP6R) and 0.408(URP4R) with a mean average of 0.264. The MI value ranged between 0.372 (URP6R) and 4.785 (M13) with a mean average of 2.269 (Table 3).

The relatedness degree among natural populations of *S. tomentosa* (Fig. 2) was calculated based on the Unweighted Pair Group Mean Arithmetic average (UPGMA) using Statistica program and Percent Disagreement Values (PDV). For the 35 *S. tomentosa* samples tested, two main clusters were revealed: one cluster involved the 29 *S. tomentosa* samples collected from Lattakia which divided further two subclusters. The first subcluster included 6 samples (L20, L21, L22, L27, L28 and L29); of which L27 and L28 were close with a PDV value of 0.10. The second subcluster was composed of the remaining 23 samples. Within Lattakia samples, the highest PDV values (0.37) were recorded between L2 and L29, L5 and L29, as well as between L6 and L29. The lowest PDV values (0.08) were recorded between L4 and L5 and between L6 and L7 samples.

The second main cluster was also separated into two subclusters, the first of which included Tartous samples T30 and T31, while the second one included T32 and T33, as well as the samples collected from Hama (H34 and H35).

Overall, the lowest PDV was 0.03 between H34 and

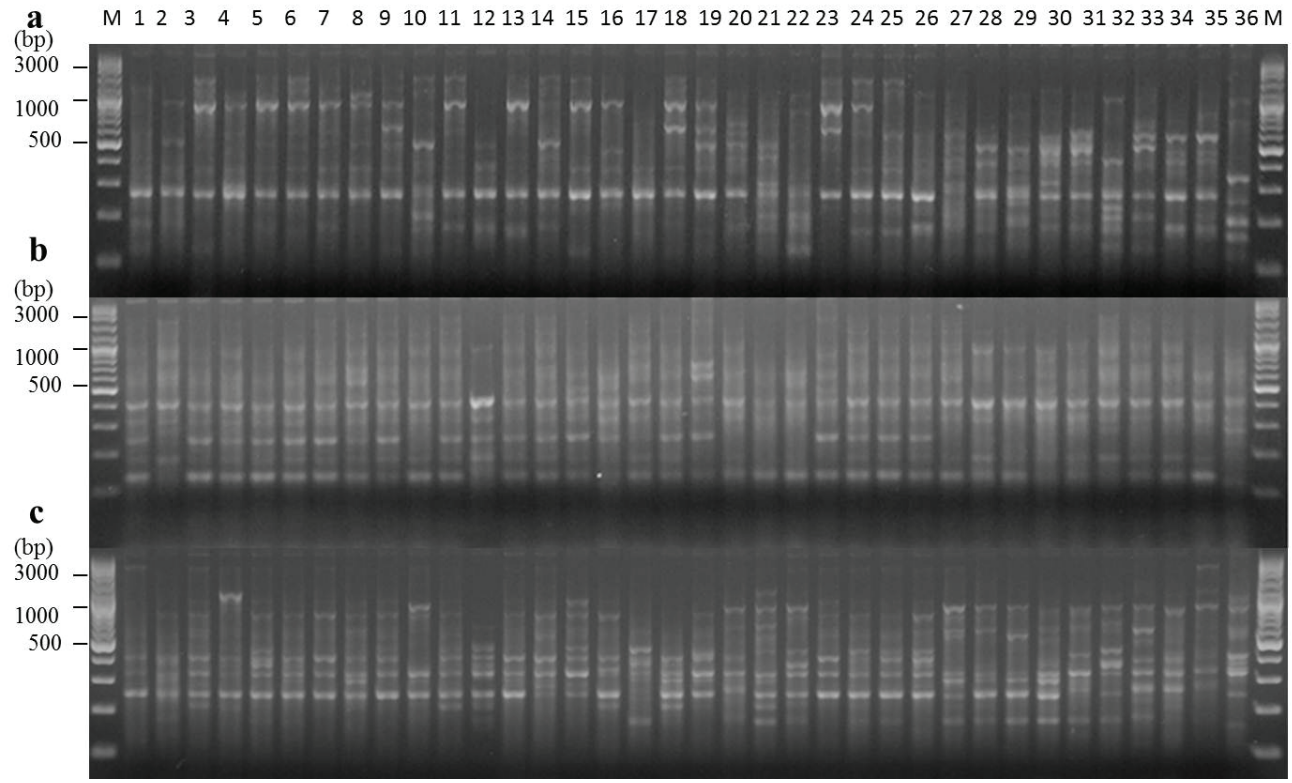

Figure 1. Td- DAMD polymorphism profile yielded by HBVb (a), URP17R (b) and HVA (c) DAMD primers for natural *S. tomentosa* population; 1-29: samples collected from Lattakia, 30-33: samples collected from Tartous and 34-35: samples collected from Hama; 36: *S. nivea* as an outside reference. M: A VC 100bp Plus DNA Ladder (Vivantis).

H35, whereas, the highest one was 0.44 between L15 and T32 followed by 0.43 between L4 and T30, L2 and T32 and between L15 and T30. These values were comparable with the mean PDV average of 0.25 observed for all tested samples. Based on the mean PDV average of 0.25, the current study could suggest that the 29 *S. tomentosa* samples collected from Lattakia were genetically distinct from the samples collected from Tartous and Hama.

Discussion

Genetic relationship investigation for 35 *S. tomentosa* samples collected from different regions in Syria has been performed using Td-DAMD markers. Td-DAMD assay produced 158 total bands (markers) of which 131 (82.911%) were polymorphic. This was in agreement with the results of Guo et al. (2002) who reported P% of 80.44% in *S. miltiorrhiza* Bge using RAPD analysis with 11 primers.

Cahill (2004) reported that DNA genetic diversity in *S. hispanica* L. samples (collected from Mexico) revealed by RAPD analysis, was higher among natural samples compared to cultivated (domesticated) once. However, Song et al. (2010) reported low genetic diversity in *S. miltiorrhiza* using SRAP and ISSR markers. Moreover, Zhang et al. (2012) applied nrDNA ITS sequences to investigate phylogenetic relationships within *S. lachnostachys* population in relation to other *Salvia* taxa. They reported that *S. miltiorrhiza* f. alba, *S. bowleyana*, *S. cavaleriei* var. *simplicifolia*, and *S. yunnanensis* were the most closely related taxa to *S. miltiorrhiza* species; they could be considered as new gene germplasm candidates for *S. miltiorrhiza*. Ince and Karaca (2012) reported a total of 17, 13, 9, 11 and 20 species-specific Td-DAMD-PCR markers for *S. sclarea, S. fruticosa, S. tomentosa, S. dichroantha* and *S. virgate,* respectively, using 22 DAMD primers.

Zhang et al. (2013) reported the high importance of DNA genetic diversity of *S. miltiorrhiza* (studied by ISSR markers) in plant breeding programs. Erbano et al. (2015) reported genetic variation in *S. lachnostachys* populations collected from Brazil using ISSR-based markers. ISSR marker yielded 159 bands of which 152 (95.6%) were polymorphic; reflecting high genetic diversity level for *S. lachnostachys.* Takano (2017) reported deference among *Salvia lutescens* var. *intermedia* and its allies based on morphological traits and molecular analyses of nuclear

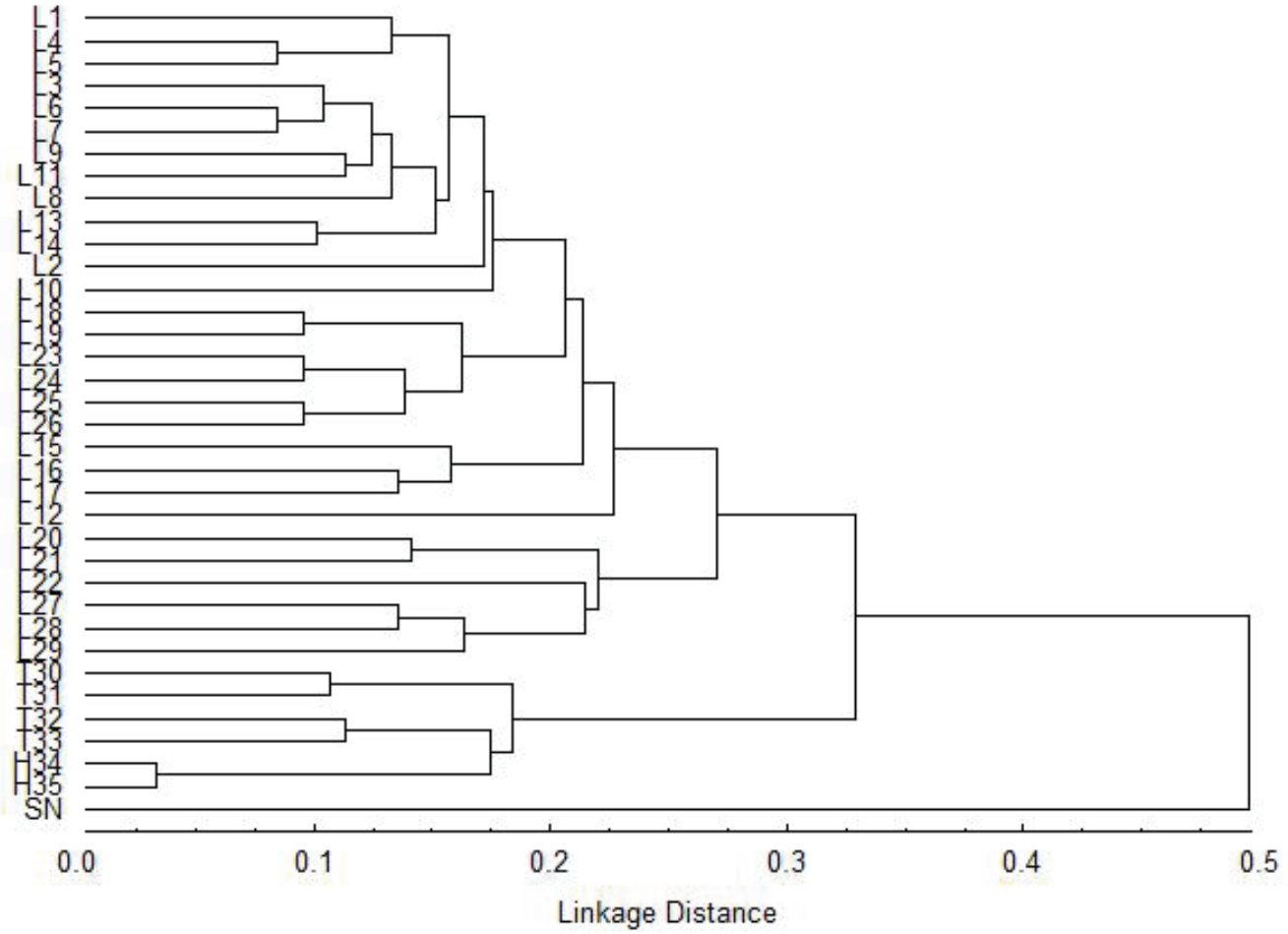

Figure 2. UPGMA cluster analysis-based on the percent disagreement value (PDV) for Td-DAMD fingerprints showing genetic relationship for natural *S. tomentosa* population.

ribosomal DNA and plastid DNA sequences.

Td-DAMD marker has been successfully employed for fingerprinting of other plant species, e.g., Deniz et al. (2013) used 10 DAMD primers and reported 454 total bands of which 245 (53.96%) were polymorphic between *Allium elmaliense* and *Allium cyrilli*. They found that *A. elmaliense* Deniz & Sumbul is a distinct species and not a synonym of *A. cyrilli* Ten. Ince and Karaca (2015) reported a total of 858 bands of which 570 (66.43%) were polymorphic and PIC ranged from 0.120 to 0.341, with an average of 0.249/primer when genetic variation of 16 carnation cultivars were studied using 12 DAMD primers. Gocer and Karaca (2016) reported 120 total bands, 42 (35%) of which were polymorphic among 26 cotton samples using 10 DAMD primers.

Recently, Fabriki-Ourang and Yousefi-Azarkhanian (2018) applied TRAP and CoRAP markers to evaluate genetic diversity among 25 *Salvia* ecotypes/species in Iran. They reported that the 6 primer combinations of TRAP gave a mean average total bands of 181 of which 15 were polymorphic with a mean PIC and MI values of 0.42 and 3.05, respectively. Whereas, CoRAP with 6 primer combinations gave a mean average total bands of 153 bands of which 15 were polymorphic with a mean PIC and MI values of 0.377 and 2.49, respectively.

It has been demonstrated that genetic structure in plant populations is influenced by various factors like, e.g., reproductive biology, gene flow, seed dispersal and natural selection (Erbano et al. 2015), of which reproductive biology plays a fundamental role (Zhao et al. 2007). On the other hand, the higher observed diversity previously observed for *Salvia* within populations was related to the outcrossing strategy (Bijlsma et al. 1994). This is particularly probable with high population densities where outcrossing rates are higher. Recently, Radosavljević et al. (2019) reported that interspecies hybridization affects genetic and epigenetic alterations through DNA methylation patterns modification. Consequently, ap-

pearance of new phenotypic variants in hybrid progeny was more originated from epigenetic compared to genetic recombination.

It worth to mention that moderate genetic variation observed within natural population of *S. tomentosa* in this study could be attributed to one or more following factors: i) big size of this population; ii) occurrence a huge number of individuals in this population; iii) outcrossing process as similarly reported for *S. officinalis* (Stojanović et al. 2015) or to spontaneous hybrids occurred in the wild or in cultivated *Salvia* (Tychonievich and Warner 2011); or to be interspecific hybrid as similarly reported in *S. divinorum* (Casselman 2016) or to reproductive biology, gene flow, seed dispersal and nature selection (Erbano et al. 2015) encouraged efficient gene flow. This observation could support by the phylogenetic study done by Walker et al. (2004) suggested that the *Salvia* was polyphyletic involved 2-3 distinct lineages. This phenomenon could lead to inbreeding expansion and higher heterozygosity, leading consequently to increase their adaptive potential. This could explain their distribution wide spare in the selected sites at different altitudes and rain full.

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