

## Morphometric analysis and sequence related amplified polymorphism determine genetic diversity in *Salvia* species

Abdul SHAKOOR<sup>1,2</sup>, Fang ZHAO<sup>1,2\*</sup>, Gul ZAIB<sup>3,4</sup>, Wuyang LI<sup>1</sup>,  
Xincan LAN<sup>1</sup>, Somayeh ESFANDANI-BOZCHALOYI<sup>5</sup>

<sup>1</sup>Henan University, College of Environment and Planning, Kaifeng, 475004, Henan, China; [abdul\\_shakoor954@yahoo.com](mailto:abdul_shakoor954@yahoo.com);  
[zhaofang@lreis.ac.cn](mailto:zhaofang@lreis.ac.cn) (corresponding author); [104753190127@henu.edu.cn](mailto:104753190127@henu.edu.cn); [lxc0113@henu.edu.cn](mailto:lxc0113@henu.edu.cn)

<sup>2</sup>Key Laboratory of Geospatial Technology for the Middle and Lower Yellow River Regions, Ministry of Education, Kaifeng 475004, Henan, China

<sup>3</sup>Yangzhou University, Institute of Epigenetics and Epigenomics, Yangzhou, China; [gulzaib\\_chsan@hotmail.com](mailto:gulzaib_chsan@hotmail.com);

<sup>4</sup>Yangzhou University, College of Veterinary Sciences, Yangzhou, China

<sup>5</sup>Shahid Beheshti University, Faculty of Life Sciences and Biotechnology, Department of Plant Sciences, Tehran, Iran; [somayehesfand@yahoo.com](mailto:somayehesfand@yahoo.com)

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### Abstract

*Salvia* species is a member of the Lamiaceae family, and it also possesses medicinal and horticulture values. The genetic diversity was assessed through sequence-related amplified polymorphism. To uncover genetic diversity and species characteristics in *Salvia* species were studied through a combination of morphological and molecular data. One hundred forty-five individuals related to 30 *Salvia* were collected in 18 provinces. A total of 157 (Number of total loci) (NTL) DNA bands were produced through polymerase chain reaction (PCR) from 30 *Salvia* species. These bands were produced with the combinations of 10 selective primers. The total number of amplified fragments ranged from 10 to 20. The predicted unbiased heterozygosity (H) varied between 0.11 (*Salvia urmiensis*) and 0.31 (*Salvia limbata*). High Shannon's information index was detected in *Salvia limbata*. The genetic similarities between 30 species are estimated from 0.46 to 0.91. Clustering results showed two major clusters. According to the SRAP (Sequence-related amplified polymorphism) markers analysis, *Salvia hydrangea* and *Salvia sharifii* had the lowest similarity. *Salvia bracteata* and *Salvia suffruticosa* were genetically dissimilar to each other. This study also detected a significant signature of isolation by distance. Present results showed that sequence-related amplified polymorphism has the potential to decipher genetic affinity between *Salvia* species. Current results have implications in biodiversity and conservation programs. Besides this, present results could pave the way for selecting suitable ecotypes for forage and pasture purposes in Iran.

**Keywords:** gene flow; labiatae; morphometric analysis; *Salvia*; sequence-related polymorphism

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### Introduction

*Salvia* species is a member of the Lamiaceae family, and the high diversity of *Salvia* L. has been reported in Western Asia, Eastern Asia, and South America (Walker *et al.*, 2004). Iran is considered one of *Salvia*'s

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important regions, and 19 species are endemic to Iran (Jamzad, 2013). The majority of species are aromatic and possess medicinal and horticultural values (Safaei *et al.*, 2016). Some of the *Salvia* species are also pharmacologically important and possess anti-inflammatory (Hosseinzadeh *et al.*, 2003) and gastro-protective properties (Mayer *et al.*, 2009). Scientific data shows that member of *Salvia* species, such as *Salvia miltiorrhiza*, is widely used in folk and traditional medicine and used in cardiovascular treatment (Wang *et al.*, 2009).

Several systematics and genetic diversity studies were carried out via molecular markers to address population structure, genetic diversity, and phylogenetic relations between the species (Robarts and Wolfe, 2014). Some experiments have been performed to determine genetic diversity within the *Salvia* genus (Song *et al.*, 2010; Erbano *et al.*, 2015). In the past, molecular methods such as Inter Simple Sequence Repeats (ISSR) and Random Amplification Polymorphic DNA (RAPD) have been used to study genetic diversity in *Salvia* (Song *et al.*, 2010; Javan *et al.*, 2012; Peng *et al.*, 2014; Erbano *et al.*, 2015). Sequence-related amplified polymorphism (SRAP) is PCR-based marker system. It is an efficient and simple marker system to study gene mapping and gene tagging in plant species (Li and Quiros, 2001). SRAP are potential markers to assess plant systematics and genetic diversity studies (Robarts and Wolfe, 2014). Previously, Wu *et al.* (2010) assessed genetic diversity and population structure in *Pogostemon cablin* with SRAP markers' aid. SRAP markers were successfully implemented in Lamiaceae family to study natural populations and variations within the family (Saebnazar and Rahmani, 2013; Talebi *et al.*, 2015). These past studies showed that molecular markers, including SRAP markers, are efficient in investigating genetic diversity analyses and the phylogenetic relationship among *Salvia* species in the Lamiaceae family. Indeed, molecular markers are efficient methods to study genetic diversity (Esfandani-Bozchaloyi and Sheidai, 2018). Genetic diversity helps plant species to survive and adapt against constantly changing environmental conditions (Pauls *et al.*, 2013).

In order to develop conservation strategies and proper utilization of plant genetic resources, it is crucial to characterize plant species based on genetic studies (Kharazian *et al.*, 2015), particularly this approach may assist in understanding genotypes of the geographically differentiated genus, such as *Salvia* (Song *et al.*, 2010; Erbano *et al.*, 2015). In Iran, most of the studies have been conducted to assess the medicinal potential of the *Salvia* species. The majority of the studies have addressed phytochemical and antimicrobial aspects. Therefore, we implemented the morphometric analysis and Sequence related amplified polymorphism to study genetic diversity. According to current knowledge, this is the first study that successfully documented genetic diversity based on novel sequence-related amplified polymorphism markers.

The present study investigated the molecular variation of 30 *Salvia* species in Iran. The study's objectives were: estimate genetic diversity; evaluate population relationships using WARD approaches. Current results have implications in breeding and conservation programs.

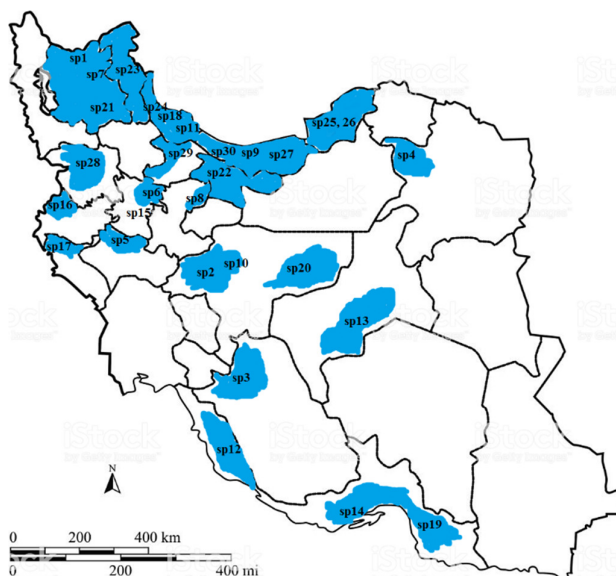
## Materials and Methods

### *Plants collection*

One hundred forty-five (145) individuals of *Salvia* were sampled. These individuals were recorded based on eco-geographical different features. Thirty *Salvia* species in East Azerbaijan, Lorestan, Kermanshah, Guilan, Mazandaran, Golestan, Yazd, Esfahan, Tehran, Arak, Hamadan, Kurdistan, Ilam, Bandar Abbas, Ghazvin, Khorasan, and Ardabil Provinces of Iran were selected and sampled during July-August 2017-2019 (Table 1). Morphometric and SRAP analyses on 145 plant accessions were carried out. One to twelve samples from each population belonging to 30 different species were selected based on other eco-geographic characteristics. Samples were stored at - 20 °C till further use. Detailed information about samples locations and geographical distribution of species are mentioned (Table 1 and Figure 1).

**Table 1.** Locality and geographical information of *Salvia* species

No.	Species names	Locations	Latitude	Longitude	Altitude
Sp1	<i>Salvia aristata</i> Aucher ex Benth.	East Azerbaijan, Kaleybar, Shojabad	38 ° 52'37"	47 ° 23' 92"	1144
Sp2	<i>S. eremophila</i> Boiss.	Esfahan, Ghameshlou, Sanjab	32°50'03"	51°24'28"	1990
Sp3	<i>S. santolinifolia</i> Boiss.	Fars, Jahrom	29°20'07"	51° 52'08"	1610
Sp4	<i>S. tebesana</i> Bunge	Khorasan, Tabas	29°20'07"	51° 52'08"	220
Sp5	<i>S. bracteata</i> Banks & Sol	Lorestan, Oshtorankuh, above Tihun village	33° 57'12"	47° 57'32"	2500
Sp6	<i>S. suffruticosa</i> Montb. & Aucher	Hamedan, Nahavand	34 ° 52'373"	48 ° 23' 92"	2200
Sp7	<i>S. dracocephaloides</i> Boiss.	East Azerbaijan, Kaleybar, Cheshme Ali Akbar	38 ° 52'373"	47 ° 23' 92"	1144
Sp8	<i>S. hydrangea</i> DC. ex Benth.	Arak, Komayjan, Pass of Chehregan village, the margin road	35°50'03"	51°24'28"	1700
Sp9	<i>S. multicaulis</i> Vahl.	Mazandaran, Haraz road, Emam Zad-e-Hashem	36°14'14"	51°18'07"	1807
Sp10	<i>S. syriaca</i> L.	Esfahan, Fereydunshahr	32°36'93"	51°27'90"	2500
Sp11	<i>S. viridis</i> L.	Guilan, Sangar, Roadside	37°07'02"	49°44'32"	48
Sp12	<i>S. mirzayani</i> Rech. f. & Esfand.	Boushehr, Dashtestan	28°57'22"	51°28'31"	430
Sp13	<i>S. macrosiphon</i> Boiss.	Yazd, Khatam	30°07'24"	53° 59'06"	2178
Sp14	<i>S. sharifii</i> Rech. f. & Esfand.	Bandar Abbas, Hormozgan	28°57'22"	51°28'31"	288
Sp15	<i>S. reuterana</i> Boiss.	Hamedan, Alvand	34°46'10"	48°30'00"	1870
Sp16	<i>S. palaestina</i> Benth.	Kermanshah, Islamabad	35° 37'77"	46° 20'25"	1888
Sp17	<i>S. sclareopsis</i> Bornm. ex Hedge	Ilam, Ilam	33°47'60"	46 °07'58"	1250
Sp18	<i>S. spinose</i> L.	Guilan, Lahijan	37°07'02"	49°44'32"	48
Sp19	<i>S. compressa</i> Vent.	Bandar Abbas, Hormozgan	28°57'22"	51°28'31"	288
Sp20	<i>S. sclarea</i> L.	Esfahan, Ghameshlou, Sanjab	32°36'93"	51°27'90"	2500
Sp21	<i>S. aethiopsis</i> L.	Azerbaijan, 78 km from Mianeh to Khalkhl.	37°38_53	48 ° 36_11	1500
Sp22	<i>S. microstegia</i> Boiss. & Bal.	Tehran, Darband	35°36'93"	51°27'90"	1700
Sp23	<i>S. xanthocheila</i> Boiss. ex Benth.	Ardabil, Khalkhal	37°38_53	48 ° 36_11	1958
Sp24	<i>S. limbata</i> C.A. Mey.	Guilan, Gole rodbar, Road side	37 ° 09 45"	49 ° 55 39 "	15
Sp25	<i>S. chloroleuca</i> Rech. f. & Aell.	Golestan, Ramian	37 ° 09 45"	55 ° 55 39 "	1320
Sp26	<i>S. virgate</i> Jacq.	Golestan, Ramian	37 ° 09 45"	55 ° 55 39 "	1320
Sp27	<i>S. nemorosa</i> L.	Mazandaran, Chalos	36°14'14"	51°18'07"	1807
Sp28	<i>S. urmiensis</i> Bunge	Kurdistan, Sanandaj	37 ° 09 45"	55 ° 55 39 "	1320
Sp29	<i>S. oligophylla</i> Aucher ex Benth.	Ghazvin to Hamedan just after Avaj	35°36'93"	51°27'90"	2100
Sp30	<i>S. verticillata</i> L.	Mazandaran Jاده Chalous	36°14'14"	51°18'07"	1807



**Figure 1.** Provinces and collection sites of *Salvia*, Iran

sp1= *Salvia aristata*; sp2= *S. eremophila*; sp3= *S. santolinifolia*; sp4= *S. tebesana*; sp5= *S. bracteata*; sp6= *S. suffruticosa*; sp7= *S. dracocephaloides*; sp8= *S. hydrangea*; sp9= *S. multicaulis*; sp10= *S. syriaca*; sp11= *S. viridis*; sp12= *S. mirzayanii*; sp13= *S. macrosiphon*; sp14= *S. sharifii*; sp15= *S. reuterana*; sp16= *S. palaestina*; sp17= *S. sclareopsis*; sp18= *S. spinose*; sp19= *S. compressa*; sp20= *S. sclarea*; sp21= *S. aethiopsis*; sp22= *S. microstegia*; sp23= *S. xanthocheila*; sp24= *S. limbata*; sp25= *S. chloroleuca*; sp26= *S. virgate*; sp27= *S. nemorosa*; sp28= *S. urmiensis*; sp29= *S. oligophylla*; sp30= *S. verticillata*

#### *Morphological studies*

Each species was subjected to morphometric analysis, and twelve samples per species were processed. Qualitative (9) and quantitative (13) morphological characters were studied. Data were transformed before calculation. Different morphological characters of flowers, leaves, and seeds were studied. Ordination analyses were conducted while using Euclidean distance (Podani, 2000).

#### *Sequence-related amplified polymorphism method*

Fresh leaves from one to twelve plants, were randomly used. These were dried with silica gel powder. Genomic DNA was extracted while following the previous protocol (Esfandani-Bozchaloyi *et al.*, 2019). SRAP assay was performed as described previously (Li and Quiros, 2001). Ten SRAP in different primer combinations (PCs) were used. A 25 $\mu$ l volume containing ten mM of Tris-HCl buffer at pH 8; 50 mM of KCl; 1.5 mM of MgCl<sub>2</sub>; 0.2 mM of each dNTP (Bioron, Germany); 0.2  $\mu$ M of single primer; 20 ng of genomic DNA and 3 U of Taq DNA polymerase (Bioron, Germany) were subjected to PCR reactions. The overall reaction volume consisted of 25  $\mu$ l. For the PCR reaction, the Techne thermocycler (Germany) was used. In the initial denaturation step, the temperature was kept at 94 °C for 5 minutes. It was followed by 40 cycles for 1 minute at 94 °C, 1 minute at 52-57 °C, and 2 minutes at 72 °C. In the final extension step, the cycle duration was kept 7-10 min at 72 °C. Staining was performed with the aid of ethidium bromide. DNA bands/fragments were compared against a 100 bp molecular size ladder (Fermentas, Germany).

#### *Data analyses*

In the current study, the UPGMA (Unweighted paired group using average) ordination method was implemented to assess morphological characters. ANOVA (Analysis of variance) was conducted to assess morphological differences among species. Principle coordinates analysis (PCoA) was implemented to identify variable morphological characters in *Salvia* species. Multivariate statistical analyses, i.e., PCoA analysis, were performed in PAST software version 2.17 (Hammer *et al.*, 2001).

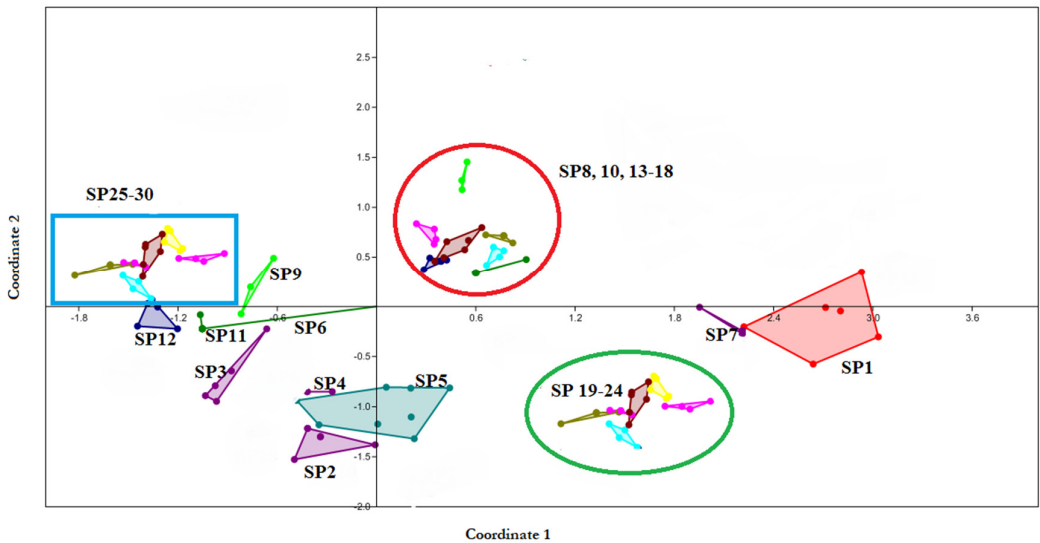
*Molecular analyses*

Sequence-related amplified polymorphism (SRAP) bands were recorded. These brands' presence and absence were scored based on the present (1) and absent (0). Total loci (NTL) and the number of polymorphism loci (NPL) for each primer were calculated. Furthermore, the polymorphic ratio was assessed based on NPL/NTL values. Polymorphism information content was calculated as previously suggested by Roldan-Ruiz *et al.* (2000). Resolving power for individual marker system was calculated as  $R_p = \sum I_b$ .  $I_b$  (band informativeness) was estimated while following equation: proposed as  $I_b = 1 - [2 \times (0.5 - p)]$ . In the equation,  $p$  indicates the presence of bands (Prevost and Wilkinson, 1999). The pairwise genetic similarity of the marker system was evaluated (Jaccard, 1908). Unbiased expected heterozygosity and Shannon information index were calculated in GenAIEx 6.4 software (Peakall and Smouse, 2006). Gene flow was conducted in POPGENE software, version 1.32 (Yeh *et al.*, 1999). Analysis of molecular variance test was conducted in GenAIEx (Peakall and Smouse, 2006). Mantel test was performed with 5000 permutations in the PAST, version 2.17 (Hammer *et al.*, 2001).

**Results**

*Morphometry*

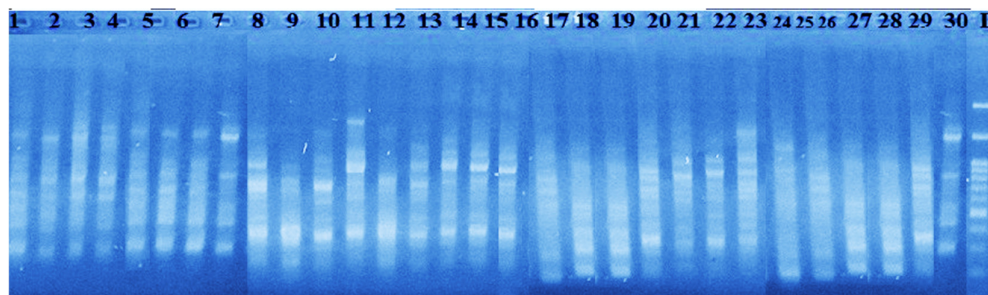
The ANOVA findings showed substantial differences ( $p < 0.01$ ) between the species in terms of quantitative morphological characteristics. Principle coordinates analysis results explained 67% cumulative variation. The first PCoA axis explained 53% of the total variation. The correlation ( $> 0.7$ ) was shown by morphological characters such as seed form, calyx shape, calyx length, bract length, and basal leaf shape. The morphological characters of *Salvia* species are shown in the PCoA plot (Figure 2). Each species formed separate groups based on morphological characters. The morphometric analysis showed a clear difference among *Salvia* species and separated each group.



**Figure 2.** Morphological characters analysis of *Salvia* species by PCoA  
 sp1= *Salvia aristata*; sp2= *S. eremophila*; sp3= *S. santolinifolia*; sp4= *S. tebesana*; sp5= *S. bracteata*; sp 6= *S. suffruticosa*; sp7= *S. dracoccephaloides*; sp8= *S. hydrangea*; sp9= *S. multicaulis*; sp10= *S. syriaca*; sp11= *S. viridis*; sp12= *S. mirzayanii*; sp13= *S. macrosiphon*; sp14= *S. sharifii*; sp15= *S. reuterana*; sp16= *S. palaestina*; sp17= *S. sclareopsis*; sp18= *S. spinose*; sp19= *S. compressa*; sp20= *S. sclarea*; sp21= *S. aethiopsis*; sp22= *S. microstegia*; sp23= *S. xanthocheila*; sp24= *S. limbata*; sp25= *S. chloroleuca*; sp26= *S. virgate*; sp27= *S. nemorosa*; sp28= *S. urmiensis*; sp29= *S. oligophylla*; sp30= *S. verticillata*

*Species identification and genetic diversity*

Ten (10) suitable primer combinations (PCs) were screened out of 25 PCs in this research. Figure 3 illustrates the banding pattern of Em4-Me1 primer by the SRAP marker profile. One hundred and forty-four (144) amplified polymorphic bands (number of polymorphic loci) were produced. These bands (fragments) had different ranges i.e., 100bp to 3000 bp. Maximum and minimum numbers of polymorphic bands were 20 and 10 for Em2-Me5 and 10 Em1-Me1, respectively. Each primer produced 14.4 polymorphic bands on average. The PIC ranged from 0.35 (Em2-Me5) to 0.51 (Em1-Me1) for the 10 SRAP primers, with an average of 0.45 per primer. The resolving power (Rp) of the primers ranged from 22.87 (Em2-Me2) to 44.23 (Em1-Me4), with an average of 35.98 per primer (Figure 3, Table 2).



**Figure 3.** Electrophoresis gel of studied ecotypes from DNA fragments produced by SRAP profile based on Em4-Me1 primer

1= *Salvia aristata*; 2= *S. eremophila*; 3= *S. santolinifolia*; 4= *S. tebesana*; 5= *S. bracteata*; 6= *S. suffruticosa*; 7= *S. dracocephaloides*; 8= *S. hydrangea*; 9= *S. multicaulis*; 10= *S. syriaca*; 11= *S. viridis*; 12= *S. mirzayani*; 13= *S. macrosiphon*; 14= *S. sharifii*; 15= *S. reuterana*; 16= *S. palaestina*; 17= *S. sclareopsis*; 18= *S. spinose*; 19= *S. compressa*; 20= *S. sclarea*; 21= *S. aethiopsis*; 22= *S. microstegia*; 23= *S. xanthocheila*; 24= *S. limbata*; 25= *S. chloroleuca*; 26= *S. virgate*; 27= *S. nemorosa*; 28= *S. urmiensis*; 29= *S. oligophylla*; 30= *S. verticillata*; L = Ladder 100 bp.

**Table 2.** SRAP primer information and results

Primer name	NTL <sup>a</sup>	NPL <sup>b</sup>	P <sup>c</sup>	PIC <sup>d</sup>	RP <sup>e</sup>
Em1-Me1	10	10	100.00%	0.51	32.24
Em2-Me2	24	19	79.00%	0.48	22.87
Em1-Me4	11	11	100.00%	0.44	44.23
Em2-Me4	16	16	100.00%	0.47	38.55
Em2-Me5	20	20	100.00%	0.35	29.65
Em3-Me4	18	12	67.00%	0.48	37.55
Em3-Me1	13	12	92.31%	0.44	43.77
Em4-Me1	12	12	100.00%	0.42	36.77
Em5-Me1	18	17	94.4%	0.43	40.46
Em5-Me2	15	15	100.00%	0.49	33.76
Mean	15.7	14.4	93.00%	0.45	35.98
Total	157	144			359.85

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)

The calculated genetic parameters of *Salvia* species are shown (Table 3). The unbiased heterozygosity (H) varied between 0.11 (*S. urmiensis*) and 0.31 (*S. limbata*) with a mean of 0.20. Shannon's information index (I) was maximum in *S. limbata* (0.35), whereas we recorded minimum Shannon's information index in *S. urmiensis* (0.12). The observed number of alleles ( $N_a$ ) ranged from 0.214 in *S. compressa* to 1.277 in *S. verticillata*. The significant number of alleles ( $N_e$ ) ranged from 1.00 (*S. compressa*) to 1.193 (*S. verticillata*).

**Table 3.** Genetic diversity parameters

SP	N	Na	Ne	I	He	UHe	P%
<i>S. aristata</i>	5.000	0.336	1.034	0.23	0.25	0.19	51.83%
<i>S. eremophila</i>	4.000	0.344	1.042	0.20	0.23	0.20	57.53%
<i>S. santolinifolia</i>	5.000	0.369	1.011	0.15	0.28	0.22	42.15%
<i>S. tebesana</i>	8.000	0.566	1.014	0.25	0.20	0.21	37.58%
<i>S. bracteata</i>	9.000	0.432	1.049	0.18	0.22	0.25	55.05%
<i>S. suffruticosa</i>	8.000	0.313	1.026	0.144	0.13	0.26	49.23%
<i>S. dracocephaloides</i>	3.000	0.297	1.024	0.23	0.15	0.17	64.30%
<i>S. hydrangea</i>	9.000	0.352	1.083	0.23	0.22	0.14	45.05%
<i>S. multicaulis</i>	8.000	0.333	1.016	0.192	0.12	0.22	48.23%
<i>S. syriaca</i>	12.000	1.155	1.190	0.271	0.184	0.192	55.91%
<i>S. viridis</i>	5.000	0.358	1.440	0.174	0.30	0.29	66.50%
<i>S. mirzayanii</i>	6.000	0.299	1.029	0.231	0.18	0.23	44.38%
<i>S. macrosiphon</i>	5.000	0.462	1.095	0.288	0.25	0.22	62.05%
<i>S. sharifii</i>	5.000	0.358	1.117	0.28	0.15	0.12	44.30%
<i>S. reuterana</i>	8.000	0.399	1.167	0.259	0.234	0.193	39.88%
<i>S. palaestina</i>	6.000	0.892	1.138	0.221	0.141	0.165	38.63%
<i>S. sclareopsis</i>	6.000	0.244	1.032	0.26	0.23	0.18	55.53%
<i>S. spinose</i>	4.000	0.314	1.044	0.16	0.18	0.23	43.38%
<i>S. compressa</i>	8.000	0.288	1.00	0.33	0.17	0.12	42.23%
<i>S. sclarea</i>	5.000	0.341	1.058	0.24	0.27	0.20	53.75%
<i>S. aethiopsis</i>	3.000	0.567	1.062	0.24	0.224	0.173	44.73%
<i>S. microstegia</i>	5.000	0.336	1.034	0.23	0.25	0.19	51.83%
<i>S. xanthocheila</i>	4.000	0.344	1.042	0.20	0.23	0.20	57.53%
<i>S. limbata</i>	5.000	0.358	1.440	0.35	0.35	0.31	69.50%
<i>S. chloroleuca</i>	10.000	0.431	1.088	0.33	0.22	0.13	57.53%
<i>S. virgata</i>	3.000	0.255	1.021	0.15	0.18	0.19	42.15%
<i>S. nemorosa</i>	3.000	0.288	1.024	0.23	0.15	0.17	64.30%
<i>S. urmiensis</i>	8.000	0.399	1.167	0.129	0.114	0.113	35.68%
<i>S. oligophylla</i>	8.000	0.333	1.016	0.172	0.12	0.22	48.23%
<i>S. verticillata</i>	12.000	1.277	1.193	0.271	0.184	0.192	55.91%

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%= The percentage of polymorphism).

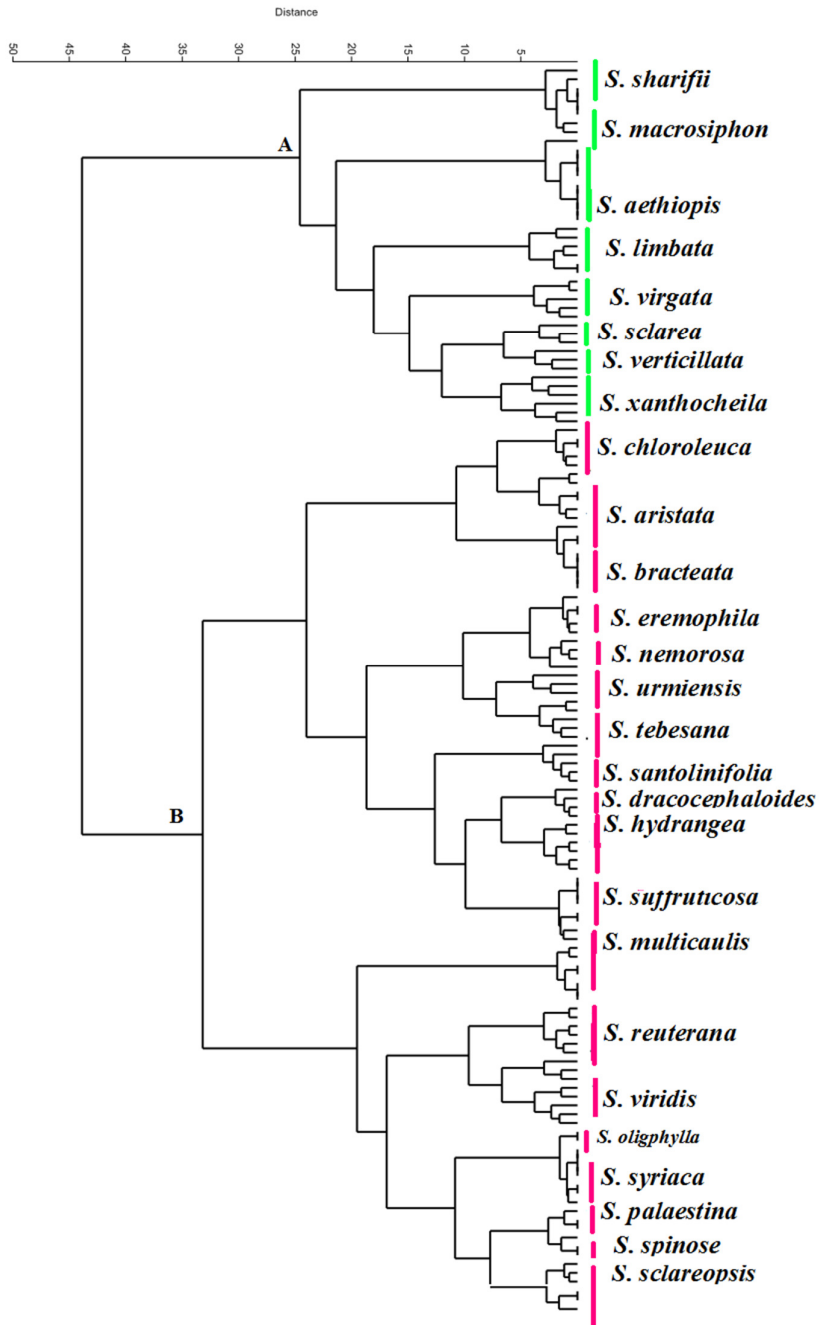
Analysis of molecular variance (AMOVA) results showed significant genetic difference ( $p = 0.01$ ) among *Salvia* species. AMOVA findings revealed that 73% of the total variation was between species, and comparatively less genetic variation was recorded at the species level (Table 4). Genetic difference between *Salvia* species was highlighted by genetic statistics (Nei's  $G_{ST}$ ), as evident by significant  $p$  values i.e. Nei's  $G_{ST}$  (0.29,  $p = 0.01$ ) and  $D_{est}$  values (0.167,  $p = 0.01$ ).

The constructed dendrogram highlighted two major clusters (Figure 4). Group A consisted of 8 species. Two sub-clusters were in the B group: eight species of *S. multicaulis*; *S. syriaca*; *S. viridis*, *S. reuterana*; *S. palaestina*; *S. sclareopsis*; *S. spinose* and *S. oligophylla*.

**Table 4.** Molecular variance analysis

Source	df	SS	MS	Est. Var.	%	$\Phi PT$
Among Pops	27	1501.364	95.789	18.154	73%	73%
Within Pops	139	334.443	3.905	2.888	27%	
Total	166	1955.807		20.060	100%	

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



**Figure 4.** Dendrograms of *Salvia* species

In the present study, a strong correlation between geographical and genetic distances ( $r = 0.19$ ,  $p=0.0002$ ) and a gene flow ( $N_m$ ) score of 0.287 was reported among species. Detailed information about genetic distances and genetic identity (Nei's) are described (Table S1, Supplementary Table). The findings suggested the significantly highest degree of genetic similarity (0.91) between *S. suffruticosa* and *S. bracteata*. On the other hand, *S. hydrangea* and *S. sharifii* (0.46) had the lowest genetic resemblance.

## Discussion

We used morphological and molecular (SRAP) data to evaluate *Salvia* species relationships in the present study. Morphological analyses of *Salvia* species showed that quantitative indicators (ANOVA test results) and qualitative characteristics are well differentiated. PCoA analysis suggests that morphological characters such as bract length, stipule length, bract shape, calyx shape, petal shape, length have the potentials to identify and delimitate *Salvia* species. Multivariate statistical analysis results suggest the utilization of morphological characters to identify and delimitate *Salvia* species. Morphological characters, comprising stipule length and petal shapes, play an important role in plant systematics and taxonomy. Our work also highlighted the significance of morphological characters and molecular data to identify and study species genetic diversity in *Salvia* species. Past research conducted on *Salvia* species has successfully linked morphological characters with molecular data to assess genetic diversity and species delimitation (Safaei *et al.*, 2016). Current morphometric and molecular methods have been implemented to study genetic diversity in *Salvia* species. For instance, morphometric analysis, coupled with molecular markers, revealed genetic diversity in *Salvia* species (Radosavljevic *et al.*, 2019). Genetic diversity is usually an important feature that helps plant species adapt to harsh environments (Tomasello *et al.*, 2015). Current *Salvia* species showed high genetic diversity; therefore, it could be argued that *Salvia* species can withstand the changing environment. In general, genetic relationships obtained from SRAP data coincides with morphometric results. This finding is in accordance with the parameters of AMOVA and genetic diversity results. SRAP molecular markers detected apparent genetic differences among species. These results indicate that SRAP has the potentials to study plant systematics and taxonomy in *Salvia* members.

Given the negative impact of biodiversity threats and overexploitation of *Salvia* plant species in Iran, it is necessary to conduct genetic diversity studies on *Salvia* species. Genetic diversity-based studies pave our understanding to develop conservation strategies (Esfandani-Bozchaloyi *et al.*, 2018). Genetic diversity studies are conducted through an appropriate selection of primers and indexes, including Polymorphic information content (PIC) and marker index (MI). These are important indexes and primers to fathom genetic variation in species (Sivaprakash *et al.*, 2004). Common logic suggests that different makers have different abilities to assess genetic diversity, and usually, genetic diversity is linked with polymorphism (Sivaprakash *et al.*, 2004). In this research, we reported PIC values of SRAP primers from 0.35 to 0.51, with a mean value of 0.45. PIC values indeed show low and high genetic diversity among genotypes. Values are ranging from zero to 0.25 show low genetic diversity; in contrast to this, 0.25 to 0.50 highlight mid-level of genetic diversity. In addition to this, values higher than 0.5 are associated with high genetic diversity (Tams *et al.*, 2005). Present results highlighted the efficiency of SRAP markers to estimate genetic diversity in *Salvia* species. In our study, SRAP markers detected an average percentage of polymorphism (93%). Current research results also described average PIC values of SRAP makers (0.45) and average RP (resolving power) values i.e. 35.98 of SRAP markers. These current reported values are higher than other reported markers on *Salvia* species (Wang *et al.*, 2009; Song *et al.*, 2010; Yousefiazar *et al.*, 2016; Etminan *et al.*, 2018). In the recent study, low gene flow ( $N_m$ ) was detected among *Salvia* species. Despite the presence of limited gene flow in *Salvia* species, two distinct ecotypes were reported previously. These ecotypes were formed due to reproductive isolation caused by altitude gradients and different niches (Moein *et al.*, 2019). The present study also depicted a significant correlation between genetic and geographical distances. Our findings revealed that isolation by distance (IBD) existed between *Salvia*

species (Mantel test results). Several mechanisms, such as isolation, local adaptation, and genetic drift, shape the species or population differentiation (Frichot *et al.*, 2013; De Kort *et al.*, 2014). The magnitude of variability among  $N_a$ ,  $N_e$ ,  $H$ , and  $I$  indices demonstrated a high level of genetic diversity among *Salvia* species. Dendrogram and principal coordinates analysis results showed a clear difference among *Salvia* species. This result indicates the high utilization of the SRAP technique to identify *Salvia* species. Our results have implications for conservation and breeding programs. Furthermore, it may identify suitable ecotypes for forage and pasture.

## Conclusions

The present study investigated the molecular variation of 30 species. The molecular and morphometric analysis confirmed the morphological and genetic differences among *Salvia* species. This study was the first attempt to assess genetic diversity through Sequence-related amplified polymorphism and morphometrics analysis in Iran. The current study reported two major clusters, and these two major groups were different from each other due to differences in morphological and genetic characters. The genetic similarities between 30 species were estimated from 0.46 to 0.91. SRAP (Sequence-related amplified polymorphism) markers analysis showed that *Salvia hydrangea* and *Salvia sharifii* had the lowest similarity. The current study also reported the correlation between genetic and geographical distances, which indicated the isolation mechanism involved in the *Salvia* species ecology. Present results indicated the potential of sequence-related amplified polymorphism to assess genetic diversity and genetic affinity among *Salvia* species. Current results have implications in biodiversity and conservation programs. Besides this, present results could pave the way for selecting suitable ecotypes for forage and pasture purposes in Iran. Future studies might include other environmental variables, including soil chemistry and geology data, to disentangle the diversity and ecology of *Salvia* species. However, this study focused on genetic diversity while incorporating morphometric analysis and a novel SRAP marker system.

## Authors' Contributions

A.S - Conceptualization, analyzed the data, mathematical processing, designed the study, prepared the initial draft; F.Z - Contributed to the conceptualization of ideas, the methodology, and the manuscripts' review, supervision, project administration, and funding acquisition. G.Z. - Genetic analysis, writing; curation, visualization, review, and editing; W.L - Literature review and contributed in mathematical processing, design; X.L - Contributed in the collection of data and analysis; S.E-B - Contributed in study design and reviewed the initial draft. All authors have read and agreed to the published version of the manuscript. All authors read and approved the final manuscript.

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## Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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