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

Abdul SHAKOOR¹², Fang ZHAO¹²*, Gul ZAIB³⁴, Wuyang LI¹, Xincan LAN¹, Somayeh ESFANDANI-BOZCHALOYI⁵

¹Henan University, College of Environment and Planning, Kaifeng, 475004, Henan, China; abdul_shakoor954@yahoo.com; zhaofang@lreis.ac.cn (corresponding author); 104753190127@henu.edu.cn; lxc0113@henu.edu.cn
²Key Laboratory of Geospatial Technology for the Middle and Lower Yellow River Regions, Ministry of Education, Kaifeng 475004, Henan, China
³Yangzhou University, Institute of Epigenetics and Epigenomics, Yangzhou, China; gulzaib_ehsan@hotmail.com
⁴Yangzhou University, College of Veterinary Sciences, Yangzhou, China
⁵Shahid Beheshti University, Faculty of Life Sciences and Biotechnology, Department of Plant Sciences, Tehran, Iran; somayehesfandi@yahoo.com

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

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*
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 | *Salvia arista* Aucher ex Benth. | East Azerbaijan, Kalybar, Shojabad | 38° 52’37” | 47° 23’92” | 1144 |
| Sp2 | *S. xeromorphia* Boiss. | Esfahan, Ghameshlo, Sanjab | 32°50’73” | 51°2’28” | 1990 |
| Sp3 | *S. xantusifolia* Boiss. | Fars, Jahrom | 29°20’07” | 51°52’08” | 1610 |
| Sp4 | *S. rebusa* Bunge | Khordan, Tabas | 29°20’07” | 51°52’08” | 220 |
| Sp5 | *S. breckense* Banks & Sol | Lorestan, Oshtorankan, above Tahun village | 33°57’12” | 47°57’32” | 2500 |
| Sp6 | *S. suffruticosa* Month. & Aucher | Hamedan, Nahavand | 34°52’373” | 48° 23’92” | 2200 |
| Sp7 | *S. dracocephaloides* Boiss. | East Azerbaijan, Kalybar, Cheshme Ali Akbar | 38°52’373” | 47°23’92” | 1144 |
| Sp8 | *S. hydrangea* DC. ex Benth. | Arak, Komayan, Pass of Chehragan village, the margin road | 35°50’03” | 51°2’28” | 1700 |
| Sp9 | *S. multicaulis* Vahl. | Mazandaran, Haraz road, Emam Zade-e-Hashem | 36°14’14” | 51°18’07” | 1807 |
| Sp10 | *S. syriaca* L. | Esfahan, Feizydelumbar | 32°36’93” | 51°2’79” | 2500 |
| Sp11 | *S. citrata* L. | Guilan, Sangar, Roadside | 37°0’02” | 49°4’32” | 48 |
| Sp12 | *S. mirzayani* Rech. f. & Esfand. | Boushehr, Dashtestan | 28°57’22” | 51°2’31” | 430 |
| Sp13 | *S. macrocephalon* Boiss. | Yazd, Khatam | 30°0’24” | 53°59’06” | 2178 |
| Sp14 | *S. sharifii* Rech. f. & Esfand. | Bandar Abbas, Hormozgan | 28°57’22” | 51°2’31” | 288 |
| Sp15 | *S. reuterana* Boiss. | Hamedan, Alvand | 34°46’10” | 48°30’00” | 1870 |
| Sp16 | *S. xanthocarpa* Benth. | Kermanshah, Islamabad | 35°57’77” | 46°20’25” | 1888 |
| Sp17 | *S. sclareopsis* Born. ex Hedge | Ilam, Ilam | 33°4’76” | 46°0’58” | 1250 |
| Sp18 | *S. spinosa* L. | Guilan, Lahijan | 37°0’02” | 49°4’32” | 48 |
| Sp19 | *S. compressa* Verne. | Bandar Abbas, Hormozgan | 28°57’22” | 51°2’31” | 288 |
| Sp20 | *S. xeromorpha* L. | Esfahan, Ghameshlo, Sanjab | 32°36’93” | 51°2’79” | 2500 |
| Sp21 | *S. aethiopis* L. | Azerbaijan, 78 km from Mianeh to Khalkhl. | 37°38_53 | 48°36_11 | 1500 |
| Sp22 | *S. microstegia* Boiss. & Bal. | Tehran, Darband | 35°36’93” | 51°2’79” | 1700 |
| Sp23 | *S. xanthocastra* Boiss. & Bal. | Ardabil, Khalkhal | 37°38_53 | 48°36_11 | 1958 |
| Sp24 | *S. limbata* C.A. Mey. | Guilan, Golroodbar, Road side | 37°09’45” | 49°55’39” | 15 |
| Sp25 | *S. chloroleuca* Rech. f. & Aell. | Golestan, Ramian | 37°09’45” | 55°55’39” | 1320 |
| Sp26 | *S. virescens* Jacq. | Golestan, Ramian | 37°09’45” | 55°55’39” | 1320 |
| Sp27 | *S. nemorosa* L. | Mazandaran, Chalos | 36°14’14” | 51°18’07” | 1807 |
| Sp28 | *S. aromatica* Bunge | Kurdistan, Sanandaj | 37°09’45” | 55°55’39” | 1320 |
| Sp29 | *S. olgyphilla* Aucher ex Benth. | Ghavzin to Hamedan just after Avaj | 35°36’93” | 51°2’79” | 2100 |
| Sp30 | *S. verticillata* L. | Mazandaran Jadeh Chalous | 36°14’14” | 51°18’07” | 1807 |
**Figure 1.** Provinces and collection sites of *Salvia*, Iran

*sp1* = *Salvia aristata*; *sp2* = *S. crenophila*; *sp3* = *S. santolinifolia*; *sp4* = *S. tebesana*; *sp6* = *S. suffruticosa*; *sp7* = *S. dracocephalodes*; *sp8* = *S. hydrangea*; *sp9* = *S. multicaulis*; *sp10* = *S. syriaca*; *sp11* = *S. viridis*; *sp12* = *S. mirzayani*; *sp13* = *S. macrosiphon*; *sp14* = *S. sharfii*; *sp15* = *S. reuterana*; *sp16* = *S. palestina*; *sp17* = *S. sclareopsis*; *sp18* = *S. spinosa*; *sp19* = *S. compressa*; *sp20* = *S. sclarea*; *sp21* = *S. aethiopis*; *sp22* = *S. microstegia*; *sp23* = *S. xanthocheila*; *sp24* = *S. limbata*; *sp25* = *S. chloroleucza*; *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 μl volume containing ten mM of Tris-HCl buffer at pH 8; 50 mM of KCl; 1.5 mM of MgCl2; 0.2 mM of each dNTP (Bioron, Germany); 0.2 μM 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 μl. 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 GenAlEx 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 GenAlEx (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](image)
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).

Table 2. SRAP primer information and results

| Primer name | NTL a | NPL b | P c  | PIC d | RP e |
|-------------|-------|-------|------|-------|------|
| Em1-Me1     | 10    | 10    | 100.00% | 0.51 | 32.24 |
| Em2-Me2     | 24    | 19    | 79.00% | 0.48 | 22.87 |
| Em1-Me4     | 16    | 16    | 100.00% | 0.47 | 38.55 |
| Em2-Me4     | 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 (Na) ranged from 0.214 in S. compressa to 1.277 in S. verticillata. The significant number of alleles (Ne) ranged from 1.00 (S. compressa) to 1.193 (S. verticillata).
Table 3. Genetic diversity parameters

| SP                | N   | Na  | Ne   | I    | He  | UHe | P%   |
|-------------------|-----|-----|------|------|-----|-----|------|
| S. aristata      | 5.000 | 0.336 | 1.034 | 0.23 | 0.25 | 0.19 | 51.83% |
| S. cremiflora     | 4.000 | 0.344 | 1.042 | 0.20 | 0.23 | 0.20 | 57.53% |
| S. santolinifolia | 5.000 | 0.369 | 1.011 | 0.15 | 0.28 | 0.22 | 42.15% |
| S. rebecasana     | 8.000 | 0.566 | 1.014 | 0.25 | 0.20 | 0.21 | 37.58% |
| S. bracteata      | 9.000 | 0.432 | 1.049 | 0.18 | 0.22 | 0.25 | 55.05% |
| S. suffruticosa   | 8.000 | 0.313 | 1.026 | 0.144 | 0.13 | 0.26 | 49.23% |
| S. dracocephaloides | 3.000 | 0.297 | 1.024 | 0.23 | 0.15 | 0.17 | 64.30% |
| S. hydrangea      | 9.000 | 0.352 | 1.083 | 0.23 | 0.22 | 0.14 | 45.05% |
| S. multicaulis    | 8.000 | 0.299 | 1.029 | 0.231 | 0.18 | 0.22 | 48.23% |
| S. syriaca       | 12.000 | 1.155 | 1.190 | 0.271 | 0.184 | 0.192 | 55.91% |
| S. viridis       | 5.000 | 0.358 | 1.440 | 0.174 | 0.30 | 0.29 | 66.50% |
| S. mirzayani     | 6.000 | 0.299 | 1.029 | 0.231 | 0.18 | 0.22 | 44.38% |
| S. Macrosiphon    | 5.000 | 0.462 | 1.095 | 0.288 | 0.25 | 0.22 | 62.05% |
| S. sharithi      | 5.000 | 0.358 | 1.117 | 0.28 | 0.15 | 0.12 | 44.30% |
| S. reuterana      | 8.000 | 0.399 | 1.167 | 0.259 | 0.234 | 0.193 | 39.88% |
| S. Palaeastina    | 6.000 | 0.892 | 1.138 | 0.221 | 0.141 | 0.165 | 38.63% |
| S. sclareopsis    | 6.000 | 0.244 | 1.032 | 0.26 | 0.23 | 0.18 | 55.53% |
| S. spinose        | 4.000 | 0.314 | 1.044 | 0.16 | 0.18 | 0.23 | 43.38% |
| S. compressa      | 8.000 | 0.288 | 1.00 | 0.33 | 0.17 | 0.12 | 42.23% |
| S. sclarea       | 5.000 | 0.341 | 1.058 | 0.24 | 0.27 | 0.20 | 53.75% |
| S. Acthiospis     | 3.000 | 0.567 | 1.062 | 0.24 | 0.224 | 0.173 | 44.73% |
| S. microstegia    | 5.000 | 0.336 | 1.034 | 0.23 | 0.25 | 0.19 | 51.83% |
| S. Xanthocheila   | 4.000 | 0.344 | 1.042 | 0.20 | 0.23 | 0.20 | 57.53% |
| S. Limbata       | 5.000 | 0.358 | 1.440 | 0.35 | 0.35 | 0.31 | 69.50% |
| S. Chloroleuca    | 10.000 | 0.431 | 1.088 | 0.33 | 0.22 | 0.13 | 57.53% |
| S. Virgata       | 3.000 | 0.255 | 1.021 | 0.15 | 0.18 | 0.19 | 42.15% |
| S. Nemorosa      | 3.000 | 0.288 | 1.024 | 0.23 | 0.15 | 0.17 | 64.30% |
| S. Urmquensis    | 8.000 | 0.399 | 1.167 | 0.129 | 0.114 | 0.113 | 35.68% |
| S. Oligphylla    | 8.000 | 0.333 | 1.016 | 0.172 | 0.193 | 0.22 | 48.23% |
| S. Verticillata  | 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. palaeastina; S. sclareopsis; S. spinose* and *S. oligphylla.*
Table 4. Molecular variance analysis

| Source        | df  | SS        | MS       | Est. Var. | %    | ΦPT |
|---------------|-----|-----------|----------|-----------|------|-----|
| Among Pops    | 27  | 1501.364  | 95.789   | 18.154    | 73%  |     |
| Within Pops   | 139 | 334.443   | 3.905    | 2.888     | 27%  |     |
| Total         | 166 | 1955.807  | 20.060   | 100%      |      | 73% |

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

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; Eetminan *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 sharitii* 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.

**Acknowledgements**

This work was funded by the National Natural Science Foundation of China (41601091) and integrated Scientific investigation of the North-South Transitional Zone of China (2017FY100900). We would also like to acknowledge the National Undergraduate Innovation and Entrepreneurship Training Program, Henan University (202010475126). Funders had no role in designing the experiments.
Conflict of Interests

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

References

De Kort H, Vandepitte K, Mergeay J, Honnay O (2014). Isolation, characterization and genotyping of single nucleotide polymorphisms in the non-model tree species Frangula alnus (Rhamnaceae). Conservation Genetics Resources 6(2):267-269. https://doi.org/10.1007/s12686-013-0083-6

Erbano M, Schiuli GSE, Santos, EPD (2015). Genetic variability and population structure of Salvia lachnostachys: implications for breeding and conservation programs. International Journal of Molecular Sciences 16(4):7839-7850. https://doi.org/10.3390/ijms16047839

Esfandani-Bozchaloyi S, Sheidai M (2018). Molecular diversity and genetic relationships among Geranium pusillum and G. pyrenacicum with intersimple sequence repeat (ISSR) regions. Caryologia 71(4):457-470. https://doi.org/10.1080/00087114.2018.1503500

Esfandani-Bozchaloyi S, Sheidai M, Kalalegh M (2019). Comparison of DNA extraction methods from Geranium (Geraniaceae). Acta Botanica Hungarica 61(3-4):251-266. https://doi.org/10.1556/034.61.2019.3-4.3

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z (2018). Morphometric and ISSR-analysis of local populations of Geranium molle L. from the Southern Coast of the Caspian Sea. Cytology and Genetics 52(4):309-321. https://doi.org/10.3103/S0095452718040102

Etminan A, Pour-Aboughadareh A, Noori A, Ahmadi-Rad A, Shooshtari L, Mahdavian Z, Yousefazar-Khanian M (2018). Genetic relationships and diversity among wild Salvia accessions revealed by ISSR and SCoT markers. Biotechnology and Biotechnological Equipment 32(3):610-617. https://doi.org/10.1080/13102818.2018.1447397

Frichot E, Schoville SD, Bouchard G, François O (2013). Testing for Associations between loci and environmental gradients using latent factor mixed models. Molecular Biology and Evolution 30(7):1687-1699. https://doi.org/10.1093/molbev/mst063

Hammer O, Harper D, Ryan P (2001). PAST: Paleontological statistics software package for education and data analysis. Palaeontologia Electronica 4(1):1-9.

Hosseinzadeh H, Haddadkhodaparast MH, Arash AR (2003). Antinociceptive, anti-inflammatory and acute toxicity effects of Salvia leriifolia Benth seed extract in mice and rats. Phytotherapy Research 17(4):422-425. https://doi.org/10.1002/ptr.1154

Jaccard P (1908). Nouvelles recherches sur la distribution florale. Bulletin de la Societe Vaudoise des Sciences Naturelles 44(163):223-270. https://doi.org/10.5169/seals-268384

Jamzad Z (2013). A survey of Lamiaceae in the flora of Iran. Rostaniha 14(1):59-67. https://doi.org/10.22092/botany.2013.10317

Javan Z, Rahmani F, Heidari R (2012). Assessment of genetic variation of genus Salvia by RAPD and ISSR markers. Australian Journal of Crop Science 6(6):1068-107. https://doi.org/10.1515/biolog-2015-0051

Kharazian N, Rahimi S, Shiran B (2015). Genetic diversity and morphological variability of fifteen Stachys (Lamiaceae) species from Iran using morphological and ISSR molecular markers. Biologia 70(4):438-452. https://doi.org/10.1515/biolog-2015-0051

Li G, Quiros CF (2001). Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in Brassica. Theoretical and Applied Genetics 103(2):455-461. https://doi.org/10.1007/s001220100570

Mayer B, Baggio CH, Freitas CS, dos Santos AC, Twardowschy A, Horst H, ... Marques MC (2009). Gastroprotective constituents of Salvia officinalis L. Fitoterapia 80(7):421-426. https://doi.org/10.1016/j.fitote.2009.05.015

Moein F, Jamzad Z, Rahiminejad M (2019). An integrating study of genetic diversity and ecological niche modelling in Salvia aristata (Lamiaceae). Acta Botanica Hungarica 61(1-2):185-204. https://doi.org/10.1556/034.61.2019.1-2.10

Pauls SU, Nowak C, Balint M, Pfenninger M (2013). The impact of global climate change on genetic diversity within populations and species. Molecular Ecology 22(4):925-946. https://doi.org/10.1111/mec.12152
Peakall R, Smouse PE (2006). GENALEX 6: Genetic Analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6(1):288-295. https://doi.org/10.1111/j.1471-8286.2005.01155.x

Peng L, Ru M, Wang B, Wang Y, Li B, Yu J, Liang Z (2014). Genetic diversity assessment of a germplasm collection of *Salvia miltiorrhiza* Bunge. based on morphology, ISSR and SRAP markers. Biochemical Systematics and Ecology 55:84-92. https://doi.org/10.1016/j.bse.2014.01.020

Podani J (2000). Introduction to the exploration of multivariate data. Backhuys, Leide, Netherlands.

Prevost A, Wilkinson MJ (1999). A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. Theoretical and Applied Genetics 98(1):107-112. https://doi.org/10.1007/s001220050146

Radosavljevic I, Bogdanovic S, Celip F, Filipovic M, Satavic Z, Surina B, Liber Z (2019). Morphological, genetic and epigenetic aspects of homoploid hybridization between *Salvia officinalis* L. and *Salvia fruticosa* Mill. Scientific Reports 9(1):3276. https://doi.org/10.1038/s41598-019-40080-0

Robarts DWH, Wolfe AD (2014). Sequence-related amplified polymorphism (SRAP) markers: A potential resource for studies in plant molecular biology. Applications in Plant Sciences 2(7):1400017. https://doi.org/10.3732/apps.1400017

Roldán-Ruiz I, Dendauw J, Van Bockstaele E, Depicker A, De Loose M (2000). AFLP markers reveal high polymorphic rates in ryegrasses (*Lolium* spp.). Molecular Breeding 6(2):125-134. https://doi.org/10.1023/A:1009680614466

Saebnazar A, Rahmani F (2013). Genetic Variation Among Salvia Species Based on Sequence-Related Amplified Polymorphism (SRAP) Marker. Journal of Plant Physiology and Breeding 3(1):71-78.

Safaei M, Sheidi M, Alianpour B, Noormohammadi Z (2016). Species delimitation and genetic diversity analysis in *Salvia* with the use of ISSR molecular markers. Acta Botanica Croatica 75(1):45-52. https://doi.org/10.1515/botcro-2016-0005

Sivaprakash KR, Prashanth SR, Mohanpy BP, Parida A (2004). Genetic diversity of black gram (*Vigna mungo*) landraces as evaluated by amplified fragment length polymorphism markers. Current Science 86(10):1411-1416.

Song Z, Li X, Wang H, Wang J (2010). Genetic diversity and population structure of *Salvia miltiorrhiza* Bge in China revealed by ISSR and SRAP. Genetica 138(2):241-249. https://doi.org/10.1007/s10709-009-9416-5

Taleb M, Rahimmalek M, Norouzi M (2015). Genetic diversity of *Thymus daenensis* subsp. *daenensis* using SRAP markers. Biologia 70(4):453-459. https://doi.org/10.1515/biolog-2015-0059

Tams SH, Melchinger AE, Bauer E (2005). Genetic similarity among European winter triticale elite germplasms assessed with AFLP and comparisons with SSR and pedigree data. Plant Breeding 124(2):154-160. https://doi.org/10.1111/j.1439-0523.2004.01047.x

Tomasello S, Alvarez I, Vargas P, Oberprieler C (2015). Is the extremely rare Iberian endemic plant species *Castrilanthemum debeauxii* (Compositae, Anthemideae) a 'living fossil'? Evidence from a multi-locus species tree reconstruction. Molecular Phylogenetics and Evolution 82:118-130. https://doi.org/10.1016/j.ympev.2014.09.007

Walker JB, Sytsma KJ, Treutlein J, Wink M (2004). *Salvia* (Lamiaceae) is not monophyletic: implications for the systematics, radiation, and ecological specializations of Salvia and tribe Mentheae. American Journal of Botany 91(7):1115-1125. https://doi.org/10.3732/ajb.91.7.1115

Wang Q, Zhang B, Lu Q (2009). Conserved region amplification polymorphism (CoRAP), a novel marker technique for plant genotyping in *Salvia miltiorrhiza*. Plant Molecular Biology Reporter 27(2):139. https://doi.org/10.1007/s11105-008-0065-0

Wu Y-G, Guo Q-S., He J-C, Lin Y-F, Luo L-J, Liu G-D (2010). Genetic diversity analysis among and within populations of *Pogostemon cablin* from China with ISSR and SRAP markers. Biochemical Systematics and Ecology 38(1):63-72. https://doi.org/10.1016/j.bse.2009.12.006

Yeh FC, Yang R, Boyle T (1999). POPGENE. Microsoft Windows-based freeware for population genetic analysis. Release 1.31. University of Alberta, pp 1-31.

Yousefiazar M, Asghari A, Ahmadi J, Asghari B, Jafari AA (2016). Genetic diversity of salvia species assessed by ISSR and rapid markers. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 44(2):431-436. https://doi.org/10.15835/nbha44210579
The journal offers free, immediate, and unrestricted access to peer-reviewed research and scholarly work. Users are allowed to read, download, copy, distribute, print, search, or link to the full texts of the articles, or use them for any other lawful purpose, without asking prior permission from the publisher or the author.

**License** - Articles published in *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* are Open-Access, distributed under the terms and conditions of the Creative Commons Attribution (CC BY 4.0) License. © Articles by the authors; UASVM, Cluj-Napoca, Romania. The journal allows the author(s) to hold the copyright/to retain publishing rights without restriction.