Introduction

Salvia genus belongs to Lamiaceae family, includes approximately 1000 species, and was considered as one of the largest plant genera (Walker et al. 2004). This genus commonly known as sage and it is considered as the largest genus within the family (Hu et al. 2018). According to Mouterde (1983), 28 Salvia species were grown along the Syrian coastline at different sea altitudes up to 900 m. Salvia judaica Boiss (Judean sage) is a perennial plant native to Mediterranean woodlands and shrublands and distributed in Turkey, Syria, Lebanon, and Palestine (Mouterde 1983). Whereas, Salvia palaestina Benth (Palestinian sage) is a perennial plant native to Palestine, Turkey, Syria, Iraq, Iran and the Sinai Peninsula and north-eastern Egypt (Loutfy 2002; Betsy 2003). It grows at wide range of habitats from 300 to 1220 m altitude.

Molecular markers have been widely and successfully used in plants genetic studies at genotypes, species and genera levels. Of which, directed amplification of minisatellite region DNA (DAMD) has been successfully employed in studies genetic variability of many plant species (Heath et al. 1993; Saleh 2019a).

More recently, Saleh (2019b) reported S. tomentosa species genetic diversity using touch-up directed amplification of minisatellite DNA (TD-DAMD) marker. The previous study reported various molecular markers that have been employed for genetic diversity of different Salvia species, e.g., random amplified polymorphic DNA (RAPD) marker in S. hispanica L. (Cahill 2004), inter simple sequence repeat (ISSR) marker in S. lachnostachys (Erbano et al. 2015), RAPD, amplified fragment length polymorphism (AFLP) (Wen et al. 2007), sequence-related amplified polymorphism (SRAP) and ISSR (Song et al. 2010) in S. miltiorrhiza Bge, nuclear ribosomal DNA and plastid DNA sequences in S. lutescens var. intermedia (Takano 2017), chloroplast simple sequence repeats (cpSSR’s) in S. divinorum (Casselman 2016), chloroplast and nuclear ribosomal DNA sequences and allozyme polymorphisms in S. japonica (Sudarmono and Okada 2008) and directed amplification of minisatellite region DNA (DAMD) in Salvia sp. (Karaca et al. 2008).

DAMD marker has been developed for the first time in common bean landraces by Ince and Karaca (2011) to touch-down directed amplification of minisatellite DNA (TD-DAMD) marker. Then this marker has been used for molecular characterization of other plant species e.g. for Salvia species (Ince and Karaca 2012); Allium sp. (Deniz et al. 2013); carnation cultivars (Ince and Karaca 2015), commercial cotton (Gocer and Karaca 2016) and S. tomentosa (Saleh 2019b).

On the basis of TD-DAMD marker, an attempt has been carried out based on increasing annealing temperature by 0.5 °C/cycle during the first 10 PCR amplification cycles in place to reducing it in TD-DAMD and thereby named as touch-up directed amplification of minisatellite DNA.
(TU-DAMD) in nouvelle test. Thereby, the current study highlights genetic diversity of S. judaica and S. palaestina species through TU-DAMD marker as a new assay for their molecular characterization.

Materials and Methods

Plants materials

Leaves samples (5-10 plants/genotype) were collected from Salvia judaica (rural Lattakia) (SJ) and Salvia palaestina (rural Damascus) (SP) species with Ballota damascena Boiss (Lamiaceae) (rural Damascus) (BD) as outside far reference, during blooming stage (Table 1). Samples were frozen in liquid nitrogen and kept at -80 °C until use.

DNA isolation

Total genomic DNA was extracted from frozen leaves samples (3 samples of S. judaica, 3 samples of S. palaestina and 1 sample of B. damascena) using CTAB (cetyltrimethylammonium bromide) method as described by Doyle and Doyle (1987).

Touch-Up Directed Amplification of Minisatellite region DNA (TU-DAMD) test

TU-DAMD test has been performed in two separated tests; in the first test (set A) the initial annealing temperature was increased from 50 °C to 55 °C by 0.5 °C/cycle during the first 10 PCR amplification cycles. Whereas, in the second one (set B), it increased from 55 °C to 60 °C by 0.5 °C/cycle during the first 10 PCR amplification cycles. Then, similar PCR amplification program was performed at annealing Tm of 55 °C for the both tests during the remaining 30 PCR amplification cycles as described by Seyedimoradi et al. (2012) for DAMD marker.

PCR products were separated on a 2% ethidium bromide-stained agarose (Bio–Rad) in 0.5 x Tris-borate-EDTA (TBE) buffer, by electrophoresis at 85 V for 2.5 h, and visualized with a UV transilluminator. PCR amplification products size was estimated with a 1 kb DNA ladder standard. Fifteen DAMD primers have been tested for each set to investigate genetic diversity in S. judaica and S. palaestina species (Table 2).

TU-DAMD data analysis

PCR products were photographed under UV, and each size class was scored as 0 or 1 for the absence or presence class, respectively. Unweighted pair group method using arithmetic averages (UPGMA) was constructed based on the estimated percent disagreement values (PDVs) using Statistica 6 (Statsoft 2003) program. Moreover, genetic similarity (GS) among examined samples was estimated according to Nei and Li (1979) index. Whereas, polymorphic information content (PIC) values were estimated for each tested primer according to the formula:

\[
\text{PIC} = 1 - \sum (P_{ij})^2
\]

Table 1. Descriptive sites of collected studied samples in the current study.

| Species          | Code | City province | Altitude (m) | Annual rainfall (mm) |
|------------------|------|---------------|--------------|----------------------|
| Salvia judaica   | SJ1  | Lattakia      | 80           | 750                  |
| Salvia judaica   | SJ2  | Lattakia      | 600          | 750                  |
| Salvia judaica   | SJ3  | Lattakia      | 680          | 1250                 |
| Salvia palaestina| SP4  | Damascus      | 920          | 260                  |
| Salvia palaestina| SP5  | Damascus      | 950          | 260                  |
| Salvia palaestina| SP6  | Damascus      | 1200         | 150                  |
| Ballota damascena| BD7  | Damascus      | 970          | 260                  |

Figure 1. TU-DAMD polymorphism profile yielded by set (A): Tm increased from 50 °C to 55 °C and set (B): Tm increased from 55 °C to 60 °C by 0.5 °C/cycle during the first 10 PCR amplification cycles using URP38F and OGRB01 DAMD primers for S. judaica (lines 1-3); S. palaestina (lines 4-6) and B. damascena (line 7). M: VC100bp Plus DNA Ladder (Vivantis) size standard.
Table 2. DAMD primers used in the current study.

| Primer number | Primer name | Primer sequence 5’-3’ |
|---------------|-------------|-----------------------|
| 1             | URP1F       | ATCCAAGTCCGAGACACCC   |
| 2             | URP2R       | CCCACAACTGATCGACACAC |
| 3             | URP4R       | AAGACTCGGATAACAGGTCCTC|
| 4             | URP9F       | ATGTGGCAGATGATGTGCTG |
| 5             | URP25F      | GATGTGGTCTGGAGGCTGT  |
| 6             | URP38F      | AGAGGCTCATCTACACACAC |
| 7             | OGRB01      | AGGGCTGGAGGAGGGGCG    |
| 8             | FVIIex8C    | CCGTGTGTGTGTGCTAT     |
| 9             | FVIIex8     | ATGGACACACACAGG       |
| 10            | HBV5        | GTGTAGAGAGGGGT        |
| 11            | 33.6        | GGAGGTGGGCA           |
| 12            | HVRc        | CCGTCTCCCTCCT         |
| 13            | URP2F       | GTGTGGCACTGGTGCTG     |
| 14            | URP6R       | GGCACGTCGTTGGAGGTAC   |
| 15            | URP17R      | AATGTTGGCACTGGTGCTG   |

TU-DAMD characterization of Salvia species

Table 3. Banding pattern of TU-DAMD amplified fragments scored.

| Set (A) | Primer name | TB | PB | P% | PIC | MI |
|---------|-------------|----|----|----|-----|----|
|         | URP1F       | 13 | 12 | 92.31 | 0.38 | 4.56 |
|         | URP2R       | 11 | 11 | 100.00 | 0.39 | 4.29 |
|         | URP4R       | 14 | 11 | 78.57 | 0.24 | 2.64 |
|         | URP9F       | 16 | 14 | 85.70 | 0.35 | 4.90 |
|         | URP25F      | 13 | 13 | 100.00 | 0.41 | 5.33 |
|         | URP38F      | 17 | 16 | 94.12 | 0.29 | 4.64 |
|         | OGRB01      | 18 | 18 | 100.00 | 0.38 | 6.84 |
|         | FVIIex8C    | 8  | 8  | 100.00 | 0.39 | 3.12 |
|         | FVIIex8     | 10 | 9  | 90.00 | 0.27 | 2.43 |
|         | HBV5        | 15 | 11 | 73.33 | 0.23 | 2.53 |
|         | 33.6        | 11 | 11 | 100.00 | 0.37 | 4.07 |
|         | HVRc        | 13 | 8  | 61.54 | 0.28 | 2.24 |
|         | URP2F       | 14 | 13 | 92.86 | 0.34 | 4.42 |
|         | URP6R       | 14 | 13 | 92.86 | 0.36 | 4.68 |
|         | URP17R      | 11 | 9  | 81.82 | 0.3 | 2.70 |
| Total   | 198         | 177 |
| Average | 13.20       | 11.80 | 89.66 | 0.33 | 3.96 |

| Set (B) | Primer name | TB | PB | P% | PIC | MI |
|---------|-------------|----|----|----|-----|----|
|         | URP1F       | 14 | 14 | 100.00 | 0.34 | 4.76 |
|         | URP2R       | 8  | 8  | 100.00 | 0.33 | 2.64 |
|         | URP4R       | 16 | 14 | 87.50 | 0.34 | 4.76 |
|         | URP9F       | 14 | 14 | 100.00 | 0.32 | 4.48 |
|         | URP25F      | 8  | 8  | 100.00 | 0.4  | 3.20 |
|         | URP38F      | 18 | 14 | 77.78 | 0.31 | 4.34 |
|         | OGRB01      | 18 | 18 | 100.00 | 0.39 | 7.02 |
|         | FVIIex8C    | 12 | 12 | 100.00 | 0.4  | 4.80 |
|         | FVIIex8     | 7  | 6  | 85.71 | 0.23 | 1.38 |
|         | HBV5        | 9  | 8  | 88.89 | 0.27 | 2.16 |
|         | 33.6        | 16 | 16 | 100.00 | 0.34 | 5.44 |
|         | HVRc        | 11 | 10 | 90.91 | 0.32 | 3.20 |
|         | URP2F       | 8  | 7  | 87.50 | 0.35 | 2.45 |
|         | URP6R       | 15 | 14 | 93.33 | 0.37 | 5.18 |
|         | URP17R      | 10 | 10 | 100.00 | 0.39 | 3.90 |
| Total   | 184         | 173 |
| Average | 12.27       | 11.53 | 94.11 | 0.34 | 3.98 |

Where $P_{ij}$ is the frequency of the $i^{th}$ pattern revealed by the $j^{th}$ primer combination, summed across all patterns revealed by the primers (Botstein et al. 1980). Indeed, marker index (MI), a universal metric to represent the amount of information obtained per experiment for a given kind of marker was also estimated for each tested primer as described by Powell et al. (1996) according to the formula:

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

Where PIC is the mean PIC value, $\eta$ the number of bands, and $\beta$ the proportion of polymorphism.

In the current study, statistical comparison based on Mantel test has been performed among TU-DAMD, A (set A), B (set B) and C (set A + B) data.

Results

TU-DAMD test has been employed to investigate genetic diversity of $S. judaica$ and $S. palaestina$ species. Polymorphism pattern including set (A) and (B) as yielded by URP38F and OGRB01 DAMD primers was presented in Fig. 1. Data showed that for set (A), total bands number ranged between 8 (FVIIex8C)-18 (OGRB01) bands with a mean average of 13.20 bands/primer. Whereas, polymorphic bands number ranged between 8 (FVIIex8C) -18 (OGRB01) polymorphic bands with a mean average of 11.80 polymorphic bands/primer. Indeed, set (A) produced 198 bands of which 177 (89.39%) were polymorphic. Whereas, PIC value ranged between 0.23 (HBV5) - 0.41 (URP25F) with a mean average of 0.33 (Table 3). As for set (B), total bands number ranged between 7 (FVIIex8) - 18 (URP38F & OGRB01) bands with a mean average of 12.27 bands/primer. Whereas, polymorphic bands number ranged between 6 (FVIIex8) - 18 (OGRB01) polymorphic bands with a mean average of 11.53 polymorphic bands/primer. Indeed, set (B) produced 184 bands of which 173 (94.02%) were polymorphic. Whereas, PIC value ranged between 0.23 (FVIIex8) - 0.40 (URP25F) with a mean average of 0.34 (Table 3).
average of 0.34 (Table 3).

Genetic diversity was separately detected in the two studied *Salvia* sp. (Fig. 2). In this regards, for set (A), P% was recorded to be 41.07, 35.48 and 82.09% with genetic similarity (GS) of 0.89, 0.91 and 0.66 for *S. judaica*, *S. palaestina* and *S. judaica* + *S. palaestina* together, respectively. As for set (B), these values were recorded to be 40.45, 42.31 and 90.00% for P% with GS of 0.87, 0.89 and 0.58 for *S. judaica*, *S. palaestina* and *S. judaica* + *S. palaestina* together, respectively.

UPGMA cluster analysis constructed (Fig. 3) based on PDV (Table 4), revealed that *B. damascena* was genetically so far from the two studied *Salvia* sp. Whereas, the two studied *Salvia* sp. were grouped into two main groups. The first group involved SJ samples; whereas, the second one involved SP samples (Fig. 3) for set (A), set (B) and set (A) + set (B) together.

Cluster analysis revealed that SJ1 and SJ3 and also SP4 and SP6 samples were the most closed samples by showing the lowest PDV value of 0.13 for set (A) (Table 4). As for set (B), SP5 and SP6 samples were the most closed samples by showing the lowest PDV value of 0.13 (Table 4).

Whereas, in set (A) and set (B) combination, SJ1 and SJ2 samples were the most closed samples by showing the lowest PDV value of 0.12 (Table 4).

Mantel test revealed a highly significant correlation among the possible combination. In this regards, very good fit (r=0.994) has been recorded between A+C and B+C data and also between A+B data (r=0.977).

**Discussion**

Genetic diversity of *S. judaica* and *S. palaestina* species through TU-DAMD marker has been highlighted based on two (A) and (B) TU-DAMD sets.

The current study revealed that P% for set (A), was recorded to be 41.07, 35.48 and 82.09%; whereas, for set (B), these values were recorded to be 40.45, 42.31 and 90.00% for *S. judaica*, *S. palaestina* and *S. judaica* + *S. palaestina* together.
Together, respectively.

This observation could suggest low genetic diversity in *S. judaica* and *S. palaestina*. Our data were coherent with Safaei et al. (2016), who reported that P% was recorded to be 38.46, 47.25, 57.14, 49.45, 42.86 and 28.57% for *S. hypoleuca*, *S. nemorosa*, *S. limbata*, *S. xanthocheila*, *S. spinosa* and *S. reuterana*, respectively, using 10 ISSR primers. Similarly, Altindal (2019) reported P% of 32.03% in *S. officinalis* using 16 ISSR primers. Similar observation has been also noted by Tonk et al. (2010) in other Lamiaceae family members, who reported that GS values ranged between 0.49 - 0.73 indicated low genetic variability in Turkish oregano (*Origanum onites* L.) species using RAPD marker. Moreover, Gocer and Karaca (2016) reported 120 total bands, of which 42 (35%) were polymorphic among 26 cotton samples using 10 DAMD primers.

However, high genetic diversity has been recorded in other Lamiaceae family members. In this regards; Swamy and Anuradha (2011) reported 96 total bands of which 80 (83.3%) were polymorphic in patchouli cultivars (*Pogostemon cablin* Benth.) using RAPD marker. Moreover, Talebi et al. (2015) reported 240 total bands of which 198 (82.5%) were polymorphic with a mean average PIC of 0.248 in *Thymus daenensis* subsp. *daenensis* using SRAP marker. Indeed, Tapeh et al. (2018) reported 198 total bands of which 184 (92.9%) were polymorphic with a mean PIC average of 0.39 in Iranian *Teucrium* (*Teucrium polium* L.) populations using ISSR marker.

More recently, Saleh (2019b) reported 158 total bands of which 131 (82.911%) were polymorphic with a mean PIC and MI values of 0.264 and 2.269, respectively, in *S. tomentosa* based on Td-DAMD marker.

Since the molecular marker efficacy depend on the produced polymorphism degree; the current study could suggest that the two sets similarly and successfully highlighted genetic diversity between the two studied *Salvia* spices by showing similar PIC and MI values of 0.33 and 0.34 and 3.96 and 3.98 between *S. judaica* and *S. palaestina* species for set (A) and set (B), respectively.

**Conclusion**

Genetic diversity in *S. judaica* and *S. palaestina* species has been highlighted based on two TU-DAMD (A) and (B) sets. This work revealed that the two employed sets gave similar highly genetic diversity between the two studied *Salvia* sp. Whereas, low genetic diversity within each species has been recorded using the two employed sets. Based on data presented herein, it worth noting to use and validate this novel assay for molecular characterization of other plant species.

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**Table 4.** Percent disagreement values (PDV) yielded by TU-DAMD data based on UPGMA routine in statistical program.

| Genotype | SJ1 | SJ2 | SJ3 | SP4 | SP5 | SP6 | BD7 |
|----------|-----|-----|-----|-----|-----|-----|-----|
| SJ1      | 0.00|     |     |     |     |     |     |
| SJ2      | 0.16| 0.00|     |     |     |     |     |
| SJ3      | 0.13| 0.17| 0.00|     |     |     |     |
| SP4      | 0.53| 0.52| 0.47| 0.00|     |     |     |
| SP5      | 0.51| 0.48| 0.44| 0.08| 0.00|     |     |
| SP6      | 0.49| 0.53| 0.42| 0.13| 0.14| 0.00|     |
| BD7      | 0.49| 0.54| 0.44| 0.52| 0.49| 0.48| 0.00|

| Genotype | SJ1 | SJ2 | SJ3 | SP4 | SP5 | SP6 | BD7 |
|----------|-----|-----|-----|-----|-----|-----|-----|
| SJ1      | 0.00|     |     |     |     |     |     |
| SJ2      | 0.12| 0.00|     |     |     |     |     |
| SJ3      | 0.14| 0.15| 0.00|     |     |     |     |
| SP4      | 0.54| 0.50| 0.47| 0.00|     |     |     |
| SP5      | 0.51| 0.47| 0.46| 0.08| 0.00|     |     |
| SP6      | 0.52| 0.51| 0.45| 0.13| 0.14| 0.00|     |
| BD7      | 0.54| 0.54| 0.49| 0.53| 0.51| 0.50| 0.00|

Set A: The initial annealing temperature (Tm) was increased from 50 °C to 55 °C and set B - Tm increased from 55 °C to 60 °C by 0.5 °C/cycle during the first 10 PCR amplification cycles.

*S. judaica* (SJ1-SJ3), *S. palaestina* (SP4-SP6) and *B. damascena* (BD7).
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