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Genetic diversity of *Origanum syriacum* L. (Lamiaceae) species through Touch-Up Direct Amplification of Minisatellite-region DNA (TU-DAMD) marker

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Abstract

Origanum syriacum L. (Lamiaceae) is a perennial species with an important role in medicinal and pharmaceutical applications. However, the attention regarding its genetic diversity is negligible worldwide. Touch-up direct amplification of minisatellite-region DNA (TU-DAMD) marker has been employed to investigate genetic diversity of natural *Origanum syriacum* L. populations, where 37 wild *O. syriacum* genotypes were collected from coastal, central and southern regions of Syria. TU-DAMD assay resulted in 188 total bands, of which 152 (80.85%) were polymorphic. The total bands number ranged between 7 and 22 with an average of 11.75 bands/primer. Polymorphic bands number ranged between 4 and 19 with an average of 9.5 polymorphic bands/primer. Moreover, polymorphic information content (PIC) value ranged between 0.051 and 0.322 with an average of 0.225. As for marker index (MI), this value ranged between 0.308 and 5.092 with an average of 2.284. Overall, the present study suggests that the *O. syriacum* samples collected from coastal region (Lattakia, Jableh, Banyas and Tartous) are genetically distinct from the ones collected from central and southern regions (Homs, Hama, Damascus, Swedah and Qunetra) based on the estimated percent disagreement values (PDV) average of 0.20. Thereby, TU-DAMD test successfully discriminate *O. syriacum* genotypes showing high genetic diversity within the studied species. In conclusion, TU-DAMD test could be considered as a new tool for studying genetic diversity of other plant species.

Keywords: genotyping; molecular marker; polymorphism; polymorphic information content (PIC); Syrian oregano

Introduction

Origanum is a genus that belongs to the Lamiaceae family and that comprises 10 distinct sections including 49 taxa and 42 species. *Origanum syriacum* is native to Europe, North Africa and much of temperate Asia. It has many synonyms e.g., Syrian oregano, *Majorana syriaca*, Bible hyssop, Biblical-hyssop and Lebanese oregano. It grows in the eastern Mediterranean, southern Turkey, Cyprus, Syria, Lebanon, Jordan, and on the Sinai Peninsula and grows at different altitude (near sea level-2000 m) on limestone in rocky soils (Ietswaart, 1980; Mouterde, 1983). Its name was mentioned in different historical records from Bilad al-Sham area. It is called za'atar Khalil in coastal regions from Syria. Two wild *Origanum* species are recorded in Syria according

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to Mouterde (1983). It has many applications in folk medicine for treating gastrointestinal problems and respiratory diseases (El-Demerdash *et al.*, 2019).

Origanum genus exhibit wide range of medicinal and pharmaceutical applications as antioxidant, antifungal, antibacterial, antiviral, antitumor and anti-inflammatory agents as well as its use in folk medicine and as food additives since ancient times (Chishti *et al.*, 2013; García-Beltrán and Esteban, 2016; El-Demerdash *et al.*, 2019), of which *O. syriacum* has anticholinesterase, antiparasite, antioxidant, antifungal, antibacterial and anti-inflammatory properties (Loizzo *et al.*, 2009; Degerli *et al.*, 2012; El-Gendy *et al.*, 2015; García-Beltrán and Esteban, 2016; El-Demerdash *et al.*, 2019; Tag *et al.*, 2020).

Previously, Ietswaart (1980) reported the morphological characterization of *Origanum* genus. The employment of molecular markers became a capital factor that could be considered in plant breeding program improvement. In this regard; Taşcıoğlu *et al.* (2018) applied sequence related amplified polymorphism (SRAP) and expressed sequence tag single sequence repeat (EST-SSR) markers to investigate genetic diversity of 22 Turkish *Origanum* species. Recently, Papaioannou *et al.* (2020) applied SSR microsatellite loci in six Greek *Origanum* taxa genetic study.

Other researches addressed the genetic diversity at *Origanum* species level; e.g. in *O. syriacum*, De Leonardis *et al.* (2007) reported its carpological and palynological characterization, while, Akeel *et al.* (2009) applied random amplified polymorphic DNA (RAPD) marker to investigate genetic variability between *O. syriacum* L. and *Origanum majorana* L. Zaghloul *et al.* (2014) applied amplified fragment length polymorphism (AFLP) marker for conservation genetics of *Origanum syriacum* subsp. *sinaicum* populations. El-Demerdash *et al.* (2019) applied AFLP marker for molecular characterization of *O. vulgare*, *O. syriacum* and *Thymus* species. Lu *et al.* (2006) applied AFLP marker for molecular characterization of *O. onites* L., Tonk *et al.* (2010) applied RAPD marker for the same purpose, and Aboukhalid *et al.* (2017) used SSR for *O. compactum* molecular characterization. Recently, Karagoz *et al.* (2020) applied inter-primer binding sites (iPBS) marker for *O. acutidens* genetic diversity assessment.

Great attention has been paid to characterize *O. vulgare*; either at the morphological (Ibrahim *et al.*, 2012) or molecular level using RAPD marker (Katsiotis *et al.*, 2009; Mutu, 2014) and AFLP and selectively amplified microsatellite polymorphic loci (SAMPL) markers (Azizi *et al.*, 2009; 2012; 2016). Moreover, Lotti *et al.* (2019) reported its morphological, biochemical, and molecular analyses (AFLP).

Recently, Saleh (2021) applied touch-up direct amplification of minisatellite-region DNA (TU-DAMD) marker as a novel technique for genetic diversity assessment of *Salvia judaica* and *Salvia palaestina* species.

In the current study, TU-DAMD marker was employed for genetic diversity assessment of *O. syriacum* species due to the lack of information of its genetic diversity of populations growing in Syria. On the other hand, comparing with other molecular markers to discover TU-DAMD assay efficacy as a novel marker that could be applied for plants molecular characterization in plant breeding programs.

Materials and Methods

Sampling

Thirty-seven leaf samples of *Origanum syriacum* L. were collected from different geographical regions, differed in terms of their altitude and annual rainfall, in Syria. Thirty-one samples of which, were collected from coastal regions (eighteen for Lattakia, four from Jableh, one from Banyas, and eight samples from Tartous); three samples central region (one from Homs and two samples from Hama) and three samples from Syrian southern region (one from Damascus, one from Swedah, and one sample from Qunetra) (Table 1). Wild

Sideritis pullulans vent. (Lamiaceae) species was collected from Lattakia and included as an outside reference. Sampling has been carried out during blooming stage (April-March 2020).

Table 1. *Origanum syriacum* collection sites

Collection site	Code	Altitude (m)	Annual rainfall (mm)
Lattakia	L1	539	1426
	L2	550	1624
	L3	420	1520
	L4	129	1516
	L5	187	1512
	L6	93	1200
	L7	190	1430
	L8	116	1477
	L9	129	1579
	L10	637	1838
Jableh	L11	428	1804
	L12	361	1377
	L13	155	1421
	L14	136	1470
	L15	704	1639
	L16	127	1528
	L17	708	1335
	L18	1081	1815
	J19	409	1778
	J20	774	1700
	J21	192	1603
Banyas	J22	459	1801
	B23	681	1619
Tartous	T24	514	1607
	T25	283	1531
	T26	58	1563
	T27	486	1923
	T28	377	1544
Homs	T29	253	1529
	T30	773	1852
	T31	852	1790
	Ho32	322	1183
Hama	Ha33	451	397
	Ha34	193	618
Damascus	D35	1614	336
Swedah	S36	955	378
Qunetra	Q37	904	553
Lattakia	SP	80	750

Genomic DNA extraction

Total genomic leaf DNA of 37 *O. syriacum* genotypes along with *S. pullulans*, was extracted by CTAB (cetyltrimethylammonium bromide) protocol according to Doyle and Doyle (1987). DNA concentration was determined using DNA fluorimeter instrument (GeneQuant-Amersham, Biosciences – England, Ser 802111-98/88411). DNA was stored at –80 °C until be used.

Touch-up direct amplification of minisatellite-region DNA (TU-DAMD) marker

Sixteen DAMD primers (Table 2) were used in TU-DAMD-PCR assay to differentiate DNA among collected samples. TU-DAMD-PCR amplification was performed as described by Saleh (2021) in a T-gradient thermal cycler (Bio-Rad, Hercules, USA) programmed as following: one cycle for four minutes at 94 °C, followed by ten cycles of pre-PCR involving of 30 seconds at 94 °C for denaturation, 45 seconds at 55 °C for annealing, and three min at 72 °C for extension. Annealing temperature was increased 0.5 °C/ cycle for the first ten cycles, then 30 cycles at a constant 55 °C as annealing temperature and followed by final extension at 72 °C for ten minutes. Final PCR products were analyzed by gel electrophoresis in 2% agarose (to which ethidium bromide was added) (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 the approximate molecular weight of TU-DAMD-PCR amplified products.

Table 2. DAMD primers employed in the present study

Primer N°	Primer name	Primer sequence 5'-3'
1	URP1F	ATCCAAGGTCCGAGACAACC
2	URP2R	CCCAGCAACTGATCGCACAC
3	URP4R	AGGACTCGATAACAGGCTCC
4	URP9F	ATGTGTGCGATCAGTTGCTG
5	URP25F	GATGTGTTCTTGGAGCCTGT
6	URP30F	GGACAAGAAGAGGATGTGGA
7	OGRB01	AGGGCTGGAGGAGGGC
8	HBV3	GGTGAAGCACAGGTG
9	HBV5	GGTGTAGAGAGGGGT
10	YNZ22	CTCTGGGTGTGGTGC
11	6.2H(+)	AGGAGGAGGGGAAGG
12	HBVb	GGTGTAGAGAGAGGGGT
13	URP6R	GGCAAGCTGGTGGGAGGTAC
14	URP17R	AATGTGGGCAAGCTGGTGGT
15	M13	GAGGGTGGCGGTTCCT
16	HVA	AGGATGGAAAGGAGGC

TU-DAMD Data analysis

Bands were photographed under UV light, scored either as present (1) or absent (0). The Unweighted Pair Group Mean Arithmetic average (UPGMA) and percent disagreement values (PDV) of the Statistica program (Statsoft, 2003), were used to construct the matrix and the genetic relationship among the studied 37 *O. syriacum* samples. Jaccard's (1908) index was used to determine genetic similarity among these samples. Polymorphic information content (PIC) was determined as described by Botstein *et al.* (1980) according to the following formula:

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

Where Pij is the frequency of the i^{th} pattern revealed by the j^{th} primer summed across all patterns revealed by the primers. Moreover, marker index (MI) was also determined as reported by Powell *et al.* (1996) according to the following formula:

$$MI = PIC \times \eta\beta \quad (2)$$

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

Results

In the current study, genetic diversity of *O. syriacum* species has been investigated using TU-DAMD marker. TU-DAMD polymorphism pattern produced by URP1F (A), URP30F (B) and HBVb (C) DAMD primers is illustrated in Figure 1.

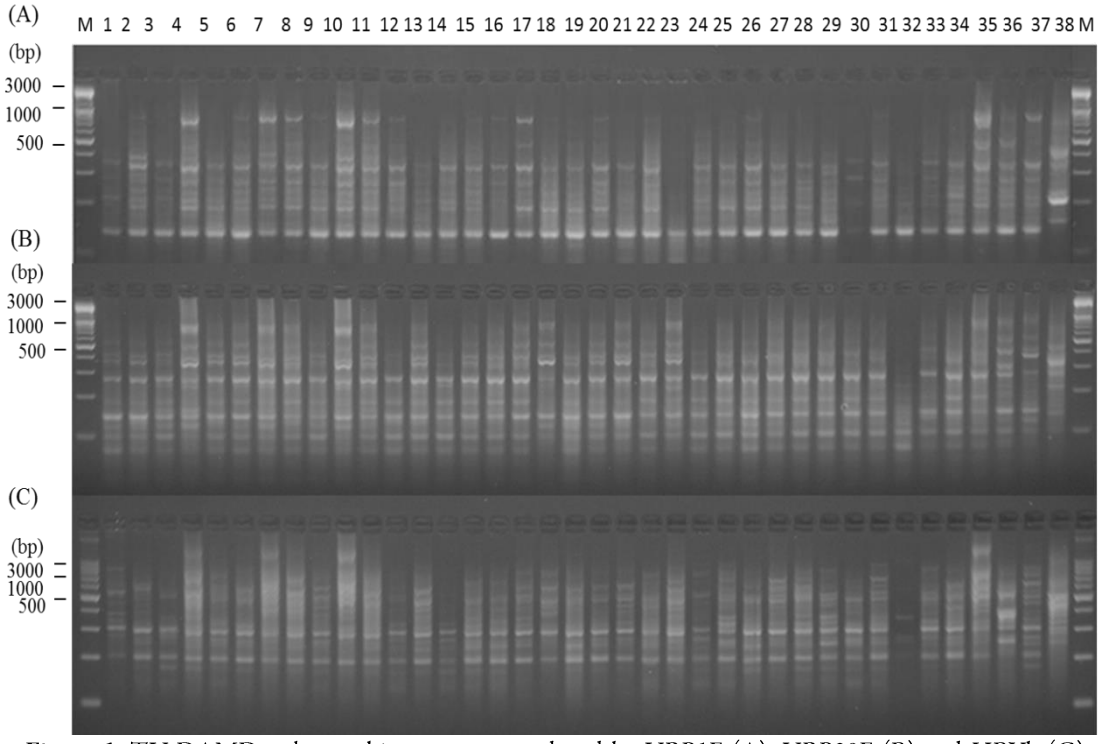

Figure 1. TU-DAMD polymorphism pattern produced by URP1F (A), URP30F (B) and HBVb (C) DAMD primers; 1-37 lanes: *O. syriacum* samples and 38 lanes: *S. pullulans* as an outside reference
M: A VC100bp Plus DNA Ladder (Vivantis) ladder standard.

PCR products-size ranged between 120-3000 bp. Data revealed that the total bands number ranged between 7 (HVA) and 22 (HBV3) with an average of 11.75 bands/primer. Polymorphic bands number ranged between 4 (HVA) and 19 (HBV3) with an average of 9.5 polymorphic bands/primer (Table 3). Polymorphism level (P%) ranged between 57.143 (HVA) and 94.118% (HBV5) with an average of 79.103%. PIC value ranged between 0.051 (OGRB01) and 0.322 (URP25F) with a mean PIC average of 0.225. As for MI, this value ranged between 0.308 (HVA) and 5.092 (HBV3) with a MI average of 2.284 (Table 3).

Table 3. TU-DAMD scoring amplified bands

Primer name	TB	PB	P%	PIC	MI
URP1F	11	9	81.818	0.227	2.043
URP2R	11	8	72.727	0.208	1.664
URP4R	10	9	90.000	0.297	2.673
URP9F	13	11	84.615	0.279	3.069
URP25F	10	9	90.000	0.322	2.898
URP30F	11	8	72.727	0.191	1.528
OGRB01	11	7	63.636	0.051	0.357
HBV3	22	19	86.363	0.268	5.092
HBV5	17	16	94.118	0.278	4.448
YNZ22	10	7	70.000	0.286	2.002
6.2H(+)	10	9	90.000	0.238	2.142
HBVb	12	10	83.333	0.259	2.590
URP6R	8	5	62.500	0.147	0.735
URP17R	15	13	86.667	0.245	3.185
M13	10	8	80.000	0.226	1.808
HVA	7	4	57.143	0.077	0.308
Total	188	152			
Mean	11.75	9.5	79.103	0.225	2.284

TB – total bands, PB – polymorphic bands, P % – polymorphic % and PIC – polymorphic information content. MI – marker index

Unweighted Pair Group Mean Arithmetic average (UPGMA) using Statistica program and percent disagreement values (PDV) were used to construct the cluster degree among *O. syriacum* 37 collected samples. UPGMA showed that the studied *O. syriacum* samples are divided into two main groups (Figure 2); the first group involves 31 samples belonging to Syrian coastal regions (Lattakia, Jableh, Banyas and Tartous, Figure 2) of which T25 and T26 were the closest samples showing the lowest PDV of 0.04 and the highest GS value of 0.91, followed by J19 and J20 with PDV value of 0.05 and GS value of 0.90. Whereas, the second group involves six samples (16.2% of total studied samples) belonging to Syrian central and southern regions (Homs, Hama, Damascus, Swedah and Qunetra, Figure 2); of which Ha33 & Ha34 were the closest samples by showing the lowest PDV of 0.10 and the highest GS value of 0.73.

Overall, the farthest genotypes were L10 and Ho32 by showing the highest PDV value of 0.39 and the lowest GS value of 0.35, followed by L7 and Ho32 with PDV value of 0.35 and GS value of 0.37.

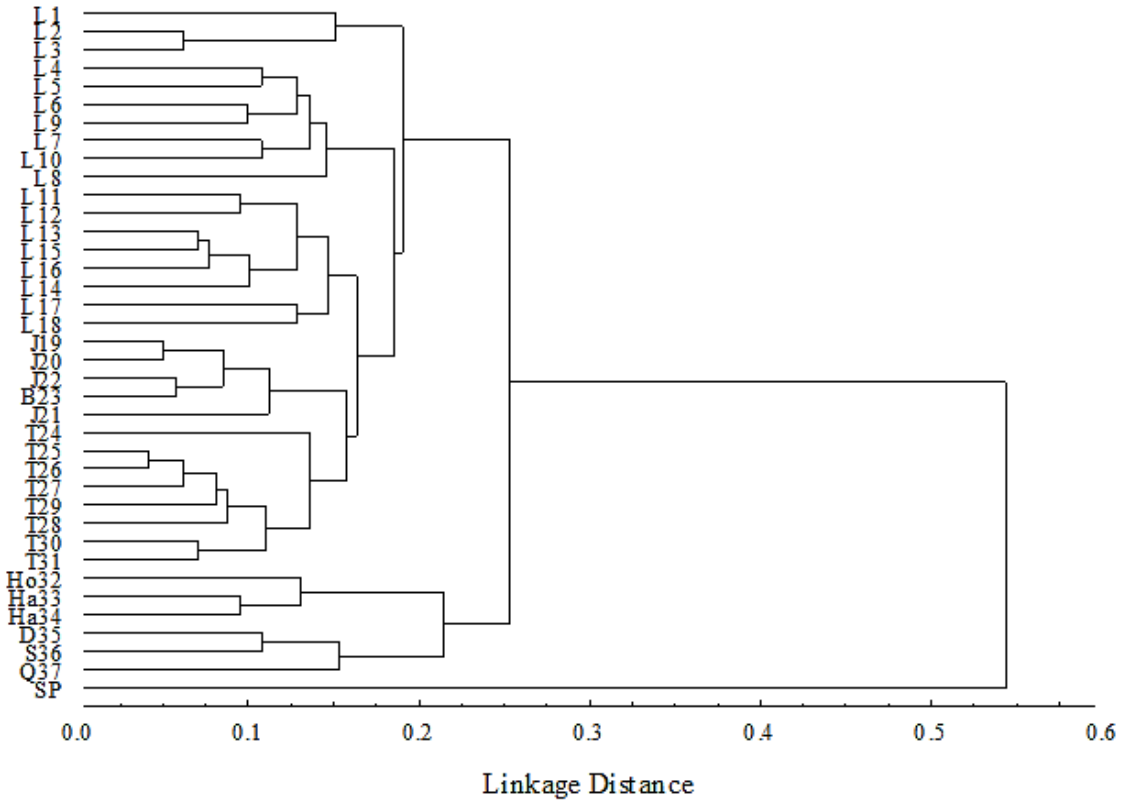

Figure 2. UPGMA cluster analysis showing grouping of *O. syriacum* studied samples using TU- DAMD marker

Discussion

Genetic diversity of *O. syriacum* L. growing in Syria has been investigated using TU-DAMD assay. Data revealed that TU-DAMD assay highlighted 188 total bands, of which 152 (80.9%) were polymorphic, reflecting high genetic diversity within the studied species. Other studies have been done on other *Origanum* species, e.g., Zaghloul *et al.* (2014) reported that polymorphism ranged between 45.77-63.18% with an average of 57.1% in *O. syriacum* subsp. *sinaicum* using AFLP marker. Whereas, Akeel *et al.* (2009) reported 1359 total bands of which 136 (10.0%) were polymorphic between *O. syriacum* L. and *O. majorana* L. using RAPD marker with similarity values varied between 0.00-0.85. They reported low genetic variability between the two studied *Origanum* species. Whereas, Mutu (2014) reported 153 total bands of which 79 (51.6%) were polymorphic in 5 *O. vulgare* genotypes using 27 RAPD primers. Azizi *et al.* (2016) reported 285 loci of which 214 (75.1%) and 287 loci of which 263 (91.64%) were polymorphic using AFLP and SAMPL markers, respectively, in *O. vulgare*.

Taşcıoğlu *et al.* (2018) applied SRAP and EST-SSR markers to investigate genetic diversity of 22 Turkish *Origanum* species. They reported that the polymorphic alleles ranged between 3-20 alleles with an average of 12.04 alleles in the case of SRAP marker. Whereas, it ranged between 2-6 alleles with an average of 4 alleles in the case of EST-SSR marker. The same study revealed that EST-SSR marker displayed higher mean genetic diversity (0.36) compared to SRAP marker (0.27). The same study revealed high genetic diversity of the Turkish *Origanum* species was with a mean Jaccard dissimilarity index of 0.76. Whereas, El-Demerdash *et*

al. (2019) reported 193 loci of which 103 (53.4%) were polymorphic among cultivated *O. vulgare* and wild *O. syriacum* using 4 AFLP primer combinations.

The previous study mentioned also that polymorphism level has been recorded to be 171 loci of which 69 (40.4%) were polymorphic in *Thymus* among wild (*T. capitatus* and *T. decassatus*) and cultivated *T. vulgaris* species using also 4 AFLP primer combinations. Furthermore, Lotti *et al.* (2019) reported 3315 total loci of which 1179 (35.6%) were polymorphic among 24 *O. vulgare* genotypes using 10 AFLP primer combinations.

Lu *et al.* (2006) reported 80.6% polymorphism level with genetic similarity ranging between 0.396 – 0.725% in Turkish oregano (*O. onites* L.) using AFLP analysis. Whereas, Karaca *et al.* (2015) reported that the PIC value ranged between 0-0.92 with a mean average of 0.307 and that the GS value ranged between 0.76-0.87 with a mean average of 0.79 in *Thymus* L. using microsatellites marker. Moreover, Tonk *et al.* (2010) reported 412 total bands of which 319 (77.427%) were polymorphic reflecting low genetic diversity in *O. onites* (GS ranged between 0.49-0.73) using RAPD marker. Whereas, Yousefi *et al.* (2015) reported that that PIC value ranged between 0.33-0.49 with a mean average of 0.42 and that the GS ranged between 0.27-0.81 with a mean average of 0.46 and mean polymorphism level of 97.305% in *Thymus* species based on inter-simple sequences repeat (ISSR) marker.

Aboukhalid *et al.* (2017) reported a P% of 82.94% among *Origanum compactum* collected from Morocco using 15 SSR PCs combinations. Recently, Karagoz *et al.* (2020) reported that total alleles ranged between 25-36 alleles with a mean average of 28.9 allele/primer; whereas, polymorphic alleles ranged between 25-36 with a mean average of 28.4 polymorphic allele/primer in *Origanum acutidens* using iPBS marker. Moreover, PIC, value ranged between 0.28-0.42 with a mean average of 0.32. Overall, iPBS marker gave a P% of 98.269%, reflecting high genetic diversity in the studied species.

More recently, Saleh (2021) reported the use of TU-DAMD for *Salvia judaica* and *Salvia palaestina* genetic diversity. The mentioned study revealed P% of 94.02%, with PIC average of 0.34 and MI average of 3.98 between the two studied species.

In the current study, high genetic diversity has been observed among the studied *O. syriacum* samples. In this regards, polymorphism level (80.851%) has been recorded in the current study among the studied *O. syriacum* samples. Our data were coherent with other studies on other species belong to lamiaceae family; e.g., this value was recorded to be 80.6% in Turkish oregano (*O. onites* L.) using AFLP analysis (Lu *et al.*, 2006); 82.94% in Moroccan *O. compactum* using SSR analysis (Aboukhalid *et al.*, 2017); 82.68 % in *Thymus caramanicus* using ISSR marker (Hadian *et al.*, 2014) and 82.911% in *S. tomentosa* using Td-DAMD analysis (Saleh, 2019). However, low genetic diversity of 57.10% has been reported in *O. syriacum* collected from Egypt using AFLP marker (Zaghloul *et al.*, 2014) and 40.45 & 42.31% in *S. judaica* and *S. palaestina* species, respectively using TU-DAMD marker (Saleh, 2021). While, Karagoz *et al.* (2020) reported high P% of 98.269% in *O. acutidens* using iPBS marker.

Overall, the present study could suggest that the *O. syriacum* samples collected from coastal regions (Lattakia, Jableh, Banyas and Tartous) were genetically distinct from the ones collected from central and southern regions (Homs, Hama, Damascus, Swedah and Qunetra).

The genetic diversity observed in *O. syriacum* population using TU-DAMD marker, might be related to the occurrence of putative hybrids that has been reported in *Origanum* (Ietswaart, 1980; Taşcıoğlu *et al.*, 2018). Where, the cross-hybridization is common in the *Origanum* genus played a significant role as key factor in its speciation (Ietswaart, 1980; Taşcıoğlu *et al.*, 2018).

Conclusions

TU-DAMD assay revealed high P% of 80.851% in the natural *O. syriacum* population. Overall, TU-DAMD as a new marker used in the current study successfully discriminate among *O. syriacum* samples collected from different geographical regions in Syria. Thereby, it is advisable to employ it for genetic diversity assessment in other plants species. The genetic diversity observed among the studied *O. syriacum* samples could serve in future *O. syriacum* breeding programs.

Authors' Contributions

The author read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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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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