# Genetic Relationship Among Some Lentil Genotypes Using ISSR Markers

**Rehab Al-Mousa* [(1)], Alaa Alshaal [(1)], Shahinaz Abbas [(1)], Nabila Ali Bacha [(1)], Yanal Al-Kudssi [(1)], Khozama Kountar [(1)] and Tony Saloom[(1)]**
(1). Department of Biotechnology, General Commission for Scientific Agricultural Research, Damascus, Syria.
(*Corresponding author: Dr.Rehab Al-Mousa, e-mail: bebo_moussa@yahoo.com, )

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**Abstract:**
The present research was conducted to detect the genetic relationship among twelve lentil lines introduced from ICARDA and two local varieties. Eighteen Inter Simple Sequence Repeats (ISSR) primers successfully amplified 167 bands in all genotypes with the average of 9.27 bands/primer. The mean value of polymorphism percentage was 78.7%. Primers NLSSR3 and 830 presented 100% polymorphism percentage. The highest number of amplification bands (124) was noticed in genotype (Ln12), while the lowest number of amplification bands (87) was noticed in genotype (Idleb5). Thirty one unique bands were obtained, out of them 9 were considered as present bands (positive) and 22 were considered as absent bands (negative). The highest number of unique bands (5) were registered in genotype (Idleb3), while genotypes (Ln9 and Ln12) did not present any unique band. The genetic distance ranged from 0.21 to 0.48. ISSR cluster analysis grouped the genotypes into two main clusters; the first one included local varieties (Idleb3 and Idleb5), while the second cluster included the remaining lines. Some genotypes showed wide divergence (Idleb5, Ln1 and Ln6), whereas genotypes (Ln11 and Ln 12) were close related.
**Keywords**: Lentil, ICARDA lines, local varieties, genetic relationship, ISSR analysis.

## Introduction:

Lentil (*Lens culinaris* Medik) belongs to the family *Leguminosae*. It is self-pollinated, diploid plant with 2n=2x= 14 (Arumuganathan and Earle, 1991).

Lentil is the third most important legume crop in the world. Lentil seeds are considered as an important source of human nutrition containing proteins (27.5-31.7%), carbohydrates, mineral, antioxidants and fibers (Migliozzi *et al*., 2015). While lentil straw is used as high quality animal feed (Lardy and Anderson, 2009). In addition, lentil plants has capacity to fix nitrogen which used as fertilizer in cereal –based cropping system (De Ron *et al*., 2017).

Lentil is cultivated only in few parts of the world. Its cultivation is expected to increase rapidly in the future due to its demand for consumption and agronomic ability to assimilate atmospheric nitrogen (Abraham, 2015) .The total lentil cultivated area in the world is estimated around 5 million hectares with annual production and productivity of 6.54 million tons and 13049 kg/ha (FAO, 2021). Canada is the leading exporting nation, while India is the leading lentil consuming and producing nation (Bedard *et al*., 2010). Production of lentil in Syria reached 200218 tons with yield of 17772 kg/ha. The area harvested of lentil in Syria amounted 112657 ha which ranked the 10$^{th}$ in the world while Canada ranked the 1$^{st}$ with about 1.7 million hectare (FAO, 2021). The main lentil-production

provinces in Syria are: Aleppo, Hasakaeh, Idleb, Hama, Dara'a and Sweida (FAO Special Report, 2021).

Knowledge of genetic diversity and population structure is a crucial step for an efficient use of available material in plant breeding and conservation programs (Mbasani-Mansi *et al*., 2019). Morphological and izozymes markers were used for identifying genetic diversity in lentil (Ahmad and McNeil, 1996). These markers are influenced by environmental conditions (Maric *et al*., 2004). Molecular markers have been widely used as they are not affected either by environmental factors or by plant development stage. The choice of molecular marker is a critical step for geneticists and breeders. Genetic diversity detection largely depends on the type of molecular markers, repeat motif nature, number of repeats and genetic relationship of the germplasm (Pandey *et al*., 2018).

Several molecular markers have been used to access genetic diversity in lentil such as Random Amplified Polymorphism DNA (RAPD) (Harb *et al*., 2019; Mbasani-mansi *et al*., 2019), Amplified Fragment Length Polymorphism (AFLP) (Idrissi *et al.,* 2016), Simple Sequence Repeats (SSR) (Pandey *et al*., 2018) and Inter Simple Sequence repeats (ISSR) (Babayeva *et al*., 2018; Harb *et al*., 2019).

Inter simple sequence repeat (ISSR) is a technique which can differentiate closely related genotypes. It is based on the amplification of a single primer containing a microsatellite ‘core’ sequence anchored at the 3′ or 5″ end by 2–4 selective, often degenerate, nucleotides. The DNA fragments amplified are flanked by inversely oriented, adjacent microsatellites (Zietkiewicz *et al.,* 1994).

Inter simple sequence repeat (ISSR) marker, in addition to its suitability to genetic diversity study, is highly polymorphic, reproducible, cost effective, and requires no prior information of the sequence (Bornet *et al.,* 2002).

The world lentil collection is held by ICARDA which obtained from ICARDA collection missions, donor institutions and ICARDA's breeding programs (Coyne and McGee, 2013). The national program in different lentil growing region including Syria are widely using ICARDA enhanced lentil germplasm.

Thus, this study aimed to detect the genetic relationship among twelve lentil lines introduced from ICARDA and two local varieties using ISSR markers.

**Materials and Methods:**

This study was performed during 2019-2021 at Molecular Genetics Laboratory, Department of Biotechnology, General Commission for Scientific Agricultural Research, Damascus, Syria.

**Plant material:**

A set of 12 lines of lentil introduced from ICARDA and 2 local varieties (Idleb 3 and Idleb 5) were used in this study (Table, 1)

**Table(1): Entries information for 12 lentil lines**

| Line No. | Pedigree | Source |
|---|---|---|
| **Ln 1** | ILL7888/ILL5782 | BARI/ICARDA |
| **Ln 2** | LC006600899Z×ILL6002 | ICARDA |
| **Ln 3** | ILL8194×ILL8006 | ICARDA |
| **Ln 4** | EP35 | ICARDA |
| **Ln 5** | ILL7012×ILL6994 | ICARDA |
| **Ln 6** | ILL5888×ILL6002 | ICARDA |
| **Ln 7** | ILL7616/ILL2501 | BARI/ICARDA |
| **Ln 8** | ILL7012×ILL6994 | ICARDA |
| **Ln 9** | ILL6994×ILL9932 | ICARDA |

| **Ln 10** | ILL6994× ILL9932 | ICARDA |
|---|---|---|
| **Ln 11** | ILL88527× Subrata | ICARDA |
| **Ln 12** | ILL6994×ILL9932 | ICARDA |

**DNA extraction:**

Young leaves were collected from lentil seedlings and used for genomic DNA isolation using the cetytrimethyl ammonium bromide CTAB method as described by (Lassner *et al*., 1989).

DNA was quantified using spectrophotometer by taking absorbance at A260 and A280.

DNA quality was checked by agarose gel electrophoresis. DNA bands without smears were considered for PCR amplification.

All genomic DNA samples were uniformed to a final concentration of 50 ng.$\mu l^{-1}$ and used for PCR amplification reactions.

**DNA amplification and visualization by ISSR analysis:**

Molecular polymorphism was assessed by a set of 20 ISSR primers (table, 2). The amplification reaction was carried out in thermocycler (Biometra modell T-1 Thermoblock) under the following conditions: initial denaturation at 95°C for 5 minute; 37 cycles of 1 minute at 94 ºC for denaturation, 1 minute for primer annealing at a (Ta) according to the primer (Table, 2), and 1.30 minute at 72 ºC for extension, with a final extension for 10 minutes at 72 ºC. The total reaction volume of PCR amplification was 25 µl containing KAPA Taq ready mix 2X, 20 pM primer and 100 ng of template DNA.

**Table(2): ISSR primers profile (name, sequence and annealing temperature (Ta))**

| Primer No. | Primer Name | Primer Sequence | Ta (C°) |
|---|---|---|---|
| **ISSR1** | 4 | CAC ACA CAC ACA CAC AAC | 48 |
| **ISSR2** | A830241 | ACT GAC TGA CTG ACT GAC TG | 44 |
| **ISSR3** | 813 | CTC TCT CTC TCT CTC TT | 50 |
| **ISSR4** | 807 | AGA GAG AGA GAG AGA GT | 50 |
| **ISSR5** | 8565 | GTC ACCACCACCACCACCACC AC | 64 |
| **ISSR6** | 866 | CTCCTCCTCCTCCTCCTC | 53 |
| **ISSR7** | W814 | CTC TCT CTC TCT CTC TTG | 45 |
| **ISSR8** | 8 | CAC ACA CAC ACA CAC AGA C | 48 |
| **ISSR9** | 862 | AGCAGCAGCAGCAGCAGC | 53 |
| **ISSR10** | 17899B | CAC ACA CAC ACA GG | 46 |
| **ISSR11** | 231 | GAG TCT CTC TCT CTC TCT C | 51 |
| **ISSR12** | 8082 | CTC TCT CTC TCT CTC TCT G | 51 |
| **ISSR13** | NLSSR3 | CAG CAG CAG CAG CAG | 53 |
| **ISSR14** | 17 | CAG CAC ACA CAC ACA CAC | 51 |
| **ISSR15** | 5 | CAC ACA CAC ACA CAC AGT | 48 |
| **ISSR16** | 830 | TGT GTG TGT GTG TGT GG | 44 |
| **ISSR17** | 811 | GAG AGA GAG AGA GAG AC | 44 |
| **ISSR18** | 812 | GAG AGA GAG AGA GAG AA | 48 |
| **ISSR19** | 8564 | CACCACCACCACCACCACCAC C | 48 |
| **ISSR20** | 16 | CGT CAC ACA CAC ACA CAC | 49 |

**Data analysis:** ISSR bands were scored in a 0-1 binary format and analyzed using Total Lab 1D software. XLSTAT software (XLSTAT, 2017) was used to calculate the Polymorphism Information

Content (PIC) for each primer, and to build of the cluster dendrogram based on the UPGMA (unweighted pair group method with arithmetical averages) algorithm.

**Results:**

A set of 20 ISSR primers were used to estimate the genetic relationship among 14 lentil genotypes. Only 18 primers amplified successfully (Fig, 1). A total of 167 amplification DNA bands, with an average of 9.27 bands/primer, were produced using 18 ISSR primers. The total number of scorable markers produced per individual primer ranged between 12 bands in primer (NLSSR3) and 6 bands in primers (862 and17) as shown in table (3). The size of amplified bands ranged from 126.67 bp in primer (862) to 1783.78 bp in primer (4).

The number of polymorphic bands was 134 with an average of 7.44 bands/primer. The highest number of polymorphic bands (12) was observed with primer (NLSSR3), while the lowest polymorphic bands (3) were noticed with primer (17). The polymorphism percentage ranged from 100% in primers (NLSSR3 and 830) to 50% in primer (17) with an average of 78.7%.

The PIC values ranged from 0.19 in primer (813) to 0.38 in primer (866) with mean value (0.29).

**Table (3): The size of amplicons, number of amplified bands (total, polymorphic, monomorphic), polymorphism percentage and PIC values among lentil genotypes as revealed by ISSR primers**

| Primers | Size range (bp) | No. of amplification | | Polymorphism% | PIC |
|---|---|---|---|---|---|
| | | Total | Polymorphic | | |
| **4** | 1783.78-297.21 | 11 | 9 | 82 | 0.31 |
| **A830241** | 742.97-267.79 | 7 | 5 | 71 | 0.28 |
| **813** | 1057.6-298.12 | 9 | 5 | 56 | 0.19 |
| **807** | 784.17-175.36 | 9 | 6 | 67 | 0.20 |
| **8565** | 770.29-236.8 | 9 | 8 | 89 | 0.36 |
| **866** | 976.88-191.76 | 11 | 9 | 82 | 0.38 |
| **8** | 715.93-256.53 | 8 | 6 | 75 | 0.34 |
| **862** | 436.39-126.67 | 6 | 4 | 67 | 0.24 |
| **17899B** | 821.16-289.45 | 11 | 9 | 82 | 0.27 |
| **231** | 710.55-253.37 | 11 | 9 | 82 | 0.30 |
| **8082** | 1200.12-275.59 | 8 | 7 | 88 | 0.32 |
| **NLSSR3** | 867.39-136.2 | 12 | 12 | 100 | 0.32 |
| **17** | 561.95-311.35 | 6 | 3 | 50 | 0.21 |
| **5** | 875.53-334.75 | 9 | 8 | 89 | 0.33 |
| **830** | 969.89-289.77 | 10 | 10 | 100 | 0.32 |
| **811** | 1133.08-276.89 | 10 | 7 | 70 | 0.24 |
| **812** | 1151.65-312.64 | 11 | 10 | 91 | 0.33 |
| **16** | 767.82-297.62 | 9 | 7 | 78 | 0.32 |
| **SUM** | | **167** | **134** | | |
| **Average** | | **9.27** | **7.44** | **78.7** | **0.29** |

The tested ISSR primers were able to identify 31 unique bands in 14 lentil genotypes (Table, 4). Out of them, 9 bands were positive (present bands) and could be considered as marker assisted selection. Genotype Ln1 generated one positive band with molecular size of 710.5bp in primer 231. Genotype Ln3 generated two positive unique bands with molecular size of 436.4bp in primer 862 and 204.3bp in primer 812. Genotype Ln4 generated one positive band of molecular size 289.7 in primer 830. Genotype Ln5 generated one positive band of molecular size 569.3 in primer 811. Genotype Ln6 generated one positive band of molecular size 746.8 in primer NLSSR3. Genotype Ln8 generated one

positive band of molecular size 1057.6 in primer 813. Genotype Ln11 generated one positive band of molecular size 568 in primer 17899B. Genotype Idleb3 generated one positive band of molecular size 244.2 in primer 813.

While genotypes Ln9 and Ln12 were not able to generate any specific band (discarded from table, 4) On the other hand, the number of unique bands (positive or negative) generated per primer ranged from 1 in primers 866, 862 and 17 to 3 in primers 8565, 5, 811 and 812, while primers A830241, 807 and 8 were not able to detect any specific band (discarded from Table, 4).

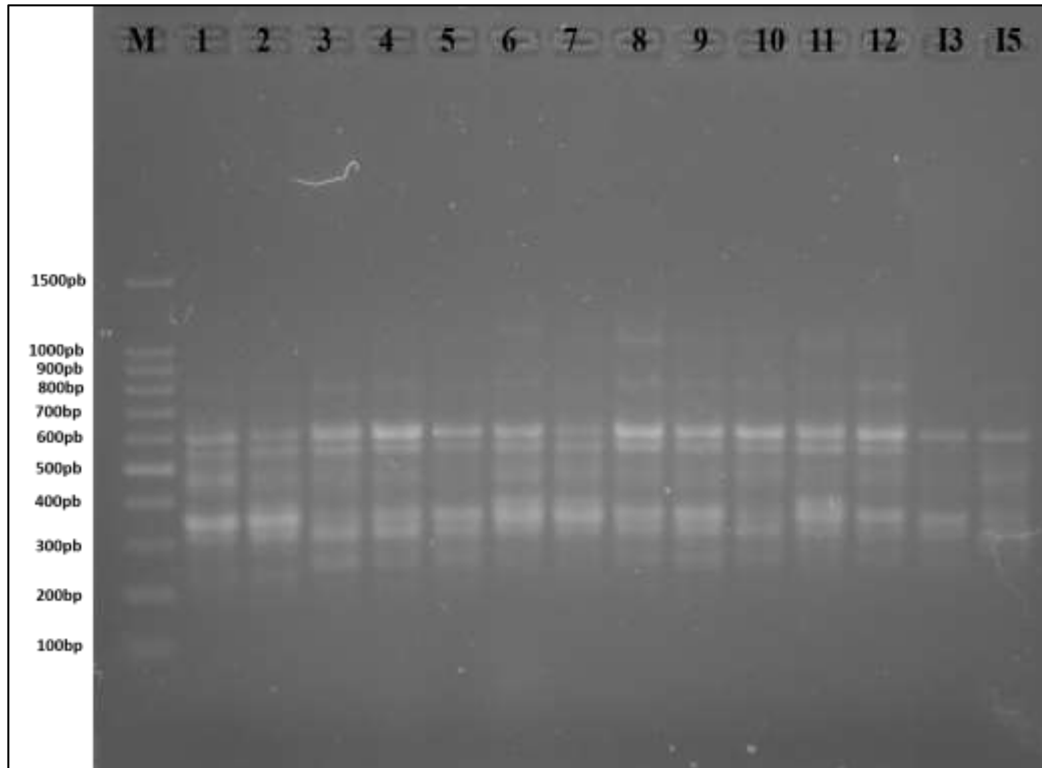


**Fig (1): ISSR profile of lentil genotypes amplified by ISSR primer 811. M:KAPA Universal Ladder (KAPA BIOSYSTEMS), lanes 1 through 14 refer to lentil lines from Ln1 to Ln12 and varieties Idleb3and Idleb 5.**

**Table (4): Molecular size (bp) of unique bands (positive+, negative-) generated from lentil genotypes with tested ISSR primers**

| Genotypes | Primer | Unique Bands | |
|---|---|---|---|
| | | Positive (Present) | Negative (Absent) |
| **Ln1** | 866 | | 421.6 bp |
| | 231 | 710.5 bp | |
| **Ln2** | 5 | | 495 bp |
| | 5 | | 441.4 bp |
| **Ln3** | 862 | 436.4 bp | |
| | 812 | 204.3 bp | 549.3 bp |
| | 16 | | 662.6 bp |
| **Ln4** | 830 | 289.7 bp | |
| **Ln5** | 17899B | | 454.1 bp |
| | 5 | | 369.2 bp |
| | 811 | 569.3 bp | |
| **Ln6** | NLSSR3 | 746.8 bp | |
| **Ln7** | 830 | | 619.3 bp |

| | | | |
|---|---|---|---|
| | 812 | | 1151.6 bp |
| **Ln8** | 813 | 1057.6 bp | |
| **Ln10** | 4 | | 405.4 bp |
| | 4 | | 297.9 bp |
| | 8082 | | 273.1 bp |
| **Ln11** | 17899B | 568.4 bp | |
| | NLSSR3 | | 459.3 bp |
| | 17 | | 544.3 bp |
| **Idleb3** | 813 | 244.2 bp | |
| | 8082 | | 824.3 bp |
| | 811 | | 845.1 bp |
| | 811 | | 605.8 bp |
| | 16 | | 330.9 bp |
| **Idleb5** | 8565 | | 645.7 bp |
| | 8565 | | 421.2 bp |
| | 8565 | | 374.3 bp |
| | 231 | | 274.4 bp |

**The dissimilarity matrix**

Table 5 showed the dissimilarity matrix of Jaccard's coefficient based on ISSR data for the 14 lentil genotypes. The genetic distance ranged from 0.21 to 0.48 with mean value of 0.41.

The lowest genetic distance (0.21) was observed between genotypes Ln11 and Ln12 suggesting their close relatedness. Whereas genotypes Idleb3 and Ln10, Idleb5 and Ln1, Idleb5 and Ln6 seemed to be the most divergent since they had exhibited the highest genetic distance (0.48).

**Table (5): Proximity matrix (Jaccard coefficient)**

| | Ln1 | Ln2 | Ln3 | Ln4 | Ln5 | Ln6 | Ln7 | Ln8 | Ln9 | Ln10 | Ln11 | Ln12 | Ideb3 | Ideb5 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Ln1** | 0 | | | | | | | | | | | | | |
| **Ln2** | **0.27** | 0 | | | | | | | | | | | | |
| **Ln3** | 0.34 | 0.28 | 0 | | | | | | | | | | | |
| **Ln4** | 0.34 | 0.29 | **0.27** | 0 | | | | | | | | | | |
| **Ln5** | 0.38 | 0.35 | 0.34 | 0.29 | 0 | | | | | | | | | |
| **Ln6** | 0.39 | 0.34 | 0.32 | 0.30 | 0.40 | 0 | | | | | | | | |
| **Ln7** | 0.39 | 0.37 | 0.33 | 0.34 | 0.41 | **0.26** | 0 | | | | | | | |
| **Ln8** | 0.39 | 0.39 | 0.39 | 0.33 | **0.29** | 0.29 | 0.33 | 0 | | | | | | |
| **Ln9** | 0.34 | 0.31 | 0.33 | 0.33 | 0.36 | 0.38 | 0.40 | 0.34 | 0 | | | | | |
| **Ln10** | 0.37 | 0.38 | 0.33 | 0.34 | 0.35 | 0.41 | 0.43 | 0.35 | 0.31 | 0 | | | | |
| **Ln11** | 0.38 | 0.38 | 0.33 | 0.30 | 0.34 | 0.33 | 0.36 | 0.35 | 0.30 | 0.33 | 0 | | | |
| **Ln12** | 0.37 | 0.36 | 0.28 | 0.26 | 0.29 | 0.28 | 0.30 | 0.27 | 0.27 | 0.30 | **0.21** | 0 | | |
| **Idib3** | 0.37 | 0.37 | 0.42 | 0.41 | 0.32 | 0.47 | 0.45 | 0.43 | 0.37 | **0.48** | 0.42 | 0.43 | 0 | |
| **Idib5** | **0.48** | 0.42 | 0.45 | 0.42 | 0.42 | **0.48** | 0.45 | 0.44 | 0.47 | 0.47 | 0.41 | 0.41 | **0.38** | 0 |

**Cluster analysis as revealed by ISSR**

UPGMA dendrogram of genetic distances between 14 lentil genotypes based on dissimilarity matrix is depicted in Fig, 2.

In this dendrogram, the 14 lentil genotypes were classified into two main clusters. The smaller cluster comprised of the two local varieties (Idleb3 and Idleb5) at a genetic distance of 0.34. Whereas, the

major cluster included the remaining lines introduced from ICARDA. This cluster was divided into two sub-clusters at 0.36 genetic distance. The first sub-cluster was made up of two genotypes (Ln1 and Ln2) at 0.27 genetic distance. The second sub-cluster was divided into two sub sub-clusters at 0.35 genetic distance; the first sub sub-cluster was made up of two genotypes (Ln6 and Ln7) at 0.26 genetic distance, the second sub sub-cluster was made up of two sub sub sub-clusters at 0.33 genetic distance; the first sub sub sub-cluster was made up of two genotypes (Ln5 and Ln8) at 0.29 genetic distance. The second sub sub sub-cluster was divided into two sub sub sub sub-clusters at 0.32 genetic distance; the first sub sub sub sub-cluster was made up of genotype Ln10, the second sub sub sub sub-cluster was divided into two sub sub sub sub sub-clusters at 0.30 genetic distance; the first sub sub sub sub sub-cluster was made up of two genotypes (Ln3 and Ln4) at 0.27 genetic distance, the second sub sub sub sub sub-cluster was divided into two sub sub sub sub sub sub-clusters at 0.28 genetic distance. The first sub sub sub sub sub-cluster was made up of genotype Ln9, the second sub sub sub sub sub-cluster was made up of genotypes (Ln11 and Ln12) at 0.21 genetic distance.

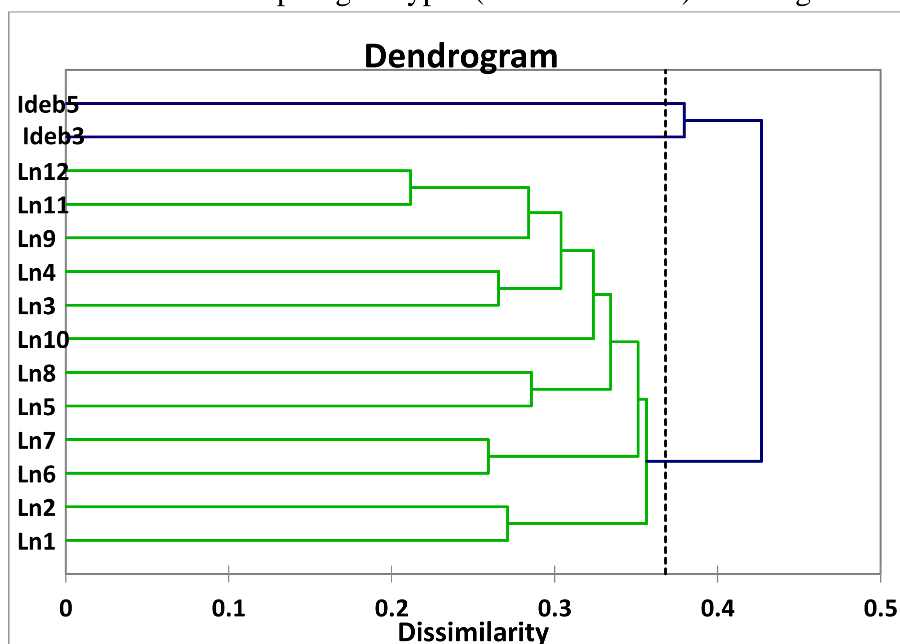

**Fig (2): Cluster analysis as revealed by ISSR data.**

**Discussion:**

Lentil is an important food legume and it has been cultivated for centuries in Syria. Therefore, improving new lentil cultivars is essential for sustainable production. We need the revelation of genetic variation among lentil genotypes to breed a new variety with the desired agronomic and commercial characters. In the present study, the genetic diversity of 14 lentil genotypes were investigated. This is one of a few studies on molecular assessment of genetic diversity in Syrian lentil genotypes. Out of the 18 ISSR primers, two primers (NLSSR3 and 830) showed 100% polymorphism. High percentage of polymorphism in this study confirms the high discriminative power of used ISSR markers in the studied lentil genotypes. This results agreed with those obtained by Duran and De La Vega, (2004) who detected 98.8% polymorphism using ISSR technique.

Primers NLSSR3 and 830 proved to be more informative primers based on polymorphism, while Primer 813 proved to be the most informative primer based on positive unique bands as it was able to detect two positive bands with molecular size of 1057.6 bp scored in genotype Ln8 and 244.4 bp scored in genotype Idleb3 .

In earlier studies, researchers observed that lentil has a low level of genetic diversity (Pandey *et al*., 2018) which agree with the results of this study, as the mean value of dissimilarity coefficient was 0.41 which is closely similar to (0.44) that was reported by Toklu *et al*. (2009)

ISSR dendrogram was able to clearly distinguish all lentil genotypes. All lines introduced from ICARDA were placed in the same cluster, however they fell into different subclusters. Lines Ln11 and Ln12 were closely related. Whereas genotypes Idleb3 and Ln10, Idleb5 and Ln1, Idleb5 and Ln6 seemed to be the most divergent.

**Conclusion:**

1. ISSR markers have been successfully applied to evaluate genetic relationship among lentil genotypes. ISSR primers NLSSR3 and 830 registered the highest polymorphism percentage (100%), while ISSR prime 813 was able to detect two positive bands. Thus, these primers are recommended in other molecular studies on lentil.
2. ISSR dendrogram was able to distinguish all tested genotypes.Genotype Idleb5 showed a wide divergence with genotypes Ln1 and Ln6; thus they are recommended to use as parents in breeding program.

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# تحديد درجة القرابة الوراثية بين بعض سلالات العدس باستخدام تقنية ISSR

**رحاب الموسى[(1)*] وآلاء الشعال[(1)] وشهيناز عباس[(1)] ونبيلة باشا [(1)] وينال القدسي[(1)] خزامة القنطار [(1)] طوني سلوم[(1)]**

(1). قسم التقانات الحيوية، الهيئة العامة للبحوث العلمية الزراعية، دمشق، سورية.

(*للمراسلة: د. رحاب الموسى، البريد الالكتروني: bebo_moussa13@yahoo.com)

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**الملخص:**

أُنجز هذا البحث لتحديد درجة القرابة الوراثية بين اثنا عشر سلالة من العدس مدخلة من ايكاردا وصنفين محليين. استطاع 18 بادئ من التكرارات التتابعية الداخلية البسيطة تضخيم 167 حزمة في كل الطرز المدروسة بمعدل 9.27 حزمة/بادئ. بلغ متوسط التعددية الشكلية 78.7%. أظهرت كل من البادئات NLSSR3 و 830 تعددية شكلية 100%. تم الحصول على أعلى عدد للحزم المتشكلة (124) حزمة في الطراز Ln12 ، بينما لوحظ أقلها (87) حزمة في الطراز ادلب 5. تم الحصول على 31 حزمة فريدة، منها 9 حزم موجودة (موجبة) و 22 حزمة غائبة (سالبة). سجل أعلى عدد للحزم الفريدة (5) في الطراز ادلب 3، بينما لم تظهر الطرز Ln9 و Ln12 أي حزمة فريدة. قسم التحليل العنقودي المعتمد على نتائج تحليل ISSR الطرز المدروسة إلى مجموعتين رئيستين؛ ضمت المجموعة الأولى الأصناف المحلية (ادلب3 و ادلب5)، بينما ضمت المجموعة الثانية باقي السلالات من ايكاردا. أظهرت بعض الطرز تباعد وراثي واسع (ادلب5 والسلالتان Ln1 و Ln6) بينما كانت الطرز Ln11 و Ln12 الأقرب وراثياً.

**الكلمات المفتاحية:** العدس، سلالات ايكاردا، أصناف محلية، قرابة وراثية، التكرارات التتابعية الداخلية البسيطة.