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Original Article

Evaluating Genetic Affinities in *Pisum sativum* Cultivars through SCoT and ISSR Marker

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Abstract

A study was conducted to examine the genetic relationships of eight pea cultivars, including four local and four introduced cultivars, used in commercial production under an irrigated farming system in Libya.. The cultivars were analyzed using PCR-based techniques to assess genetic diversity. Seven Start Codon Targeted (SCoT) markers and five Inter-Simple Sequence Repeat (ISSR) markers were employed. These primers revealed several polymorphic genetic bands with varying molecular weights. The SCoT markers generated 54 genetic bands, averaging 7.71 bands per primer, while the ISSR markers produced 35 genetic bands, with an average of 7 bands per primer. The genetic diversity for SCoT markers was 0.29, while ISSR markers showed a diversity of 0.31. The polymorphic information content (PIC) was relatively low, with an average of 23.0% for SCoT and 24% for ISSR. The dendrogram analysis revealed that the four local cultivars formed a distinct subgroup, closely related due to their genetic proximity. The high similarity indices suggest that the local pea plant populations share a strong genetic relationship, which may be a result of hybridization and the genetic closeness of their parent lines.

Keywords: Genetic variation, ISSR, molecular markers, PIC, *Pisum sativum*, and SCoT.

Introduction

The pea plant (*Pisum sativum* L.) is a member of the legume family (Fabaceae) which encompasses over 490 genera and approximately 12,000 species (Griga and Novak, 1990). Pea plants are cultivated for the purpose of obtaining fresh green seeds, tender green pods, dry seeds, and their vegetative parts (Duke, 1981). Green peas are commonly consumed as a vegetable, whereas dry peas are utilized as a pulse crop. Among vegetables, green peas rank third in global cultivation, following onions and tomatoes. In the pulse category, dry peas stand as the fourth most essential crop after dry beans, chickpeas, and cowpeas. Nutritional research indicates that incorporating peas into one's diet leads to improved intake of fiber, protein, vitamins, and minerals (Javaid *et al.*, 2022).

Pea plants primarily engage in self-pollination, and there is always the possibility of out-crossing, albeit at low rates. Consequently, any outcrossing in the cultivar is generally mechanized, and over time, this intermixing can accumulate, resulting in the loss of specific traits

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of the individual cultivar (Smýkal *et al.*, 2018). Through the examination of natural and genetic characteristics using DNA fingerprinting, it becomes feasible to maintain the cultivar's purity from one generation to the next, and the intensive utilization of a limited number of parents in competitive breeding programs significantly impacts the genetic diversity among pea cultivars (Baranger *et al.*, 2004). Ensuring the genetic diversity of any specific crop type serves as a valuable foundation for enhancing crops, providing essential data for guiding the selection of parent lines and designing appropriate breeding programs (Maesen, 1990). The use of molecular markers is a precise and strong technique for assessing relationship among entries of a germplasm based on the genetic similarity estimates; numerous researchers have directed their investigations towards evaluating genetic diversity in peas using biochemical and morphological indicators. However, these characteristics prove inadequate in distinguishing the cultivars due to their susceptibility to environmental fluctuations. Molecular markers based on DNA assist plant breeders to directly estimate genetic variation among the relative plants without effect of environmental factors (Nguyen et al., 2004). The SCoT and ISSR fingerprinting technique has found application in identifying cultivars across various crops, including Pea (*Pisum sativum*) (Osman and Ali, 2021), Chickpea (*Cicer arietinum* L) (Bhagyawant *et al.*, 2015), Faba bean (*Vicia faba* L) (Avramidou *et al.*, 2023), Common Bean (*Phaseolus vulgaris* L) (Hromadová *et al.*, 2023), and Soybean (*Glycine max* L) (Vivodik *et al.*, 2023).The objective of this research is to assess the genetic diversity among eight pea cultivars, employs two molecular marker techniques, namely Start Codon Targeted Polymorphism (SCoT) and Inter-Simple Sequence Repeat (ISSR) markers. These differences can be used to increase diversity, which is essential in crop breeding for accurate genetic assessments. This method can help in crossbreeding genetically distant varieties to produce hybrids with greater vigor.

Materials and Methods

Eight cultivars of *Pisum sativum* L. plants used in commercial production under the irrigation system in Libya were studied. Four of them are local cultivars (Dafnia, Zahraa 1, Ambassador, and MG103256) and the other four are imported cultivars (Alderman, Mezzarama, Sugar Bon, and Television). Eight random leaf samples were collected and washed, one gram of each sample was cut into small pieces, ground into fine powder using pre-washed, distilled, and sterilized sand. DNA was then extracted using the CTAB solution according to the protocol by Clarke *et al.*,. (1989) Twleve primers were used in the PCR amplification process, five ISSR and seven SCoT primers (Table 1).

The amplification was carried out using an eppendorf mastercycler thermal cycler. twenty microliter blend was assembled, comprising the following components: 2 microliters (5 nanograms) of total DNA, Taq polymerase enzyme supplied by iNtRON Biotechnology,

pre-packaged in tubes with a combination of nucleotides and regulatory solution in the Maxime PCR PreMix Kit i-Taq, One microliter of primer at a 5 micromolar concentration, and 17 microliters of nucleic acid-free, distilled, sterilized water. The amplification of ISSR primers was conducted in the thermal cycler under the following conditions: Initial denaturation cycle at 95ºC for 10 minutes, 35 cycles, each comprising a denaturation step at 95ºC for 30 seconds, followed by an annealing step at a temperature specific to each primer for 30 seconds, and an extension step at 72ºC for one minute and final extension cycle at 72 ºC for 10 minutes. The PCR product was then stored at -4 ºC in the thermal cycler until it was either stored in a freezer or directly injected into the gel.

Table 1. Sequences and melting temperature (Tm) of five ISSR and seven SCoT primers.

No	Primer	Primer sequences (5′–3′)	CG%	Optimal Tm °C
1	BT10	(CA) 6GG	57.14	44
2	BT12	(CA) 8A	47.06	50
3	BT22	(GA) 6GG	57.14	44
4	BT27	(GT) 6CC	57.14	44
5	BT28	(GT) 6GG	57.14	44
1	SCoT 01	CAACAATGGCTACCACCA	50	53.9
2	SCoT 02	CAACAATGGCTACCACCC	55.56	56.1
3	SCoT 03	CAACAATGGCTACCACCG	55.56	56.1
4	SCoT 04	CAACAATGGCTACCACCT	50	53.9
5	SCoT 05	CAACAATGGCTACCACGC	55.56	56.1
6	SCoT 07	CAACAATGGCTACCACGT	50	53.9
7	SCoT 08	CAACAATGGCTACCAGCA	50	53.9

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minute and final extension cycle at 72ºC for 10 minutes. The PCR product was then stored at -4ºC in the thermal cycler until it was either stored in a freezer or directly injected into the gel.

SCoT primers amplification was carried out with the following parameters: Initial denaturation cycle at 94ºC for 4 minutes, 38 cycles, each comprising a denaturation step at 94ºC for 30 seconds, followed by an annealing step at a temperature specific to each primer for 60 seconds, and an extension step at 72ºC for one minute and final extension cycle at 72ºC for 8 minutes. The PCR product was then stored at -4ºC in the thermal cycler until it was either placed in a freezer or directly introduced into the gel.

Statistical analysis: ISSR and SCoT bands were scored as present (1) or absent (0) to generate a binary data matrix. They were computed in PAST software V 1.91 (Hammer *et al.*, 2001) to identify the relationships between the cultivars. This matrix was employed to discern the phylogenetic relationships existing among the genotypes. The Hamming genetic similarity coefficient is employed in constructing the similarity tree. The same coefficient was also used in calculating and drawing the Principal Coordinate Analysis (PCoA).

The program Power Marker version 3 (Liu and Muse, 2005) was used to calculate genetic diversity and Polymorphic Information Content (PIC), based on the frequency of varying alleles within the population.

Results and discussion

SCoT technique: The results indicate that the seven SCoT primers used in amplification produced 54 bands, with an average of 7.71 bands per primer. The number of bands ranged from 5 bands with primer SCoT 04 to 14 bands with primer SCoT 05. All the bands obtained represent sources of genetic variation among the studied pea cultivars, with an average of 76% polymorphic percentage ranging from 33.33% in SCoT 07 to 100% in SCoT 01, SCoT 02 and SCoT 05 (Table 2). In comparison to the findings on the genetic relationships among various subspecies of Pisum sativum reported by Osman and Ali (2021), which identified 105 bands with an average of 13 bands per primer (ranging from 10 bands in SCoT 1 and SCoT 3 to 20 bands in SCoT 15), our study observed a higher percentage of polymorphic fragments at 81.5%, surpassing their reported value of 75.24%. This suggests a greater genetic diversity within the samples analyzed in our research.

The primer SCoT01 showed 6 bands with varying molecular weights; however the heaviest band was approximately 800 base pairs, while the lightest was 300 base pairs (Figure, 1). The primer SCoT02 displayed 8 bands with varying molecular weights; the heaviest band was approximately 700 base pairs with the Ambassador cultivar, while the lightest was 200 base pairs with the Alderman cultivar. The primer did not attach and amplify the DNA for the MG103256 cultivar; however SCoT03 primer produced 6 bands with varying

molecular weights, the heaviest bands were around 900 base pairs with the cultivars MG103256, Eldafniah, Ambassador, Zahraa 1, and Sugar Bon, while the lightest was 350 base pairs with the Sugar Bon cultivar.

Table 2. Amplified DNA bands and polymorphism percent of SCoT primers on eight peas cultivars.

No.	Primer	No. of bands	Polymorphic	Non Polymorphic	Percentage
1	SCoT 01	6	6	0	%100
2	SCoT 02	8	8	0	%100
3	SCoT 03	6	3	3	%50
4	SCoT 04	5	3	2	%60
5	SCoT 05	14	14	0	%100
6	SCoT 07	6	2	4	%33.33
7	SCoT 08	9	8	1	%88.88
Total		54	44	10	
Avarage		7.71	%81.5	%18.5	%76.03

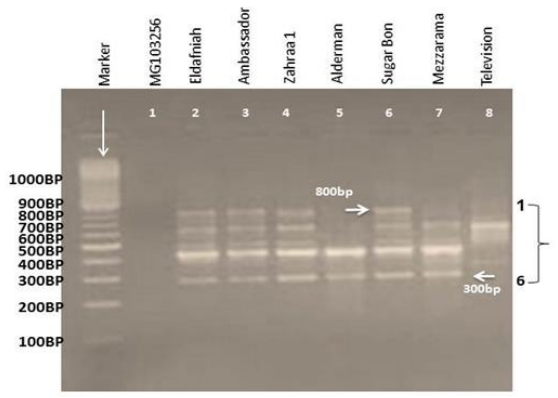

Figure 1: Polymorphism in banding patterns of eight Pisum sativum cultivars generated by the SCoT 01 primer.

The primer did not attach and amplify the DNA for the Alderman and Television cultivars, whereas.primer SCoT04 produced 5 bands, the molecular weights varied from 1000 to 400 base pairs. In this study, SCoT markers were utilized due to their advantages over other molecular markers, including the simpler development of species-specific primers compared to SSRs, cost-effectiveness relative to AFLP (Jiang *et al.*, 2014), and higher reproducibility than RAPD (Xiong *et al.*, 2011). Numerous studies have demonstrated the effectiveness of SCoT markers in assessing genetic diversity and relationships within the Fabaceae family, including Vicia faba (Avramidou *et al.*, 2023), Phaseolus vulgaris (Hromadová *et al.*, 2023), and Glycine max (Vivodik *et al.*, 2023).

Primer SCoT05 generated 14 bands, with the largest band around 950 base pairs and the smallest at 250 base pairs (Figure 2). In contrast, SCoT07 produced 6 bands, with molecular weights ranging from 900 to 550 base pairs, and SCoT08 produced 9 bands, with molecular weights ranging from 800 to180 base pairs. However, SCoT08 did not amplify DNA for the Ambassador cultivar.

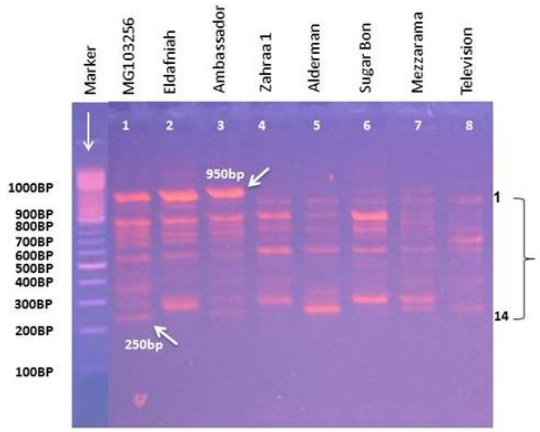

Figure 2: Polymorphism in banding patterns of eight Pisum sativum cultivars generated by the SCoT 05 primer.

ISSR technique: The five ISSR primers produced 35 bands, with an average of 7 bands per primer. The highest was 10 with primer BT 10, and the lowest was 4 bands with primer BT 28. 75.5% of the obtained bands represent morphological diversity for genetic variation among the studied pea cultivars based on the primers used in the study. This diversity varies depending on the used primers, with a 60% polymorphic percent recorded with primer BT 12, while 87.5% was recorded with primer BT 27. (Table 3). In contrast, Osman and Ali (2021) reported that eight ISSR primers produced 71 bands, averaging 8.9 bands per primer, with a maximum of 14 bands from primer 812 and a minimum of 5 from 807. This discrepancy in band production rates may be attributed to variations in the genetic makeup of the studied plants and differences in primer selection.

The primer BT10 showed 10 bands with varying molecular weights. The heaviest band was approximately 1000 base pairs with the Ambassador cultivar, while the lightest was 150 base pairs with the Mezzarama cultivar (Figure, 3). Numerous studies have utilized ISSR markers to assess genetic diversity and relationships within the Fabaceae family, including *Vicia faba* (Yilmaz, 2020), *Phaseolus vulgaris* (Sakhravi *et al.*, 2023), and *Cicer arietinum* (Sadhu *et al.*, 2020). Approximately 75.5% of the observed bands indicate diversity among the studied pea cultivars, depending on the specific primers used.

Table 3. Amplified DNA bands and polymorphism percent of ISSR primers on Eight peas cultivars.

No.	Primer	No. of bands	Polymorphic	Non Polymorphic	Percentage
1	BT 10	10	8	2	%80
2	BT 12	5	3	2	%60
3	BT 22	8	6	2	%75
4	BT 27	8	7	1	%87.5
5	BT 28	4	3	1	%75
Total		35	27	8	
Avarage		7	%77	%22.9	%75.5

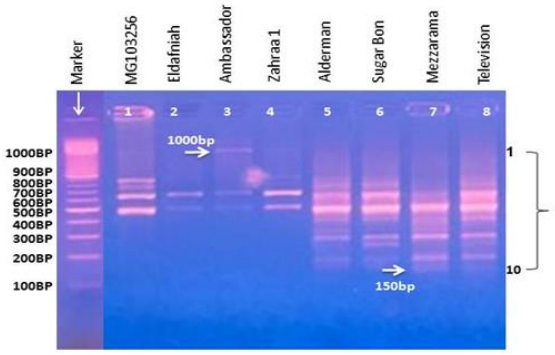

Figure 3: Polymorphism in banding patterns of eight Pisum sativum cultivars generated by the BT10 ISSR primer.

Polymorphic percentages varied, with BT 12 showing 60% and BT 27 recording 87.5%. These findings align with Nautiyal and Panwar (2016), who reported polymorphism ranging from 50% to 100% in their chickpea study using seven ISSR primers. In contrast, Avramidou *et al.*, (2023) found polymorphism in 53 *Vicia faba* genotypes ranged from 21% to 57%. Polymorphism reflects biological diversity and genetic variation, arising from multiple alleles at the same chromosomal locus, which facilitates genetic and phenotypic diversity.

The primer BT12 displayed 5 bands with molecular weights ranging from 600 to280 base pairs (Figure, 4). While BT22 showed 8 bands with molecular weights ranging from 800 to 150 base pairs, although BT27 primer generated 8 bands with molecular weights ranging from 600 to180 base pairs, however primer BT28 showed 4 bands with molecular weights ranging from 250 to 750 base pairs.

Genetic Diversity and (PIC) : The average gene diversity for the different studied pea cultivars, based on the combined results of 12 primers (the seven primers for ScoT in addition to the

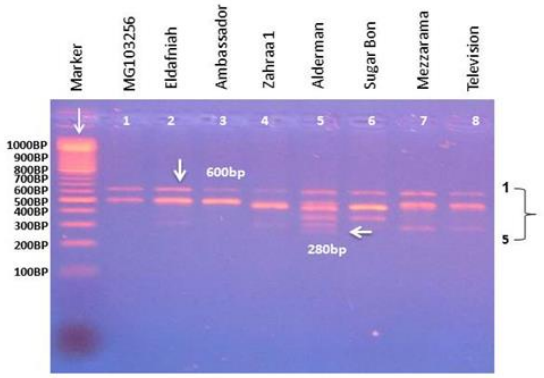

Figure 4: Polymorphism in banding patterns of eight Pisum sativum cultivars generated by the BT12 ISSR primer.

five primers for ISSR), approached 30%. Of this, 29% was attributed to ScoT primers, while ISSR primers recorded a genetic diversity of 31%. This finding is consistent with Osman and Ali (2021), who reported a gene diversity of 29% in Pisum sativum using SCoT techniques. In contrast, studies on wild soybean using SSR markers revealed a gene diversity rate of 77% (Li *et al.*, 2010), attributed to the diverse origins of the samples. The lower gene diversity observed in this study reflects the effects of self-pollination in peas, which reduces genetic variation.

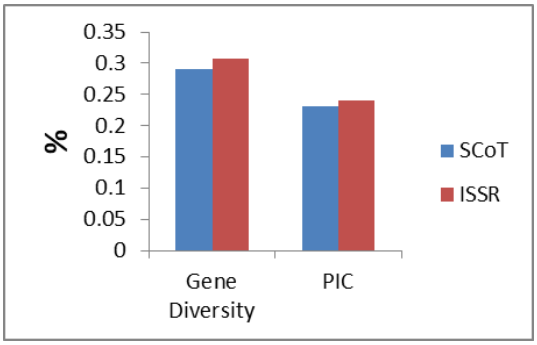

Figure 5: The genetic diversity and the polymorphism information content (PIC) content for eight cultivars of peas.

PIC is considered low, with an average of 23.5% for all primers used in the study. This includes the seven ScoT primers with an average of 23%, in addition to the five ISSR primers with an average of 24% (Figure 5). This finding aligns with Yadav *et al.*, (2022), who reported an average PIC value of 0.27 for *Pisum sativum* L. using 31 polymorphic SSR markers. Similarly, Osman and Ali (2021) found PIC averages of 0.23 and 0.21 with SCoT and ISSR markers, respectively. In contrast, El-Fatah and Nafea (2020) reported a PIC of 0.39 for 22 pea genotypes using phenotypic characters and SSR markers. These values are lower than those from studies on various plants; for example, Mohamed *et al.*, (2023) observed a PIC of 0.7 in endangered Tunisian peas and Turkish accessions using ISSR markers. Variability in PIC values can be attributed to factors such as marker type, primer quality, and genetic similarity, with the self-pollination of pea plants contributing to increased genetic similarity and lower PIC values.

Dendrogram of Genetic Similarity:The Hamming genetic similarity coefficient was used to construct the dendrogram of genetic similarity among the eight different cultivars of pea plants as shown in (Figure 6) the shared primers of ScoT and ISSR were able to divide the studied pea plant cultivars into two main groups. The first group includes the local cultivars, while the second group contains the imported cultivars. The results also indicate that the four local cultivars formed a relatively closed independent group due to their genetic proximity. This suggests that they are closely related, possibly due to their relatively low genetic diversity.

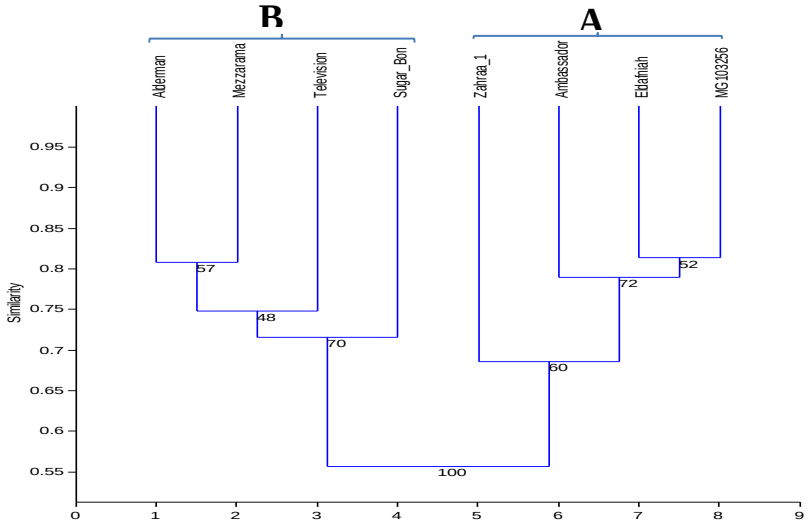

Figure 6. Dendrogram based on the Hamming coefficient analysis showing relationships of SCoT and ISSR loci among Eight Pisum sativum cultivars.

The funding was confirmed by the results of the Principal Coordinate Analysis (PCoA), The cultivars were divided into two main groups, with the first group consisting of the four local cultivars in red, while the imported cultivars overlapped in blue in the opposite direction (Figure, 7). Genetic examination indicates that the four native cultivars exhibit a close relationship, potentially attributable to their limited genetic diversity. This observation is corroborated by both the Hamming genetic similarity coefficient and the Principal Coordinate Analysis (PCoA).

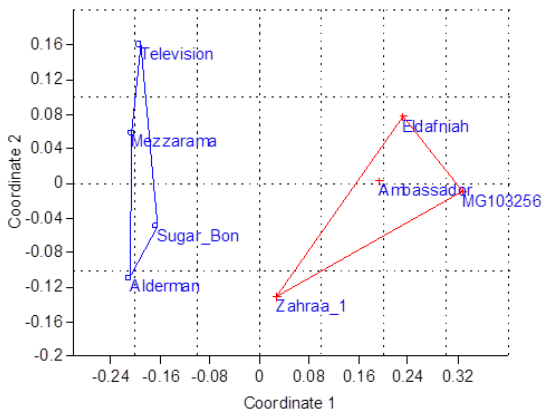

Figure 7. Scatter plot of Principle Coordinate analysis (PCoA analysis) of SCoT and ISSR loci among Eight Pisum sativum cultivars.

The genetic difference matrix indicates that the lowest genetic difference was 18.6% between the MG103256 and Eldafniah cultivars, both of which are local cultivars (Table 4). This was followed by a value of 19.2% between the Mezzarama and Alderman cultivars, and then in third place with a percentage of 19.7% between the Ambassador cultivar and Eldafniah. These results indicate significant genetic similarity among individuals of local pea cultivars, possibly resulting from the exchange of genetic material through hybridization in breeding and improvement processes. The largest difference values were 56.5% between MG103256 and Mezzarama cultivar, followed by a difference of 55% between the same local MG103256 cultivar and the imported Television cultivar (Table 4). This demonstrates the genetic diversity and variation between local and imported cultivars, as illustrated by the dendrogram. To ensure precise outcomes in genetic analysis, it is recommended to incorporate a broader array of primers.

Table 4. The genetic similarity matrix of eight pea cultivars.

	MG103256	Dafnia	Ambassador	Zahraa 1	Alderman	Sugar Bon	Mezzarama	Television
MG103256	0							
Dafnia	**0.18667**	0						
Ambassador	0.22388	0.19753	0					
Zahraa 1	0.37333	0.32584	0.24691	0				
Alderman	0.53623	0.50602	0.45333	0.3012	0			
Sugar Bon	0.54667	0.44944	0.39506	0.32584	0.26506	0		
Mezzarama	**0.56522**	0.43373	0.42667	0.3253	0.19277	0.28916	0	
Television	0.55072	0.46988	0.42667	0.38554	0.3012	0.3012	0.20482	0

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The results indicate significant genetic similarity among individuals within the indigenous pea cultivars, likely due to genetic exchange through hybridization during breeding. To maximize vigor in hybrids, it is advisable to cross genetically distant cultivars when hybridization is necessary.

Conclusion

The study utilized SCoT and ISSR techniques to analyze genetic diversity among pea cultivars. The results revealed considerable genetic variation, with SCoT primers showing an average polymorphism of 76%, while ISSR primers exhibited 75.5% polymorphism. The combined gene diversity for all primers was 30%, with ISSR showing slightly higher diversity than SCoT. The low PIC values suggest limited genetic variation, likely due to the self-pollinating nature of peas. Genetic analysis via dendrogram and Principal Coordinate Analysis (PCoA) identified two main groups: local and imported cultivars, with local cultivars exhibiting close genetic relationships. These findings highlight the importance of using genetically distant cultivars for hybridization to enhance vigor and genetic diversity.

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تقييم التقارب الوراثي في أصناف الجلبان باستخدام تقنية الواسمات الجزيئية SCoT و ISSR

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المستخلص

تم دراسة ثمانية أصناف من نبات الجلبان *Pisum sativum* L. ، المستخدمة في الإنتاج التجاري في ليبيا تحت نظام الزراعة المروية، تشمل أربعة أصناف محلية وأربعة أصناف مستوردة، تم استخدام تقنيات PCR لدراسة درجة القرابة الوراثية، حيث استخدمت سبع بادئات بتقنية (SCoT) وخمس بادئات بتقنية (ISSR)، أظهرت البادئات العديد من الحزم الوراثية متعددة الأشكال ذات أوزان جزيئية متباينة، حيث أظهرت تقنية SCoT عدد 54 حزمة وراثية بمتوسط 7.71 حزمة لكل بادئ، بينما أظهرت تقنية ISSR عدد 35 حزمة وراثية بمتوسط 7 حزمة لكل بادئ، كما أظهرت بادئات SCoT تنوع وراثي 0.29، في حين سجلت بادئات ISSR تنوع وراثي بقيمة 0.31. كان محتوى المعلومات المتعدد الأشكال منخفضًا نسبيًا، بمتوسط 23.0٪ لبادئات SCoT و24٪ لبادئات ISSR. كما دلت نتائج شجرة القرابة إلى أن الأصناف المحلية الأربعة شكلت مجموعة فرعية متميزة ومرتبطة ارتباطًا وثيقًا نسبيًا بسبب قربها الجيني. وتدل مؤشرات التشابه العالي إلى أن أفراد عشائر نباتات الجلبان المحلية لديها علاقة وراثية وثيقة وهذه العلاقة قد تكون مرتبطة بعمليات التهجين وتقارب الآباء وراثياً.

الكلمات الدالة: التنوع الجيني، ISSR، العلامات الجزيئية، محتوى المعلومات المتعدد الأشكال ، الجلبان و SCoT.

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