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ASSESSMENT OF GENETIC DIVERSITY OF SOME COWPEAS (*VIGNA UNGUICULATA* L.) CULTIVARS GROWN IN EGYPT BASED ON START CODON-TARGETED (SCOT) MARKERS

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Abstract

The different techniques of molecular markers have proved to play a considerable role in assessed the genetic diversity between and within different species. The genetic diversity among the seven cowpea cultivars was characterized using the twelve SCoT markers. The total number of amplicons was 169 with an average of 16.9. Among 169 amplicons, 121 were polymorphic with a level of polymorphism of 71.60%. All primers (12) were successful to identify each of the seven cowpea genotypes by unique positive and/or negative markers. Tiba cultivar showed the highest (15) number of unique markers. The presence of SCoT unique markers among the cowpea cultivars indicates the utility of the approach for fingerprinting purposes. Moreover, the dendrograms constructed from UPGMA divided the seven genotypes into two clusters. The first cluster comprises the cultivar Tiba, Dokki- 331 and Kaha-1. While the second cluster divided into two subclster the Kareemy-7 (subcluster) was separated from other remaining 3 cultivars. The second subcluster contained Balady, Sudan and Kafr-El-Shikh, two cultivars that clustered together (Sudany and Balady).

Key words: Cowpea, Cultivars,Genetic relationships, SCoT molecular makers

Introduction

Cowpea (*Vigna unguiculata* L. Walp.) is one of the most important foods and economic vegetable crops in the world (Ezzat, *et al*., 2019). Cowpea is a legume crop that is resilient to hot and drought prone climates and a primary source of protein in sub Saharan Africa and other parts of the developing world. It is characterized by a chromosome number of 2n =2x= 22 and an estimated genome size of 620 Mb (Chen *et al*., 2007). According to the statistics of FAO (2018), the world's cultivated area is about 16 million hectares, Egypt cultivated area is about 1841 hectares with productivity 38,609hg/ha .Different studies added further information on the genetics of important traits in cowpea include the inheritance of qualitative and quantitative traits (Singh, 2002).

Traditionally, diversity in cowpea is estimated by measuring the variation in phenotypic or qualitative traits such as flower colour, growth habit, or quantitative agronomic traits such as yield potential and stress tolerance. DNA based markers have been used for characterization and assessing genetic diversity in legume crops including cowpea(*Vigna unguiculata* L. Walp). This generated a vast amount of information to be used in crop genetic improvement and breeding programs. However, it is crucial to choose suitable markers for genome analysis (Igwe, *et al*., 2017 and Alghamdi, *et al*., 2019). Genetic diversity in cultivated crops indicates gene pool richness. It is the greatest resource for plant breeders to select lines that enhance food security (Wamalwa *et al*., 2016).

Molecular markers possess many advantages over phenotypic traits, as they are stable and detectable in all tissues, besides, they are notaffected by environmental effects (Mondini *et al*., 2009 and Sonnino, 2017). Molecular markers techniques such as RFLP (restriction fragment length polymorphism), RAPD (random amplified polymorphic DNA), SSR (simple sequence repeats) and AFLP (amplified fragment length polymorphism) were routinely being used in ecological, evolutionary, taxonomical, phylogenic and genetic studies of plant sciences (Agrawal *et al*., 2008). To date, different molecular markers platforms are available with variable

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properties, such as SSR, RAPD, ISSR, SCoT and AFLP to study the genetic diversity of different plants (Legesse *et al.,* 2007; Adnan and Katsuhiko, 2011; Gorji *et al*., 2011; Abdel-Lateif and Hewedy, 2018 andMostafa *et al*., 2020). Several authors, who assessed the genetic diversity in cowpea using different markers such as RAPD (Zannou *et al*., 2008) and Malviya *et al*., 2012), SSR (Xu *et al*., 2010 and Chen *et al*., 2017), AFLP (Fang *et al.,* 2007 *and* Al-Hinai*, et al.,* 2018) and ISSR (Tantssawat, *et al*., 2010, Mahfouz, 2015 and Araújo *et al*., 2019).

Start codon-targeted (SCoT) molecular markers method is a simple, low-cost, highly polymorphic, provides extensive genetic information in plants, where primers were designed based on plant universal gene composition (Gorji *et al*., 2011). It is markers that were developed to target the conserved regions of genome flanking the ATG start codon in plant genes reported by (Joshi *et al*., 1997 and Sawant *et al*., 1999). These advantages have been validated through studies on genetic diversity in olive (Alsamman *et al*., 2017), mango (Luo *et al*., 2010), tomato (Shahlaei *et al*., 2014), potato (Gorji *et al*., 2011) and cowpea (Igwe *et al*., 2017). Also, the SCoT marker has been of great importance in the characterization and identification of genetic variation in both irradiated cowpea cultivars (Ezzat, *et al*., 2019).

The aim of this work to study the genetic diversity among seven different cowpea cultivars grown in Egypt based on the SCoT markers.

Materials and Methods

Plant materials

The plant material used in the present investigation was comprised of theseven cowpeacultivars (*Vigna unguiculata* L. Walp.) table 1.

Table 1: Number, name, seed colour and source of the seven cowpea cultivars.

No.	Cultivar	Seed Colour	Source
1	Tiba	White with brown eye	"Horticulture Research Institute, Agriculture Research Center (ARC), Giza, Egypt."
2	Kaha-1	Yellowish-white	
3	Dokki-331	White with black eye	
4	Sudany	Black with yellowish eye	
5	Balady	Black with yellowish eye	
6	Kareem-7	Yellowish-white (Creamy)	
7	Kafr-El-Shikh	Yellowish-white	

Methods

DNA isolation

In the present study, two different approaches were employed for the characterization of the seven varieties under study based on molecular or DNA markers (SCoT and SSR markers). The total genomic DNA was isolated from young leaves of cowpea plants using the CTAB method according to (Murray and Thompson, 1980).

Start-Codon Targeted (SCoT) analysis

A set of twelve SCoT primers was employed in the present study, the nucleotide sequence of the primers as shown in table 2. Synthesis of SCoT primers is carried out by HVD Vertriebs-Ges. m.b.H. (Vienna, Austria).

The amplification reaction was carried out in the total volume 20 µl, containing 1X reaction buffer, 1.5 mM MgCl2, 0.2 mM of each dNTPs, 0.4 µM of a single primer; 50 ng genomic DNA and 1U of *Taq* DNA polymerase (Qiagen Ltd., Germany). The SCoT amplifications were carried out with a preliminary cycle of 4 min at 94°C, followed by 35 cycles of the 40s at 94°C, 50s at 50°C and 1min at 72°C and a final cycle of 10 min at 72°C.The amplified products were resolved on 1.5% agarose gel electrophoresis in 1 x TBE buffer, stained with ethidium bromide (0.5 mg/mL) and visualized under UV light.

Data analysis

The banding patterns generated by SCoT markers were compared to determine the genetic relationships among the different cowpea cultivars. Clear and distinct amplification products were scored as (1) for present and (0) for absent bands for all samples. Bands of the same mobility were scored as identical. SCoT banding patterns were compared to determine the genetic relationships among the different varieties, using Phortix nonlinear dynamics (UK) software Version 10.

Result and Discussion

The Start codon-targeted markers (SCoT) markers used in a wide range in the identification, characterizationand genetic comparison between many

Table 2: Nucleotide sequences of the twelve primers assayed in SCoT-PCR.

No.	Primer Code	Sequences	No.	Primer Code	Sequences
1	SCOT-13	5'ACGACATGGCGACCATCG3'	7	SCOT-34	5'ACCATGGCTACCACCGCA3'
2	SCOT-14	5'ACGACATGGCGACCACGC3'	8	SCOT-52	5'ACAATGGCTACCACTGCA3'
3	SCOT-24	5'CACCATGGCTACCACCAT3'	9	SCOT-61	5'CAACAATGGCTACCACCG 3'
4	SCOT-26	5'ACCATGGCTACCACCGTC3'	10	SCOT-70	5'ACCATGGCTACCAGCGCG 3'
5	SCOT-31	5'CCATGGCTACCACCGCCT3'	11	SCOT-71	5'CCATGGCTACCACCGCCG3'
6	SCOT-33	5'CCATGGCTACCACCGCAG 3'	12	SCOT-77	5'CCATGGCTACCACTACCC3'

plant varieties and were validated in grape (Zhang *et al*., 2011), mango(Luo *et al*., 2010), olive (Alsamman *et al*., 2017) and *Echinacea* genus (Jedrzejczyk, 2020). SCoT markers can be useful for a breeder for selective genotypes and specific traits in breeding programs in chickpea (Ahmad and Talebi 2017). Moreover, SCoT markers have great potential in cultivar identification and can reproducibly amplify polymorphic bands in cultivars that are closely related (Mahdy, 2018).

Table 3: Total number of amplicons, monomorphic amplicons, polymorphic Amplicons and polymorphism percentage as revealed by SCoT analysis among the seven cowpea genotypes.

Name Primer	Total Ampl-icons	Monom-orphic Amplicons	Polym-orphic Amplicons	% Polymo-rphism
SCoT-13	22	5	17	77.27
SCoT-14	19	5	14	73.68
SCoT-24	6	-	6	100%
SCoT-26	13	6	7	53.85
SCoT-31	12	6	6	50.00
SCoT-33	6	1	5	83.33
SCoT-34	14	7	7	50.00
SCoT-52	16	1	15	93.75
SCoT-61	10	7	3	30.00
SCoT-70	11	3	8	72.72
SCoT-71	19	7	12	63.15
SCoT-77	21	-	21	100
Total	169	48	121	-
Average	16.9	4.8	12.1	71.60

Polymorphism among the seven cowpea cultivars as detected by SCoT molecular markers

In the present study, the genetic relationships among the seven cowpea cultivars were determined using twelve SCoT primers table 2. The total number of amplicons produced by the twelve primers was 169 with an average of 16.9 amplicons/primers. The highest number of amplicons (22) was obtained with primer SCoT-13, while the lowest number of amplicons (6) was amplified with primers SCoT-24 and SCoT-33.The number of polymorphic amplicons ranged from 3 to 21 with an average of 12.1. Primer SCoT-77 amplified the highest number of polymorphic amplicons (21) and showed the highest percentage (100%) of polymorphism. Moreover, the different primers showed different levels ofpolymorphism, ranging from 30.00% with the primer SCoT-61 to 100% with the primers SCoT-24 and SCoT-77 with an average of 71.60.% table 3 and Fig. 1.

In this respect, Igwe *et al*., (2017) stated that the SCoT markers are more efficient in resolving genetic diversity and relatedness than ISSR in cowpea and they found that the polymorphism information contents (PIC) ranged from 0.6304 to 0.9210.They suggested that the polymorphic SCoT markers can be used as stock for novel gene exchange in crop breeding, Alsamman *et al*., (2017) thirty – nine SCoT primers have used the analysis of the genetic diversity on ten olive varieties. The total number of polymorphic amplicons (PA) was 642. 1). Thus, the range of % P was from 12.5% (SCoT-16) to 86.70% with an average percentage of polymorphism was 59.5%.

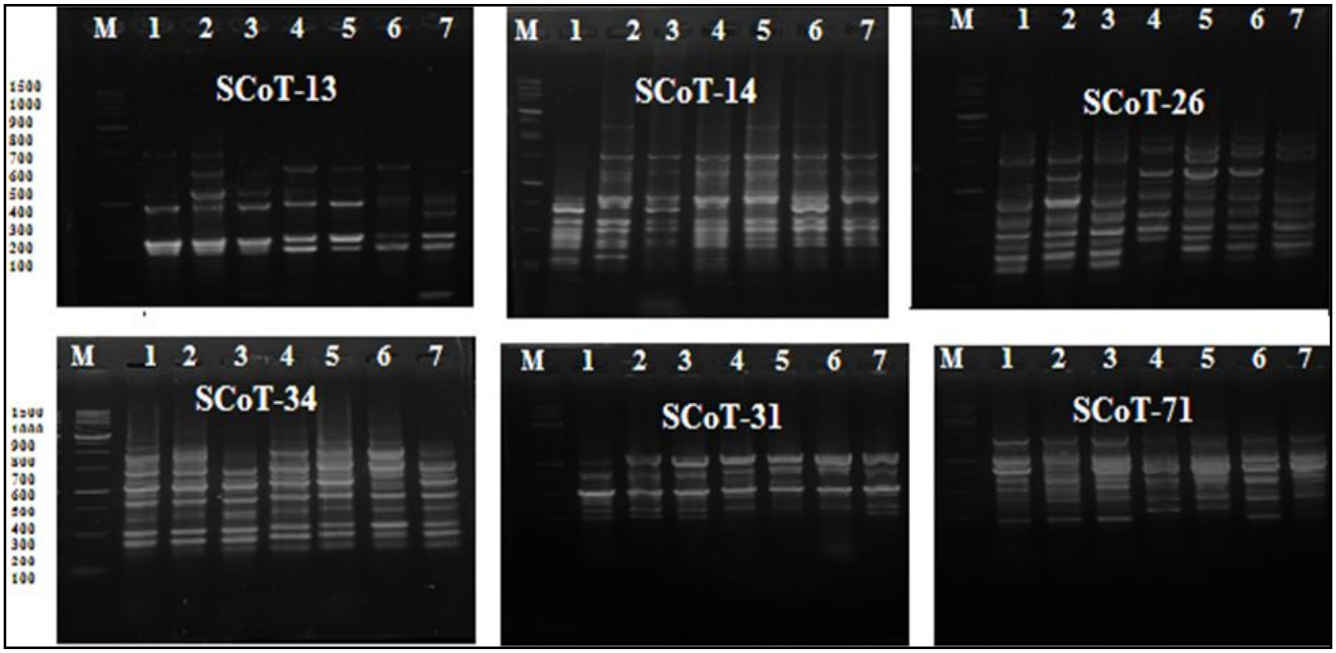

Fig. 1: SCoT profiles of the seven cowpea cultivars. Lane 1 to 7 represent: Tiba, Kaha-1, Dokki-331, Sudany, Balady, Kareem-7 and Kafr-El-Shikh, respectively. M: Molecular Marker (1Kbp ladder).

Moreover, (Mahdy, 2018) seventeen SCoT primers were screening and evaluation with the DNA of ten accessions of *Vigna*. The ten accessions produced a total number of 153 fragments with an average of 9 bands per primer. The average percentage of polymorphism was 69.93%. Also, Ezzat *et al*., (2019) found that the analysis of two varieties of cowpea and six radiation treatment using fifteen SCoT primers yielded 219 fragments, of which 101 were polymorphic. On the other hand, Nosair (2016) obtained the one hundred and eighty-three bands (183) were produced by SCoT markers generating (93.99) polymorphism in a study of genetic relationships among the nine Fabaceae species using the ten SCoT markers. Polymorphism percentage of banding patterns was (93.99). Therefore, this significant number of polymorphic bands indicates the powerful of SCoT marker as a fingerprinting and diversity analyzer.On the other hand,our results showed that the percentage of polymorphism is 71.60.% this result is disagreement with Mahfouze (2015) obtained the low (26.37 and 28.27% for RAPD and ISSR, respectively) degree of polymorphism among the four cultivars of cowpea (Kareem 7, Dokki 331, Kaha 1 and Kafer El-Sheikh 1). Meanwhile, the study of the genetic diversity of 20 peanut accessions using 18 SCoT primers revealed a polymorphism of 38.22% (Xiong *et al.*, 2011).

Identification of cowpea cultivars using SCoT unique markers

Unique markers are defined as bands that specifically identify a variety from the other by their presence or absence. If the bands that are present in one variety but not found in others are termed positive unique markers in contrast to the negative unique markers. In the present study, genotype-specific SCoT unique markers could distinguish seven cowpea varieties table 4. These unique markers are useful as variety-specific ones. Fifty-one unique markers were generated from 12 primers. Tiba

Table 4: Genotypes characterized by unique positive SCoT markers, marker size and total number of markers identified in each genotype for the seven cowpea cultivars.

-Genotypes	Unique	positive markers	Total# of makers genotypes	Unique	negative markers	Total# of makers genotypes	Grand total markers
Tiba	SCoT-13	1631		SCoT-13	516		
	SCoT-31	1757,1618		SCoT-14	2293,1455,		
	SCoT-34	2500,1500	7		1191,243	8	15
	SCoT-71	521		SCoT-52	1368,1075,890		
	SCoT-77	2023					
Kaha-1	SCoT-13	2168,686		SCoT-14	279		
	SCoT-14	302	4	SCoT-26	1460	4	8
	SCoT-52	452		SCoT-52	517		
				SCoT-71	827		
Dokki-331	SCoT-14	779					
	SCoT-52	1124	3	SCoT-24	786	1	4
	SCoT-77	1380					
Sudany	SCoT-13	1765					
	SCoT-31	669	5	SCoT-26	374	1	6
	SCoT-70	832					
	SCoT-71	1508,673					
Balady	SCoT-13	2488					
	SCoT-14	910		SCoT-26	299		
	SCoT52	1450	5	SCoT-71	1005	2	7
	SCoT-70	809					
	SCoT-77	1747					
Kareem-7	SCoT-26	1543					
	SCoT52	642	4	SCoT-61	1839,287	2	6
	SCoT-71	1956					
	SCoT-77	1342					
Kafr-El-Shikh	SCoT-13	1239 ,724,326	4	SCoT-13	1130	1	5
	SCoT-33	1528					
Total			32			19	51

cultivar was characterized by the highest number of unique markers 15 (7 positive and 8 negative). This was followed by Kaha-1 which showed 8unique markers (4 positive and 4 negative). On the other hand, Dokki-331 wascharacterized by the lowest number of unique markers (4) three unique positive and only one negative. Primers SCoT-24 and SCoT-33revealed the lowest number of unique markers onenegative and one positive, respectively. Certain primers, were more informative than the others e.g, SCoT-13, SCoT-52 and SCoT-77 since they identified 5 genotypes.In this respect,El Saied et al. (2012) found that only seven unique ISSRs bands out of seventy-one detected bands. These unique bands were useful as variety-specific markers which distinguished 5 foreign olive varieties and 2 local olive varieties. Mahfouz (2015) successfully identified nine unique bands with polymorphism ranged from 16.67% to 55.56% from RAPD markers. Also, nineteen specific ISSR markers were each corresponding uniquely to with a higher level of polymorphism than RAPD among the DNA samples of the studied cowpea cultivars. Alsamman *et al*., (2017) detected 111 unique markers out of 383 amplicons obtained from 10 olive varieties. Moreover, twenty SCoT unique bands were detected to distinguish ten accessions of cowpea plants .These bands can be considered as potential markers to identify germplasm (Mahdy, 2018).

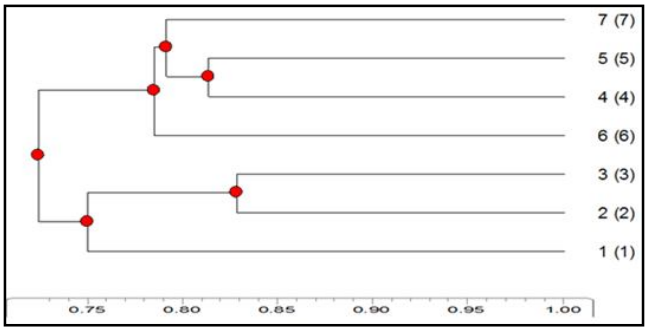

Fig. 2: UPGMAdendrogram of seven cowpea cultivars based on SCoT markers (1= Tiba, 2 = Kaha-1, 3 = Dokki-331, 4 = Sudany, 5 = Balady, 6 = Kareem-7 and 7 = Kafr-El-Shikh).

Table 5: SCoT-based similarity matrices among the seven cowpea cultivars.

Varieties	Tiba	Kaha -1	Dokki -331	Sud-any	Bal-ady	Kar-eem-7	Kafr-El -Shikh
	1						
Kaha-1	73	1					
Dokki-331	77	83	1				
Sudany	70	69	76	1			
Balady	69	69	70	81	1		
Kareem-7	71	69	76	79	78	1	
Kafr-El-Shikh	73	75	80	77	81	78	1

The UPGMA-dendrogram based on SCoT markers Fig. 2 classified the seven cultivars into two main clusters. The first cluster contained three cultivarsTiba, Kaha-1 and Dokki-331, two cultivars that clustered together (Kaha-1 and Dokki-331). The second cluster divided into two subclster the Kareemy-7 (subcluster) was separated from other remaining 3 cultivars. The second subcluster contained Balady, Sudan and Kafr-El-Shikh, two cultivars that clustered together (Sudany and Balady). Our results are in agreement with Mahfouz, (2015) that Kaha-1 and Dokki-331 cultivars clustered together in each of the ISSR, proteins and combined dendrograms. Igwe *et al*., (2017) studied 18 accessions of cowpea genotypes by using 10 SCoT and 10 ISSR primers the which separated the accessions into five major clusters for both markers.

The SCoT-based similarity matrices among the seven varieties ranged from 0.69 to 0.83 table 5. It was 0.83 between Dokki-331 and Kaha-1. Meanwhile, it was 0.69 between cultivars Balady and Tiba and between cultivars Kareem-7 and Kaha-1. In this respect, Mahfouz (2015) found that the similarity value between Dokki-331 and Kaha-1, Kafer-El-Sheikh and kream-7 were 0.78 and 0.86, respectively in ISSR analysis. Meanwhile, RAPD markers showed the genetic similarity of the same cultivars are 0.69 and 0.92. Therefore, Nosair, (2016) reported that the SCoT markers are efficient inassessing the genetic diversity among fabaceae.

Conclusion

The assessment and maintenance of genetic diversity using the molecular markers is important because it provides a depository for adaptation to different changes. In the current work, we assessed the genetic diversity of the seven cowpea cultivars through SCoT makers. The percentage of polymorphism was record (71.6 %) among cultivars in this study. Also, it is successfully distinguished each cultivar with different unique markers, which reflects the added value of this marker provides interesting tools for breeding new cultivars of Egyptian cowpea. However, further studies are required to elucidate the genetic relationships at the molecular level by advanced techniques for more accurate characterization of germplasm.

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