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Genetic relationship of some *Pisum sativum* subspecies using different molecular markers

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Abstract

Field pea (*Pisum sativum*) is a member of Viciae tribe and considers the basic model of modern plant genetics because it has been utilized in Mendel's laws of inheritance. Different molecular markers such as Random Amplified Polymorphic DNA (RAPD), Inter-Simple Sequence Repeats (ISSR) and Start Codon Targeted (SCoT) were utilized for determining the genetic relationships among six *Pisum sativum* subspecies (subsp. *asiaticum*, subsp. *abyssinicum*, subsp. *elatus*, and three subsp. *sativum* convarietes *axiphium*, *medullare* and *speciosum*). It was found that SCoTs marker gave the maximum values of polymorphism (75.24%) and gene diversity (0.29), while minimum values of polymorphism (62.7%) and gene diversity (0.23) were reported in RAPD markers. It has been observed that the dendrogram (UPGMA) results of combined data were identical to SCoT dendrogram (UPGMA) results. Therefore, SCoT was the most informative marker compared to ISSR and RAPD markers for discrimination and identification of studied *P. sativum* subspecies. It could be concluded from this study that the genetic relationship among the studied subspecies was as follows: subsp. *sativum* covariates, *speciosum*, *medullare* and *axiphium* are the most closely related to each other, then subsp. *elatus* which is more related to subsp. *abyssinicum* than subsp. *asiaticum*

Keywords:*Pisum sativum*, Genetic relationships, molecular markers, RAPD, ISSR, SCoT

1. Introduction

Fabaceae family is one of the largest families in higher plant after Gramineae and Brassicaceae and it is commonly called legumes or pulses. This family has more than 727 genera and 20,000 species (Gepts *et al.*, 2005). Legumes have an economic importance; they have some of beneficial food crops such as soybeans, peas, beans, lentils and peanuts. Other genera of this family are significant sources of green compost or animal nutrition such as cassia, lupins, soybean, alfalfa and clover. Some genera like Laburnum, Gleditsia, Robinia, Mimosa, Acacia and Delonix are ornamental trees and shrubs. Still, other genera of this family have medicinal or insecticidal properties (for insect Derris) or yield valuable substances like tannin dyes, gum arabic, or resins. The tribe Vicieae includes three genera such as *Vicia* L., *Lathyrus* L., and *Pisum* L. (Young *et al.*, 2003).

Field pea (*Pisum sativum*) was found in the Middle East and cultivated from 10,000 years ago (Blixt, 1972; Zohary, 1996; Mithen, 2003). Cultivated *Pisum* is prevailed by *P. sativum*, but *P. sativum* ssp abyssinicum (referred to as *P. abyssinicum*) is an independently derived cultivated type. *Pisum sativum* is perhaps considered as a species complex with multiple subspecies (Vershinin *et al.*, 2003; Tar'an *et al.*, 2005). Pea is considered the basic model of modern plant genetics because it has been utilized in Mendel's laws of inheritance discover. It has a chromosome number of 2n = 14. The climate changes and the recent technologies make pea breeders perform more effective selection methods and take benefit of the large genetic diversity present in the *Pisum sativum* (Smýkal *et al.*, 2012).

Modern gene technological methods based upon induced mutants are becoming widespread in *P. stivum* subsp *sativum*. The induced variations in flower, pod, seed, leaf and stem characters have led to various attempts to classify the intraspecific diversity (Govorov, 1937; Makaševa, 1979; Lehmann and Blixt, 1984). Many convarieties and botanical varieties had been produced and described, a few like: convar. *speciosum* (Dierb.) Alef., the field peas, used now mainly as grain forage, convar. *axiphium* Alef., is known as the sugar pea with edible pods, convar. *sativum*, common pea, the dry and green seeds used, and convar. *medullare* Alef., only the green seeds useful.

Molecular markers were considered powerful tools to determine the genetic diversity through DNA sequence variations. These markers have an advantage by requiring a little amount of DNA and are fast to analyze (Sharma *et al.*, 2008). 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). Several molecular markers were used to evaluate the extent of genetic variability, such as Random amplified polymorphic DNA (RAPD) (Pérez de la Vega, 1997, which has been widely applied for examining genetic diversity and genetic relationships among several legumes including pea (Simioniuc *et al.*, 2002; Baranger *et al.*, 2004; Tar'an *et al.*, 2005; Yadav *et al.*, 2010; Kwon *et al.*, 2012). Inter-simple sequence

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repeats (ISSR) marker is more reliable than the RAPD marker because it is simple, fast, cheap and highly discriminating, so it is considered an informative tool for estimating genetic relationships (Ci *et al.*, 2008; Crespe *et al.*, 2009; Zhang and Dai, 2010; Uysal *et al.*, 2010). Nowadays, Start codon targeted (SCoT) markers are considered novel alternative and reproducible tools. SCoT-PCR technique depends on the short conserved region surrounding the ATG translation start (or initiation) codon in plant genes. (Collard and Mackill, 2009; Amirmoradi *et al.*, 2012; Hamidi *et al.*, 2014). SCoTs are used due their importance as constructing markers helping in breeding programs than RAPDs, ISSRs. These markers have some properties as being fast, easy to use and cheap (Mulpuri *et al.*, 2013).

The purpose of the current study is to figure out the genetic relationships among different *Pisum sativum* subspecies based on polymorphism of RAPD, ISSR and SCoT markers.

2. Materials and Methods

2.1. Plant materials:

Seeds of *Pisum sativum* subspecies, *asiaticum* Gov, *abyssinicum* (A. Braun) Berger, *elatus* (M. Bieb) schmalb, and three convarities of subsp. *sativum* (*axiphium* Alef "Dvargsabel", *medullare* and *speciosum* (Dieb) Alef) were received from Leibniz Institute of Plant Genetics and Crop Plant Research (IPK, Gatersleben, Germany).

2.2. DNA extraction and purification:

Young leaves from each seedling plant of studied *P. sativum* subspecies were collected and genomic DNA was extracted and purified. Plant Genomic DNA Purification Kit (Gene Jet Mini Kit, Cat no. K0791, Thermo- Fisher Scientific, Germany) was used for extraction and purification of genomic DNA. The extracted DNA was checked on agarose gel (1%) using horizontal electrophoresis and diluted to working concentration 50 ng/µl by nuclease-free water and stored at -20 °C.

2.2.1. RAPD-PCR Amplification:

Six 10 bp random primers (Table 1) were used for performing RAPD-PCR. Amplification of purified DNA was achieved in PCR microtubes (Eppendorf 0.2 ml) containing 12.5 µl PCR Master Mix 2X (Dream Taq Green, Thermo-Fisher Scientific, Germany), 1 µl primer (Metabion, Germany) 10 pmol and 1 µl purified DNA (50 ng/µl) with total volume 25 µl of the reaction mixture. Thermocycler (Bio-Rad, USA) was programmed as follows: one cycle (94 °C for 5 min), 35 cycles (94 °C for 1 min, 38 °C for 45 sec and 72 °C for 45 sec), one cycle (72 °C for 5min) and finally held at 4°C. Afterward, 5µl of each PCR product were loaded in agarose gel (1%) in TAE buffer, together with 3 µl DNA Ladder 100 bp (H3 RTU, Cat No. DM003-R500, GeneDirex) in horizontal electrophoresis (Cleaver, UK) for about 2 hours at 100 V. The gel was stained with ethidium bromide and photographed by gel documentation (Bio-Rad, USA).

Table 1. Represented RAPD, ISSR and SCoT primers sequence.

Ser. No.	RAPD primers sequence			ISSR primers sequence			SCoT primers sequence		
	Primers	Sequence (5-3)	Tm (°C)	Primers	Sequence (5-3)	Tm (°C)	Primers	Sequence (5-3)	GC%
1	OPA1	CAGGCCCTTC	38	ISSR2M	(CA)8 AAGCT	61	SCoT-1	CAACAATGGCTACCACCA	50
2	OPA4	AATCGGGCTG	38	807	(AG)8 T	55	SCoT-3	CAACAATGGCTACCACCG	56
3	OPA11	CAATCGCCGT	38	812	(GA)8 A	54	SCoT-6	CAACAATGGCTACCACGC	56
4	OPB3	CATCCCCCTG	38	818	(CA)8 G	55	SCoT-9	CAACAATGGCTACCAGCA	50
5	OPH7	CTGCATCGTG	38	842	(GA)8 CTG	58	SCoT-10	CAACAATGGCTACCAGCC	56
6	OPH12	ACGCGCATGT	38	848	(CA)8AAGG	61	SCoT-11	AAGCAATGGCTACCACCA	50
7	-	-	-	857	(AC)8 CTG	54	SCoT-14	ACGACATGGCGACCACGC	67
8	-	-	-	866	(CTC)6	61	SCoT-15	ACGACATGGCGACCGCGA	67

2.2.2. ISSR-PCR Amplification:

ISSR-PCR was carried out by using eight ISSR primers (Table 1). Amplification of purified DNA was achieved in PCR microtubes (Eppendorf 0.2 ml) containing 12.5 µl PCR Master Mix 2X (Dream Taq Green, Thermo-Fisher Scientific, Germany), 1 µl primer (Metabion, Germany) 10 pmol and 1 µl purified DNA (50 ng/µl) with total volume 25 µl of the reaction mixture. Thermocycler (Bio-Rad, USA) was programmed as follows: one cycle (93°C for 20 sec), 40 cycles (94 °C for 20 sec, Tm (54-61) °C for 1 min and 72 °C for 20 sec), one cycle (72 °C for 6 min) and finally held at 4°C. Afterward, 5µl of each PCR product was loaded in agarose gel (1.5%) in TAE buffer and electrophoresis was run as described above.

2.2.3. SCoT-PCR Amplification:

SCoT-PCR was achieved by using eight SCoT primers (Table 1). Amplification of purified DNA was achieved in PCR microtubes (Eppendorf 0.2 ml) containing 12.5 µl PCR Master Mix 2X (Dream Taq Green, Thermo-Fisher Scientific, Germany), 1 µl primer (Metabion, Germany) 10 pmol and 1 µl purified DNA (50 ng/µl) with total volume 25 µl of the reaction mixture. Thermocycler (Bio-Rad, USA) was programmed as follows: one cycle (94 °C for 3 min), 35 cycles (94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min), one cycle (72 °C for 5 min) and finally held at 4 °C. Afterward, 5µl of each PCR product was loaded in agarose gel (1.2%) in TAE buffer and electrophoresis was run as described above.

2.2.4. Data analysis and molecular dendrograms construction:

Total Lab program was used to analyze images of RAPD, ISSR and SCoT assays for detecting the molecular size of each fragment and compare their presence or absence among studied species, and these data were analysed using MVSP (Multi-Variant Statistical Package, Kovach, 1998). The similarity matrix was studied and dendrogram (UPGMA, using Jaccard's coefficient) was constructed to indicate the genetic relationships between the studied species.

Polymorphic information content (PIC) values reflected gene diversity of studied species; it could be calculated with the following equation (Anderson *et al.*, 1993):

$$PICi = 1 - \sum_{f=1}^{n} (Pij)^2$$

Where, n is the number of marker alleles for marker *i* and *Pij* is the frequency of the *j* the allele for marker *i*.

The summary statistics including the number of alleles per locus, major allele frequency, gene diversity, polymorphism information content (PIC) values were calculated using Power Marker version 3.25 (Liu and Muse, 2005).

3. Results

The genetic relationships of the studied six *Pisum sativum* subspecies were determined by RAPD, ISSR and SCoT markers, the patterns of the amplified DNA products by their primers are shown in Figures 1, 2 and 3, respectively. The phylogenetic dendrograms by UPGMA of RAPD, ISSR, SCoT and combined data of three markers together were illustrated in Figure 4.

3.1. Random amplified polymorphic DNA (RAPD) analysis

The results of RAPD amplification are present in Table 2. The six RAPD primers produced good reproducible patterns, that screened the polymorphisms between the different *Pisum sativum* subspecies (Figure 1).

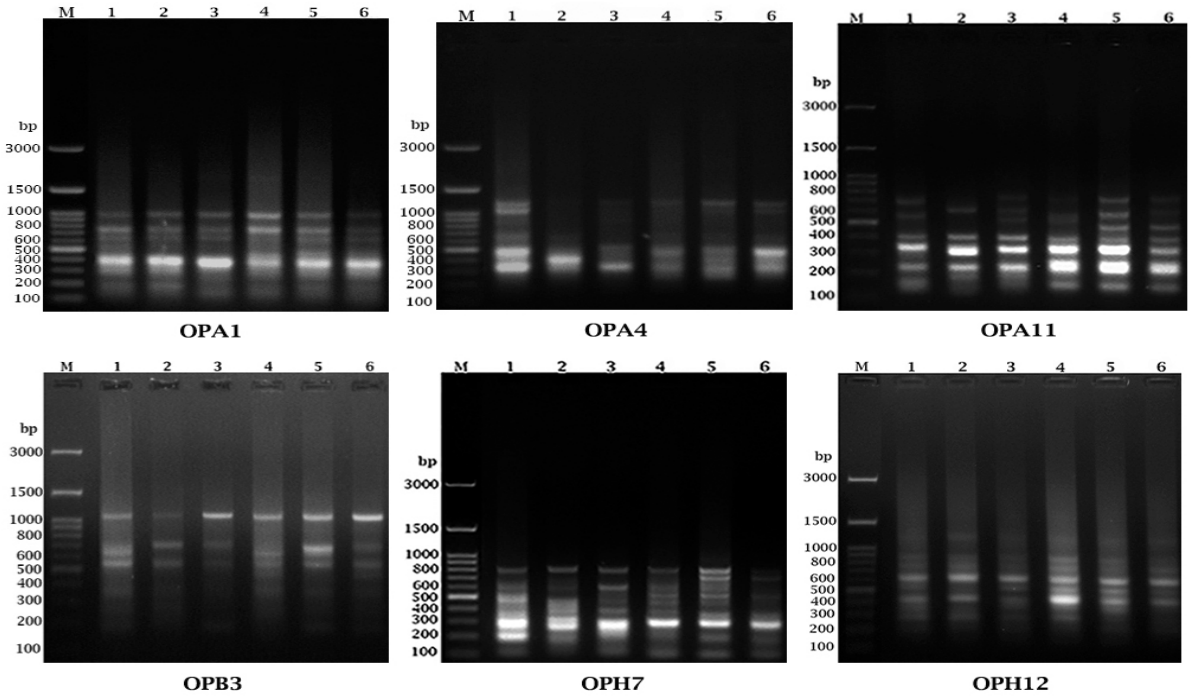

Figure 1. Electrophoresis of RAPD-PCR amplification for *Pisum sativum* subspecies.

M: DNA ladder 100 bp

1- *P. sativum* subsp. asiaticum 2- *P. sativum* subsp. abyssinicum 3- *P. sativum* subsp. elatus 4- *P. sativum* subsp. sativum con var. axiphium" 5- *P. sativum* subsp. sativum con var. medullare 6- *P. sativum* subsp. sativum con var. speciosum

A total number of 59 amplicons were produced, among them 37 bands were polymorphic. The polymorphism ranged between 33.33% with primer OPH12 and 80% with primer OPA1 with an average 62.71%. Polymorphic information content (PIC) and Gene diversity ranged from 0.11 and 0.14 in primer OPH12 to 0.14 and 0.32 in primer OPA1 with an average of 0.18 and 0.23, respectively. All of the studied subspecies had 9 positive unique bands (band present only in one subspecies while absent in others) and 8 negative unique bands (band absent only in one subspecies while present in others) (Table 2). The phylogenetic dendrogram showed two main clusters. The first cluster split into two sub-clusters, (A) contained subsp. *sativum* convar. *medullare* and convar. *speciosum* while the second sub-cluster (B) contained convar. *axiphium* only. The second cluster split into two sub-clusters, (C) contained subsp. *asiaticum* and subsp. *elatus* while the second sub-cluster (D) contained only subsp. *abyssinicum* (Figure 4a).

The highest similarity value 0.79 was found between subsp. *sativum* convar. *medullare* and *speciosum* then 0.75 between convar. *axiphium* and *medullare*, which indicates that these three convarities are more closely related. It has been observed that subsp. *elatus* is more related to subsp. *asiaticum* than subsp. *Abyssinicum*, while the lowest value observed was 0.50 between subsp. *abyssinicum* and subsp. *sativum* convar. *Speciosum* (Table 3).

Table 2. Total number of bands polymorphism, gene diversity and PIC as revealed by RAPD-PCR amplification of *Pisum sativum* subspecies.

Primer	Total of fragments	Polymorphic fragments	Monomorphic fragments	Polymorphism (%)	+ve Unique band	-ve Unique band	Gene Diversity	PIC	Allele size range (bp)
OPA1	10	8	2	80.00%	1	1	0.32	0.26	170-1000 bp
OPA4	8	5	3	62.50%	2	2	0.20	0.17	280-1185 bp
OPA11	11	7	4	63.63%	3	2	0.21	0.17	140-720 bp
OPB3	12	9	3	75.00%	3	0	0.30	0.24	190-1040 bp
OPH7	9	5	4	55,55%	0	2	0.22	0.17	100-800 bp
OPH12	9	3	6	33.33%	0	1	0.14	0.11	185-1100 bp
Total	59	37	22	-	9	8	-	-	-
Average	9.83	6.166	3.66	62.71%	-	-	0.23	0.18	-

Table 3. Similarity matrix among examined *Pisum sativum* subspecies estimated based on Jaccard' Coefficient as revealed by RAPD marker.

***P. sativum* subsp. asiaticum**	1					
***P. sativum* subsp. abyssinicum**	0.70	1				
***P. sativum* subsp. elatus**	0.71	0.70	1			
***P. sativum* subsp. sativum con var. axiphium**	0.67	0.59	0.67	1		
***P. sativum* subsp. sativum con var.medullare**	0.71	0.61	0.61	0.75	1	
***P. sativum* subsp. sativum con var. speciosum**	0.633	0.50	0.60	0.60	0.79	1
	***P. sativum* subsp. asiaticum**	***P. sativum* subsp. abyssinicum**	***P. sativum* subsp. elatus**	***P. sativum* subsp. sativum con var. axiphium**	***P. sativum* subsp. sativum con var.medullare**	***P. sativum* subsp. sativum con var. speciosum**

3.2. Inter-simple sequence repeats (ISSR) analysis

The amplified products of eight ISSR primer are present in Table (4). The primers gave a reproducible patterns that showed the polymorphisms among studied *P. sativum* subspecies (Figure 2).

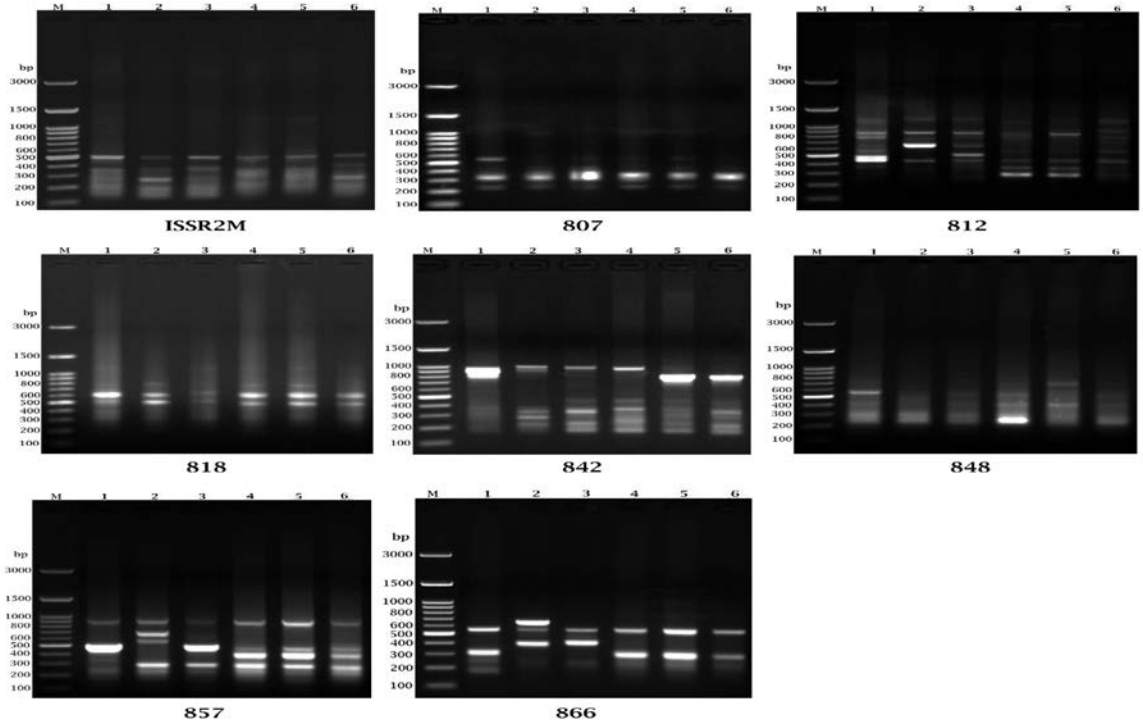

Figure 2. Electrophoresis of ISSR-PCR amplification for *Pisum sativum* subspecies.

M: DNA ladder 100 bp

1- *P. sativum* subsp. asiaticum 2- *P. sativum* subsp. abyssinicum 3- *P. sativum* subsp. elatus 4- *P. sativum* subsp. sativum con var. axiphium 5- *P. sativum* subsp. sativum con var. medullare 6- *P. sativum* subsp. sativum con var. speciosum

Table 4. Total number of bands polymorphism, gene diversity and PIC as revealed by ISSR-PCR amplification of *Pisum sativum* subspecies.

Primer	Total of fragments	Polymorphic fragments	Monomorphic fragments	Polymorphism (%)	+ve Unique band	-ve Unique band	Gene Diversity	PIC	Allele size range (bp)
ISSR2M	12	10	2	83.33%	6	3	0.27	0.23	140-1220 bp
807	5	1	4	20.00%	0	0	0.09	0.07	210-1050 bp
812	14	12	2	85.71%	3	4	0.30	0.24	290-1200 bp
818	7	3	4	42.86%	0	0	0.19	0.15	320-1100 bp
842	12	11	1	91.66%	3	3	0.32	0.26	210-1000 bp
848	6	5	1	83.33%	0	0	0.38	0.29	240-680 bp
857	8	5	3	62.5%	1	2	0.26	0.21	230-900 bp
866	7	5	2	71.43%	2	0	0.27	0.22	190-650 bp
Total	71	52	19	-	15	12	-	-	-
Average	8.875	6.5	2.375	73.24	-	-	0.26	0.21	-

A total number of 71 amplified amplicons was obtained, among which 52 amplicons were polymorphic. The polymorphism ranged from 20 % with primer 807 and 91.66 % with primer 842. The average percentage of polymorphism was 73.24 %. The maximum polymorphic information content (PIC) and Gene diversity were 0.29 and 0.38, respectively with primer 848 while the minimum PIC and Gene diversity were 0.07 and 0.09, respectively with primer 807. The average of PIC and Gene diversity were 0.21 and 0.26, respectively. All investigated *Pisum sativum* subspecies had 15 positive and 12 negative unique bands. (Table 4). The phylogenetic dendrogram showed two main clusters; (A) contained only subsp. *asiaticum*, while the second cluster (B) contained all other subspecies which was subsequently split into two sub-clusters; (C) contained subsp. *abyssinicum* and subsp. *elatus*, while the second sub-cluster (D) contained the other three subspecies which is subsequently split into two groups, (E) contained only subsp. *sativum* convar. *speciosum*, and the second group (F) contained convar. *axiphium* convar. *medullare* (Figure 4b).

The highest similarity value 0.69 was reported between subsp. *abyssinicum* and subsp. *elatus*, then 0.68 between subsp. *sativum* convar. *axiphium* and convar. *medullare*, and between convar. *medullare* and convar. *speciosum* as well. While, the lowest value observed was 0.48 between subsp. *asiaticum* and subsp. *sativum* convar. *axiphium* (Table 5).

Table 5. Similarity matrix among examined *Pisum sativum* subspecies estimated based on Jaccard' Coefficient as revealed by ISSR marker.

***P. sativum* subsp. asiaticum Gov**	1					
***P. sativum* subsp. abyssinicum**	0.52	1				
***P. sativum* subsp. elatus**	0.55	0.69	1			
***P. sativum* subsp. sativum con var. axiphium**	0.48	0.62	0.68	1		
***P. sativum* subsp. sativum con var.medullare**	0.54	0.57	0.66	0.68	1	
***P. sativum* subsp. sativum con var. speciosum**	0.49	0.57	0.61	0.62	0.67	1
	***P. sativum* subsp. asiaticum**	***P. sativum* subsp. abyssinicum**	***P. sativum* subsp. elatus**	***P. sativum* subsp. sativum con var. axiphium**	***P. sativum* subsp. sativum con var.medullare**	***P. sativum* subsp. sativum con var. speciosum**

3.3. Start codon targeted Polymorphism (SCoT) analysis

The results of SCoT amplification were shown in Table (6). The eight primers gave reproducible patterns that screened the polymorphisms among the studied *Pisum sativum* subspecies (Figure 3).

A total of 105 bands were obtained, out of which 79 bands were polymorphic bands. The polymorphism ranged from 50% with SCoT-3 and 100% with SCoT-15. The average percentage of polymorphism was 75.24 %. The maximum polymorphic information content (PIC) and Gene diversity were 0.32 and 0.40, respectively with SCoT-15, while the minimum PIC and gene diversity were 0.15 and 0.19, respectively with SCoT-3. The average of PIC and Gene diversity were 0.23 and 0.29, respectively. All investigated subspecies had 20 positive unique and 10 negative unique bands. (Table 6). The phylogenetic dendrogram showed two main clusters; (A) contained subsp. *abyssinicum* and subsp. *elatus*, while the second cluster (B) contained all other subspecies. The second cluster was split into two sub-clusters; (C) contained only subsp. *asiaticum*, while the second sub-cluster (D) contained the other three species. The second sub-cluster split into two groups, (E) contained only subsp. *sativum*

convar. *axiphium*, while the second group (F) contained subsp. *sativum* convar. *medullare* and convar. *speciosum* (Figure 4c).

The highest similarity value 0.70 was reported between subsp. *sativum* convar. *medullare* and convar. *speciosum*, then subsp. *sativum* convar. *medullare* and convar. *axiphium* (0.66), and it was observed that subsp. *abyssinicum* and subsp. *elatus* are more related to each other (0.67) than to subsp. *asiaticum*. While the lowest value observed was 0.42 between subsp. *asiaticum* and subsp. *abyssinicum* (Table 7).

Table 6. Total number of bands polymorphism, gene diversity and PIC as revealed by SCoT-PCR amplification of *Pisum sativum* subspecies.

Primer	Total of fragments	Polymorphic fragments	Monomorphic fragments	Polymorphism (%)	+ve Unique band	-ve Unique band	Gene Diversity	PIC	Allele size range (bp)
SCoT-1	10	7	3	70.00%	2	1	0.27	0.22	420-1700 bp
SCoT-3	10	5	5	50.00%	2	0	0.19	0.15	320-1965 bp
SCoT-6	16	12	4	75.00%	3	3	0.28	0.23	400-2470 bp
SCoT-9	12	10	2	83.33%	1	2	0.33	0.26	440-1575 bp
SCoT-10	13	7	6	53.85%	2	0	0.24	0.18	400-2720 bp
SCoT-11	11	8	3	72.73%	2	1	0.29	0.23	260-1320 bp
SCoT-14	13	10	3	76.92%	2	2	0.30	0.24	250-1215 bp
SCoT-15	20	20	0	100%	6	1	0.40	0.32	125-2850 bp
Total	105	79	26	-	20	10	-	-	-
Average	13.125	9.875	3.25	75.24%	-	-	0.289	0.228	-

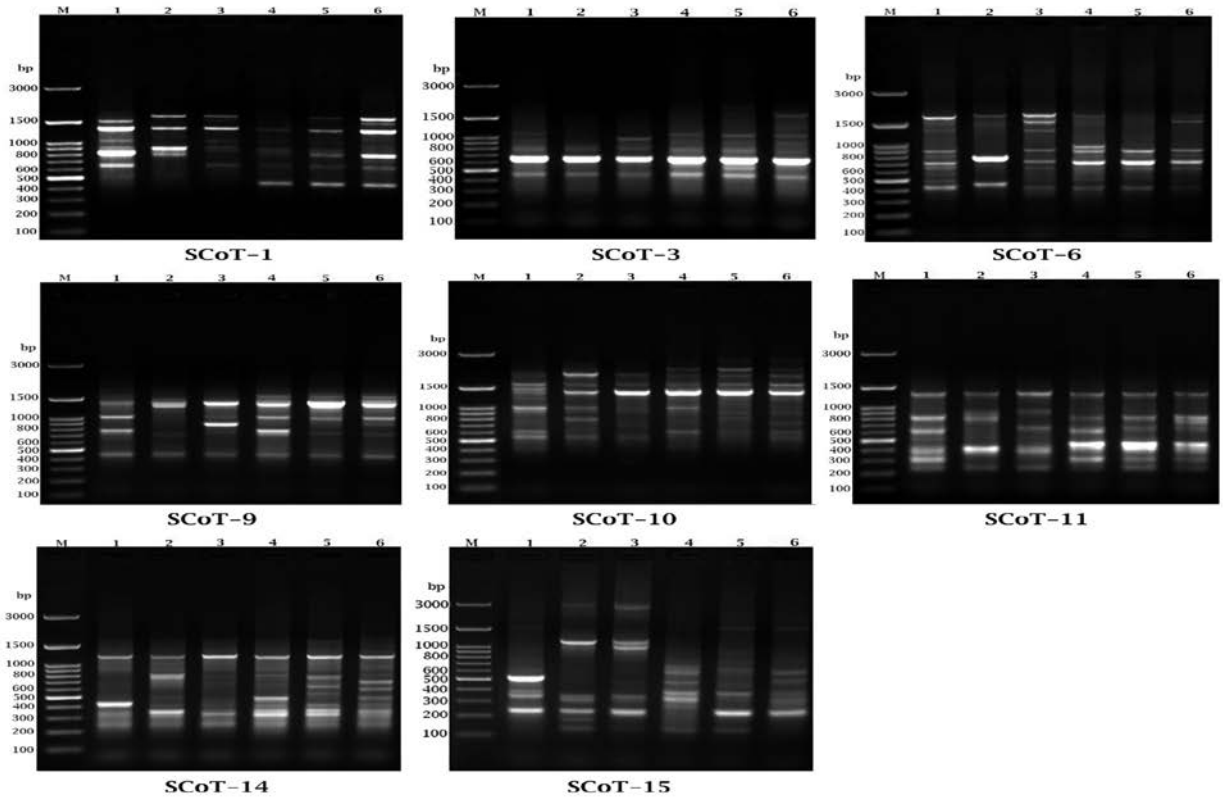

Figure 3. Electrophoresis of SCoT-PCR amplification for *Pisum sativum* subspecies.

M: DNA ladder 100 bp

1- *P. sativum* subsp. asiaticum 2- *P. sativum* subsp. abyssinicum 3- *P. sativum* subsp. elatus 4- *P. sativum* subsp. sativum con var. axiphium 5- *P. sativum* subsp. sativum con var. medullare 6- *P. sativum* subsp. sativum con var. speciosum

Table 7. Similarity matrix among examined *Pisum sativum* subspecies estimated based on Jaccard' Coefficient as revealed by SCoT marker.

***P. sativum* subsp. asiaticum**	1					
***P. sativum* subsp. abyssinicum**	0.42	1				
***P. sativum* subsp. elatus**	0.57	0.67	1			
***P. sativum* subsp. sativum con var. axiphium"**	0.58	0.44	0.53	1		
***P. sativum* subsp. sativum con var. medullare**	0.52	0.42	0.49	0.66	1	
***P. sativum* subsp. sativum con var. speciosum**	0.52	0.43	0.51	0.65	0.70	1
	***P. sativum* subsp. asiaticum**	***P. sativum* subsp. abyssinicum**	***P. sativum* subsp. elatus**	***P. sativum* subsp. sativum con var. axiphium**	***P. sativum* subsp. sativum con var.medullare**	***P. sativum* subsp. sativum con var. speciosum**

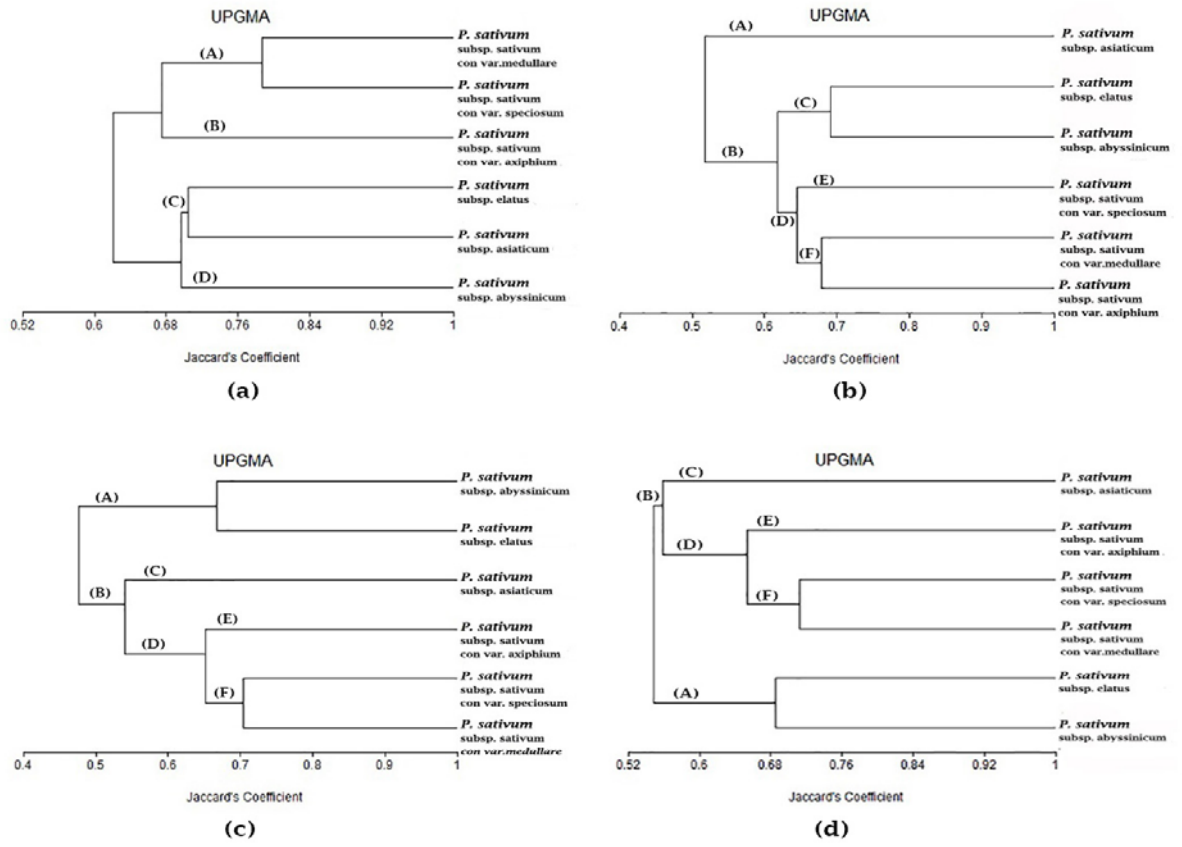

Figure 4. The constructed dendrogram by UPGMA and similarity matrix depending on Jaccard' Coefficient using (a) RAPD, (b) ISSR, (c) SCoT and (d) combined data from the *Pisum sativum* subspecies

3.4. Genetic similarity based on the combined data of RAPD, ISSR and SCoT markers:

It was observed by combining the obtained data from the three markers RAPD, ISSR and SCoT that the maximum polymorphism, PIC and Gene Diversity were detected with SCoT marker, while the minimum polymorphism, PIC and Gene Diversity were detected with RAPD marker (Table 8). The dendrogram showed two main clusters; (A) contained subsp. *abyssinicum* and subsp. *elatus*, while the second cluster (B) contained all other. The second cluster was split into two sub-clusters; (C) contained subsp. *asiaticum*, while the second sub-cluster (D) contained the other three subspecies. The second sub-cluster split into two groups, (E) contained only subsp. *sativum* convar. *axiphium* and the second group (F) contained subsp. *sativum* convar. *medullare* and convar. *speciosum* (Figure 4d). Likewise, it was observed that UPGMA results of combined data are similar to the

UPGMA of SCoT marker; this indicates that SCoT marker is an informative marker for discrimination and identification of different studied subspecies.

The highest similarity value (0.71) was reported between subsp. *sativum* convar. *medullare* and convar. *speciosum*, then subsp. *sativum* convar. *medullare* and convar. *axiphium* (0.69), and subsp. *abyssinicum* and subsp. *elatus* are more related to each other (0.69) than to subsp. *Asiaticum*,while the lowest value observed was 0.50 between subsp. *abyssinicum* and both of subsp. *sativum* convar. *speciosum* and convar. *axiphium* (Table 9).

Table 8. Comparison of genetic parameters between RAPD, ISSR and SSR analysis.

Molecular Parameter	Value		
	RAPD	ISSR	SCoT
Total number of amplicon	59	71	105
Number of polymorphic amplicon	37	52	79
+ve Unique Bands	9	15	20
% of polymorphism per assay	62.71%	73.24%	75.24%
PIC	0.19	0.21	0.23
Gene Diversity	0.23	0.26	0.30

Table 9. Similarity matrix between studied *Pisum sativum* subspecies estimated based on Jaccard' Coefficient as revealed by combined data.

***P. sativum* subsp. asiaticum**	1					
***P. sativum* subsp. abyssinicum**	0.52	1				
***P. sativum* subsp. elatus**	0.59	0.69	1			
***P. sativum* subsp. sativum con var. axiphium**	0.57	0.50	0.60	1		
***P. sativum* subsp. sativum con var.medullare**	0.57	0.52	0.57	0.69	1	
***P. sativum* subsp. sativum con var. speciosum**	0.54	0.50	0.56	0.62	0.71	1
	***P. sativum* subsp. asiaticum**	***P. sativum* subsp. abyssinicum**	***P. sativum* subsp. elatus**	***P. sativum* subsp. sativum con var. axiphium**	***P. sativum* subsp. sativum con var.medullare**	***P. sativum* subsp. sativum con var. speciosum**

4. Discussion

There are various types of markers as RAPD, ISSR and SCoT, which have been used for the proper characterization and management of germplasm to detect the genetic diversity and genetic relationships among plant taxa. RAPD marker is considered a useful molecular tool because of its simplicity and low cost; however, this technique could have problems with reproducibility (Waugh and Powell, 1992). ISSR marker target microsatellite regions, does not need information about gene sequence; it is more reproducible than RAPD as it can be performed with higher stringency (high annealing temperature). ISSR markers have been used widely for genetic diversity analyses of black gram, chick pea and jojoba plants (Souframanien and Gopalakrishna, 2004) and both types of marker (RAPD and ISSR) were useful for identifying relationships at the cultivar and species level (Karuppanapandian *et al.*, 2007; Rao *et al.*, 2007; Sharma *et al.*, 2008). Therefore, RAPD and ISSR techniques are widely used because they are fast, cheap and do not need more knowledge about DNA sequence but need a small amount of template DNA. Start Codon Targeted (SCoT) Polymorphism was designed dependent on the short conserved region next to the ATG start codon in plant genes (Collard and Mackill, 2009). Commonly SCoT markers are reproducible; however, it is proposed that annealing temperature and primer length are not the only considerations controlling reproducibility. SCoT markers were used in this study due to a number of advantages compared to other molecular markers. They provide easier development of species-specific primers than SSR and are cheaper than AFLP (Jiang *et al.*, 2014) and offer higher reproducibility than RAPD (Xiong *et al.*, 2011). Many studies were performed to evaluate the genetic diversity and relationship using SCoT markers with different plants as Egyptian *Glycine max* cultivars, *Elymus sibiricus* and *Vigna unguiculata* (Zhang *et al.*, 2015; Igwe *et al.*, 2017; Rayan and Osman, 2019). Also, Teshome et al., (2015) used EST-SSR markers in resolving the inconsistency in the taxonomic status of the different subspecies of genus *Pisum*. Stavridou *et al*. (2020) documented in their results that different landraces are more closely related to *P. sativum* subsp. *elatius* than *P. abyssinicum* and *P. fulvum* species using ISSR markers and DNA barcoding.

It was observed that high level of genetic variability among the studied pea subspecies using RAPD, ISSR and SCoT markers. SCoT markers gave maximum values of polymorphism (75.24%), PIC (0.23) and gene diversity (0.29) while ISSR markers had a polymorphism (73.24%), PIC (0.21) and gene diversity (0.26). The minimum values of polymorphism (62.7%), PIC (0.18) and gene diversity (0.23) were reported in RAPD markers. The results of molecular dendrogram (UPGMA) of combined data gave two main clusters; the first cluster included *P. sativum* subsp. *abyssinicum* and subsp. *elatus*, while the second cluster included all other subspecies. The second cluster was split into two sub-clusters; the first sub-cluster contained only *P. sativum* subsp. *asiaticum*, whereas the second sub-cluster contained the other three subspecies. The second sub-cluster was split into two groups; the first group contained only *P. sativum* subsp. *sativum* convar. *axiphium* and the second group contained subsp. *sativum*

convar. *medullare* and convar. *speciosum*. This result was identical with the results of SCoT molecular dendrogram (UPGMA). So, we can conclude that SCoT markers are more informative markers than ISSR and RAPD markers for discrimination and identification of studied species, this agreed with Zeng *et al.* (2014) and Tiwari *et al.* (2016) who mentioned that SCoT marker has a greater capability than other markers to polymorphism, for identification and discrimination among species and subspecies.

The fingerprinting and genetic relationships among studied *Pisum sativum* subspecies was performed using three types of molecular markers, RAPD, ISSR, and SCoT. It was found from RAPD, SCoT and combined observation that the most genetically related subspecies were subsp. *sativum* convar. *medullare* and convar. *speciosum* (0.79, 0.70 and 0.71, respectively); this relationship has not been verified by ISSR (subsp. *abyssinicum* and subsp. *elatus* by 0.69).

By combining the similarity matrix between the studied *Pisum sativum* subspecies as computed by Jaccard' Coefficient from these three markers (Table 14), it could be concluded that the most related subspecies are subsp. *sativum* convar. *medullare* and convar. *speciosum* (0.71), then comes subsp. *sativum* convar. *axiphium* and convar. *medullare* (0.69), Afterword comes subsp. *abyssinicum* and subsp. *elatus* (0.69). According to these observations, the genetic relationship among the studied subspecies in an order is as follows: subsp. *sativum* convar. *speciosum* is more related to convar. *medullare*, then comes convar. *axiphium*, followed by the three *P. sativum* subspecies, subsp. *abyssinicum*, subsp. *elatus* then subsp. *asiaticum*, where subsp. *elatus* has high relationship with subsp. *sativum* convar. *axiphium* (0.60%).

5. Conclusion

The genetic relationships among the six *Pisum sativum* subspecies were studied using three types of molecular markers, RAPD, ISSR and SCoT. It was found that SCoT marker is the most informative marker for the discrimination of the studied *P. sativum* subspecies. It could be concluded that the genetic relationships among the studied subspecies in an order is as follows: subsp. *sativum* convar. *speciosum* is more related to convar. *medullare* then comes convar. *axiphium* and followed by subsp. *elatus* which is more related to subsp. *abyssinicum* than subsp. *asiaticum* .

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