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ESTIMATION OF GENETIC VARIATIONS IN DIFFERENT TAXA IN BRASSICACEAE BY RAPD AND ISSR ANALYSIS

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ABSTRACT

Twelve species from Brassicaceae family were studied using two different molecular techniques: RAPD and ISSR; both of these techniques were used to detect some molecular markers associated with the genotype identification. RAPD results, from using five random primers, revealed 241 amplified fragments, 62 of them were polymorphic (26%).

ISSR results showed that out of seven primers, three (ISSR3, UBC807, UBC811) could not amplify the genomic DNA; other primers revealed 183 amplified fragments, 36 of them were polymorphic (20%). The Similarity evidence and dendrogram for the genetic distances of the incorporation between the two techniques showed that the highest similarity was 0.897 between the varieties red cabbage and red ornamental cabbage, meanwhile the lowest similarity index was between the varieties red radish and Green ornamental cabbage (0.169); thus these RAPD and ISSR markers have the possibility for the identification of species or varieties and the description of genetic variation within the varieties. Furthermore, it could be concluded that the Brassicaceae taxa have a suitable amount of genetic variance and a wide range in the genetic principle of the studied genotypes which can be used for output improvement.

Keywords: Brassicaceae, Genetic similarity, ISSR, Polymorphism, RAPD.

INTRODUCTION

Although many of families in Flora of Iraq have been examined morphologically and phytochemically in some detail (Harborne *et al.,* 1971), there are many open inquiries concerning the classification of particular genera within tribes and subfamilies. Molecular information enhances or even permits the illustration of phylogeny, and provides the fundamental information for comprehension taxonomy, breeding, and advancement of plants. Molecular techniques are being used increasingly in plant systematic (Soltis *et al*., 1992). In addition; the improvement of PCR-based molecular marker manner has prompted expanding the utilization of molecular marker technologies in many fields of science, including systematic studies. In this relation randomly amplified polymorphic DNA (RAPD) and Inter-Simple Sequence Repeat (ISSR) techniques have drawn much attention in a wide variety of organisms, especially in plants. Therefore, this study chooses the Brassicaceae family because it is typical of families to compare it with other molecular and evolutionary studies (Franzke *et al.* 2011) for the following reasons. Firstly the consideration of *Arabidopsis thaliana* (L.) Heynh. which is a protrude model amongst the most vital plants in molecular studies (Meinke

et al. 1998) as well as many studies on *Brassica* spp.; secondly it investigated for different sides of botany, for example, biotic and abiotic stress tolerance, genome development (Vekemans *et al*. 2014), thirdly it encountered entire genome duplications and organismal radiation in its underlying formative history (Edger *et al*. 2015).

Mustard family (Brassicaceae or Cruciferae) is a fourth large and natural family which can be easily morphologically diagnosed by scrupulous flowering Actinomorphic form, Cruciform corolla and Silique fruits. It has a global apportionment predominately in the moderate area of the northern hemisphere (Al-Shehbaz, 1984). Most of the members of the understudied family are economically important and include vegetables such as cabbage, cauliflower and broccoli, oilseed from *Brassica napus* L., and *B.rapa* L., fodder, ornamental mainly in *B.oleracea* var. *acephala* (ornamental cabbage), and condiment plants like *Brassica nigra (*L) W.D.J.Koch. and *B.juncea* (L.) Czern, (Gomez-Campo and Prakash 1999).

Brassicaceae are divided into three to nineteen tribes and twenty to thirty subtribes. The phylogeny and ranking of the familial level like genera and tribe are still suspicious (Appel and Al-Shehbaz, 2003). This obstruction was performed in a lack of understanding of the number and limits of phylogenetically established tribes and genera and gave rise to various distinct classification systems which have been submitted previously (Warwick *et al*. 2010). Later, Al-Shehbaz (2012) estimated the number of tribes as approximately 51 which included 321 genera and 3660 species. Description of the plant with the utilization of molecular markers is a typical way to memorize plant genetic assets and molecular depiction helps to determine the breeding behavior of species (Prasad, 2014). In this way the objective of the present investigation is to estimate of degrees of relationship between different varieties and species of different taxa within the Brassicaceae by using random primers in RAPD and ISSR techniques a further important use of these markers are to distinguish between genotypes

MATERIALS AND MATHODS

Plant materials and extraction of genomic DNA: Twelve taxa which used in this study are listed in Table (1) and Plate (1). Fresh leaves, clean and free from dirt and bacterial and fungal infections were selected then treated with liquid nitrogen carefully in mortar and pestle until being a fine powder. Genomic DNA was isolated from leaves according to the method protocol of the Genomic DNA Mini Kit (Geneaid Biotech. Ltd; Taiwan Company).

Checking of the RAPD and ISSR primers: The amplification reactions were performed by five decamer oligonucleotide primers from (OPC2, OPC8, OPC14, OPB11 and OPB18 for RAPD analysis) and seven oligonucleotide primers (BH10, BH11, BH14, ISSR1, ISSR3, UBC807 and UBC811 for ISSR analysis) (Tab. 2).

Polymerase chain reaction: 25 µl a final volume of amplification reactions containing 5 µl of DNA, 1µl of primer (50 pmol), 12.5 µl of the master mix including (dNTPs, PCR buffer, Taq DNA polymerase (5U/µl), MgCl2) and 6.5µl deionized water. PCR reaction was carried out in Thermal cycler PCR System (Verity, Applied Biosystem). The subsequent different trials for upgrading the best fitting conditions, a program for PCR response was standardized with following settings according to Table (3). Then amplified DNA were separated by electrophoresis in 1.3 % agarose gels (stained with 0.3 µl of ethidium bromide at 3-4 hr on 70V).

Data analysis: RAPD and ISSR markers produce DNA amplification signals that can be changed over into a measurement of similarity or dissimilarity DNA electrophoretic pattern

containing visible bands at a specific position in the individual lane. The banding patterns were transformed into binary characters where the appearance of a band was given the number (1) while the absence of the band was given the number (0).

A square symmetric matrix of similarity was acquired using Jaccard's similarity coefficient to draw the dendrograms. The unweighted pair group method with arithmetic average (UPGMA) was applied for cluster analysis using past software ver. 1.92 (Hammer *et al*., 2001), and calculated the percentage of polymorphism, efficiency and discriminating power of primer as the following equation:

Polymorphism% = (No. the polymorphic bands of random primer / the total number of bands of the same primer) × 100.

Efficiency of primer =(No. the polymorphic bands to each primer / total number of bands to the same primer) × 100

Discriminating power of primer = No. the polymorphic band to each primer /total number of the polymorphic band to all primer X 100 %. according to (Grudman *et al*., 1995).

Table (1): Species and varieties included under study from Brassicaceae family with common names and tribes.

No.	Scientific name	Common name	Tribe
1.	*Raphanus sativus* var. *longipinnatus* L. H. Bailey	White radish	Brassiceae
2.	*Raphanus sativus* L.	Red radish	Brassiceae
3.	*Raphanus raphanistrum* L.	Wild radish or FIHAILA	Brassiceae
4.	*Lepidium sativum* L.	Cress or RASHAD	Lepidieae
5.	*Cardaria draba* (L.)Desv.	Hoary Cress or QUNAIBRA	Lepidieae
6.	*Sisymbrium irio* L.	Rocket Mustard	Sisymbrieae
7.	*Brassica oleracea* var. *botrytis* L.	Cauliflower or QARNABÎT	Brassiceae
8.	*Brassica oleracea* var. *italic* L.	Broccoli	Brassiceae
9.	*Brassica oleracea* var. *capitata* L.	Green Cabbage-LAHANA	Brassiceae
10.	*Brassica oleracea* var. *capitate* L.	Red Cabbage	Brassiceae
11.	*Brassica oleracea* var. *acephala* DC.	Green ornamental cabbage	Brassiceae
12.	*Brassica oleracea* var. *acephala* DC.	Red ornamental cabbage	Brassiceae

Plate (1): Species and varieties included under study from Brassicaceae family.

Table (2): List of primers are used in RAPD and ISSR techniques and their sequences.

Primer		Primer sequences 5' to 3'	Primer		Primer sequences 5' to 3'
RAPD	OPC2	GTGAGGCGTC	ISSR	BH10	GAGAGAGAGAGACC
	OPC8	TGGACCGGTG		BH11	GTGTGTGTGTGTCC
	OPC14	TGCGTGCTTG		BH14	CTCCTCCTCGC
	OPB11	GTAGACCCGT		ISSR1	TCTCTCTCTCTCTCTCC
	OPB18	CCACAGCAGT		ISSR3	GGGTGGGGTGGGGTG
				UBC 807	AGA GAG AGA GAG AGA GT
				UBC 811	GAG AGA GAG AGA GAG AC

Table (3): Condition of PCR amplification RAPD and ISSR.

Name of Primer	Initial denaturation		Denaturation		Annealing		Extension		Final extension	
	Temp Cº	Time (s)	Temp Cº	Time (s)	Temp Cº	Time (s)	Temp Cº	Time (s)	Temp Cº	Time (s)
OPC2	1		44						1	
OPC8	94	60	94	30	40	60	72	120	72	300
OPC14	1		40						1	
	94	240	94	60	36	60	72	120	72	120
OPB11	1		45						1	
OPB18	95	300	94	60	36	60	72	120	72	240
BH10	1		40						1	
BH11 BH14	٩٤	240	94	60	40	60	72	120	72	300
ISSR1	1		35						1	
ISSR3	94	300	94	35	47	40	72	35	72	300
UBC 807	1		40						1	
UBC 811	94	300	94	60		45	72	60	72	300

RESULTS AND DISSCUSION

Genetical variation indicates any alteration in nucleotides, gene, chromosomes or entire plant's genome (Kurane *et al.,* 2009). This variation in DNA sequence (polymorphism) can result in different banding patterns which are assessed by agarose gel electrophoresis (Williams *et al*. ,1990). Thus, twelve primers were used to twelve individuals of Brassicaceae family for DNA amplification. The outcomes demonstrated various primers made distinctive fragment numbers and length of DNA amplification products as seen in Table (4) and (5).

An overall of 413 polymorphic amplified products was gained from five RAPD primers and seven ISSR primers distributed into 241 and 183 bands respectively. The total number of the amplified RAPDs produced by each primer varied from a minimum number of 21 amplified products by primer OPC14 to a maximum of 76 amplified products by primer OPC8. The polymorphic fragments number varied between 12 in OPC2, OPC8 and OPB18 to 13 in OPC14 and OPB11 from total 62 fragments were polymorphic thus generating 26%

polymorphism and found the lower value of Primer efficiency is 9% in OPC14 and higher value is 32% in OPC8, also the results appeared the same value of primer discriminatory power in all RAPD primers approximately 19% except primer OPC14 is 21%. (Tab. 4 and Pl. 2).

The ISSR profiles of the amplification products showed out of seven primers, three (ISSR3, UBC807, UBC811) could not amplify the genomic DNA, The primer ISSR1 accord the lowest number of fragments (11), while the largest number of fragments (67), was amplified with primer BH11. The minimum number of polymorphic bands was 5 with BH10, which appeared the polymorphism (8%) and 6 polymorphic bands in ISSR1 that represented (55%), BH11 and BH14 showed the largest number of polymorphic bands 12 and 13 in 18% and 33% respectively. And converged percentages of the efficiency of the primers BH10 and BH11 by 33% and 36%, and decreased ratio to 22% in BH14. On the contrary, the Primer discriminatory power in the last primer has a higher value to 36% but a lower value was 14% in BH10. It is remarkable that all used primers do not contain monomorphic bands except the primer BH10 which explained three bands have the same genotype (Tab. 4 and Pl. 2). Also from Table (5) and Diagram (1) explained maximum number of polymorphism bands appeared in Green Cabbage and Green ornamental cabbage but minimum bands in Red and White radish, Some polymorphisms were easy to register whereas other bands appeared to produce nuclear fragments (Williams *et al.* ,1990). The best primers will output more than three clear bits. The number of fragments produced depends on the primer sequence rather than the nucleotide length. In general, show spacious whilst various studies have reported that of the vegetable Brassicas parental lines have emerged from a limit genetic base (Gray, 1993).

Furthermore, Kurane *et al.* (2009) emphasis the ISSR markers proved to be very useful for accurate plant identification by recognized the Intra and Interspecies difference. ISSR strategies are almost indistinguishable to RAPD strategies exclude that sequences of ISSR primer are non-randomly outlined from microsatellite regions and the annealing temperatures applied are higher than utilized for RAPD markers. A difference of results was normal since just seven ISSR primers were utilized against five primers in the RAPD data analysis; notwithstanding, the average number of bands amplified per ISSR primer was higher.

Table (4): Details of RAPD and ISSR amplifications in twelve species and varieties in Brassicaceae.

Primer		No. of total amplified bands	No. of polymorphic bands	No. of Monomorphic bands	Polymorphism %	Primer efficiency (%)	Primer discriminatory power (%)
RAPD	OPC2	60	12	0	20	25	19
	OPC8	76	12	0	16	32	19
	OPC14	21	13	0	62	9	21
	OPB11	33	13	0	39	14	19
	OPB18	51	12	0	24	21	19
	Total	241	62	0	26		
ISSR	BH10	65	5	3	8	36	14
	BH11	67	12	0	18	37	33
	BH14	40	13	0	33	22	36
	ISSR1	11	6	0	55	6	17
	ISSR3	0	0	0	0	0	0
	UBC 807	0	0	0	0	0	0
	UBC 811	0	0	0	0	0	0
	Total	183	36	3	20		
Total RAPD+ISSR		424	98	3	-	-	

Table (5): Details of RAPD and ISSR band sharing in twelve species and varieties in Brassicaceae.

markers	Common name/primes	White radish	Red radish	Wild radish	Cress or RASHAD	QUNAIBRA	Rocket Mustard	Cauliflower	Broccoli	Green Cabbage	Red Cabbage	Green ornamental cabbage	Red ornamental cabbage	Total
RAPD	OPC2	4	2	9	0	8	6	5	6	7	3	7	3	60
	OPC8	4	3	6	8	2	9	7	7	8	7	8	7	76
	OPC14	0	0	0	3	0	6	0	0	7	0	5	0	21
	OPB11	0	0	7	8	0	2	0	2	5	1	6	2	33
	OPB18	2	0	5	6	4	3	4	5	7	4	7	4	51
	Total	10	5	27	25	14	26	16	20	34	15	33	16	241
ISSR	BH10	7	5	7	5	3	5	6	5	5	6	5	6	65
	BH11	5	3	6	6	6	6	4	6	9	4	8	4	67
	BH14	1	1	7	5	3	4	3	2	5	2	5	2	40
	ISSR1	1	1	1	0	0	4	0	0	2	0	2	0	11
	ISSR3	0	0	0	0	0	0	0	0	0	0	0	0	0
	UBC 807	0	0	0	0	0	0	0	0	0	0	0	0	0
	UBC 811	0	0	0	0	0	0	0	0	0	0	0	0	0
	Total	14	10	21	16	12	19	13	13	21	12	20	12	183
Total RAPD+ISSR		24	15	48	41	26	45	29	33	55	27	53	28	424

In this screening, RAPD markers were effectively exercised to distinguish 12 taxa of Brassicaceae from each to another and data from molecular markers display a good basis for better conservation approaches (Prasad, 2014). Thus, on the basis of RAPD, the discoveries of this investigation are closely resembling the notice of Hu and Quiros (1991) were demonstrated the use of RAPD-PCR in comparison of broccoli and cauliflower cultivar.

The put markers of RAPD and ISSR together found a rising rank of distinction in the genetic relationship. The similarity coefficients extend from 0.169 (between varieties Red

radish and Green ornamental cabbage) to 0.897 (between varieties Red cabbage and Red ornamental cabbage) (Tab. 6).

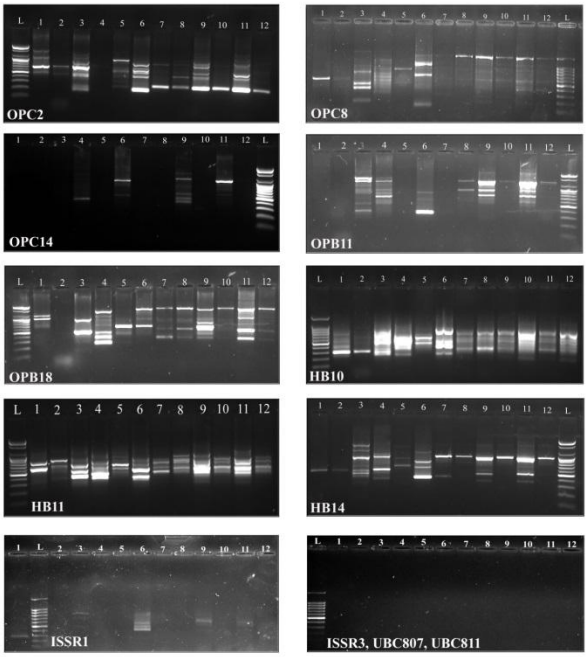

Plate 2: Banding patterns of RAPD and ISSR fragments of Brassicaceae individuals.

Lane L molecular size marker one step 100 bp ladder (Promega). Lane 1-12 species under study (1- White radish, 2- Red radish, 3- Wild radish or FIHAILA, 4- Cress or RASHAD, 5- Hoary Cress or QUNAIBRA, 6- Rocket Mustard, 7- Cauliflower , 8- Broccoli, 9- Green Cabbage-LAHANA, 10- Red Cabbage, 11- Green ornamental cabbage, and 12- Red ornamental cabbage.

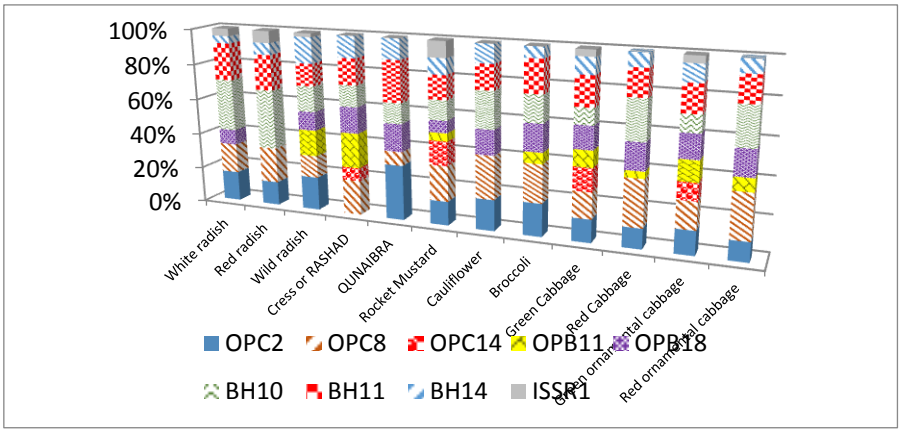

Diagram 1: RAPD and ISSR band sharing in twelve species and varieties in Brassicaceae.

Table (6): Similarity Matrix computed with Jaccard coefficient.

	1	2	3	4	5	6	7	8	9	10	11	12
1	1	0.393	0.309	0.275	0.282	0.255	0.359	0.326	0.234	0.275	0.238	0.268
2		1	0.260	0.217	0.206	0.250	0.294	0.263	0.186	0.235	0.169	0.229
3			1	0.348	0.321	0.310	0.351	0.373	0.355	0.271	0.360	0.288
4				1	0.218	0.229	0.273	0.345	0.391	0.308	0.397	0.327
5					1	0.291	0.341	0.439	0.306	0.293	0.290	0.317
6						1	0.396	0.368	0.316	0.309	0.303	0.281
7							1	0.771	0.424	0.697	0.431	0.629
8								1	0.517	0.765	0.554	0.694
9									1	0.439	0.758	0.431
10										1	0.473	0.897
11											1	0.439
12												1

An indistinguishable outcome was expressed in the dendrogram where two noticeable bunches were gotten The tree-cluster analysis explains the allocation of genotypes in two main clusters and genetic similarity among the 12 species ranged from 0.169 to 0.897 (Diag. 2). Cluster I which was divided into group and subgroup. The first group included three sub-groups, Red Cabbage and Red ornamental cabbage were represented as the first sub-group, The second sub-group consisted of genotypes labeled as Cauliflower and Broccoli, and Third sub-group included Green Cabbage and Green ornamental cabbage. Then Cress or RASHAD and Wild radish combined together with the first group, Then QUNAIBRA and Rocket Mustard attached with the previous group respectively. Cluster II comprised of two genotypes including White and Red radish. Thus the dendrogram showed the genetic relationships among species *Brassica* seem very closely related together which return to Brassiceae tribe. For the results of the current study agreed with the study Yang *et al.* (1999) that Confirmed the genus *Brassica* is a monophyletic group within the Brassicaceae very closely related to model crucifer plant *Arabidopsis thaliana* except their genomes are more complex because of nature of polyploidy. Also the combined RASHAD which belong to Lepidieae tribe with the *Brassica* group concurred with Zunk, *et al.* (1999) that indicated the current molecular systematic research reveals that exclusive Brassiceae and Lepidieae's tribes might to be counted a naturalistic composition. so the White and Red radish seem closely related together in the tree relationship because they have the same morphological and taxonomic feature and agreed with Cruz *et al.* (2014) when demonstrated that RAPD and ISSR biochemical and molecular markers are effective and promising when differentiating cultivars of the radish

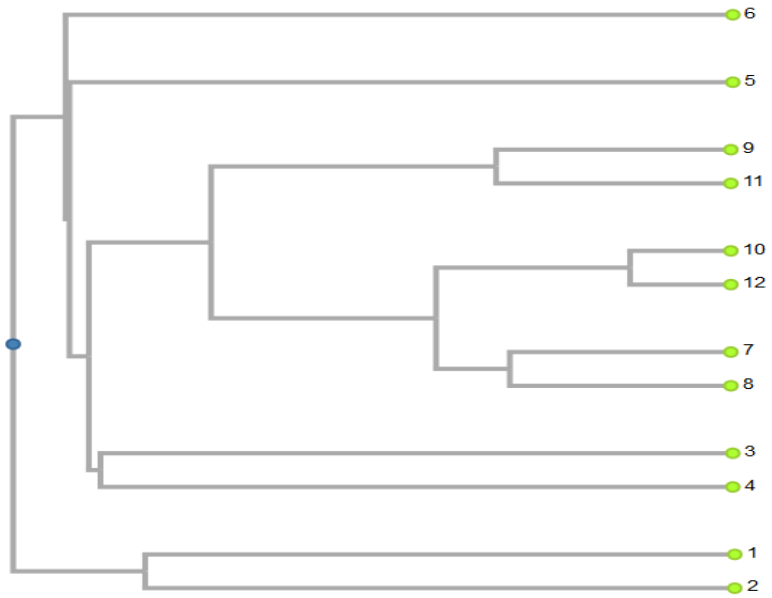

Diagram 2: UPGMA dendrogram indicating the genetic relationships among Brassicaceae taxa.

Based on RAPD and ISSR markers. 1-12 species under study (1- White radish, 2- Red radish, 3- Wild radish or FIHAILA, 4- Cress or RASHAD, 5- Hoary Cress or QUNAIBRA, 6- Rocket Mustard, 7- Cauliflower, 8- Broccoli, 9- Green Cabbage-LAHANA, 10- Red Cabbage, 11- Green ornamental cabbage and 12- Red ornamental cabbage.

CONCLUSIONS

Our investigation revealed significant variation in terms of RAPD and ISSR fingerprinting among the closely related species thought to be devoid of molecular variation and there by successfully drawing the interspecific phylogenetic relationships.

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تقدير التغايرات الوراثية لمراتب تصنيفية مختلفة من العائلة الصليبية باستخدام تحليل RAPD و ISSR

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تاريخ الاستلام: ٢٠١٧/١٢/١٣ **تاريخ القبول: ٢٠١٨/٠١/٠٩**

الخلاصة

درست اثنا عشر نوعا من العائلة الصليبية باستخدام اثنين من التقنيات الجزيئية المختلفة RAPD و ISSR؛ إذ استخدمت كل من هذه التقنيات للكشف عن بعض الواسمات الجزيئية المرتبطة مع تحديد النمط الجيني اذ اظهرت نتائج تضاعف العشوائي المتعدد الاشكال لسلسلة الدنا RAPD، من استخدام خمسة بادئات عشوائية، تمييز ٢٤١ حزمة مضخمة، ٦٢ منها كانت متعددة الأشكال (٢٦٪).

أظهرت النتائج تكرار التسلسلات البسيطة ISSR من أصل سبعة بادئات، ثلاثة (ISSR3، UBC807، UBC811) لم يظهر اي تضخيم للحامض النووي الجيني، وكشفت البادئات الأخرى ١٨٣ حزمة مضخمة، ٣٦ منها كانت متعددة الأشكال (٢٠٪) وتبين ان مؤشرات التشابه والمخطط التشجيري للمسافات الوراثية عند الجمع بين الطريقتين أن أعلى التشابه كان ٠,٨٩٧ بين أصناف الملفوف الأحمر والملفوف الزينة الأحمر، بينما ظهر أدنى مؤشر للتشابه بين أنواع الفجل الأحمر والملفوف الزينة الأخضر (٠,١٦٩)؛ اي ان علامات RAPD و ISSR لديها القدرة على تحديد الأنواع / الأصناف وتوصيف الاختلاف الجيني داخل الأصناف أيضا يمكن أن نستنتج أن انواع العائلة الصليبية لها كمية كافية من التنوع الوراثي ومجموعة واسعة في القاعدة الوراثية للتراكيب المستخدمة في الدراسة والتي يمكن استخدامها لتحسين المحاصيل.