GENETIC DIVERSITY IN INTRODUCED CASSAVA USING INTER SIMPLE SEQUENCE REPEAT MARKERS (ISSRS)

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Abstract

Four Introduced genotypes of cassava were used in the present study, Indonesian, Brazilian, Thai (Rayong 60) and Thai (Huay Bong 60) cassava genotypes. Ten primers for ISSR were successful in generating reproducible and reliable amplicons for the four the imported cassava genotypes. The aim of this study was to measure genetic distance and to generate molecular profile for imported cassava using ISSR markers. The total number of bands from the ten primers was 79 bands, distributed as 43 polymorphic bands and 36 monomorphic bands. The nine unique bands were given from interaction among ten primers ISSR and four cassava genotypes. The polymorphism level was differing from primer to another reflecting the primer ability to detect the diversity between cassava genotypes. The 14A ISSR-primer revealed higher level of polymorphism than the rest of ISSR primers followed by 98A which produced 80% polymorphism. The lowest polymorphism was produced by HB09 followed by HB14 with values 17 and 25. The highest value was similarity between Brazilian Cassava and Thai Cassava (Huay Bong 60) (82.3%) followed by Thai Cassava (Rayong 60) and Thai Cassava (Huay Bong 60) (81.1%) then Brazilian Cassava and Thai Cassava (Rayong 60) (81%). The lowest similarity value appeared between Indonesian Cassava and Brazilian Cassava (75%) followed by Thai Cassava (Rayong 60) and Indonesian Cassava (75.4%). The far genetic distance is between Indonesian Cassava and Thai Cassava (Huay Bong 60).

Key words: Cassava, ISSR, Genetic Diversity.

INTRODUCTION

Cassava is regarded as one of the most important staple crops and food for about 800 million people in tropics and sub tropics (FAO, 2007). It contributes to the world food supply in many ways either as eaten roots for human and animals or as starch or flour. Cassava starch is used in food industry too. Cassava (*Manihot esculenta* Grantz) belongs to the family Euphorbiaceae, which are characterized by lactiferous vessels composed of secretory cells. Its relatives in the euphorbiaceae family include several commercially important plants, such as rubber trees (*Heveabrasiliensis*), castor

oil plants (*Ricinuscomunis*) and ornamental plants (*Euphorbia* spp.). It's believed that cassava originated by hybridization between two wild *Manihot* species, followed by vegetative reproduction of the hybrid. The center of origin of cassava was first reported to be Central America including Colombia, Venezuela, Guatemala and Southern Mexico due to the large number of genotypes present there (Sauer, 1952 and Roger, 1965).

Cassava is generally propagated with stem cuttings, thereby maintaining a genotype.Under natural conditions as well as in plant breeding, propagation by seed is common and farmers in Africa are known to occasional use of spontaneous seedlings for subsequent planting (Silvestre and Arraudeaus, 1983).Cassava generally has a diploid genome (2n=36). However, some authors have described it as a segmental allotetraploid with basic chromosome number x=9. (Jos and Nair, 1979).

A lot of 1000 cassava seeds was introduced by the senior author from Brazil in 1986 to Egypt. Growing plants were subjected to selection through 12 consecutive years, and resulted in selecting two clones that were productive. One of these clones was used in intercropping trials with groundnut in sandy soil of Northern Egypt region, a typical temperate climate. A high productivity of intercropped cassava was achieved. It reached 21 t fed^{-1} i.e., 49.98 t ha^{-1} when planted in spring (April) (Sherif and Nassar, 2010).

Techniques based on molecular marker analysis (*i.e.* RFLP, RAPD, ISSR-PCR) may provide a more efficient and accurate screening method biochemical genetic analysis. Simple sequence repeats comprise short oligonucleotide sequences, two to six bases long, repeated in tandem array, which occur very frequently in eukaryotic genomes (Tautz and Renz, 1984, Beckmann and Soller 1990 and Lagercrantz *et al.* 1993). The ISSR-PCR technique uses primers that are complementary to a single SSR and anchored at either the 5′ or 3′ end with a one- to three-base degenerate oligonucleotide ('anchor') (Zietkiewicz *et al.,* 1994).The aim of this study was to investigate the genetic distance as well as the molecular profile in imported cassava using ISSR markers.

MATERIALS AND METHODS

Plant Material

Four Introduced genotypes of cassava were used in the present study (Table 1).
Table 1. Cassava code, Common Name, Chromosome no., and origin.

Code	Common Name	Chromosome no.	Origin
A_1	Indonesian Cassava	36	Indonesia
A_2	Brazilian Cassava	36	Brazil
A_3	Thai Cassava (Rayong 60)	36	Thailand
A_4	Thai Cassava (Huay Bong 60)	36	Thailand

Genomic DNA extraction and purification: extraction of total DNA was performed using methods for medicinal and aromatic plants according to Anna *et al.* (2001). To remove RNA contamination, RNase A (10 mg/ml, Sigma, USA) was added to the DNA solution and incubated at 37°C for 30 min. Estimation of the DNA concentration in different samples was done by measuring optical density at 260 nm according to the following equation: Conc. (ug/ml) = OD_{260} X 50 X dilution factor.

Table 2. List of the ten primer names and their nucleotide sequences used in the study for ISSR procedure.

No	Name	Sequence	No	Name	Sequence
1	14 A	5` $(CT)_8$ TG 3`	6	HB 09	5` $(GT)_6$ GC 3`
2	44 A	5` $(CT)_8$ AC 3`	7	HB 10	5` $(GA)_6$ CC 3`
3	98 A	5` $(CA)_7$ 3`	8	HB 11	5` $(GT)_8$ CC 3`
4	44 B	5` $(CT)_8$ GC 3`	9	HB 13	5` $(GAG)_3$ GC 3`
5	49 B	5` $(CA)_6$ GG 3`	10	HB 14	5` $(CTC)_3$ GC 3`

Inter simple sequence repeats (ISSRs)

Ten primers for ISSR were used in the study and were successful in generating reproducible and reliable amplicons for the four imported cassava accessions. Names and sequences of the selected primers are shown in **Table (2)**. The amplification reaction was carried out in 25 µl reaction volume containing 1x PCR buffer, 4 mM $MgCl_2$, 0.2 mM dNTPs, 20 pmole primer, 2 units Taq DNA polymerase and 25 ng template DNA. PCR amplification was performed in a Perkin Elmer 2400 thermocycler (Germany), programmed to fulfill 40 cycles after an initial denaturation cycle for 4 min at 94°C. Each cycle consisted of a denaturation step at 94°C for 1 min, an annealing step at 40°C for 2 min, and an extension step at 72°C for 2 min, following by an extension cycle for 7 min at 72°C.

Detection of PCR Products

The products of ISSR-based PCR analyses were detected using agarose gel electrophoresis (1.2% in 1X TBE buffer), stained with ethidium bromide (0.3 ug/ml) and then visually examined with UV transilluminator and photographed using a CCD camera (UVP, UK).

Data analysis

Clear, unambiguous and reproducible bands were considered for scoring. Each band was considered a single locus. Data were scored as (1) for the presence and (0) for the absence of a given DNA band. Band size was estimated by comparing with 1-kb ladder (Invitrogen, USA) using Totallab, TL120 1D v2009 (nonlinear Dynamics Ltd,

USA). The binary data matrices were entered into the NTSYSpc (Ver. 2.1) and analyzed using qualitative routine to generate similarity coefficient and used to construct a dendrogram using unweighted pair group method with arithmetic average (UPGMA) and sequential hierarchical and nested clustering (SHAN) routine (Nei, 1973 and 1978).

RESULTS AND DISCUSSION

Evaluation of ISSR-PCR Markers

Plate (1) depicts the DNA banding patterns obtained with ten ISSR primers (Table 2) for the four genotypes, i.e., Indonesian Cassava, Brazilian Cassava, Thai Cassava (Rayong 60) and Thai Cassava (Huay Bong 60). The total bands from ten primers were 79 bands, these bands were distributed 43 polymorphic bands and 36 monomorphic bands. The nine unique bands were given from interaction among ten primers ISSR and four cassava genotypes. The polymorphism level was differing from one primer to another that reflects the primers ability to detect diversity between cassava genotypes as shown in Table 5 and Plate 1. The 14A ISSR-primer revealed higher level of polymorphism than the rest of ISSR primers followed by 98A which produced 80% polymorphism. The lowest polymorphism was produced by HB09 followed by HB14 with values 17 and 25 (Table 5). Moreover, the primers was differing in the produce bands the primer 44A and HB10 give 11 bands which were highest values in all of them, while, the lowest bands produced is 5 bands by primer 98A. The unique bands were founding from ISSR-PCR primers 14A, 44A, 49B, HB10, HB11 and HB13. These results agree with those of JingRu *et al*, (2012) ten pairs of ISSR primers. A total of 70 clear electrophoretic bands were amplified, each pair of primers had amplified 3-9 electrophoretic bands, with an average of 7, and the length of amplified bands ranged from 150 to 2,000 bp, cluster analysis showed that 39 cassava genotypes (lines) were clustered into two categories by the similarity coefficient of 0.67, in addition, the genetic distance among cassava was very narrow, with genetic similarity coefficients ranging between 0.80 and 1.00.

Tanya *et al* (2011) employed inter-simple sequence repeats (ISSRs) to assess genetic variation among 30 accessions of jatropha. Genetic relationships were evaluated using 27 of 86 ISSR markers, yielding 307 polymorphic bands with polymorphism contents ranging from 0.76 to 0.95 for IMPN 1 and UBC 807 markers, respectively. Dice's genetic similarity coefficient ranged from 0.39 to 0.99, which clearly separated the plant samples into seven groups at the coefficient of 0.48.

Table 3. Present and absent bands ISSR-PCR products by ISSR-primers in four imported cassava genotypes.

Primer	MW	14A				Primer	MW	HB09			
No. Band	bp	C 1	C 2	C 3	C 4	No. Band	bp	C 1	C 2	C 3	C 4
1	1000	-	-	-	+	1	500	+	+	+	+
2	600	+	-	-	-	2	450	+	+	+	+
3	500	+	-	-	+	3	400	+	+	+	+
4	300	+	-	-	+	4	350	+	+	+	+
5	270	+	-	-	+	5	300	+	+	+	+
6	230	+	-	+	+	6	220	+	+	-	-
7	200	+	-	-	+	Primer	MW	HB10			
Primer	MW	44A				No. Band	bp	C 1	C 2	C 3	C 4
No. Band	bp	C 1	C 2	C 3	C 4	1	500	+	+	+	+
1	700	-	+	+	+	2	450	+	-	+	-
2	500	-	+	+	+	3	400	-	+	-	+
3	450	+	+	+	+	4	370	+	-	+	-
4	400	-	+	+	+	5	320	-	+	-	+
5	380	-	+	+	-	6	300	-	+	+	+
6	350	-	+	-	-	7	280	+	-	-	-
7	320	+	+	+	+	8	220	+	+	-	-
8	310	+	-	-	-	9	200	+	+	+	+
9	250	+	+	+	+	10	180	+	+	+	+
10	200	+	+	+	+	11	110	+	+	+	+
11	180	+	+	+	+	Primer	MW	HB11			
Primer	MW	98A				No. Band	bp	C 1	C 2	C 3	C 4
No. Band	bp	C 1	C 2	C 3	C 4	1	600	-	+	-	+
1	400	+	+	+	+	2	500	+	+	-	-
2	350	+	+	-	-	3	400	-	+	-	-
3	300	+	+	-	+	4	350	+	+	+	+
4	280	+	+	-	-	5	300	+	+	+	+
5	200	+	+	-	+	6	280	+	+	+	+
Primer	MW	44B				Primer	MW	HB13			
No. Band	bp	C 1	C 2	C 3	C 4	No. Band	bp	C 1	C 2	C 3	C 4
1	1000	+	-	+	+	1	600	+	+	+	+
2	450	+	+	+	+	2	500	+	+	-	-
3	380	+	+	+	+	3	400	+	+	+	+
4	330	+	-	+	+	4	370	+	+	+	+
5	310	+	+	+	+	5	300	+	+	+	+
6	300	+	+	+	+	6	280	+	+	+	+
7	280	+	+	+	+	7	180	-	-	-	+
8	250	+	+	+	+	Primer	MW	HB14			
9	200	+	-	+	+	No. Band	bp	C 1	C 2	C 3	C 4
Primer	MW	49B				1	800	-	-	+	+
No. Band	bp	C 1	C 2	C 3	C 4	2	600	+	+	+	+
1	1500	-	+	+	+	3	450	+	+	+	+
2	950	+	+	+	+	4	400	-	-	+	+
3	700	-	+	-	-	5	320	+	+	+	+
4	500	-	+	+	+	6	300	+	+	+	+
5	450	+	-	-	-	7	280	+	+	+	+
6	400	-	+	+	+	8	200	+	+	+	+
7	280	-	+	+	+						
8	250	-	+	+	+						
9	200	+	+	+	+						

Where: C 1 = Indonesian, C 2 = Brazilian, C 3 = Thai - Rayong 60 and C 4 = Thai - Huay Bong 60.

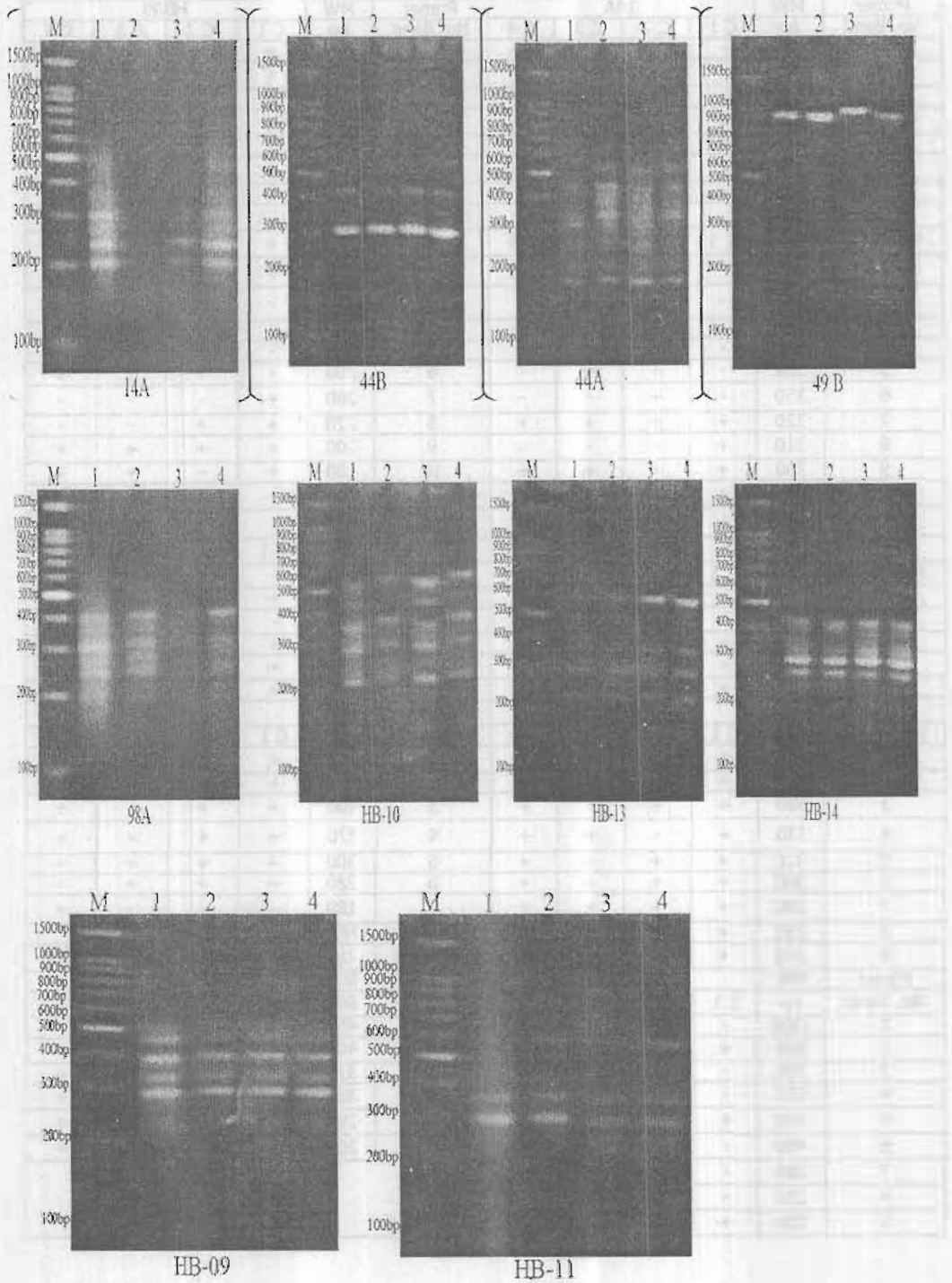

Plate: 1. Illustration of ISSR-PCR reactions using 10-primers,14A, 44B, 44A, 49B, 98A, HB-10, HB-13, HB-14, HB-09 and HB-11 with four imported cassava genotypes ,1= (Indonesian Cassava), 2= (Brazilian Cassava), 3= (Thai Cassava - Rayong 60) and 4 =(Thai Cassava - Huay Bong 60).

Similarity and Dissimilarity

Similarity indices (table 4) among the four imported cassava varieties based on ISSR analysis revealed that the highest value was between Brazilian Cassava and Thai Cassava - (Huay Bong 60) (82.3%) as well as Thai Cassava (Rayong 60) and Thai Cassava (Huay Bong 60) (81.1%) followed by Brazilian Cassava and Thai Cassava - (Rayong 60) (81%). The lowest similarity value appeared between Indonesian Cassava and Brazilian Cassava (75%) followed by Thai Cassava - (Rayong 60) and Indonesian Cassava (75.4%) (Table 4). These results agree with results obtained by JingRu *et. al.*, (2012). The similarity coefficient was 0.67, in addition, the genetic distance among cassava was very narrow, with genetic similarity coefficients ranging between 0.80 and 1.00. Tanya *et al*, (2011) estimates of Dice's genetic similarity coefficient ranged from 0.39 to 0.99, which clearly separated the plant samples into seven groups at the coefficient of 0.48.

Table 4 Similarity indices among the four imported cassava genotypes based on ISSR analysis.

Proximity Matrix				
Case	Matrix File Input			
	Indonesian Cassava	Brazilian Cassava	Thai Cassava (Rayong 60)	Thai Cassava (Huay Bong 60)
Indonesian Cassava		0.750	0.754	0.770
Brazilian Cassava			0.810	0.823
Thai Cassava (Rayong 60)				0.811
Thai Cassava (Huay Bong 60)				

Dendrogram and cluster analysis

As shown Fig. (1) the cassava genotypes were distributed in two clusters. The cluster had Indonesian Cassava only while the second cluster included two sub clusters. The first sub cluster included the Brazilian Cassava and the sub cluster two had the Thai Cassava (Rayong 60) and the Thai Cassava (Huay Bong 60). Therefore, the genetic distance between both the Thai Cassava (Rayong 60) and the Thai Cassava (Huay Bong 60) is very low as shown in Table (4) and the far genetic distance between Indonesian Cassava and Brazilian Cassava. The origin of the genotypes may be explain the causes of genetic distance.

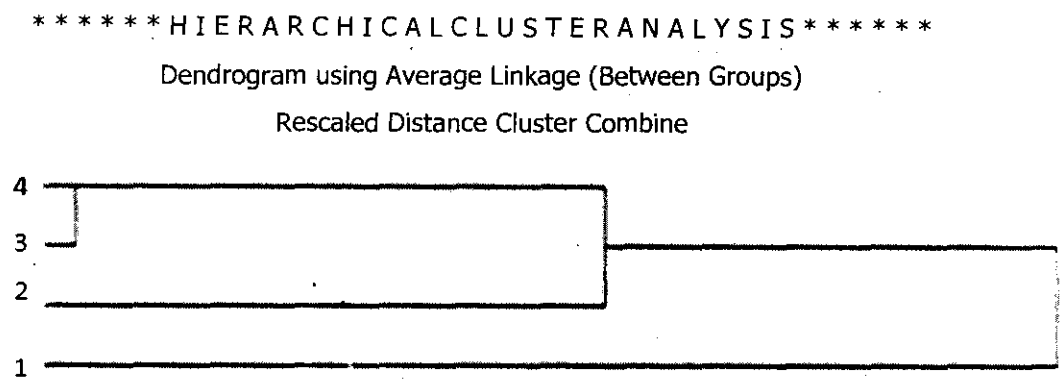

Fig.1. Dendrogram of the genetic distances between imported Cassava genotypes based on ten primers ISSR-PCR, The name of the genotypes from one to four, (1 = Indonesian Cassava), (2 = Brazilian Cassava), (3 = Thai Cassava - Rayong 60) and (4 = Thai Cassava - Huay Bong 60).

Table 5. Primer name, total number of bands, monomorphic bands, polymorphic bands, polymorphism ratio, unique bands and genotypes code, (1 = Indonesian Cassava), (2 = Brazilian Cassava), (3 = Thai Cassava - Rayong 60) and (4 = Thai Cassava - Huay Bong 60).

No.	Primer name	Monomorphic band	Polymorphic band	Total band	Polymorphism %	Unique bands		
						No.	Genotype code	bp
1	14A	0	7	7	100	2	4	1000
							1	600
2	44B	5	4	9	44	0	-	-
3	44A	5	6	11	55	2	2	350
							1	310
4	49B	2	7	9	78	2	2	700
							1	450
5	98A	1	4	5	80	0	-	-
6	HB09	5	1	6	17	0	-	-
7	HB10	4	7	11	64	1	1	280
8	HB11	3	3	6	50	1	2	400
9	HB13	5	2	7	29	1	4	180
10	HB14	6	2	8	25	0	-	-
Total		36	43	79	54	9	1,2,4	

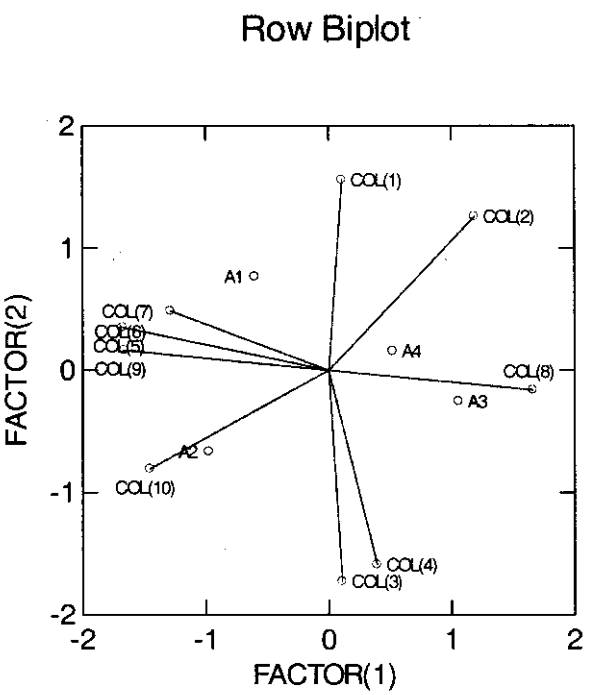

Fig. 2. GGE Biplot Analysis: Linera multidimenstial Scaling analysed as Similairities dimenstions with four genotypes from A1 to A4 (See Table 1) and Col1 (primer 1) to Clo 10 (See Table 2).

The relative location of the points can be interpreted. Points that are close together correspond to observations that have similar scores on the components(Primers ISSR-PCR,Col.) displayed in the plot. To the extent that these components fit the data well, the points also correspond to observations that have similar values on the variables. The genotypes were distributed in spirate points which reflects of the ability of markers to measure variation according to Gabriel (1971, 2002) and Yan *et al* (2008).

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التنوع الوراثي لأربعة تراكيب وراثية مستوردة من الكاسافا بإستخدام تقنية ISSRS ما بين التتابعات القصيرة المتتالية

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إستخدم أربعة من التراكيب الوراثيه المستورده من الكاسافا في هذه الدراسة وهي: الكاسافا الاندونيسية، الكاسافا البرازيلي، الكاسافا التايلاندية (Rayong 60) و (Huay Bong 60). واستخدمت عشرة من البادئات لـــ ISSR في الدراسة . كانت البادئات الوراثيه ناجحة في توليد amplicons متضاعف للأربعة تراكيب وراثيه من الكاسافا. وتهدف هذه الدراسة لقياس التباين الوراثى وكذلك التوصيف الجزيئى في الكاسافا باستخدام المعلمات ISSR.

وأوضحت النتائج أننا حصلنا من مجموع البادئات الجزيئية العشرة على ٧٩ حزمة جزيئية ، توزعت الحزم ما بين ٤٣ حزمه متعددة المظهرية الجزيئية و ٣٦ حزمة monomorphic. كما كان من الحزم ٩ حزمة فريدة من التفاعل بين ISSR ذو البادئات الجزيئية العشرة ، والأربعة تراكيب وراثية من الكاسافا المستورده. يدل هذا المستوي من تعدد الأشكال الجزيئية المختلفة علي قدرة المعلم الجزيئ وبادئاته العشره لاظهار التنوع بين التراكيب الوراثيةالأربعة من الكاسافا وقد أظهر البادئ الجزيئ ISSR 14A أعلى معدل من التباين (١٠٠%) يليه البادئ الجزيئ ISSR 98A الذى أظهر نسبة تباين٨٠%، كما أظهرت البادئات الجزيئة أدنى تعدد من الأشكال الجزيئية بالبادئ الجزيئ HB-9 ويليه HB-14 مع القيم ١٧ و ٢٥. كما ظهر أن أعلى قيمة من التشابه الوراثي بين الكاسافا البرازيلي والكاسافا التايلاندية (Huay Bong 60) هى ٨٢,٣٪، وكذلك الكاسافا التايلاندية (Huay Bong 60) والكاسافا التايلاندية (Rayong 60) ٨١,١٪، يليه الكاسافا البرازيلي والكاسافا التايلاندية (Rayong 60) ٨١ ٪. كما أظهرت النتائج ان ادنى نسبة تشابه كانت بين الكاسافا الاندونيسية والكاسافا البرازيلي ٧٥٪، يليه الكاسافا التايلاندية (Rayong 60) والكاسافا الاندونيسية ٧٥,٤٪ فى حين ان ابعد مسافة جينية كانت بين الكاسافا الاندونيسية والكاسافا التايلاندية (Huay Bong 60).