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Genetic Identification of Some Guava Seedling Trees

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Abstract

This research was conducted in two main techniques: The first was Molecular genetic identification for twelve guava seedling trees. RAPD and ISSR based on PCR techniques five primers were used had successfully generated reproducible polymorphic products to study the genetic variability between the twelve guava seedling trees. Data cleared that, a total bands of guava seedling trees with RAPD - ISSR primers were recorded 94 band where the total polymorphic bands was recorded 48 with polymorphic percentage 51.06%. While, combining RAPD and ISSR, construct classify twelve guava seedling trees. Results from this analysis showed two main groups. The first main group was between seedling trees 9 and 12 and the second main group was divided into two sub main groups: the first sub main group included genotype 1 alone and the second sub main group was divided into two sub sub group which included the remained seedling trees. The second was: Protein electrophoresis analysis for the twelve guava seedling trees revealed a total number of 16 bands eight of them were polymorphic with 50% polymorphism. While, peroxidase and polyphenyl Oxidase analysis for the twelve guava seedling trees, all of bands illustrated were characterized for all the guava seedling trees studied, which all of them (monomorphic) with differences in their banding patterns densities

Keywords: Guava seedling trees, *Psidium guajava*, RAPD and ISSR.

Introduction

Guava "*Psidium guajava*, Linn." Is the most important fruit species of the family "Myrtaceae". Guava fruits are delicious, rich in vitamin 'C', carotene, thiamine, antioxidants, pectin and minerals like calcium, phosphorus and iron. Guava fruits are consumed as fresh fruits and industrial as jam, jelly, nectar etc**. (Boora, 2012).** It is believed to be native to the area between Mexico and Peru, from where it has spread to almost all **(Chandler, 1958).**

As for horticulturists, guava is admired as being of low cultural requirements. For instance, the trees are successfully grown on various kinds of soils, rich or poor; dry or moist **(Popenoe, 1988).**

In Egypt, the total cultivated area of guava trees reached about 37398 Feddan and fruiting orchards about 33512 Feddan that producing about 307535 tons. **(Anonymous, 2019).**

Identification of plant has usually been carried out by morphological and agronomical characteristics. The environment often influences these characters. Therefore it is advisable to use alternative methods, which are less affected by the environment. Several modern techniques such as RAPD, ISSR, SDS protein, isozyme analysis…etc., are being used for more effective means of identification of the diversity within many fruit trees such as guava **(Hassan *et al.*, 1998 and Tijet *et al.*, 2000),** citrus **(Cabrita *et al.*, 2001),** olives **(Cresti *et al.*, 1997).**

When comparing several methods, such as SDS protein patterns, isozyme indices, and RAPD analysis, it was found that SDS pattern or isozyme indices did not stand alone to provide sufficient polymorphic expressions to distinguish between guava cultivars in a unique pattern. On the other hand, RAPD analysis proved to be more powerful in discriminating between the studied guava cultivars **(Hassan *et al.*, 1998).**

Accordingly, the present study aimed to throw some lights on some techniques of finger printing analysis and determining whether is more informative one in assessment of the genetic similarity and diversity in such selected genotypes (seedling trees).

Materials and Methods

Finger printing analysis: Finger printing analysis techniques for studying the genetic diversity of the twelve selected guava seedling trees (genotypes).

Leaf protein and isozyme electrophoreses:

SDS-polyacrylamide gel electrophoresis was performed in 12 % acrylamide slab gels following the system of **Laemmli (1970)** to identify their protein profiles. Fresh and young leave samples for each strain and location were used separately for isozymes extraction. The utilized isozymes are Peroxidase (Px), Polyphenyl Oxidase (PPO) Protein extraction was conducted by mixing 0.2 g of young leaves of guava with an equal weight of pure, clean, sterile fine sand. The samples then ground to fine powder using a mortar and pestle and homogenized with 1 M Tris-HCl buffer, pH 6.8 in clean eppendorf tube and left in refrigerator overnight. Then centrifuged at 10.000 rpm for 10 min. The supernatant of each sample (contains protein extract) was kept in deep-freeze until use for electrophoretic analysis. Then boil for 5 minutes in water bath before loading in the gel. Native-polyacrylamide gel electrophoresis (Native-PAGE) was conducted to identify isozyme variations among

studied strains using three isozyme systems according to **Stegemann *et al*. (1985).**

PCR analysis based on RAPD and ISSR polymorphism.

DNA was extracted from young leaves of guava plants by Cetyltrimethyl Ammonium Bromide (CTAB) according to **Doyle and Doyle (1990).** The DNA amplifications were performed in an automated thermal cycle (model Techno 512) programmed for one cycle at 94º C for 4 min followed by 45 cycles of 1 min at 94º C, 1 min at 57º C, and 2 min at 37º C. the reaction was finally stored at 72º C for 10 min.

Table 1. List of the primer names and their nucleotide sequences used in the study for RAPD procedure

	Name	Sequence
1	**OP-A2**	5´ GTG ATC GCA G3`
2	**OP-A07**	5´ GAA AGG GGT G 3`
3	**OP- B9**	5` CTCACCGTCC 3`
4	**OP-C13**	5` GGACCCAACC 3`
5	**OP-D1**	5´ ACC GCG AAG G 3`

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

No	Name	Sequence
1	14A	5 CTC TCT CTC TCT CTC TTG 3`
2	HB-8	5` GAG AGA GAG AGA GG 3`
3	HB-12	5`CAC CAC CAC GC 3`
4	HB-14	5´ CTC CTC CTC GC 3`
5	HB-15	5´ GTG GTG GTG GC 3`

Gel preparation procedure

1- Agarose (1.50 gm) was mixed with (100ml) l x TBE buffer and boiled in microwave.
2- Ethidium bromide (5µl) was added to the melted gel after the temperature became 55°C.
3- The melted gel were poured in the tray of mini-gel apparatus and comb was inserted immediately, then comb was removed when the gel become hardened.
4- The gel was covered by the electrophoretic buffer (1 x TBE).
5- DNA amplified product (15 µl) was loaded in each well
6- DNA ladder (100bp) mix was used as standard DNA with molecular weights of 3000, 1500,1000,900, 800, 700,600, 500, 400, 300, 200 and 100 bp. The run was performed for about 30 min at 80 V in mini submarine gel BioRad .

Data analysis

All date obtained during both 2018 & 2019 experimental seasons were subjected to analysis of variance and significant as factorial experiments in completely randomize blokes difference among means were determined according to **(Snedecor and Cochran, 1980).** In addition, significant difference among means were distinguished according to the Duncans multiple test range **(Duncan, 1955)** using letters for differentiating the values of each investigated measurement.

The similarity matrices were done using Gel works ID advanced software UVP-England Program. The relationships among genotypes as revealed by dendrograms were done using SPSS windows (Version 10) program. DICE computer package was used to calculate the pairwise difference matrix and plot the phenogram among strains **(Yang and Quiros, 1993).**

Results and Discussion

1. Biochemical genetic evaluation using SDS-Protein and isozymes electrophoresis banding patterns:

1. A. SDS-Protein electrophoresis.

Leaf protein electrophoresis provides valuable evidence for taxonomic and evolutionary relationships of plant species. It is worthy to note that leaf protein profiles are often species – specific, highly stable and unlikely to be not influenced by environmental conditions and seasonal fluctuations **(Yates *et al*., 1990).**

The electrophoretic banding pattern of protein extracted from leaves of *Psidium guajava* genotypes are shown in **Figure 1**. Results of leaves SDS-PAGE revealed a total number of 16 bands with molecular weight (MW) ranging from about 3.6 to 192 KDa. Analysis of data showed eight common bands (monomorphic bands) while, the remaining eight bands were polymorphic with 50 % polymorphism. On the other hand, there were three unique bands two of them were negative unique bands: the first with genotype 1 at 192KDa and the second was with genotype 12 at 68KDa and the last one was positive unique bands with genotypes 12 at 112 KDa.

Figure 1: SDS- leaf protein banding patterns of the twelve guava seedling trees

1.B. Peroxidase electrophoresis banding patterns:

Figure 2 illustrated leaf peroxidase electrophoresis banding patterns of the twelve *Psidium guajava* genotypes. A total of four bands were characterized for the studied 12 guava seedling trees which three of them (monomorphic) were represented in all 12 guava seedling trees with differences in their banding patterns densities and they could be considered as common bands. The remaining band (polymorphic) was absent in only one strain 4 with relative mobility 0.6 and present in the other genotypes.

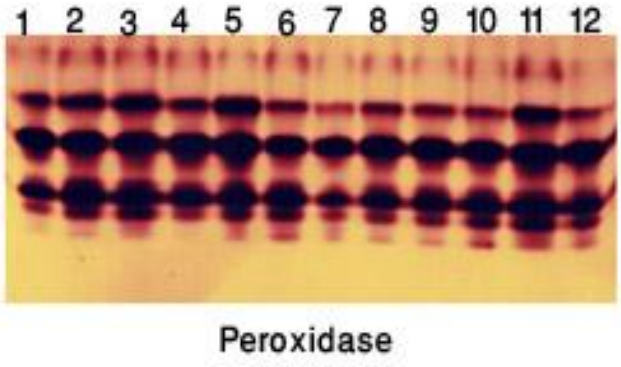

Figure 2: Leaf peroxidase isozyme banding patterns of the twelve guava seedling trees.

1.C. Polyphenyl oxidase electrophoresis banding patterns:

Figure 3 demonstrated Poly Phenyl Oxidase (PPO) banding patterns among examined leaves of the twelve *Psidium guava* genotypes. Obtained patterns exhibited four bands with relative mobiliteis 0.25, 0.35, 0, 50 and 0.60 all of them were present in all 12 trees (monomorphic) with differences in banding patterns density which could be considered as common band for all the twelve guava seedling trees.

These techniques could detect enough polymorphism in the *Psidium guava* genotypes to distinguish each genotype from the others by at least band or group of combined banding pattern. Furthermore, the use of these data in the future is important for *Psidium guajava* germplasm management, improvement as well as for the selection strategies of parental lines that facilitate the prediction of crosses in order to produce hybrids with higher performance **(Mansoor *et al.*, 1998; 1999 and Hassan *et al.*, 1998).**

Several isozymes have been found to detect useful amounts of variation in species. Isozymes analysis has also revealed some cases of obvious mislabelling of plant material. The differences between electrophoretic patterns could be observed with simple visual analysis, but the smaller differences were revealed only by densitometer analysis. The method of biochemical markers allow to solve whole number of problems for selection such as identification of genotypes, analysis of hybrids, selection of valuable genotype. The most investigations of enzyme polymorphism for fruits were carried out for evaluation of genotypes.**(Biruk and Kazlovskaya,2008).**

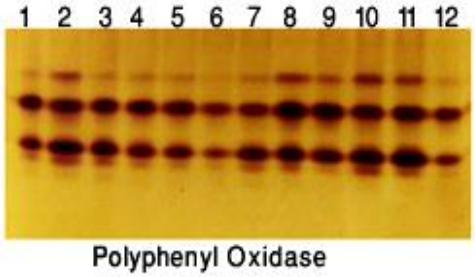

Figure 3: Leaf polyphenyl oxidase isozyme banding patterns of the twelve genotypes of *Psidium guajava*

2. Molecular genetic diversity or similarity of the 12 selected guava seedling trees based on RAPD and ISSR:

2. A. Molecular genetic similarity of *Psidium guajava* genotypes based on RAPD:

Initially, the application of molecular marker techniques was hampered by the difficulty in extracting genomic DNA from mucilaginous tissues **(Griffith and Porter, 2003).** However, researchers have demonstrated that RAPD patterns can be obtained from cacti using primers OPA-11 **(De La Cruz *et al.*, 1997),** and OPA-12 **(Tel-Zur *et al.*, 1999).** RAPD profiles have been used to verify the maternal origin of apomictic seedlings in cactus pear (**Mondragón, 2002).**

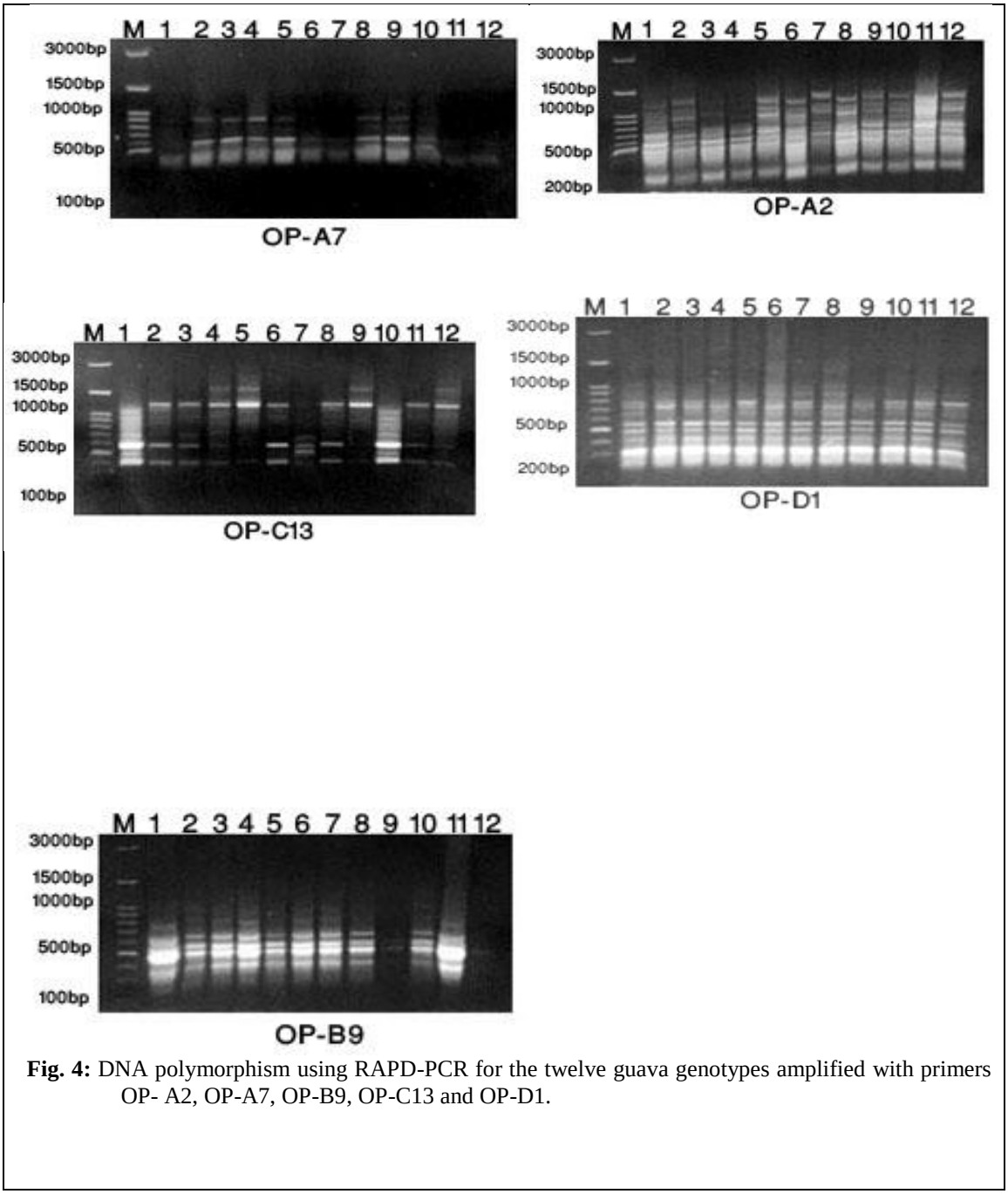

Fig. 4: DNA polymorphism using RAPD-PCR for the twelve guava genotypes amplified with primers OP- A2, OP-A7, OP-B9, OP-C13 and OP-D1.

Table 3. List of RAPD primers of twelve genotypes of *Psidium guajava*. Percentage of polymorphism and Unique bands (SM)

	Sequence	Total Band	Monomorphic Band	Polymorphic Band	Unique band	Polymorphic %
OP-A2	5´ GTG ATC GCA G3`	14	8	6	3	42.58%
OP-A7	5´ GAA AGG GGT G 3`	7	2	5	1	71.42%
OP-B9	5` CTCACCGTCC 3`	10	3	7	-	70.00%
OP-C13	5` GGACCCAACC 3`	11	5	6	-	54.56%
OP-D1	5´ ACC GCG AAG G 3`	9	5	4	1	44,44%
Total		**51**	**23**	**28**	**5**	**54.90%**

-Genetic relationship between twelve *Psidium guajava* genotypes based on RAPD analysis.

The similarity between the twelve selected seedling trees of *Psidium guajava* ranged from 0.07 to 1.00 and the highest similarity was between genotypes 1and 10 at 1.00 and the lowest similarity was between genotypes 3 and 4. UPGMA cluster and analysis based on genetic similarity values for RAPD markers from all the *Psidium guajava* genotypes was used to construct classify twelve genotypes of *Psidium guava* **Table 13** and **Figure 6.** Results from this analysis showed two main groups. The first main group was genotype 1 alone and the second main group was divided into two sub main groups: the first sub main group included genotypes 9 and 12 and the second sub main group was divided into two sub sub group: the first sub sub group was included genotypes 10 and 11, and the second sub sub group was included other seven genotypes 2, 5, 4, 3, 8, 7 and 6.

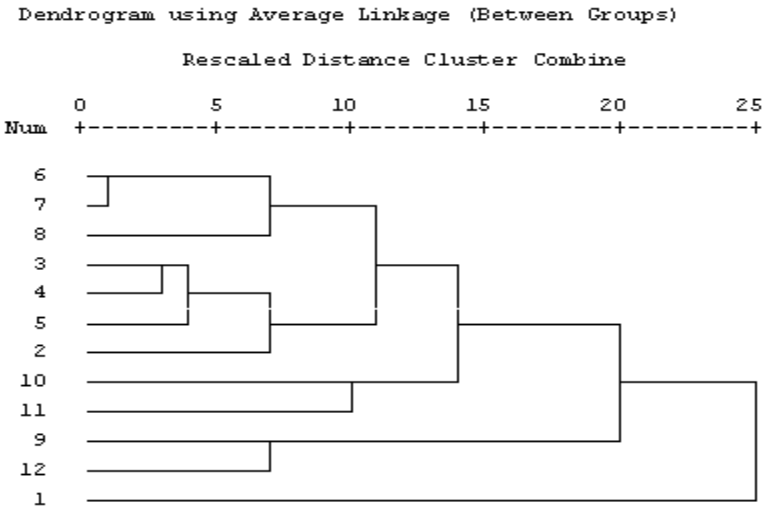

Fig.5: Unweighted pair group method with arithmetic mean (UPGMA) dendrogram illustrating the genetic relationships between twelve *Psidium guajava* genotypes based on RAPD analysis

2.B. Molecular genetic evaluation of *Pisidium guava* genotypes based on ISSR:

Besides the use of ISSR markers in micro evolutionary studies, this technique has also been used in the estimation of genetic diversity and differentiation among individuals and closely related plant species (**Rana *et al.*, 2012).** For instance, **Luna-Paez *et al.*, (2007)** employed this technique for variety distinction, whereas **Alves *et al.*, (2009)** used it to develop strain-unique bands. This PCR-based technique generates multilocus highly polymorphic dominant genomic markers without the need of prior DNA sequence knowledge **(Mishra *et al.*, 2003).**

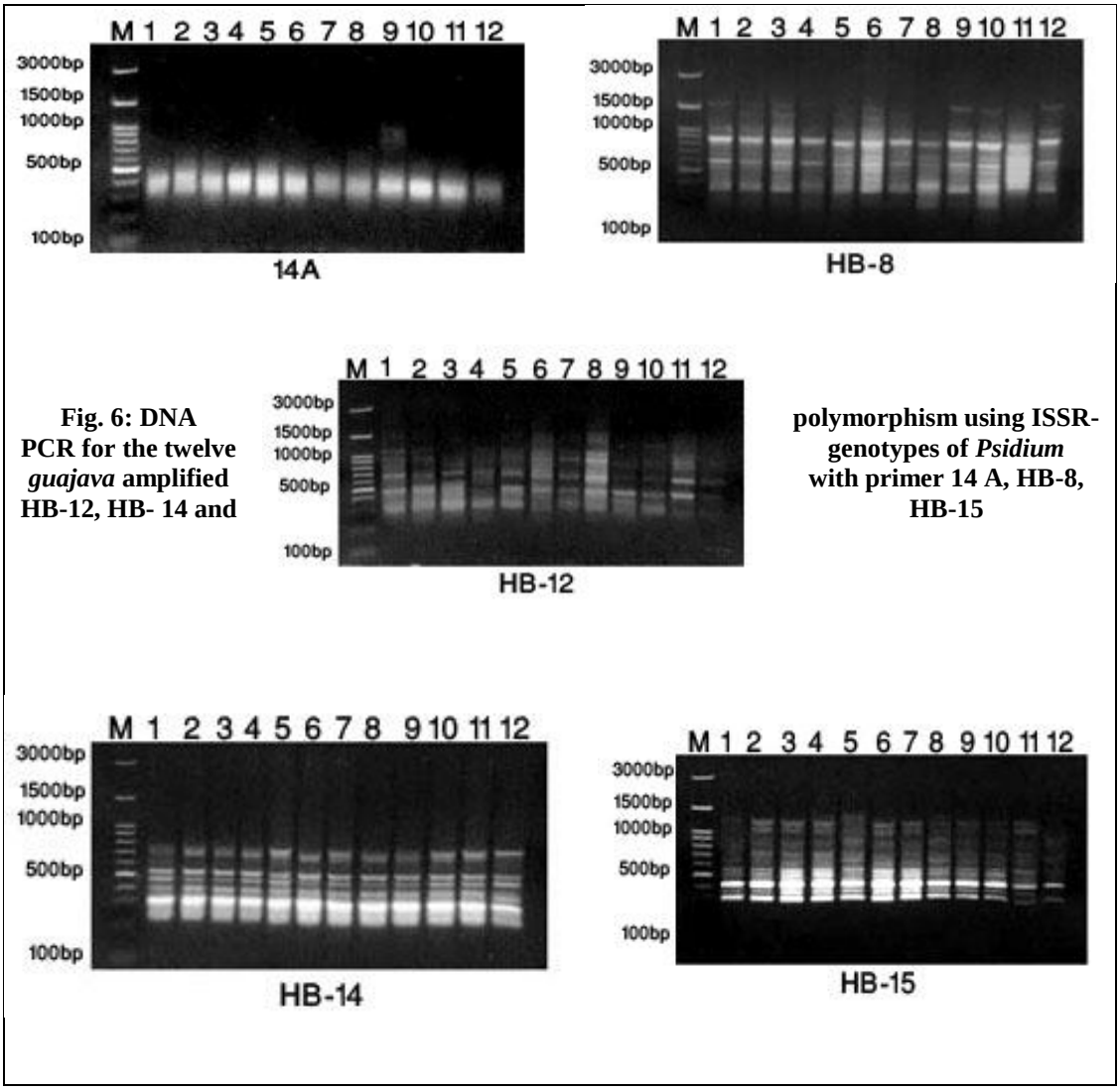

Fig. 6: DNA polymorphism using ISSR-PCR for the twelve genotypes of *Psidium guajava* amplified with primer 14 A, HB-8, HB-12, HB- 14 and HB-15

Table 4. List of ISSR primers of twelve genotypes of *Psidium guajava* Percentage of polymorphism and Unique bands (SM)

Primer Name	Sequence	Total Band	Monomorphic Band	Polymorphic Band	Unique Band	Polymorphic %
14A	5 CTC TCT CTC TCT CTC TTG 3`	4	3	1	-	25.00%
HB-8	5` GAG AGA GAG AGA GG 3`	11	5	6	2	54.54%
HB-12	5`CAC CAC CAC GC 3`	12	3	9	2	75.00%
HB-14	5´ CTC CTC CTC GC 3`	7	6	1	-	14.28%
HB-15	5´ GTG GTG GTG GC 3`	9	6	3	2	33.33%
Total		43	23	20	7	46.51%

A total number of 43 bands were produced from ISSR analysis and visualized across the investigated twelve genotypes of *Psidium guajava*, the results obtained 43 total bands with molecular weight from 235 to 2240 bp, 20 of total amplified bands were polymorphic (46.51%) and the highest polymorphic percentage was 75% produced with primer HB-12 and the lowest polymorphic percentage was 14.28% produced with primer HB-14. On the other hand, total of 23 monomorphic bands were appeared and seven unique bands in five primers. Finally, primer HB-12 was the highest amplified bands (12 bands) whereas, primer 14-A were lowest amplified bands (4bands).Two genotypes-unique bands were detected among the polymorphic bands obtained with primer HB-78; one as a positive marker for genotype 8 at 1305 bp also, the other one as negative marker for genotype 7 at 630bp. Two genotypes -unique bands were found, among the polymorphic bunds obtained with primer HB-12 one of them was positive markers

with genotype 8 at 2240 bp and the other was detected as negative marker with genotype 9 at 525bp. In addition two genotypes-unique bunds were also obtained by the HB-15 primer one as positive marker for genotype 5 at 1365bp, but second as negative for genotype 1 at 685bp.

On the other hand, no genotype unique (SM) bunds were obtained by the two other ISSR primers i.e., (14-A and HB-14)**.**

Similar results were reported in castor **(Gajer *et al*., 2010).** However, both of the markers worked effectively in the assessment of genetic diversity in canola as they exhibited more than 60% polymorphism. Knowledge of genetic similarity (distance) between genotypes and among individuals or populations is useful in a breeding program because it permits organization of germplasm and provides more efficient sampling of genotypes to cross for the development of populations **(Afiah *et al*., 2007).**

-Genetic relationship between *Psidium guajava* genotypes based on ISSR analysis.

The similarity between the twelve genotypes of *Psidium guajava* ranged from 0.07 to 1.00 and the highest similarity was between genotypes 2 and 9 at 1.00 and the lowest similarity was between genotypes 3 and 4**.** UPGMA cluster and analysis based on genetic similarity values for RAPD markers from all the *Psidium guajava* genotypes was used to construct classify twelve genotypes of *Psidium guajava* **(Fig.7).** Results from this analysis showed two main groups. The first main group was between genotypes 9 and 12 and the second main group was divided into two sub main groups: the first sub main group included genotype 5 alone and the second sub main group was divided into two sub sub group: the first sub sub group was included genotype 6 alone and the second sub sub group was included other genotypes 1, 11, 8, 7, 2, 3, 10 and 4.

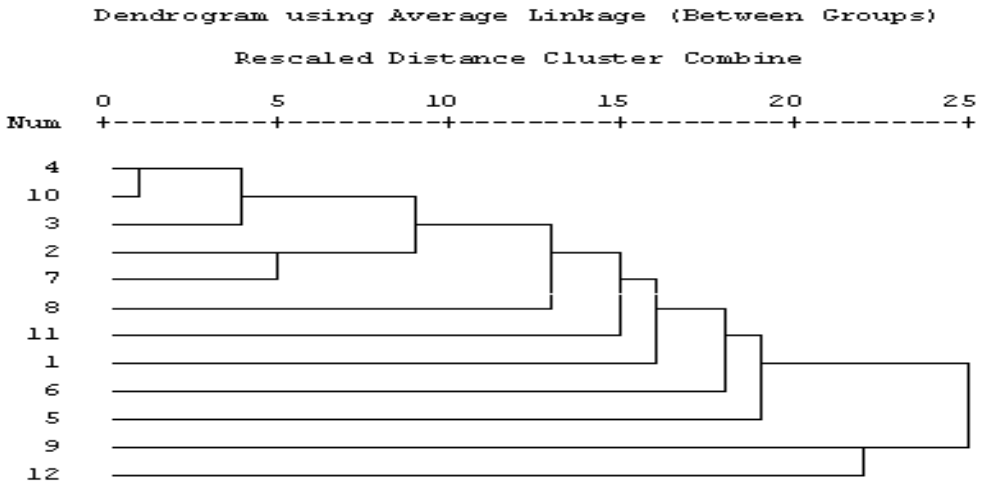

Fig. 7: Unweighted pairs group method with arithmetic mean (UPGMA) dendrogram illustrating the genetic relationship between twelve *Psidium guajava* genotypes based on ISSR analysis

ISSRs have been successfully used to estimate the extent of genetic diversity in a wide range of crop species which include sugarcane **(Srivastava** and **Gupta, 2008 and Da Costa *et al*., 2011).** In addition to that advantages (inexpensive, easy to generate), F-ISSR markers are more powerful and efficient in detecting polymorphisms within and among populations and/or species. The present study also utilized the F-ISSR markers previously used to investigate genetic diversity and relationships within and among the nineteen groups of sugarcane varieties of India. Molecular marker-based analysis of genetic diversity in plant species have become an important tool in crop improvement and conservation purpose **(Weising *et al*., 2005).**

2. C. Molecular genetic similarity of *Pisidium guava* genotypes based on combining between RAPD and ISSR

Table 5 investigated twelve genotypes of *Psidium guajava*, across five primers of RAPD and five primers of ISSR, the results obtained 94 total bands with molecular weight from 190 to 2240 bp, 48 of total amplified bands were polymorphic 51.06% and the highest polymorphic percentage was 54.90% produced with RAPD primers and the lowest polymorphic percentage was 46.51%% produced with ISSR primers. On the other hand, total of 46 monomorphic bands were appeared and twelve unique bands in ten primers.

Table 5. List of RAPD and ISSR primers of twelve genotypes of *Psidium guajava*. Percentage of polymorphism and Unique bands (SM)

Primer Name	Total band	Monomorphic band	Polymorphic band	Unique Band	Polymorphic %
RAPD	51	23	28	5	54.90%
ISSR	43	23	20	7	46.51%
Total	94	46	48	12	51.06%

Genetic relationship between *Psidium guajava* genotypes based on combining RAPD and ISSR analysis.

By combining RAPD and ISSR, the similarity between the twelve genotypes of *Psidium guajava* ranged from 0.06 to 1.00 and the highest similarity was between genotypes 1 and 9 at 1.00 and the lowest similarity was between genotypes 3 and 4 **(Table, 6)**. UPGMA cluster and analysis based on genetic similarity values for combining RAPD and ISSR markers from all the *Psidium guajava* genotypes was used to construct classify twelve genotypes of *Psidium guajava* **(Fig. 8).** Results from this analysis showed two main groups. The first main group was between genotypes 9 and 12 and the second main group was divided into two sub main groups: the first sub main group included strain 1 alone and the second sub main group was divided into two sub group: the first sub sub group was included two genotypes 10 and 11 and the second sub sub-group was included other genotypes 8, 7, 6, 2, 5, 4, and 3.

Table 6. Similarity coefficient between twelve genotypes of *Psidium guajava* genotypes based on combining RAPD and ISSR analysis

	1	2	3	4	5	6	7	8	9	10	11	12
1	1											
2	0.44	1										
3	0.60	0.23	1									
4	0.58	0.22	0.06	1								
5	0.69	0.26	0.13	0.20	1							
6	0.73	0.46	0.25	0.24	0.58	1						
7	0.82	0.30	0.16	0.32	0.44	0.25	1					
8	0.73	0.46	0.17	0.24	0.42	0.25	0.33	1				
9	1.00	0.96	0.49	0.55	0.60	0.71	0.80	0.57	1			
10	0.49	0.50	0.37	0.21	0.40	0.37	0.30	0.37	0.53	1		
11	0.70	0.43	0.30	0.29	0.48	0.37	0.53	0.45	0.75	0.27	1	
12	0.84	0.94	0.59	0.85	0.62	0.67	0.83	0.81	0.38	0.55	0.58	1

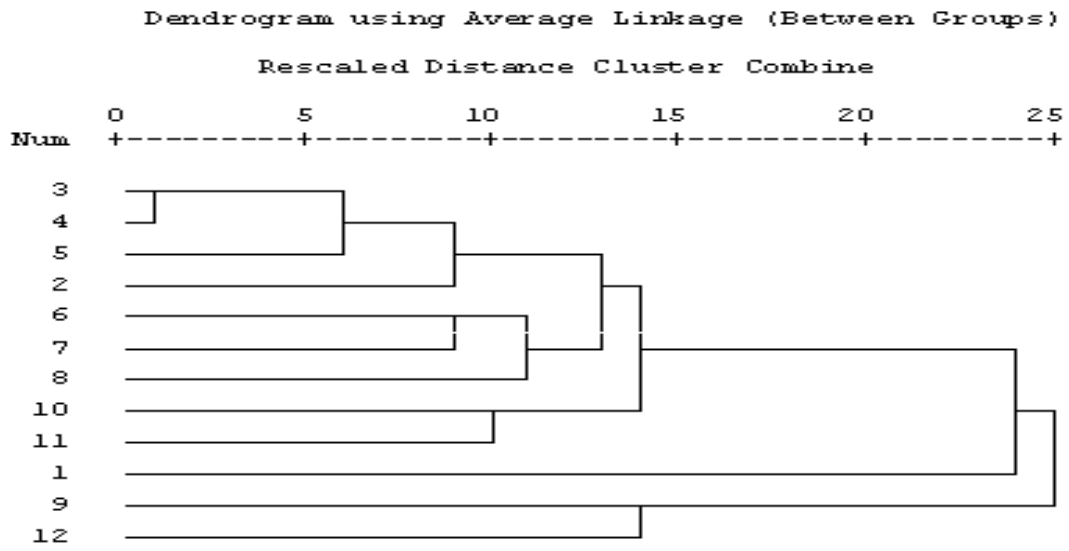

Fig.8: Unweighted pair group method with arithmetic mean (UPGMA) dendrogram illustrating the genetic relationships between twelve genotypes of *Psidium guava* based on combining RAPD and ISSR analysis.

In this study, RAPD and ISSR markers based on the polymerase chain reaction (PCR) were applied. The value of RAPD analysis for efficient germplasm management in plants is already known **(Young, 2000).** The technique is quick, easy and required less time. This detects nucleotide sequence polymorphisms using a single primer of arbitrary nucleotide sequence (**Williams *et al*., 1993).** ISSR or microsatellite are currently becoming the preferred technique for the molecular characterization of different plant species because of higher repeatability, codominant nature, specificity and having multiple alleles **(Plieske and Struss, 2001).** ISSRs are based on tandem repeats of short (2 to 6 bp) DNA fragments scattered throughout the genome that lie between conserved sequences. It permits detection of polymorphisms in inter-microsatellite loci, using a primer designed from dinucleotide or trinucleotide simple sequence repeats.

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التعريف الوراثى لبعض اشجار الجوافة البذرية

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اشتملت الدراسة على تقنيتين رئيسيتين: التقنية الأولى هى التمايز الوراثى الجزيئى بين اشجار الجوافة الأثنى عشرة تحت الدراسة باستخدام خمسة بوادئ RAPD وخمسة بوادئ ISSR. وأظهرت النتائج تباينات مختلفة بين اشجار الجوافة البذرية. وأوضحت نتائج التحليل ظهور 94 حزمة من بينها 48 حزمة متباينة وكانت نسبة التباين 51.06 %.

بينما اوضحت النتائج المجمعة لكلا التقنيتين بالنسبة لتحليل شجرة القرابة الوراثية التى قسمت الاشجار الى مجموعتين رئيستين: المجموعة الأولى والتى ضمت كلا من الشجرتين 9 و 12 والمجموعة الثانية قسمت الى تحت مجموعتين: تحت المجموعة الأولى شملت الشجرة رقم 1 منفردة بينما تحت المجموعة الثانية اشتملت على باقى الاشجار. التقنية الثانية اشتملت على: تحليل التفريد الكهربائى للبروتين لاشجار الجوافة البذرية تحت الدراسة انتجت 16 حزمة ووجود 8 حزم كانت متباينة بنسبة تباين 50% . ومن ناحية اخرى اظهرت نتائج تحليل التفريد الكهربائى لمشابهات الأنزيمات (البيروكسيديز و البولى فينيل اوكسيديز) لاشجار الجوافة البذرية تحت الدراسة وجود الحزم فى كل الاشجار مع وجود اختلاف فى كثافة الحزم بين الاشجار.