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Phylogenetic Relationship of Four *Ficus* Species Using Random Amplified Polymorphic DNA (RAPD) and Inter-simple Sequence Repeat (ISSR) Markers

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Abstract: Characterization of plants using molecular markers is an ideal approach for improvement and conservation of plant genetic resources. Random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) molecular fingerprinting markers were employed as genetic markers for the assay of the genetic relationship of four *Ficus* cultivars namely, benjamina, hawaii, stipulata and nitida. In RAPD analysis, 10 selected primers displayed a total of 340 amplified fragments, in which 212 (62.4%) were polymorphic fragments. The number of polymorphic bands scored per primer ranged from 6 (primer Z20) to 38 (primer Z18). Sixty-two out of 340 RAPD-PCR fragments were found to be useful as cultivar-specific markers. The largest number of RAPD-PCR markers was scored for FB (21 markers), while the lowest (11 markers) was scored for FH and FS. In the meantime, the largest number of RAPD-PCR cultivar-specific markers was generated by primer Z11 (9 markers), while the lowest number of RAPD-PCR specific markers (3 markers) was generated by primer Z06. In ISSR analysis, 11 of the tested ISSR primers generated variable banding patterns. A total of 179 out of 299 ISSR fragments were polymorphic. Fifty DNA amplified fragments were considered as cultivar-specific markers. Genetic similarities among the four *Ficus* cultivars were estimated according to the RAPD and ISSR data. Cultivars distribution on the consensus tree according to the banding patterns of RAPD differed from that based on ISSR. This may be due to the possibility that each technique of amplified different parts of the genome. Therefore, it was better to use the combination of the banding patterns of the two technique in order to use more segments sites of the genome that increase the validity of the consensus tree. Results of the combined data exhibited that the most two closely related cultivars were FH and FS with the highest similarity index (0.618). On the other hand, the two most distantly related cultivars were FS and FN with low similarity index (0.387). In conclusion, RAPD and ISSR polymorphisms could be used as efficient tools for the detection of similarities and phylogenetic relationships of the studied genotypes, which could be useful in the breeding programs.

Key words: DNA fingerprinting, genetic relationship, molecular markers, ornamental plants and genus *Ficus*

INTRODUCTION

Genus *Ficus*, is part of the family Moraceae. It is made up of about 1,000 species from pantropical and subtropical origins[21]. Several of which are desirable interior foliage plants. *Ficus* includes a large number of indoor ornamental plants and garden and roadside trees such as *F. elastica* Roxb. ex Hornem., *F. religiosa* L., *F. stipulate* and *F. microcarpa* L.

The application of DNA technology in agricultural research has progressed rapidly over the last twenty years, especially in the area of cultivar identification[16]. Characterization of plants with the use of molecular marker is an ideal approach for conservation of plant genetic resources and improvement[19]. In addition, molecular markers not only provide a useful method for cultivars characterisation, but they also allow genetic relatedness among cultivars to be assessed and determined more accurately[5].

Random Amplified Polymorphic DNA (RAPD) markers, utilizing PCR amplification from single arbitrary primer, were developed by Williams and his co-workers[22]. Dominant RAPD markers have been used for the identification of different plant species, as well as for assessing genetic diversity[10,11,14]. Inter-Simple Sequence Repeat Markers (ISSR, anchored

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microsatellites) use simple sequence repeats anchored at the 5' or 3' end by a short arbitrary sequence as PCR primers[23]. ISSRs are ideal as markers for genetic mapping and population studies because of their abundance, and the high degree of polymorphism between individuals within a population of closely related genotypes[8]. Those properties indicate their potential role as good supplements for RAPD-based genome analysis[12,17].

The objective of this study is to identify the genetic relationship of four *Ficus* species using random amplified polymorphic DNA (RAPD) and Inter-simple sequence repeat (ISSR) markers.

MATERIALS AND METHODS

Plant Materials: Four commercial *Ficus* cultivars, namely, Benjamina, Hawaii, Nitida and Stipulata used in this study were provided by Department of Botanical Garden Research, Antoniades Branch, Horticulture Research Institute, A.R.C. These cultivars represent three different species. Their codes, scientific names, origins and economic uses are shown in Table (1).

Methods:

DNA Extraction: Genomic DNA was extracted from fresh leaves of single adult trees using plant DNA miniprep kit (OMEGA Bio-Tek).

RAPD and ISSR Analysis:

Selection of Primers: Ten RAPD primers were chosen as potentially useful, from twenty, 10-mer oligonucleotides with arbitrary sequence. The codes and sequences of the used primers are shown in Table (2). Fifteen primers based on dinucleotide, tetranucleotide or pentanucleotide repeats were used in ISSR analysis. Eleven ISSR primers that produced clear and reproducible fragments were selected for the amplification of all DNA samples (Table, 3).

PCR Reaction: The PCR reaction mixture consisted of 20ng genomic DNA, 5X PCR buffer (Promega), 25mM/L $MgCl_2$ (Promega), 100µM/L of each dNTP (Promega), 66ng/µl Primer and 5 U/µl *Taq* polymerase in a 25µl volume. The amplification protocol was carried out according to Jie Shen *et al*.,[9], with some modifications. The reaction mixtures were pre-denatured at 94 ºC for 5min, followed by 5 cycles of 92 ºC for 30 Sec, 35 ºC for 2min and 72 ºC for 90 sec, followed by 35 cycles of 92 ºC for 30 Sec, 40 ºC for 30 Sec and 72 ºC for 90 Sec, with a final extension at 72 ºC for 5 min, and eventually stored at 4 ºC.

The amplified products were electrophoresed in 1% agarose gel with 0.5x TBE buffer. After the gel had been stained with ethidium bromide, banding patterns were visualized with a UV transilluminator.

Data Analysis: RAPD and ISSR data were scored as presence (1) or absence (0) bands by using of the Phoretix 1D image analysis system (Phoretix International, London) to integrate the data. Similarity indices were calculated and consensus tree was developed based on the banding patterns of the four cultivars in RAPD and ISSR analysis using SPSS statistical analysis program (Version 10). The genetic relationships among the four cultivars, at the molecular level, were determined.

RESULTS AND DISCUSSION

RAPD Analysis: Table (4) and Figure (1) show the results of total amplified fragments (TAF), amplified fragments (AF) and specific markers (SM) for each cultivar of *Ficus* using RAPD-PCR analysis with ten random primers. A total number of 340 DNA fragments were detected, in which 212 (62.4%) were polymorphic fragments. However, 18 bands were common (monomorphic) for all cultivars. The lowest number of polymorphic fragments was detected for primer Z20 (6 out of 25 amplified bands), while the highest number of polymorphic fragments was detected for primer Z18 (38 out of 44 amplified bands). Cultivar-specific markers generated from RAPD-PCR analysis are shown in Table (4). Sixty-two out of 340 RAPD-PCR fragments were found to be useful as cultivar-specific markers. The largest number of RAPD-PCR markers was scored for FB (21 markers), while the lowest (11 markers) was scored for FH and FS. In the meantime, the highest number of RAPD-PCR cultivar-specific markers was generated by primer Z11 (9 markers), while the lowest number of RAPD-PCR specific markers (3 markers) was generated by primer Z06. In conclusion, all of the ten primers used allowed enough distinction among the cultivars under study. These cultivar-specific markers can be used in subsequent experiments to detect molecular markers for polymorphic genes with economic importance among these and other cultivars. Similar finding were obtained in mints by Hassan[6] and Momeni *et al*.[13] and in other genera[4,3].

ISSR Analysis: ISSR-results as shown in Table (5) using eleven primers out of fifteen produced reproducible banding patterns. A total number of 299 DNA fragments were amplified with different lengths overall the four cultivars under investigation. The results showed that 17 DNA amplified fragments were monomorphic in the four cultivars and 179 amplified fragments were polymorphic. Figure (2) represents some ISSR banding patterns. Fifty DNA amplified fragments were considered as cultivar-specific markers. Among the samples studied, the highest

Table 1: List of the four *Ficus* cultivars; their scientific names, origin and economic uses.

Code	Scintific Name	Origin	Economic uses
FB	*Ficus benjamina*	- South and southeast Asia and Australia (Randall, 1998).	- Popular tree worldwide cultivated for ornamental purposes. - Bonsi, container or above-ground planter, hedge. - Suitable for growing indoors. - In its native range, its small fruit are a favorite food of some birds
FH	*Ficus retusa* Hawaii	- grown widely in many tropical regions of the world.	- Suitable for growing indoors
FN	*Ficus retusa* Nitida Thunb.	- grown widely in many tropical regions of the world.	- Has a long history of use as interior tree. - Suitable for street tree. - used as a park tree, tolerates trimming and can be shaded and sheared into a hedge, screen or barrier. - It also makes a wonderful shade tree on large properties.
FS	*Ficus Stipulata* Thunb, *F. pumila*	- Grown in warm tropical areas of the world.	- The plant grows as a vine and can adhere to rock, concrete and other surfaces by means of a rubbery substance which exudes from aerial roots (Neal 1965). - It is often planted along rock walls, on sides of buildings, and on other trees.

Table 2: Code and sequence of ten different random primers (10-mer oligonucleotides)

No.	Oligo Name	SEQUENCE
1	Z-05	5'-TCC CAT GCT G-3'
2	Z-06	5'-GTC CCG TTC A-3'
3	Z-08	5'-GGG TGG GTA A-3'
4	Z-11	5'-CTC AGT CGC A-3'
5	Z-12	5'-TCA ACG GGA C-3'
6	Z-13	5'-GAC TAA GCC C-3'
7	Z-17	5'-CCT TCC CAC T-3'
8	Z-18	5'-AGG GTC TGT G-3'
9	Z-19	5'-GTG CGA GCA A-3'
10	Z-20	5'-ACT TTG GCG G-3'

Table 3: Code and sequence of the eleven different ISSR primers

No.	Oligo Name	Code	SEQUENCE
1	ISSR 844B	S3	5'-CTC TCT CTC TCT CTC TGC-3'
2	ISSR 17898A	S4	5'- CAC ACA CAC ACA AC -3'
3	ISSR 17899A	S6	5'- CAC ACA CAC ACA AG-3'
4	ISSR 17899B	S7	5'- CAC ACA CAC ACA GG-3'
5	ISSR HB-8	S8	5'- GAG AGA GAG AGA GG -3'
6	ISSR HB-8	S9	5'- GTG TGT GTG TGT GG -3'
7	ISSR HB-10	S10	5'- GAG AGA GAG AGA CC -3'
8	ISSR HB-11	S11	5'- GTG TGT GTG TGT CC -3'
9	ISSR HB-12	S12	5'- CAC CAC CAC GC -3'
10	ISSR HB-13	S13	5'- GAG GAG GAG GC -3'
11	ISSR HB-15	S15	5'- GTG GTG GTG GC -3'

Table 4: Summary of data obtained by RAPD analysis for the four *Ficus* cultivars using ten RAPD primers.

Primer	TAB	PB	% PB	Cultivars								TSM	Common bands
				FB		FH		FS		FN			
				AB	SM	AB	SM	AB	SM	AB	SM		
Z-05	23	12	52.2	3	0	7	2	4	1	9	4	7	1
Z-06	34	23	67.7	9	0	4	0	9	2	12	1	3	3
Z-08	48	26	54.2	19	5	7	0	9	1	13	2	8	4
Z-11	28	19	67.9	12	7	6	1	9	1	1	0	9	0
Z-12	35	17	48.6	13	4	6	0	6	1	10	1	6	3
Z-13	44	22	50.0	14	2	11	2	7	1	12	1	6	4
Z-17	30	24	80.0	5	1	11	2	9	3	5	0	6	0
Z-18	44	38	86.4	11	0	10	1	6	0	17	5	6	0
Z-19	29	25	86.2	8	1	8	2	4	1	9	0	4	0
Z-20	25	6	24.0	5	1	4	1	6	0	10	5	7	3
Total	340	212	62.4	99	21	74	11	69	11	98	19	62	18

TAB= Total amplified bands, PB= Polymorphic bands, %PB= %Polymorphic bands
TSM= Total specific markers, AB= Amplified band and SM= Specific marker
FB= *Ficus Benjamina*, FH= *Ficus hawaii*, FS= *Ficus stipulate* and
FN= *Ficus Nitida*

Table 5: Summary of data obtained by ISSR analysis for the four *Ficus* cultivars using eleven ISSR primers.

Primer	TAB	PB	% PB	Cultivars								TSM	Common bands
				FB		FH		FS		FN			
				AB	SM	AB	SM	AB	SM	AB	SM		
Pr.S3	33	17	51.5	9	0	8	1	6	1	10	2	4	3
Pr.S4	24	17	70.8	2	0	9	1	6	1	7	1	3	1
Pr.S6	29	19	65.5	4	0	8	0	3	0	14	1	1	1
Pr.S7	35	30	85.7	10	0	8	1	6	0	11	1	2	1
Pr.S8	22	7	31.8	7	1	4	0	5	1	6	1	3	3
Pr. S9	13	5	38.5	6	3	4	1	1	0	2	0	4	1
Pr.S10	30	18	60.0	4	0	6	0	9	2	11	4	6	2
Pr.S11	37	20	54.1	7	0	7	0	6	1	17	8	9	2
Pr.S12	31	23	74.2	11	2	3	0	7	1	10	1	4	1
Pr.S13	26	11	42.3	4	0	4	0	4	1	14	10	11	1
Pr.S15	19	12	63.2	5	0	5	1	2	0	7	2	3	1
Total	299	179	61.2	69	6	66	5	55	8	109	31	50	17

TAB= Total amplified bands, PB= Polymorphic bands, %PB= %Polymorphic bands
TSM= Total specific markers, AB= Amplified band and SM= Specific markerFB= *Ficus Benjamina*, FH= *Ficus hawaii*, FS= *Ficus stipulate* and FN= *Ficus Nitida*

number of cultivar-specific marker was generated with primer S13 (11 markers), while the lowest number of cultivar-specific marker was 1 marker generated with primer S6 for FN. On the other hand, the highest number of ISSR markers was scored for FN (31 markers), while the lowest number (5 markers) was scored for FH.

The variation of the polymorphism in the different cultivars can be explained by the hypothesis that the microsatellites whose sequences are complementary to the primer, are abundant or rare in the genome of the studied cultivar, these microsatellites occupy some sites sufficiently distant not allowing the synthesis of sequences that separates them[5].

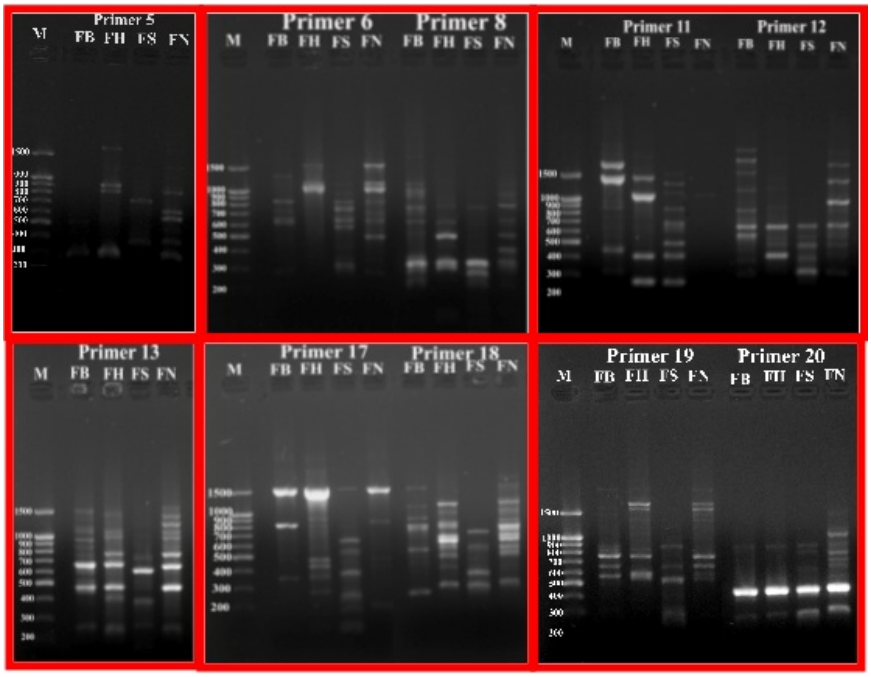

Fig. 1: Agarose gel (1%) in TBE buffer stained with ethidium bromide showing RAPD-PCR polymorphism of DNA for four *Ficus plants* (FB: *Ficus Benjamina,* FH: Ficus hawaii, FS: *Ficus stipulate* and SN: *Ficus nitida*) using random primers. M refers to 100 bp Ladder.

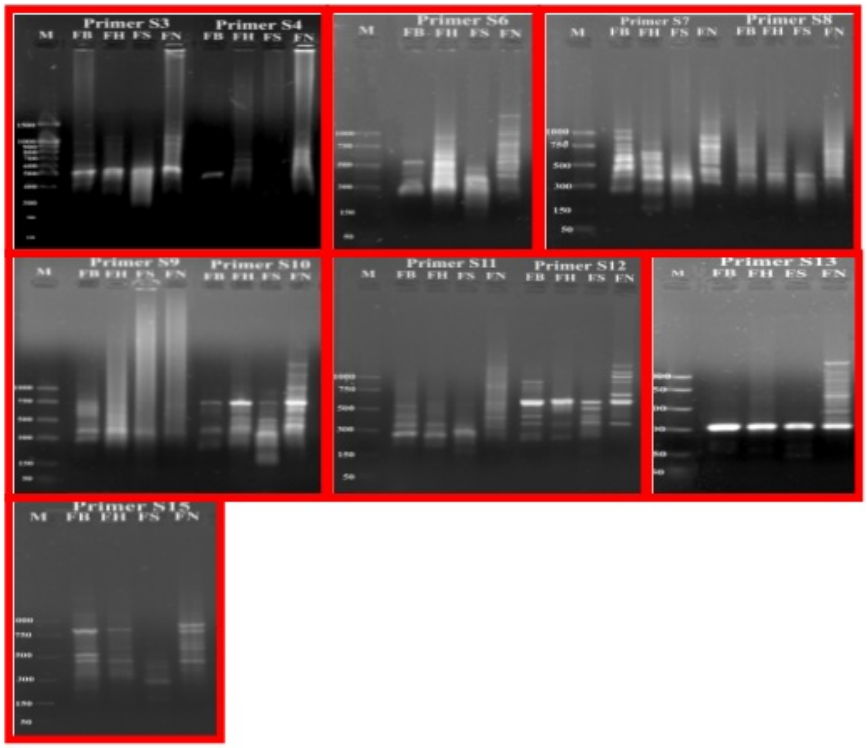

Fig. 2: Agarose gel (1%) in TBE buffer stained with ethidium bromide showing ISSR-PCR polymorphism of DNA for four *Ficus plants* (FB: *Ficus Benjamina,* FH: *Ficus hawaii*, FS: *Ficus stipulate* and SN: *Ficus nitida*) using ISSR primers. M refers to 250 bp Ladder.

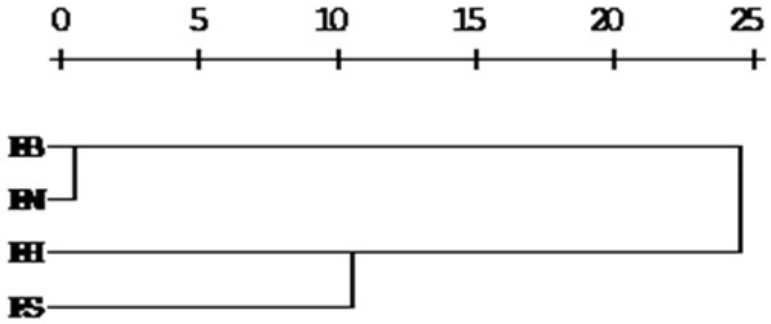

Fig. 3: Consensus tree for four ficus cultivars developed on the basis of their banding patterns with RAPD primers. (FB: *Ficus benjamina,* FH: *Ficus hawaii*, FS: *Ficus stipulate* and SN: *Ficus nitida*).

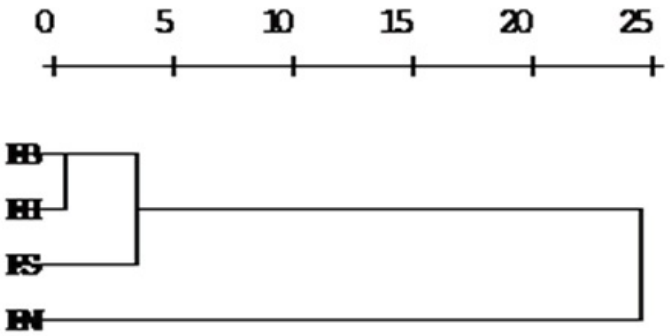

Fig. 4: Consensus tree for four ficus cultivars developed on the basis of their banding patterns with ISSR primers. (FB: *Ficus benjamina*, FH: Ficus hawaii, FS: *Ficus stipulate* and SN: *Ficus nitida*).

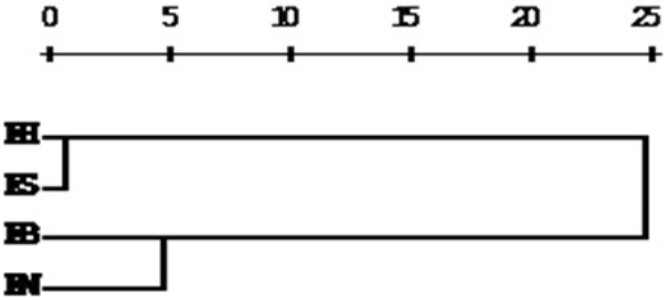

Fig. 5: Consensus tree for four ficus cultivars developed on the basis of their banding patterns with combination of RAPD and ISSR. (FB: *Ficus benjamina*, FH: *Ficus hawaii*, FS: *Ficus stipulate* and SN: *Ficus nitida*).

Genetic similarities among the four *Ficus* cultivars were estimated according to the RAPD data. Table (6) showed that the most two closely related cultivars were FB and FN with the highest similarity index (0.618). On the other hand, the results indicated that the two most distantly related cultivar were FS and FN with low similarity index (0.473). The results of the consensus tree indicated that tree was divided into two clusters, the first included cultivars FB and FN, the second cluster included cultivars FH and FS (Fig., 3).

According to ISSR results, the most two closely related cultivars were FB and FH (Table, 7) with the highest similarity index (0.677). On the other hand, the most two distantly related cultivars were FS and FN with low similarity index (0.303), the two cultivars located very far. Figure (4) indicated that the dendrogram revealed one main group of three cultivars including two subgroups. Subgroup 1 included both FB and FH and subgroup 2 included cultivar FS only. The remaining cultivar represented distant sequences.

Cultivars distribution on the consensus tree according to the banding patterns of RAPD differed from that based on ISSR banding patterns, which may be due to that each technique, amplified different parts of the genome. So, it is better to use the combination of the banding patterns of the two techniques to use more segments of the genome that will increase the validity of the consensus tree. Results of the combined

Table 6: Similarity indices for the four *Ficus* cultivars on the basis of their banding patterns with RAPD.

Cultivars	FB	FH	FS
FH	.509		
FS	.545	.582	
FN	.618	.600	.473

Table 7: Similarity indices for the four *Ficus* cultivars on the basis of their banding patterns with ISSR.

Cultivars	FB	FH	FS
FH	.677		
FS	.636	.657	
FN	.566	.485	.303

Table 8: Similarity indices for the four *Ficus* cultivars on the basis of combination of the banding patterns with RAPD and ISSR.

Cultivars	FB	FH	FS
FH	.598		
FS	.588	.618	
FN	.603	.544	.387

data as shown in Fig (5) and Table (8) exhibited that the most two closely related cultivars were FH and FS with the highest similarity index (0.618). On the other hand, the two most distantly related cultivars were FS and FN with low similarity index (0.387). The results of the consensus tree indicated that the tree divided the cultivars into two main clusters, the first included cultivars FH and FS. The second one included cultivars FB and FN.

This study provides evidence that RAPD and ISSR polymorphisms could be used as efficient tools for the detection of similarities and phylogenetic relationships of the studied genotypes. The same conclusion was obtained by several authors[2,1,7].

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