AFLP MARKER IN GENETIC DIVERSITYASSESSMENT OF FIG (*Ficus carica* L.) POPULATIONS IN KURDISTAN REGION – IRAQ.

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ABSTRACT

In this study, the genetic relatedness of 12 cultivars of fig from different populations in Kurdistan region- Iraq were analyzed using eleven AFLP primers pairs combinations by using the technology of molecular analysis the DNA. Genetic similarity matrices were produced for the AFLP data to calculate genetic distances among their cultivars. Genetic similarity coefficient ranged from 0.1261 to 0.3905. The lowest genetic similarity was observed between Kola and Gala Zard (0.1261). The Hejeera Rash and Shela cultivars were most similar ones with a coefficient of 0.3905. Clustering based on AFLP data for the 12 fig cultivars was identified at the 0.32 similarity level. In the developed dendogram two main groups were found, the first one combined Ketek and Shela together, while the second group contained two sub group Shingaly and Benatty combined together, while in the other sub group cluster three other sub-group were identified. The results of this study may help in the formulation of appropriate strategies for conservation and cultivar improvement in figs, for which limited knowledge of the genetic diversity is available.

Key words: Molecular Biology, DNA markers, genetic diversity, Fig.

علامات تباين اطوال قطع الدنا المتضعف لتقييم التنويع الوراثي للتين --- في كردستان العراق

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المستخلص

في هذه الدراسة ، تم تحليل العلاقة الوراثية لـ 12 صنف من التين من مجموعات سكانية مختلفة في إقليم كردستان – العراق باستعمال أحد عشر أزواج من البائدات (Primers من (تباين اطوال قطع الدنا المتضعف (AFLP باستعمال تقنية التحليل الجزيئي الدنا. تم إنتاج مصفوفات التشابه الوراثي لبيانات AFLP لحساب المسافات الجينية بين ألاصناف المنتجة. معامل التشابه الوراثي تراوح من0.1261 إلى 0.3905 . وقد لوحظ أقل التشابه وراثي بين قولى و كالا زارد (0.1261). كانت هجيرا راش و شيلا أكثر تشابهًا مع معامل 0.3905 . وتم تحديد التكتل على أساس بيانات AFLP لأصناف التين الـ 12 عند مستوى التشابه 0.32. تم العثور على مجموعتين رئيسيتين في الشعبة المتقدمة ، الأولى ضمت كتك و شيلا معًا ، بينما احتوت المجموعة الثانية على مجموعتين فرعيتين شنكالي و بيناتي مجتمعة ، بينما في المجموعة الفرعية الأخرى تم تحديد ثلاث مجموعات فرعية أخرى. دراسة هذه النتائج قد تساعد في صياغة استراتيجيات مناسبة للحفظ وتحسين الأصناف المنتجة من التين ، ضمن محدودية معرفة وتوفرالمعلومات بالتنوع الجيني.

الكلمات المفتاحية: الوراثة الجزائية، مؤشرات الدنا، التباين الوراثي، التين.

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INTRODUCTION

The fig *Ficus carica* L. (2n= 2x =26 chromosome) (7). Fig belongs to Family: Moraceae (32), in literature it has several common names such as common fig, edible fig (22). The genus *Ficus* is made up of about 1,000 species from pan-tropical to subtropical origins (32). Fig plants are all woody in the family, from trees and shrubs to climbers (22). The name carica is named after the Caria place in Asia Minor, home of the fig. (11, 22) *F. carica* is presumed to originate from Western Asia and spread to the Mediterranean by humans (9). Today, it is considered as one of an important world crop, because of their nutritional, medicinal, food industry and ornamental values (13, 15). According to FAO reports, the planet generates more than one million tons of figs per annum (12). Large edible fig producers include Turkey, Egypt, Morocco, Spain, Greece, California, Italy, Brazil and other usually mild winters and hot dry summers (29). The available methods for fig plants diversity analysis include the classical research methods which mainly include morphological and agronomical traits, biochemical markers and cytological such as cell karyotype analysis and isoenzymes (4, 14, 19). These methods are considered as sensitive to environmental factors and the number of markers is limited, thus the research of fig diversity has been limited. Molecular marker techniques such as RFLP, ISSR, RAPD, and AFLP have vastly improved knowledge on genome structure, organization, and evolution of many cultivates plants (1, 2, 5, 10, 18, 21, 24). AFLP analysis has been used to detect DNA polymorphisms and the genetic relationships of many economically important plants including fig genotypes (8, 25). However, few applications of AFLP technology to the genetic analysis and fingerprinting of fig cultivars have been reported. AFLP technique was introduced as a reliable and reproducible marker system (31). It was favored over other DNA-based markers mainly because of its high multiplex ratio and prior sequence information is not needed (34). Distribution of fig plants throughout Iraq's Kurdistan region in rocky mountain slopes, valleys, hill sides and road sides, depleted forests of Oak and Pine. The figs were also grown in irrigated orchards in dry vineyards and as house plants (27). Most fig populations in Kurdistan have received very little attention from scientists, so they are not aware whether they are native trees or new varieties that the local people have introduced to the region for many years. The objectives of the present study were the application of AFLP markers to reveal DNA polymorphism among populations and between individuals and to determination of genetic relationships between the populations or cultivars of fig in Kurdistan region of Iraq.

MATERIALS AND METHODS

Samples collection

Samples (fresh leaves) from (12) of fig cultivars were collected from different districts in the Kurdistan Region – Iraq. These samples were obtained from the Ministry of Agriculture fields at Duhok, Erbil and Sulimania cities. The cultivars of Fig selected for this study were (Shingaly, Benatty, Ketek, Rebwary Rash, Henjeer Rash, Rash khomali, Rehan Rash, Rehan Zard, Zarda Roon, Shela, Kola and Gala Zard).

DNA Extraction

Genomic DNA was extracted from fresh healthy tissue as mentioned by Weigand, et al (33). Fresh tissue (3g) was homogenized to powder with 40 ml in liquid nitrogen. The fine powder was dissolved in a pre-heated (60 C^o) 2x CTAB extraction buffer (2x CTAB, 5M NaCl, 1M Tris-HCl, 0.5 M EDTA), and incubated at 60^{o}C in a water bath with shaking for 30 min. The mixture was extracted with an equal volume of choloroform / isoamyl alcohol (24:1, v/v) (20). The mixture was then centrifuged at 4000 rpm for 30 min. The aqueous phase was transferred into fresh tube and precipitated with 0.66 volume of isopropanol. Precipitated nucleic acids were then dissolved in 500µl Tris EDTA TE-buffer (1 ml of 1M tris-HCl (PH8.0) 0.2µl of 0.5M EDTA.

PCR Amplification of AFLP- primers

The AFLP procedure was performed as described by Vos, et al (31) as follows; 500ng of DNA from each sample was double digested with 5U each of the two restriction enzymes, *MseI* (recognition site 5'T↓TAA3') and *PstI* (recognition site 5'CTGCA↓G3'). The digestion reaction was prepared in 30µl

final volume containing, 1x one-phor all buffer (Pharmacia Bioteh, Uppsala, Sweden), and incubated for three hours at 37^{o}C. DNA fragments, were then ligated to *Pst I* and *MseI* adapters by adding 50 pmol of *MseI*-adapter, 5 pmol *PstI*-adapter in a reaction containing 1U of T4-DNA ligase, 1mM rATP and 1x of one-phore-buffer and incubated for 3hr. at 37C. After ligation, the reaction mixture was diluted to 1:5 using sterile distilled water. Pre selective PCR amplification was performed in a reaction volume of 20 µl containing 50ng of each of the primers (P00, M00) corresponding to the *MsI* and *Pst I* adapters, 2µl of template-DNA, 1U *Taq* DNA polymerase, 1x PCR buffer and 5mM dNTPs. PCR amplification was performed in WMG thermal cycler using the following program: 30 cycles of 30s at 94 ºC, 1min at 60ºC, 1min at 72 ºC. Pre-amplification products were then diluted to 1:5 and 2µl were used as template for selective amplification. Selective amplification was conducted using *MseI* and *Pst1* selective primer combinations, (Table 1). Amplification was performed using a selective program of 36 cycles with the following profile: a 30sec. DNA denaturation step at 94ºC, 30sec. annealing step, and a 1 min extension step at 72ºC. The annealing temperature in this program varied in the first cycle where it was 65ºC and in each subsequent cycle for the next 12 cycles it was reduced by 0.7ºC (touchdown PCR). Then for the remaining 23 cycles, it was 56ºC. Selective amplification products were loaded onto 6% polyacrylamid gels, and DNA fragments were visualized by silver staining kit (Promega, Madison, Wis) as described by the supplier. Silver – stained gels were scanned to capture digital images of the gels after air drying.

Table 1. Represents the sequences of Pre-selective and Selective primers combinations used in this study

	Sequences '5--------3'
Pre-selective primer combinations Poo + Moo	**GACTGCGTACATGCAG** **GATGAGTCCTGAGTAA**
Selective primer combinations	
1- P_{GGGA} /M_{GATA}	**GACTGCGTACATGCAGGGA** **GATGAGTCCTGAGTAAGATA**
2- P_{GATA} /M_{TAAG}	**GACTGCGTACATGCAGGATA** **GATGAGTCCTGAGTAATAAG**
3- P_{TACC} /M_{GATA}	**GACTGCGTACATGCAGTACC** **GATGAGTCCTGAGTAAGATA**
4- P_{GATA} /M_{GATA}	**GACTGCGTACATGCAGGATA** **GATGAGTCCTGAGTAAGATA**
5- P_{GATA} /M_{TACA}	**GACTGCGTACATGCAGGATA** **GATGAGTCCTGAGTAATACA**
6- P_{TACC} /M_{TAAG}	**GACTGCGTACATGCAGTACC** **GATGAGTCCTGAGTAATAAG**
7- P_{GATA} /M_{TCAG}	**GACTGCGTACATGCAGGATA** **GATGAGTCCTGAGTAATCAG**
8- P_{TACC} /M_{AACC}	**GACTGCGTACATGCAGTACC** **GATGAGTCCTGAGTAACC**
9- P_{GGGA} /M_{TACA}	**GACTGCGTACATGCAGGGA** **GATGAGTCCTGAGTAATACA**
10- P_{AATA} /M_{GATA}	**GACTGCGTACATGCAGAATA** **GATGAGTCCTGAGTAAGATA**
11- P_{GATA} /M_{AACC}	**GACTGCGTACATGCAGGATA** **GATGAGTCCTGAGTAACC**

Data analysis

The digital photographs of gels were used to score the data for AFLP analysis starting from the higher molecular weight product to lowest molecular weight product. Presence of a product was identified as (1) and absence was identified as (0). Data were scored for all genotypes, their amplification product and primers. The data then entered into NTSYS-PC (Numerical Taxonomy and multivariate

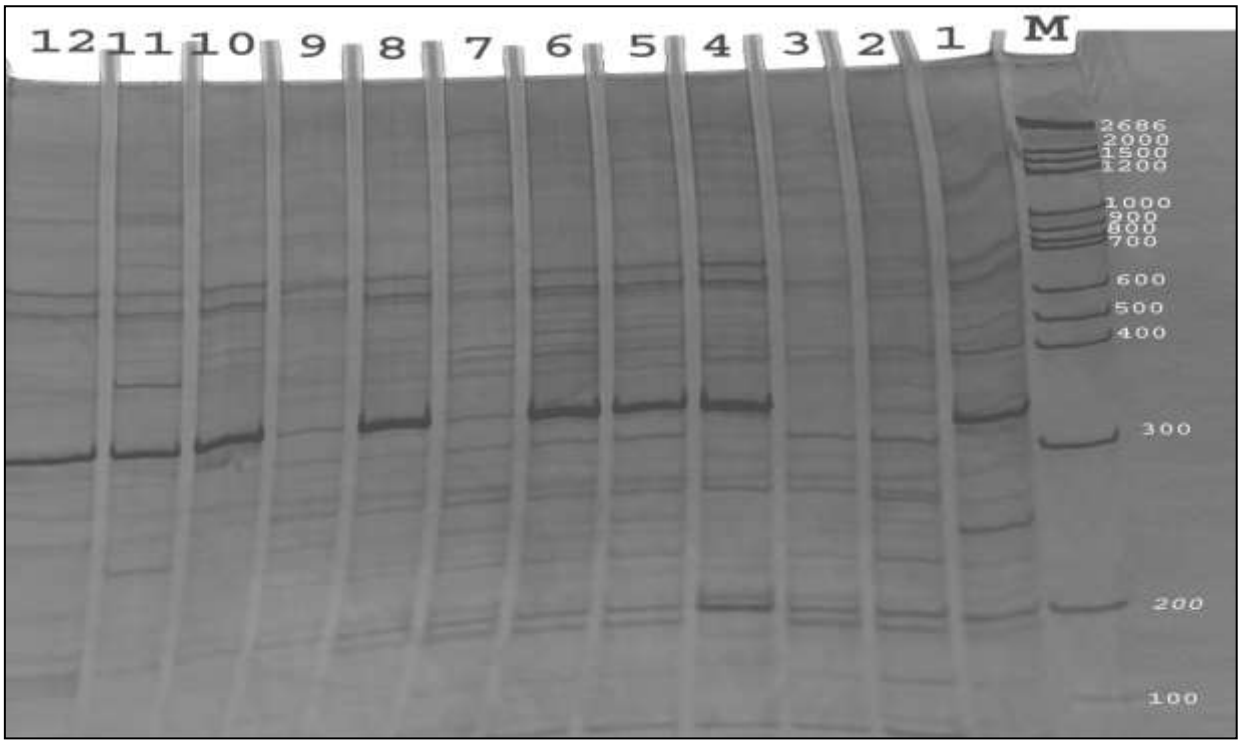
Analysis System), Version 2.1 (Applied Biostatistics) program (26) using the program editor. The data were analyzed using SIMQUAL (Similarity for Qualitative Data) routine to generate genetic similarity index (23).

RESULTS AND DISCUSSION

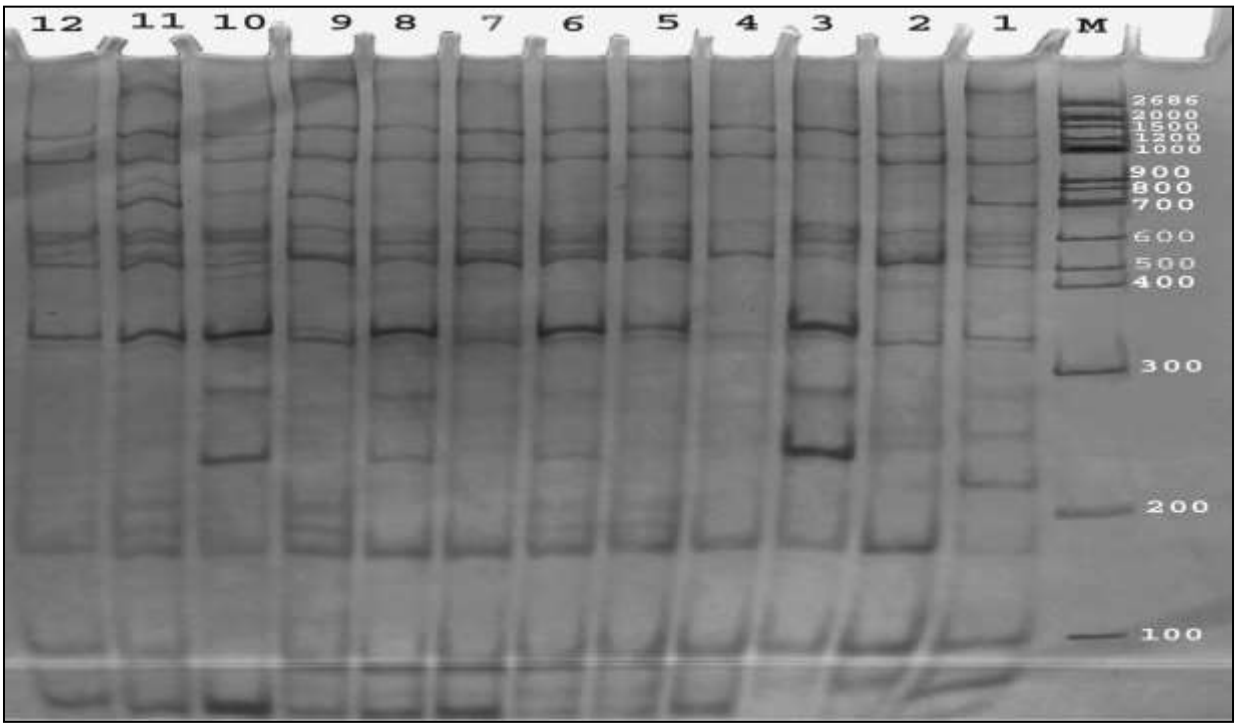
The results of selective primer amplifications are shown in (Figure 1 A,B) and the presence of AFLP bands across all 12 Fig cultivars clearly indicate the successful application of AFLP marker technology. AFLP analysis (Table 2) was used eleven selective primer combinations, the experiments generated a total of 301 fragments (bands of DNA), in the rate of 27.36 band for each combination. The number of polymorphic bands was 226 representing a level of polymorphism of 75.07% and in the rate of 20.54 for each combination. Also in this study the greatest Discrimination Power appeared for the combination (P_{GGGA}/M_{GATA}) where it reached 15.04% and the smallest where 3.98% appeared for the combination (P_{GATA}/M_{AACC}) which shows the lowest number of polymorphic bands. Also the results of this study have produced the greatest number and size of bands by the two combinations (P_{GGGA}/M_{GATA}) and (P_{GATA}/M_{TAAG}). These combinations were produce 41 bands, whereas the lowest bands 19 and 17 were produce by two combinations (P_{AATA}/M_{GATA}) and (P_{GATA}/M_{AACC}). What supports any study is the appearance of polymorphic bands or bands with different sizes that provide the database with the ability to make it eligible to carry out the necessary genetic analyzes that are consistent with the objective of the study (30, 3). So the importance of primer combinations is measured by the number of polymorphic bands, it stands out for the discrimination Power for each combination, it is compared to the total product polymorphic bands that showed by all combinations that used in any study. Another important variation by using AFLP marker as in all molecular markers is the differences in molecular weight (bp) for bands, those present on gel. In currently study the size of the AFLP amplified fragments ranged from 50bp. to 1500bp. Other study was reported by Laddomada, et al (17) to assess polymorphism and relationships among 24 fig accessions using AFLP markers; 553 amplification products of which 535 were polymorphic among the analyzed genotypes. A high degree of polymorphism was revealed by these primer combinations. The results showed (6) that using AFLP marker with Tunisian fig germplasm is characterized by having a large genetic diversity at the deoxyribonucleic acid level, as most of AFLP bands were detected. In fact, 351 (342 polymorphic) were detected using AFLP primers. AFLP markers showed the highest effective multiplex ratio (56.9). It was not accurate to identification of varieties depending on morphological traits only. May be a variety have many names in different plantation and genetically different varieties may have the same name (28). There were several different DNA marker analysis techniques that have been used to identify and characterize fruits to determine genetic diversity (16).

A- P_{GATA}/M_{TAAG}

B- P_{GATA}/M_{TCAG}



A- P_{GATA}/M_{TAAG}



B- P_{GATA}/M_{TCAG}

Figure 1 A,B. Results of twelve fig cultivars using AFLP primer combinations (A- P_{GATA}/M_{TAAG}) & (B- P_{GATA}/M_{TCAG}). Lanes from1 to 12 represent *Ficus* cultivars: 1= Shingaly, 2= Benatty, 3= Ketek, 4= Rebwary Rash, 5= Hejeer Rash, 6= Rash khomali, 7= Rehan Rash, 8= Rehan Zard, 9= Zarda Roon, 10= Shela, 11= Kola, 12= Gala Zard. Lane M refers to molecular marker 100-2686 bp.

Table 2. Estimates of genetic diversity between *Ficus* samples studied

Primer combinations	Total number of Bands	Number of polymorphic bands	Polymorphic percentage %	Primer Efficiency %	Discrimination Power %
1-P_{GGGA}/M_{GATA}	41	34	82.92	13.62	15.04
2-P_{GATA}/M_{TAAG}	41	25	60.97	13.62	11.06
3- P_{TACC} /M_{GATA}	30	28	93.33	9.96	12.38
4- P_{GATA} /M_{GATA}	28	24	85.71	9.30	10.61
5-P_{GATA} /M_{TACA}	27	20	74.07	8.97	8.84
6- P_{TACC} /M_{TAAG}	25	20	80.00	8.30	8.84
7-P_{GATA}/M_{TCAG}	25	18	72.00	8.30	7.96
8-P_{TACC} /M_{AACC}	24	20	83.33	7.97	8.84
9-P_{GGGA}/M_{TACA}	24	16	66.66	7.97	7.07
10-P_{AATA}/M_{GATA}	19	12	63.15	6.31	5.30
11-P_{GATA}/M_{AACC}	17	9	52.94	5.64	3.98
Average	27.36	20.54	75.07		
Total	301	226			

Genetic Similarity

Genetic similarity matrices were produced for the AFLP data to calculate genetic distance. As shown in (Table 3) genetic similarity coefficient ranged from 0.1261 to 0.3905. The lowest genetic similarity was observed between Kola and Gala Zard (0.1261). The Hejeera Rash and Shela populations were most similar ones with coefficient of 0.3905. These data were used to generate a dendogram.

Cluster analysis

Dendogram was established with UPGMA cluster analysis based on the AFLP data using 11 combination primers. Clustering based on AFLP data for the twelve figs was identified at the 0.32 similarity level (Figure 2). In this dendogram there was two main groups, the first one combined C3 Ketek and C10 Shela together, while the second group contain two sub group C1 Shingaly and C2 Benatty combine together, while the other sub group cluster there are three other sub-group, the first one C8 Rehan Zard and C9 Zarda Roon clustered together, the other sub-group dived to more sub group which first include C5 Hejeer Rash and C6 Rash khomali together in one cluster, second C7 Rehan Rash cluster alone. The third sub group also contains C4 Rebwary Rash clustered alone. The analyzed data illustrates a good variability in the genetic pool of the common local fig making it a valuable source for incorporation into potential breeding programs for the region. The most important advantage of these markers for use in genetic diversity such as fig is that they can be used without any prior knowledge of the target template DNA sequence. These results of this study conclude the usefulness of AFLP marker characterization fig populations compared with other PCR- based techniques.

Table 3. The genetic similarity between *Ficus* samples studied

	Shingaly	Benatty	Ketek	Rebwary Rash	Hejeera Rash	Rash khomali	Rehan Rash	Rehan Zard	Zarda Roon	Shela	Kola	Gala Zard
Shingaly	0.0000											
Benatty	0.1419	0.0000										
Ketek	0.3328	0.2453	0.0000									
Rebwary Rash	0.2102	0.2116	0.3291	0.0000								
Hejeera Rash	0.2507	0.2817	0.3661	0.1817	0.0000							
Rash khomali	0.2355	0.2508	0.2855	0.2240	0.1358	0.0000						
Rehan Rash	0.2412	0.2711	0.3218	0.1948	0.1386	0.1510	0.0000					
Rehan Zard	0.2991	0.2931	0.2509	0.2589	0.2211	0.1438	0.1449	0.0000				
Zarda Roon	0.3141	0.2998	0.3135	0.2649	0.2694	0.2331	0.1972	0.1678	0.0000			
Shela	0.3645	0.3417	0.3177	0.3690	0.3905	0.2727	0.3076	0.2322	0.2632	0.0000		
Kola	0.2398	0.3123	0.3790	0.2563	0.2254	0.2348	0.2188	0.2613	0.2373	0.3124	0.0000	
Gala Zard	0.2313	0.2673	0.3611	0.2614	0.2237	0.2465	0.2239	0.2664	0.2842	0.3248	0.1261	0.0000

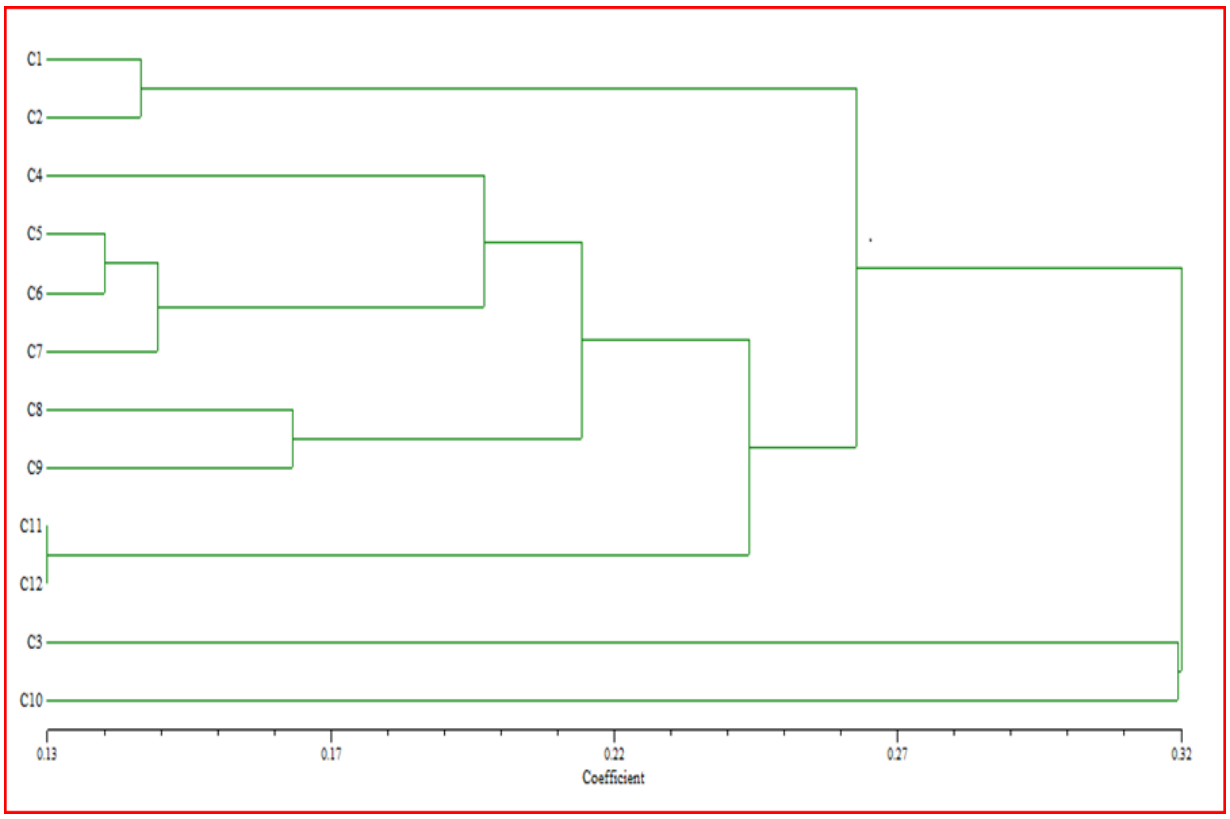


Figure 2. UPGMA dendrogram of twelve fig varieties showing genetic relationships of estimated from AFLP data. The 12 *Ficus* cultivars are: 1= Shingaly, 2= Benatty, 3= Ketek, 4= Rebwary Rash, 5= Hejeer Rash, 6= Rash khomali, 7= Rehan Rash, 8= Rehan Zard, 9= Zarda Roon, 10= Shela, 11= Kola, 12= Gala Zard

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