RESEARCH PAPER

Determination of genetics relationships by moleculars markers of SSR type, for some varieties of apricot (*Prunus armeniaca* L.) in the area of the Hodna, (M'sila), Algeria

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Received 13 Jun 2016; Revised 29 Jun 2016; Accepted 30 Jun 2016

Abstract

For long time studies of the genetic diversity of the apricot in Algeria are mainly based on phenotypic criteria. Some of these criteria are limited to well-defined stages of development that are highly sensitive to environmental factors. Indeed, these criteria are unstable, so it became necessary to consider more reliable traits to better characterize real taxonomic diversity. Markers with high mutation rates, such as microsatellites (SSR) used in our study have the ability to be a high polymorphism between individuals, so they are the markers of choice for studies of genetic diversity. In this study eight apricot genotypes were analyzed with 11 microsatellite primer pairs. PCR amplification of microsatellite regions was obtained polymorphic, clear and easily interpretable profiles. These primers produced 100 bands were used to study the genetic diversity of eight genotypes. For the 100 bands obtained, 40% of these fragments were polymorphic and 60% were monomorphic. The size of the bands generated by the 11 primers is variable; it ranges from 60 bp to 360 bp. The analyses of different outcomes have to classify the eight varieties into three distinct groups. These results indicate a strong genetic similarity between varieties, despite their different geographic origins sometimes. These results confirm the usefulness of microsatellites markers for the study of genetic diversity in apricot.

Keywords: Apricot, molecular markers, PCR, microsatellite, genetic diversity.

Détermination des relations génétiques par marqueurs moléculaires de type SSR, pour quelques variétés d'abricotier (Prunus armeniaca L.,) dans la région de Hodna (M'sila), Algérie

Résumé

Les études de la diversité génétique de l'abricotier en Algérie se sont basées essentiellement sur des critères phénotypiques, certains de ces critères sont limités à des stades de développements bien déterminés qui sont fortement sensibles aux facteurs environnementaux. En effet, ces critères sont instables, il devenait donc nécessaire de prendre en considération des caractères génétiques plus fiables pour mieux caractériser les diversités taxonomiques réelles. Les marqueurs à forts taux de mutations, comme les microsatellites (SSR) employés dans notre étude, ont la capacité de révéler un polymorphisme élevé entre différents individus. Dans la présente étude 8 génotypes dabricotier ont été analysés avec 11 paires d'amorces microsatellites. L'amplification PCR (Réaction en chaîne de la polymérase) des régions microsatellites a révélés des profils polymorphes clairs et facilement interprétables. Ces amorces ont produit 100 bandes qui ont été employées pour étudier la diversité génétique des 8 génotypes. Pour les 100 bandes obtenus, 40% de ces fragments se sont avérés polymorphes et 60% monomorphes. La taille des bandes générées par ces 11 amorces est variable, elle oscille entre 60 pb à 360 pb. L'analyse de différents résultats ont permis de classer les huit variétés en trois groupes distincts. Ces résultats obtenus indiquent une forte proximité génétique entre les variétés, malgré leurs origines géographiques parfois très différentes. Ces résultats confirment l'utilité des marqueurs microsatellites pour l'étude de la diversité génétique chez l'abricotier.

Mots-clés: *L'abricotier, Marqueur moléculaire, PCR, Microsatellite, diversité génétique.*

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I-Introduction:

Apricot (Prunus armeniaca L.,) is a species that has an adaptation to scoring contrasting environments. All apricot varieties are diploid with eight pairs of chromosomes (2n = 16, X = 8) (Lyne and al., 1996).

Apricot is grown in the region of M'sila decades; this is a very suitable species for arid climate of the region, dozens of varieties whose origin remains unknown.

The study of genetic diversity was performed using as a tool for analysis of polymorphism, microsatellite molecular markers SSR. These markers have high allelic polymorphism (Weber, 1990), annexed to the PCR technique, which was proposed simultaneously by Williams et al., 1990, based on the amplification of DNA using a primer small SSR types. These PCR primers, which are manipulated and visualization, can be automated through the use of automatic sequencers (Mort-Gaudry and Briat 1992).

The aim of our work is the determination of the genetic relationships among the eight varieties of apricot by technical SSR to determine whether there is a certain polymorphism and a relationship between the eight varieties through the construction of a phylogenetic tree.

II. Materials and Methods:

II.1 Plant Material:

Our plant material is formed of fresh leaves from eight varieties of apricots (Bulida, Louzi Red, Biche, Ben Sarmouk Red, Pavit Red, Ben Sarmouk White, Laarbi and Polonais), we obtained the sample of young leaf stage at April 17, 2013. All experiments and analyses were conducted in the laboratory of genetics, biochemistry and plant biotechnology at the University of Constantine1, Algeria.

II.2 Extraction DNA by CTAB method:

The study of genetic variability was made on the DNA extracted from the leaves of the studied individuals. The DNA extraction was conducted using modified CTAB method (Doyle and Doyle, 1990). DNA is extracted from 140 mg of fresh leaf stored at -80 C°, finely cut then crushed in a mortar previously cooled with liquid nitrogen.

Using spatula, transferred the homogenate in 20 ml tube, then added 900 µl of CTAB 2x supplemented with beta-mercapptoethanol pre-heated at 65°C, this buffer is a mixture of CTAB 2% (w/v), tris 100 Mm, EDTA 20 Mm, Nacl 1,4M, PVP40 1% (p/V), mixture was homogenized with vortex for a few seconds, then after incubation for 60 min in 65°C water bath with agitation, after 15 min of centrifugation at 10,000 rpm at 4°C, the supernatant is recovered 800 µl in a new eppendorffe tube of 2ml, then add 800 µl (1vol) chloroform/isoamyl alcohol (24:1), the mixture was stirred for 45 min at low speed, on gets the upper aqueous phase and is placed in a new tube eppendorffe, then added 3 to 5 µl RNAase (10 mg / ml), stirred by inversion and incubated for 30 min at 37C°, and then added 540 µl (2/3vol) of cold isopropanol (- 20 C°) the tubes are inverted gently until the appearance of a white ball, the tube is left -2C° for 10 min to precipitate the DNA and then centrifuged 10min at 10,000 rpm at 4C°.

To remove the supernatant, 500 µl of wash solution 1 (asitate sodium 200mm, 76% ethanol) is added, 15 min then allowed to add the wash solution 2 (10 mM ammonium asitate, ethanol 76%), it is avoid incubated over 5 min, after removal of supernatant, dried DNA outdoors for 15 min, the DNA pellet was dried and suspended with 100 µl of pure water for 20 min at 37°C. The extracted DNA was stored at 4C° overnight before dosing.

II.3 Evaluation of the quality of extracted DNA:

The quality of the extracted DNA was first checked on agarose gel test 0.8%, in fact, 5ng of DNA and 2 µl of loading buffer are then deposited in the gel, which were migrated to TBE buffer for 35 min at 80 V, the gel was then visualized by the gel imaging system for molecular biology.

Moreover, the concentration of DNA is determined by NANODROP2000. Another measurement of the OD at 280 nm taken has also assess the amount of protein contained in the DNA suspension. Thus, the relationship between these two measures: OD260/ OD280 is an evaluative digital means to assess the quality or purity of our DNA extract. The more this ratio tends to 2 plus the DNA extract is called pure.

II.4 Amplification DNA of apricot tree by PCR:

II.4.1 Analysis PCR / SSR:

The principle of PCR is based on an enzymatic amplification of DNA (Vekemans and Jacquemart 1997, Claros and Quesada 2000). For the SSR technique, quality of DNA doesn't have great importance in

the success of his PCR amplification, unlike other methods like AFLP (Konate, 2007).

The SSR analysis is carried out with the use of the 11 primers (Table 1).

II.4.2 The reaction mixture:

The reaction mixture of a final volume of 20 µl includes 10 µl of Taq gold 2X master mix, 0.8 µl of buffer, 2 µl primers F and R (5 µM) 4.8 H2O µl UP, 4 µl of DNA (5 ng/µl).

We introduced the reaction mixture at the level of the wells that are a PCR plate, we puts the extracted DNA from eight varieties tested with order meet along a line, each primer is injected into a column, at the end is added to all column of the plate a mixture that contains 10 µl of amp Taq master mix 2 X gold, 0.8 µl of buffer, 4.8 µl H2O UP.

At the end, the plate was well covered, then we placed the plate in the PCR machine during all cycles.

Amplification reactions were conducted on a thermal cycler program indicated in the table below (Table 2):

The amplification products were analyzed by electrophoresis on Agarose gel 3%, and DNA bands were detected by Ethidium Bromide and visualized under UV radiation.

II.5 Electrophoresis of PCR:

II.5.1 Preparation of samples:

The samples were prepared, 2 µl of buffer 5X charge added to 10 µl of the extract was mixed amplification and finally marker.

II.5.2 Preparation of gels:

The carrier consists of an Electrophores Agarose gel 3%, 1X TBE buffer in the presence of BET (Table 3).

I.5.3 Visualization:

The Agarose was poured into the mold with the comb and allowed to cool, then the comb was removed and

Table 1: Characterization of the used primer, T: hybridization Temperature

T	Vol	Concentration	Sequence 5'- 3'	Primer
BPPCT001F	AATTCCCAAAGGATGTGTATGAG	100	185	52,86°C
BPPCT001R	CAGGTGAATGAGCCAAAGC	100	221	52,34°C
BPPCT004F	CTGAGTGATCCATTTGCAGG	100	230	52,18°C
BPPCT004R	AGGGCATCTAGACCTCATTGTT	100	211	54,88°C
BPPCT011F	TCTGAGGGCTAGAGTGGGC	100	237	55,64°C
BPPCT011R	TGTTTCAGGAGTCGAACAGC	100	224	53,84°C
BPPCT017F	TTAAGAGTTTGTGATGGGAACC	100	197	52,37°C
BPPCT017R	AAGCATAATTTAGCTAACCAAGC	100	174	52,90°C
BPPCT020F	GGTGGATGGTCAAGATGC	100	241	50,53°C
BPPCT020R	ATTGACGACTTACAGGTG	100	211	53,53°C
BPPCT032F	TTAAGCCACAACATCCATGAT	100	211	51,38°C
BPPCT032R	AATGGTCTAAGGAGCACACG	100	213	53,28°C
UDP96001F	AGTTTGATTTTCTGATGCATCC	100	217	51,46°C
UDP96001R	TGCCATAAGGACCGGTATGT	100	224	54,04°C
UDP98021F	AAGCAGCAATTGGCAGAATC	100	210	53,01°C
UDP98021R	GAATATGAGACGGTCCAGAAGC	100	191	54,79°C
UDP98408F	ACAGGCTTGTTGAGCATGTG	100	226	54,71°C
UDP98408R	CCCTCGTGGGAAAATTTGA	100	237	50,76°C
UDP98410F	AATTTACCTATCAGCCTCAAA	100	214	48,34°C
UDP98410R	TTTATGCAGTTTACAGACCG	100	229	49,40°C
UDP98414F	AAAAGGCACGACGTTGAAGA	100	201	54,03°C
UDP98414R	TTCAGATTGGGAATTTGCAG	100	220	49,80°C

Table 2: Program of specific PCR for the apricot tree with SSR technique

The PCR step	took the stage	Temperature	
initial denaturation	5 min	94°C	
Denaturation	30 sec	94°C	10 cycles
Hybridization	30 sec	60°C	
Elongation	30 sec	72°C	
Denaturation	30 sec	95°C	30 cycles
Hybridization	30 sec	57°C	
Elongation	30 sec	72°C	
Denaturation	30 sec	95°C	10cycles
Hybridization	30 sec	50°C	
Elongation	30 sec	72°C	
Final elongation	10 min	72°	

Table 3: Components of Agarose gel 3%.

8.1g	Agarose
270 ml	TBE buffer 1X
3 drops	BET

the mold was placed in the vessel, the samples (12 µl of the mixture) were poured into the wells, they migrated according to their molecular weight in a current of 300 volts for 40 minutes. The DNA bands were detected by Ethidium Bromide and visualized under UV radiation; molecular sizes were estimated by Gene marker express Ruler DNA Ladder 100 bp.

When an electric field through a gel of pH is neutral, the negatively charged phosphate groups of the DNA cause migration towards the anode (Westermeier, 1997). Photograph of each gel is formed by the imaging system to gel molecular biology.

II.6 Data Analysis:

Analysis of the electrophoretic profiles of the different PCR products was performed by visual inspection of electrophoresis runs. The presence of the fragments was determined visually and entered into a matrix of binary data 0 /1 (presence of tape as represented by 1/absence represented by 0) Matrices and set for different markers generated by different primer were analyzed using PAST software. A dendrogram was constructed from the matrix aggregation group on the basis of the method of unweighted UPGMA (Unweigheted Pair Groups Method of Analysis) average link.

III - Results and Discussion:

III.1 Extraction of genomic DNA of apricot tree by the CTAB method:

The CTAB technique (Doyle and Doyle 1990) is very efficient and commonly used to extract genomic DNA from plants by using the CTAB method in the apricot tree we could obtain a large amount of DNA with little protein contamination.

This technique gave a report DNA/protein extracted DNA more than 2, for all eight varieties of apricot tested, sign of a little contaminated DNA. The largest amount of DNA registered was 572.1 ng, extracted from leaf variety Louzi red, not far from two varieties Biche and Ben Sarmouk White with respectively 560.1 ng and 537.8ng, a quantity average in both Polonais and Ben Sarmouk Red varieties with respectively 297.2 ng and 198.2 ng, the remaining three varieties showed low values with 117.7 ng for Pavit Red and 116.5 ng for Laarbi and only 108.2 ng for Bulida (Table 4).

This classic CTAB method gave us an idea about the apricot varieties best suited to this technique, and the amount of DNA obtained refers to it.

Table 4: Quantity and quality of DNA extracted from leaves of eight varieties of apricot using the CTAB protocol (Doyle and Doyle 1990) modified.

Report 260/230	DNA concentration (ng/µl)	Variety
2.55	108.2	Bulida
2.01	572.9	Louzi Red
2.12	560.1	Biche
2.61	198.2	Ben Sarmouk Red
2.25	117.7	Pavit Red
2.03	537.8	Ben Sarmouk White
2.54	116.5	Laarbi
2.45	297.2	Polonais

Table 5: List of SSR primers, their sequences, repeat type, size of polymorphic band (P) and monomorphic (M).

Primer Name	Sequence (5'-3')	Size bands (bp)	Polymorphisme (%)	Number of alleles	
				M	P
BPPCT001	AATTCCCAAAGGAGTTGTATGAGCAGGTGAATGAGCCAAAGC	115	0	8	0
BPPCT004	CTGAGTGATCCTTTTGCAGGAGGGCATCTAGACCTCATTGTT	200 -210	22.22	7	2
BPPCT011	TCTGAGGGCTAGAGTGGGCTGTTTCAGGAGTCGAACAGC	200	0	7	0
BPPCT017	TTAAGAGTTTGTGATGGGAACCAAGCATAATTTAGCATAACCAAGC	75 – 130	81.25	3	13
BPPCT020	GGTGGATGGTCAAGATGCATTGACGTGGACTTACAGGTG	70 – 80	22.22	7	2
BPPCT032	TAAAGCCACAACATCCATGATAATGGTCTAAGGAGCACACG	100 – 630	71.42	2	5
UDP96001	AGTTTGATTTTCTGATGCATCCTGCCATAAGGACCGGTATGT	100 – 130	0	8	0
UDP98021	AAGCAGCAATTGGCAGAATCGAATATGAGACGGTCCAGAAGC	100- 140	54.54	5	6
UDP98408	ACAGGCTTGTTGAGCATGTGCCCTCGTGGGAAAATTTGA	350 – 550	44.44	5	4
UDP98410	AATTTACCTATCAGCCTCAAATTTATGCGATTTACAGACG	60 – 130	25	6	2
UDP98414	AAAAGGCACGACGTTGTTGAAGATTCAGATTGGGAATTTGCAG	100 – 340	75	2	6
			M : 36*M	60	40

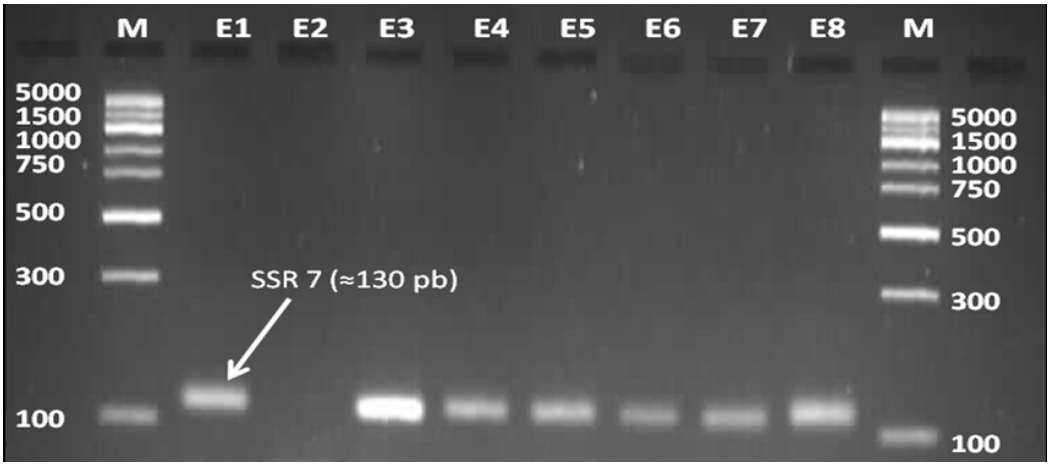


Figure 1: Checking the amplification of the marker (UDP69001) Agarose gel 3% for the eight varieties of apricots.

III.2 Characterization of SSR primers:

The primers used in number 11 belong to two families of primers (BPPCT and UDP), the following base sequences different, and each primer generated bands of different size base pair, which we will reveal the percentage of polymorphism for each primer. The results from each primer Agarose gel for all eight varieties are summarized in the table below (Table 5).

III.3 Molecular characterization of apricot varieties using SSR:

After DNA extraction of the eight varieties of apricot by the CTAB method we performed for the amplification of DNA by PCR by using 11 SSR primers to know the response of these eight varieties about these primers.

After PCR was carried out electrophoresis on Agarose gel 3%, these leaders responded positively to the appearance of a certain number of bands per primer, each band corresponds to the amplification of a DNA sequence.

A matrix evaluation was performed for each primer for the eight varieties tested to highlight different characteristics such as the number of alleles and the rate of polymorphism and that for each primer (Figure 1).

III.4. General characterization of SSR markers:

A total of 100 amplified DNA fragments were produced in eight varieties of apricots from 11 used primers. 40 of these fragments were polymorphic with a percentage of 40% and 60 fragments monomorphic with a percentage of 60%. The BPPCTO17 marker gave the highest rate of polymorphism with 81.25%, with five polymorphic alleles. The markers BPPCT004 and BPPCT020 are the least polymorphic with 22.22% and only one polymorphic allele.

Markers BPPCT001, BPPCT011, UDP96001 are not polymorphic; they are considered monomorphic, with 1 or 2 monomorphic alleles.

II.5 The multivariate analysis, Structuring groups:

The Factorial Correspondence Analysis (FCA):

The correspondence factorial analysis pointed to 3 groups:

-The first group is formed by Louzi Red, Ben Sarmouk Red, Pavit Red, Ben Sarmouk White, Laarbi and Polonais varieties, these varieties are genetically very similar.

-The second group, is formed by a single variety, Biche variety, but who is closer to the fist group as the third group.

-The third group, is formed by the Bulida variety, this variety is genetically far compared to other varieties

These results are based on the given indicator matrix by the absence or presence of band of each of 11

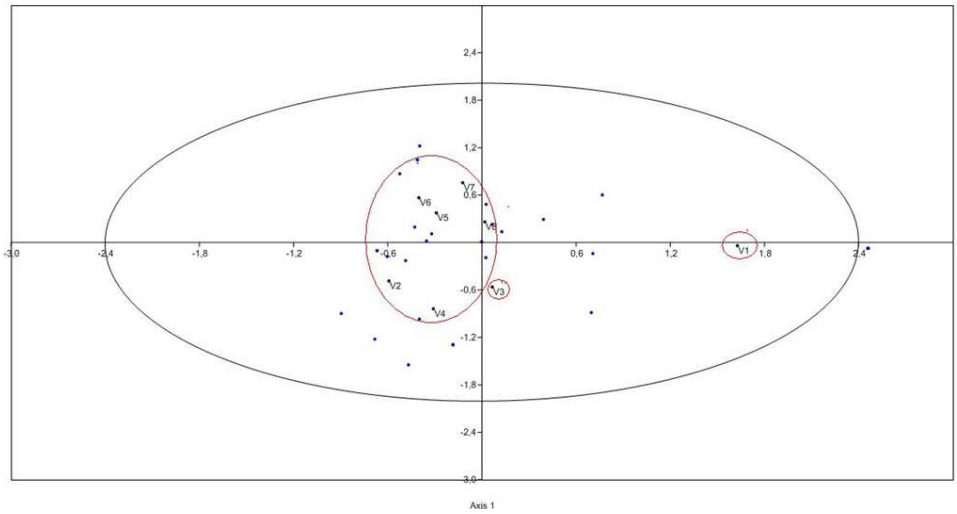
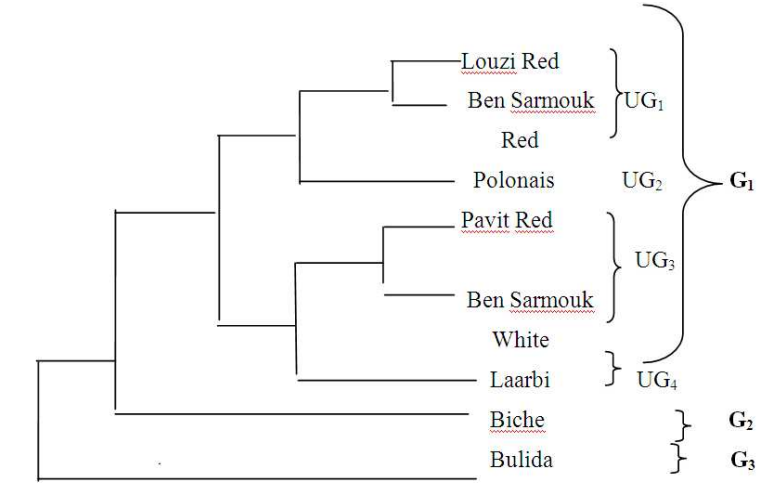


Figure 2: Pin 1 and 2 of the factorial Correspondence Analysis (FCA) of the diversity of the eight varieties of apricot by PAST.



UG: under Group, G: Group

Figure 3: Dendrogram generated by analysis of SSR markers.

primers and for each of the eight varieties of apricot trees (Figure 2).

III.6 The phylogenetic relationship between apricot varieties:

The eleven primers used in this analysis have generated a total of 100 polymorphic bands and they were used to establish a dendrogram of similarity. This dendrogram allowed us to study the genetic variability within eight tested apricot varieties. The principle of this method is to successively link the taxa that are closest according to the criterion of minimum evolution defined by Saitou and Nei (1987). A first step was to choose the closest taxa and link them by a node representing the common ancestor; the second step was to recalculate the distance between the node obtained and all other taxa (Penny 1982).

The dendrogram of similarity produced by SSR approaches was able to differentiate all the genotypes analyzed and classified into three groups of genotypes genetically close. The first group consists of four under groups, the first under group consists of varieties Louzi Red and Ben Sarmouk Red, the second under group is formed by the Polonais variety, in the third under group is formed by the varieties Pavit Red and Bensarmouk White, and the fourth under group consists the variety Laarbi, the second group is formed by the variety Biche and the third group is the last group is the Bulida variety (Figure 3).

IV-Conclusion:

In this study we investigated the genetic diversity using molecular markers of the SSR technique for eight genotypes of apricot tree. The deeply modified CTAB method was used to obtain, from the fresh leaves of apricot tree, a large amount of DNA ranging from 108.2 to 572.9 ng/µl. With average quality report D260/D280 ranging 2.01 to 2.62.

The analysis of eight varieties of apricot by SSR markers through the use of the 11 primers was eloquent, indeed the results of this method helped to significantly differentiate eight varieties.

In total 100 amplified DNA fragments were obtained in eight varieties of apricots, 40% of these fragments were polymorphic and 60% are monomorphic.

The size of the bands generated by the 11 primers in apricot is variable; it ranges from 60 bp to 360 bp.

This study allowed classifying the eight varieties into three groups of genotypes genetically very close.

The first group is formed by four under groups, the first under group is formed by the varieties Louzi Red and Ben Sarmouk Red, the second under group is formed by Polonais variety, the third under group is formed by Pavit Red and Ben Sarmouk White varieties, and the fourth under group is formed by Laarbi variety, the second group is formed by Biche variety, against by the third group is formed by Bulida variety.

This preliminary study needs to be continued on a larger range of varieties of apricot, using polymorphic primers, since it is desirable to use gel electrophoresis polyachrylamid kind to profile more intense and clear bands.

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