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Molecular comparison of some variety of apple (*Malus domestica* L.) "Douce Djerba" by ISSR and RAPD markers

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Abstract
In this study, two markers (RAPD and ISSR) were evaluated for potential use in fingerprinting and determination of the similarity degree between 3 accessions of apple "Douce Djerba" and 8 grafting between Douce Djerba and Anna.
A total of 4 ISSR primers was used and 28 polymorphic alleles were amplified. Four RAPD primers yielded a total of 20 bands, of which 4 (20%) were polymorphic. all accessions were easily distinguishable employing both methods. The similarity coefficient between accessions ranged from 0.692 et 0.923 for ISSR analysis and from 0.875 et 0.933 using the RAPD methodology.
This study indicates that the results obtained based on the RAPD, and ISSR techniques are not significantly correlated. The marker index, based on the effective multiplex ratio and expected heterozygosity, was calculated for both analyses (MI = 2.9 for RAPD and MI = 6.7 for ISSR assays). The ISSR markers were found to be useful for cultivar identification and assessment of phenetic relationships, revealing advantages, due to higher reproducibility, over other RAPD.

Keywords: apple; accession identification; Inter-Simple Sequence Repeats; germplasm; marker comparison; RAPD

Introduction
Apple (*Malus domestica* L.) is economically the most important fruit tree crop of the temperate zones, and a high number of commercial cultivars are available, as the result of open-pollinated seedlings, controlled crosses in breeding programs, and exploitation of naturally or induced somatic mutations in adapted cultivars. An accurate characterisation of the existing cultivars is essential to successful breeding programs, patent protection and nursery control [1].
Molecular markers have been replacing or complementing traditional morphological and agronomic characterisation, since they are virtually unlimited, cover all the genome, are not influenced by the environment and, particularly in the case of fruit trees with long juvenile period, can be less time consuming for the characterisation of new hybrids. Several molecular markers studies on apple have been published using techniques such as RFLP (Restriction Fragment Length Polymorphism) [2, 3], RAPD (Random Amplified Polymorphic DNA) [4, 5, 6, 7], AFLP (Amplified Fragment Length Polymorphism) [7], and SSR (Simple Sequence Repeats) [1, 8]. The increasing development and generalised use of a large number of methodologies during the last years, requires comparative studies in order to choose the best DNA marker technology to be used in fingerprinting and in diversity studies, considering reproducibility, costs, sensibility and level of polymorphisms detection. Molecular technique comparisons have become important because, depending on the objective of the study, one technique can be more appropriate than another, as well as different techniques being informative at different taxonomic levels. However, the congruence among the different molecular techniques began to be discussed with opposite results among authors. Several works report comparable results among different markers [9, 10], while other show considerable differences [11, 12]. A phenetic characterisation of forty-one apple cultivars, comparing RAPD and AFLP markers was reported previously [7].
Random Amplified Polymorphic DNA (RAPD) markers [13], and ISSR markers [14], are two molecular typing approaches that have been used to detect the variation among the plants. Each method has been used extensively to identify and determine the relationships at the species and cultivar levels [15]. ISSR analysis has been used for the cultivar identification in numerous plant species, including apple [7] and strawberry [16]. ISSR-PCR gives multilocus patterns which are very reproducible, abundant and polymorphic in plant genomes [14]. Comparison of ISSR and other PCR-based markers have shown their efficiency in plant breeding [15, 16].
In that study the results obtained were not correlated, and differences were observed between the UPGMA dendrograms originated from the two methods. However, these two techniques show some disadvantages regarding reproducibility. In fact, the use of short primers and low annealing temperatures makes RAPD markers extremely sensitive to the reaction conditions and therefore irreproducible among different laboratories.
This study has the purpose of investigating possible advantages of the use of two molecular methods (ISSR and RAPD), for identification and estimation
of phenetic similarities among accessions of apples.

Materials and methods
Plant material
In this work 3 accessions (*Malus domestica.*L*)* variety "Douce Djerba" and 8 grafting between "Douce Djerba" and "Anna" are selected from Institute of Dry Area from Médenine (South Tunisia) were used. The accessions numbers, country of origin are listed in Table 1.

Table 1: Apple accessions studied with their origin.

code	accession	origin
C1	Meski	Djerba
C2	Grifting (Meski X Anna)	Djerba
C3	Grifting (Meski X Anna)	Djerba
C4	Grifting (Meski X Anna)	Djerba
C5	Grifting (Meski X Anna)	Djerba
C6	meski	Medenine
C7	meski	tataouine
C8	Grifting (Meski X Anna)	Medenine
C9	meski	Zarzis
C10	Grifting (Meski X Anna)	Zarzis
C11	Grifting (Meski X Anna)	Zarzis

DNA isolation

Total DNA was isolated from fresh leaves as described by [17] with some modification. DNA concentration was determined by spectrophotometry at 260 nm and by 2% agarose gel electrophoresis.

ISSR analysis

The four ISSR primers used are listed in Table 2. Amplification reactions were carried out in volumes of 20 µl containing 30 ng template DNA, 1 unit of Taq DNA polymerase 0.25 mM each dNTP (Gibco BRL) and 1 µMprimer (Gibco BRL), in 1 × reaction buffer (50 mM KCl, 1.5 mM MgCl2, 10 mM Tris HCl pH 9.0). PCR reactions were performed in a Biometra thermal cycler under the following conditions: 4 min at 94 .C for initial denaturation, 40 cycles of 45 sec at 94 .C (denaturation), 40 sec at 52 .C (annealing) and 1 mn at 72 .C (extension), followed by 7 min at 72 .C for final extension of the single strands. ISSR amplified fragments were separated on 2% agarose gel, stained with ethidium bromide and visualised on UV. The gel was photographed using gel doc camera.

Table 2: ISSR primers used in this study.

primer	*Sequence of primer (5'- 3')*
I2	CTCTCTCTCTCTCTCTG
I3	TCTCTCTCTCTCTCTCA
I5	AGAGAGAGAGAGAGAGYT
I6	CTCTCTCTCTCTCTCTRG

RAPD analysis

RAPD analysis was carried out with 10 decamer random primers from Operon molecular for life (Table3). PCR amplifications were carried out also with 3 accessions. The primers that gave clear and polymorphic amplification patterns (4 primers) were used for further analysis with all the accessions. For each primer, a 20 µl amplification reaction contained: 100 ng of genomic DNA, 5 mM of $Mgcl_2$, 1U of Taq DNA polymerase and 4µl de buffer (Taq Buffer avec $(NH_4)_2SO_4$ 10X). PCR amplifications were performed in a gen-Amp PCR 9700 thermal cycler system. The PCR conditions included initial denaturation at 94°C for 5 min, followed by 45 cycles: denaturation at 92°C for 1 min, annealing at 50 °C for 2 min, extension at 72°C for 2 min with final extension at 72°C for 7 min.

Table 3: RAPD primers used in this study.

Primer	*Sequence of primer (5'- 3')*
OPE-01	-CCCGCCGTTG-
ORB-07	-CCAGCGTATT-
OPA-18	-AGTCGACCTT-
OPD-02	-CACCCCCTGC-

Data analysis

Clear and well-marked bands were coded in a binary form by '0' and '1', for absence or presence in each cultivar, respectively. For ISSR analysis, only fragments between 100bp and about 2000bp were scored, although a very high number of larger fragments existed. However, the proximity between these markers made them very difficult to score without errors.

The information content of each marker system was determined according to the indices of Powell *et al*. (1996): Effective Multiplex Ratio (number of polymorphic products from a single amplification reaction), Expected Heterozygosity (H = 1- pi2, where pi is the allele frequency for the ith allele) and Marker Index (the product of Effective Multiplex Ratio and Expected Heterozygosity). These calculations were performed for ISSR and RAPD data obtained in this work,

Similarity values were estimated based on the fraction of bands common to each pair of cultivars, according to[18], coefficient, and cluster analysis was performed to construct dendrograms, using the unweighted pair-group method with arithmetic averages (UPGMA) from the similarity data matrices.

The bands were considered reproducible and scorable only after observing and comparing them in three separate amplifications for each primer. Clear and intense bands were scored while faint bands against background smear were not considered for the further analysis. Band informativeness (Ib) and Resolving power (Rp) were calculated as given by Prevost and Wilkinson (1999). The formulae used for the above mentioned parameters are:

- Band informativeness of a given band: **Ib = 1 - (2 x 0.5-p)** where p is the proportion of the total genotypes containing the band.
- Resolving power of a primer: **Rp = ∑ Ib**

Results

Identification and evaluation of RAPD markers for diversity estimates in apples accessions

Out of 10, decamer random primers used for initial screening with three representative genotypes, while only 4 primers amplified polymorphic patterns. These primers were then used for RAPD analysis of all accessions. Amplification products yielded a total of 20 scorable bands, which 4 polymorphic (Table 4). The highest number of bands (8) was obtained with primer OPA-18, while the lowest number (3) was obtained with primer OPE-01.The 4 polymorphic primers exhibited variation with regard to average band informativeness (AvIb) and resolving power (Rp). The primer ORB-07 showed the lowest AvIb (0.22) while the highest AvIb of 0.63 and Rp (0.90) values were exhibited by the primers OPA-18.

Table 4: Polymorphism exhibited by RAPD primers.

Primer	TM (°C)	RAPD-PCR bands			Resolving Power (RP)	Average of informativeness bands (IB)
		Total	polymorphic	% of polymorphism		
OPE-01	45	3	1	33.33	0.72	0.34
ORB-07	45	4	0	0	0.81	0.22
OPA-18	45	8	2	25	0.90	0.63
OPD-02	45	5	1	20	0.36	0.59
Total		20	4	20	M= 0.69	M= 0.44

Identification and evaluation of ISSR primers for diversity estimates in apples accessions

A total of 10 primers consisting of di and tri repeat motifs were used for initial screening with 3 genotypes. Out of these, 6 primers gave no amplification at all, while only 4 primers were found to give clear and polymorphic patterns, and were subsequently used to analyze the entire set of 11 accessions. These ISSR primers amplified a total of 28 bands out of which 14 bands were polymorphic. These primers showed variation in the percentage of polymorphism, band informativeness (Ib) and resolving power (Rp). Average band informativeness (AvIb), is a measure of closeness of a band to be present in 50% of the genotypes under study and resolving power (Rp) is the sum of Ib values of all the bands amplified by a primer. The percentage of polymorphism is 50%, average Ib ranged from 0.15 to 0.81 while Rp ranged from 0.54 to 0.90 (Table 5). The primer I2 showed the highest values of average Ib (0.81) and Rp (0.90).

Table 5. Polymorphism exhibited by ISSR primers.

Primer	TM (°C)	Bands			Resolving Power (RP)	Average of informativeness bands (IB)
		Total	polymorphic	% of polymorphism		
I2	32	6	1	16.66	0.90	0.81
I3	32	5	5	100	0.76	0.15
I5	32	9	5	55.55	0.60	0.28
I6	32	8	3	37.5	0.54	0.31
Total		28	14	50	M=0.7	M= 0.38

Combined RAPD and ISSR analysis

The genetic similarity matrix data generated using RAPD and ISSR systems were compared. Mantel test for congruence of RAPD and ISSR data matrices indicated a bad of fit (r = 0.27158) indicating no good correlation between the two molecular marker systems (table 6).

Table 6: Comparison of the results obtained with ISSR and RAPD methodologies

PCR methodology	RAPD	ISSR
Number of primer used	4	4
Total number of bands scored	20	28
Number of bandes per primer set/primer	5	7
Number of polymorphic bands	4 (20%)	14 (50%)
Ranges in DNA diversity	0.31- 0.89	0.52-0.97

Similarity matrices were calculated independly from ISSR and RAPD data and the UPGMA- based dendrograms obtained are shown in figures1.

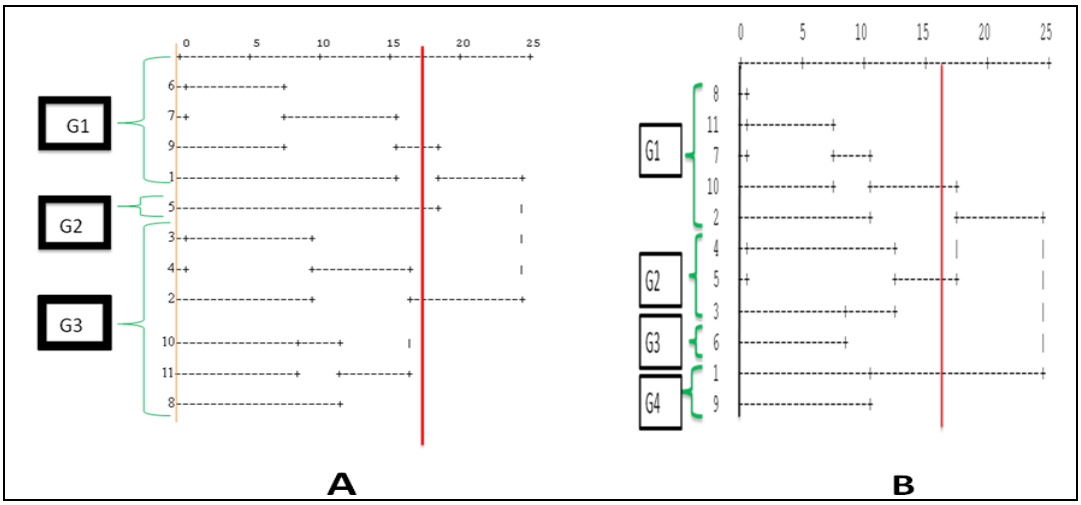

Fig 1: Dendrogram based en Nei & Li's similarity index, representing phonetic relationships among the 11apple accessions analysed by ISSR marker (A) and RAPD markers (B).

The phonetic classification obtained and illustrated in the dendrograms was not similar for the 2 methods. In the ISSR dendrogram the four accessions Meski (Douce Djerba) from distinct origin (Medenine, Djerba, Zarzis and Tataouine) are appeared in the same cluster, however in the RAPD dendrogram these accessions are not included in a same cluster whish represent a materiel geographically distinct form.

The ISSR marker showed higher expected heterozygosity than the RAPD, this indicates a higher percentage of band sharing in RAPD (table 7).Although the highest marker index was obtained with ISSR analysis sems to be a best marker due to it simplicity and higher reproducibility in apple.

Table 7: Comparison of the marker information obtained with RAPD and ISSR methodologies in apple.

	Effective multiplex ratio	Expected heterozygosity	Marker index
RAPD	7.4	0.58	2.9
ISSR	12.0	0.7	6.7

Discussion

Assessment of the genetic variability withen a cultivated crop has important consequences in plant breeding and the conservation of genetic resources. It is particulary useful in the characterisation of individual accessions and cultivars, in detecting duplications of genetic material in germplasm collections and as a general guide in the choice of parents for breeding hybrids [19]. In *Malus domestica* L traditional methods like horticultural traits are relatively less reliable and inefficient for precise discrimination of closely related genotypes [20]. Hence, selection based on genetic information using neutral molecular markers is essential as it is more reliable and consistent. Among different marker systems available, RAPD and ISSR markers became popular in diversity studies because of simplicity, rapid, inexpensive and applicable to any genome without any prior information regarding the genome of the plant.

In the present study, a set of 11 accessions of *Malus domestica* L were analyzed using 4 RAPD and 4 ISSR markers to describe the genetic structure among the accessions. The RAPD primers revealed 20% polymorphism with 5 polymorphic bands/primer, while ISSR primers revealed 50% polymorphism with 7 polymorphic bands/primer indicating wide genetic variation among the accessions. ISSR primers detect more polymorphism than RAPD primers because of variability in microsatellite loci due to DNA slippage [13].

The RAPD markers cover the entire genome in coding and non-coding regions including repeated or single-copy sequences, while ISSR markers disclose polymorphism from sequences between two microsatellite primer sites [21]. The ISSR method has been reported to be more reproducible [22] and produces more complex marker patterns than the RAPD approach reported that is an advantageous when differentiating closely related cultivars. Both the marker techniques provides a useful approach for evaluating genetic differentiation, significantly in those species that are poorly known genetically and are propagated vegetatively like monocot genus in Musa [23].

If the objective is assessment of phenetic similarities, high multiplex ratio markers are the more reasonable choice. Although the highest marker index was obtained with ISSR analysis sems to be a best marker due to it simplicity and higher reproducibility in apple. In this case, ISSR is advantageous over RAPD analysis due to its higher percentage of polymorphism. Furthemore, an easy and reliable transfer of information across laboratories is possible.

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