Which Molecular Markers are Best Suited to Identify Fig Cultivars: a Comparison of RAPD, ISSR and Microsatellite Markers

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Abstract

The efficiency of RAPD, ISSR and microsatellite markers for the molecular characterisation of 30 fig cultivars was determined by evaluating information content (expected heterozygosity), number of loci simultaneously analysed per experiment (multiplexing ratio), identification efficiency of a primer or locus (discriminating power). Microsatellite markers have the highest expected heterozygosity (0.64), while ISSR markers have the highest multiplexing ratio (2.85). According to discriminating power (which ranged from 0.186 to 0.961) and to optimal combination of primers necessary to identify all accessions analysed, ISSR and microsatellite markers are the most efficient tools for fig varietal identification. Advantages and limits of these molecular markers are discussed taking into account practical issues and effectiveness in analysing the genetic structure of fig germplasm.

Abbreviations: RAPD (random amplified polymorphism DNA), ISSR (intersimple sequence repeats)

INTRODUCTION

Varietal identification which is necessary for seed production and management of germplasm collections, is usually performed using morphological descriptors. In fruit species, pomological descriptors are the most discriminating characters and verifying the conformity of propagated material plant is very limited because of the absence of fruit. Despite of the discriminating power of pomological descriptors, several confusions of denomination remain unresolved, especially for fruit species used in traditional cultivation, because of the limited number of discriminating descriptors and the influence of environmental conditions on phenotypic expression. Such problems of genotype identification are encountered in fig tree which is an under-utilised fruit species. Because of traditional cultivation, fig orchards are mostly characterised by several, unknown cultivars. Furthermore, several cases of synonymy (several denominations for one genotype) and homonymy (several genotypes under one denomination) do occur. The limits of the use of morphological descriptors which are detailed in Roger & Khadari (submitted) could be overcome by using molecular markers. Advances in molecular biology have introduced a complementary toolto morphological description for genotype identification, DNA markers.

Based on the detection of polymorphism by assaying subsets of the total amount of DNA sequence variation in a genome, several molecular technologies are available and can be classified into two groups. The first one correspond to fingerprint markers which are not specific to one locus because of the use of arbitrary sequences (RAPD, AFLP, ISSR...) and

are dominant markers: the presence of a band correspond to heterozygotic (Aa) and to homozygotic individuals (AA). The second group corresponds to monolocus markers which are revealed by the use of a specific primer or probe (RFLP, SSR or microsatellites) and are co-dominant markers since a heterozygotic individual (Aa) can be differentiated from a homozygotic one (AA).

As a first step for exploring the usefullness of molecular markers in fig cultivar identification, we compared the information obtained by RAPD, ISSR and microsatellite analyses of 30 fig cultivars originated from two germplasm collections. Our aim was to select the most performing molecular technology for a reliable identification of fig cultivars as a complementto morphological descriptors. We compared the different marker systems for three parameters: information content (expected heterozygosity), number of loci simultaneously analysed per experiment (multiplexing ratio) and identification efficiency of a primer or locus (discriminating power). We discuss the advantages and limits of these marker systems taking into account practical issues and effectiveness in analysing the genetic structure of fig germplasm.

MATERIALS AND METHODS

Plant Material and DNA Extraction

The fig cultivars analysed are listed in Table 1. DNA was extracted from 100 mg of frozen material (leaves) according to Dneasy Plant Mini Kit (Quiagen) with the following modification: 1% of Polyvinylpyrrolidone (PVP 40,000) was added to buffer AP1.

Molecular Analysis

RAPD analysis was performed as described by Khadari *et al.* (1995) using the following primers purchased from MGW: A11, A16, A18, K17, X05, X09, Y04, Y11 and Z12. For ISSR analysis, 10 primers from MGW were tested on a subset of 5 fig cultivars and 4 were selected for the complete analysis: ISSR5 (CACACACACACACACACG), ISSR8 (GAGAGAGAGAGAGAGAGT), ISSR 9 (GAGAGAGAGAGAGAGACG) and ISSR 12 (CACACACACACACACATG) were used. The PCR amplification was performed in 10 mM Tris-HCl (pH 9,0), 50 mM KCl, 1.5mM $MgCl_2$, 0.1% TritonX100, 0.02% gelatin, with 200 µM of each dNTP, 1 µM of primer, 2 U of Taq DNA polymerase (Appligene-Oncor) and 200 ng of DNA in a final volume of 30µl. Amplifications were realised in a 96-weels thermocycler MJ Research PTC100 using the following conditions : initial denaturation at 94°C for 5 min., followed by 35 cycles (94°C 30 sec., 50°C 1 min, 72°C 1 min) and a final extension at 72° for 7min. The amplification products were electrophoresed on 2 % agarose gel at 120 volts followed by staining with ethidium bromide and photographed on Polaroïd 667 film under ultra-violet light. Microsaltellite analysis was performed as described by Khadari *et al.* (accepted) using the eight loci characterised in this study.

Data Analysis

For RAPD and ISSR data, polymorphic bands were scored as present (1) or absent (0) assuming that each band position corresponds to a locus with two alleles, presence and absence of the band, respectively. Expected heterozygosity He is calculated from the sum of the squares of allele frequencies (Nei, 1973): $He = 1 - \sum p_i^2$ where p_i is the allele frequency for the i-th allele. The arithmetic mean heterozygosity He (av) is calculated for each marker class: $He(av) = \sum He/n$ where n is the number of markers (loci) analysed. The effective multiplexing ratio E is calculated as the average of the number of polymorphic bands revealed per experiment for each marker class. The marker index MI is calculated as the product of the effective multiplex ratio E and the average expected heterozygosity He (av). The discriminating power is calculated as follows: $Dj = \sum p_i (Np_i - 1) / N - 1$ where p_i is the frequency of the i-th molecular pattern revealed by primer or locus j. Under the hypothesis of independence between markers, the probability of confusion of one individual with the molecular pattern i is calculated as the product of the allele frequency

for each marker of the pattern i: $Pi = \prod fj$.

RESULTS

For RAPD analysis, each of the 9 primers produced 1 to 5 markers with an average of 2.2 markers / primer (Table 3). The analysis of 30 fig cultivars using 20 RAPD markers allowed to distinguish 28 different banding patterns. Each of two accessions pairs had the same banding pattern: El Khal / Jeblia and Ambar El Khal / Dauphine. For ISSR analysis, the primers produced 4 to 11 markers with an average of 7 markers / primer (Table 3). All 30 fig cultivars were distinguished using 28 ISSR markers. The microsatellite analysis of 30 fig cultivars allowed to identify 2 to 6 alleles per locus with an average of 4 alleles / locus (Table 3). The use of 8 loci and 32 alleles allowed to distinguish all 30 fig cultivars.

Based on the average expected heterozygosity He (av), the highest levels of polymorphism are detected with microsatellite markers and the lowest with RAPD markers (Table 2). Taking into account the effective multiplexing ratio which is the measure of the number of polymorphic markers in the germplasm set analysed per experiment and the level of polymorphism, ISSR markers appear to be the most efficient tools in detecting polymorphism as showed by the highest value of the multiplexing ratio (Table 2).

The discriminating power for each primer or locus is given in Table 3. Except for RAPD primer Y04, the highest discriminating power is obtained with ISSR markers (ranging from 0.961 to 0.793) and the lowest with RAPD markers. For the microsatellite loci MFC1, MFC2, MFC3 and MFC6, the discriminating power is relatively high (ranging from 0.885 to 0.798). Taking into account the discriminating power and the confusion probability with a given molecular pattern, we have identified the optimal combination of primers or loci for each marker system to distinguish all the 30 fig cultivars with a confusion probability below $5.\ 10^{-3}$ (Table 4). The optimal combination is IMA 5 and IMA 12 for ISSR analysis and MFC1, MFC2, MFC3, MFC4, MFC6 and MFC7 for microsatellite analysis.

DISCUSSION

Previous studies have compared different marker systems in the analysis of genetic relationships between cultivars (Thormann et al., 1994; Powell et al., 1996). Our study focused on a comparison between fingerprint markers (RAPD and ISSR) and microsatellite markers for a reliable identification of fig cultivars in a complementary approach to morphological characterisation. Despite of the limited number of cultivars assayed, the analysis of the 30 fig cultivars originated from two germplasm collections (CBNM Porquerolles and Moroccan germplasm) allows to draw valid conclusions on large fig germplasm.

The use of 9 RAPD primers which correspond to 9 different experiments are not sufficient to distinguish all the 30 fig cultivars while only two experiments are sufficient for ISSR analysis. A reliable identification has been obtained using two ISSR primers (IMA 5 and IMA 12) with a low confusion probability under the hypothesis of independence between markers ($4.\ 10^{-4}$). These results are mainly explained by the effective multiplexing ratio since one ISSR experiment is equivalent to more than 3 RAPD experiments (Table 2). Consequently, ISSR markers are more efficient tools than RAPD markers for identification of fig cultivars. This comparison is based only on the effective multiplex ratio but it is valid because the two markers systems have similar expected heterozygosity. This is not true for comparison between microsatellite and fingerprint markers. One microsatellite experiment corresponds to one locus analysis while one experiment allows to analyse several loci for fingerprint methods. Conversely, several alleles can be identified for one microsatellite locus while only two alleles are recognised for fingerprint markers (presence and absence of band). For these reasons, comparison between microsatellite and fingerprint markers has to be based either on the effective multiplexing ratio or on the expected heterozygosity. Taking into account these two parameters, Powell et al. (1996) have proposed an estimate of marker utility, the marker index (MI). Microsatellite and RAPD markers have similar marker index values but contrasting results for fig identification. The use of 9 RAPD

primers is not sufficient to distinguish all the 30 fig cultivars while a reliable identification has been obtained using 6 microsatellite loci with a low confusion probability under the hypothesis of independence between markers (2. 10^{-3}). Consequently, the marker index seems to be insufficient for comparison between marker systems and results of fig cultivar identification have to be considered. To compare the efficiency of the markers in cultivar identification, Tessier et al. (1999) proposed the discriminating power which is an extension of the polymorphism information content available from the frequencies of the different banding patterns (Anderson et al., 1993). Based on either the marker index, the discriminating power and the optimal combination of primers, ISSR markers are the most efficient tool for fig identification but microsatellite markers are also suited for this application. These two marker systems have different molecular and genetic proprieties and can provide complementary information for fig identification and genetic analysis.

Although presenting the highestmarker index and discriminating power, ISSR markers as other fingerprint markers (RAPD, AFLP...) have some disadvantages related to reproducibility of results and genetic information. Because of the use of arbitrary sequences, examination of the same loci depends strongly on amplification conditions and problems of result repeatability can occurred between experiments. Such problems can be resolved by standardising experiment conditions in one laboratory but it is difficult to standardise analytical protocols between several laboratories in different regions or countries. Conversely, no problems of result reproducibility are encountered for microsatellite markers since each primer pair allows to amplify one locus which is previously well identified and characterised. Consequently, the same microsatellite analytical protocol can be used by different laboratories with comparable results. Because of the dominant markers, fingerprint methods provide limited genetic information. Parentage and genetic relationships between cultivars are analysed using co-dominant markers like microsatellite (Bowers et al., 1999). Moreover, to provide a background genetic knowledge for a rational management of genetic resources of fig germplasm, it is essential to analyse the domestication process and fig origin by analysing the genetic structure of cultivars in comparison with supposedly related natural populations. For such studies the use of nuclear co-dominant markers and cytoplasmic markers is required. In this context, microsatellite analysis of fig cultivars allows to identify different accessions but corresponds also to a first step in the investigation of the domestication process.

Our study showed that ISSR markers as a fingerprint method and microsatellites as co-dominant markers are complementary tools for a reliable fig identification.

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Tables

Table 1. List of the 30 fig varieties used in the study with indications of their collection origin

varieties	Collection origin
Violette de Soliès	CBNM Porquerolles
Sultane	CBNM Porquerolles
Douqueira negra	CBNM Porquerolles
Nefiach	CBNM Porquerolles
Col de dame noir	CBNM Porquerolles
Longue d'Août	CBNM Porquerolles
Abicou	CBNM Porquerolles
Pastilière	CBNM Porquerolles
Précoce ronde de Bordeaux	CBNM Porquerolles
Dauphine	CBNM Porquerolles
Dorée	CBNM Porquerolles
Jaune	CBNM Porquerolles
Brunswick	CBNM Porquerolles
Grise de la saint Jean	CBNM Porquerolles
Mtioui	INRA Ain Taoujdate
Hamra - 35	INRA Ain Taoujdate
Baouidi	INRA Ain Taoujdate
Ham Rhamam – 91	INRA Ain Taoujdate
Ambar El Khal – 21	INRA Ain Taoujdate
Nabout – 42	INRA Ain Taoujdate
Lamtel – 4	INRA Ain Taoujdate
Rhoudane – 24	INRA Ain Taoujdate
Fassi – 33	INRA Ain Taoujdate
El Khal – 84	INRA Ain Taoujdate
Jeblia – 8	INRA Ain Taoujdate
Filaliya – 34	INRA Ain Taoujdate
Oud Elma – 51	INRA Ain Taoujdate
Farkouch Jmel – 13	INRA Ain Taoujdate
Assal – 92	INRA Ain Taoujdate
Ournakssi - 5	INRA Ain Taoujdate

Table 2. Comparison of the average expected heterozygosity for polymorphic markers He(av), the effective multiplexing ratio E and the marker index MI for each marker class, calculated on the basis of experimental data obtained from 30 fig cultivars (Table 1).

Marker system	Number of primers or loci	Number of bands	He(av)	SD	E	MI
IMA	4	28	0.41	0.09	7	2.85
SSR	8	32	0.64	0.10	1	0.64
RAPD	9	20	0.37	0.12	2.22	0.82

Table 3. Primer discriminating power calculated on the subsample of 30 fig varieties

Primer or locus	Number of markers	Number of banding patterns	D (discriminating power)
IMA 5	11	23	0.961
IMA 12	9	13	0.961
RAPD – Y04	5	14	0.922
IMA8	4	13	0.908
MF-6C6	6	10	0.885
MF-4B12	5	11	0.844
MF-6E2	6	8	0.825
MF-4E12	4	8	0.798
IMA 9	4	7	0.793
RAPD – K17	3	6	0.784
MF-4B3	3	5	0.729
RAPD – A18	2	4	0.717
RAPD – X05	3	3	0.683
MF-6H2	2	3	0.605
RAPD – Z12	2	4	0.540
RAPD – A16	2	4	0.531
RAPD – A11	1	2	0.508
MF-6G8	3	4	0.434
RAPD – X09	1	2	0.370
MF-4F7	3	3	0.342
RAPD – Y11	1	2	0.186

Table 4. Efficiency of a primer / locus combination and confusion probability under the hypothesis independence

	Number of indistinguishable pairs		Confusion probability under the independence hypothesis
	Experimentally observed	Expected under the independence hypotheses	
IMA 5	18	17	
IMA 5 + IMA 12	0	0.4	$2.\ 10^{-8} - 4.\ 10^{-4}$
IMA 5 + IMA 12 + IMA 8 + IMA 9	0	0	$4.\ 10^{-10} - 7.\ 10^{-6}$
MFC6	50	50	
MFC6 + MFC3	9	7.8	
MFC6 + MFC3 + MFC1	3	1.4	
MFC6 + MFC3 + MFC1 + MFC2	1	0.3	
MFC6 + MFC3 + MFC1 + MFC2 + MFC7	0	0.7	$9.\ 10^{-9} - 6.\ 10^{-3}$
MFC6 + MFC3 + MFC1 + MFC2 + MFC7 + MFC4	0	0	$2.\ 10^{-9} - 2.\ 10^{-3}$
MFC6 + MFC3 + MFC1 + MFC2 + MFC7 + MFC4 + MFC8 + MFC5	0	0	$2.\ 10^{-11} - 7.\ 10^{-5}$
Y04	34	34	
Y04 + K17	9	7.3	
Y04 + K17 + A18	4	2.1	
Y04 + K17 + A18 + X05	3	0.6	
Y04 + K17 + A18 + X05 + A11	2	0.3	$8.\ 10^{-6} - 2.\ 10^{-3}$
Y04 + K17 + A18 + X05 + A11 + Z12 + A16 + X09 + Y11	2	0	$8.\ 10^{-6} - 2.\ 10^{-3}$