Microsatellite Markers as Reliable Tools for Fig Cultivar Identification

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ABSTRACT. Accurate and reliable cultivar identification of crop species is essential to ensure plant material identity for registration and for cultivar protection. In this article, we proposed six simple sequence repeat (SSR) loci as a sufficient tool to characterize fig (*Ficus carica* L.) germplasm in Morocco maintained in an ex situ collection. A set of 17 microsatellite loci was used to characterize 75 accessions representing eight caprifigs, 51 local accessions, 11 foreign accessions, and five accessions of unknown origin. Eighty-five alleles with a mean number of six alleles per locus were observed in 62 distinct genotypes. Suspected synonyms and homonyms were confirmed, some of which maybe resulted from somatic mutation. Based on genetic criteria, including linkage disequilibrium, discrimination power, and molecular criteria as polymerase chain reaction conditions of loci multiplexing, we proposed a key identification set using six microsatellite markers to discriminate all genotypes present in the ex situ collection. Our selected SSR loci set can be used for larger genetic studies of fig germplasm, and a similar approach can be adopted for other fruit species.

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The common fig is an emblematic and characteristic fruit species of the Mediterranean region. Its domestication may have been contemporary to cereal crops (Poacea) suggesting very early cultivation [9000 to 12,000 BCE (Kislev et al., 2006)]. In Morocco, the fig tree is one of the main fruit species and it is cultivated over wide areas (El Bouzidi, 2002). Fig is especially important in traditional agroecosystems such as oasis and mountain agroecosystems. Morphological descriptions of fig cultivars have been published as monograph in Algeria (Mauri, 1942), whereas such data for Morocco are still scarce. Despite the high potential of fig culture in Morocco, it remains

a low value-added crop. One of the main constraints is the lack of knowledge of plant material. Some studies on Moroccan germplasm characterization have been published recently (Ater et al., 2008; Oukabli et al., 2003).

Because fig is mainly cultivated in traditional agroecosystems, the orchards present diversified plant material with several cultivars per orchard. Such traditional production remains marginal with low average yield [i.e., less than 3 Mg·ha⁻¹ per year (Ouaouich and Chimi, 2005)]. Currently, the main goal in Morocco is to develop fig production by focusing on the establishment of modern orchards (5 to 8 Mg·ha⁻¹ per year) by improving the technology of drying and by ensuring the quality of products.

The Moroccan Institut National de la Recherche Agronomique (INRA) has to provide selected plant material for multiplication in nurseries as a starting point for the development of fig production (Oukabli et al., 2006). Concurrently, nurseries have to ensure the identity of plant cuttings. Until now, only morphological descriptors are in use to reach this goal. However, morphological descriptors are not adequate for identifying young plants produced by cuttings because discriminate characters are mainly associated with fruit traits. For this purpose, the use of DNA polymorphism is a complementary analysis for reliable fig characterization.

Isozymes were the first markers used for plant genotype characterization (Tanksley and Orton, 1983). However, the number of these markers is very limited and they display low polymorphism (few alleles per locus). Furthermore, in some cases, isozyme polymorphism is influenced by environmental conditions. Since then, new genetic markers based on DNA polymorphisms have been largely developed and used for germplasm characterization [restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and microsatellites or simple sequence repeat (SSR)]. RAPD and AFLP markers are dominant and present limited reproducibility (Jones et al., 1997). As a result of their high, locus-specific polymorphism, reproducibility, and transferability, microsatellites have become markers of choice for fingerprinting. The efficiency of the use of microsatellite markers for testing the identity of individuals has been demonstrated in several domains, including forensic science paternity test (Balding and Nichols, 1995) and traceability of food products such as tracking beef from stable to shop (Gaudy et al., 2000). Paternity analysis is also used in plants, for example, to identify pollen donors in an olive (*Olea europea* L.) grove (Mookerjee et al., 2005) and to determine the parental source of superior cacao (*Theobroma cacao* L.) seedlings (Schnell et al., 2005). Microsatellite analysis has also been used in the agrofood industry for identification of virgin olive oil (Pasqualone et al., 2004).

In Morocco, fig germplasm is maintained in an ex situ collection at INRA Meknes. It was established from prospecting Moroccan areas, especially in the north center (Oukabli et al., 2003). Using six SSR and eight intersimple sequence repeat (ISSR) loci, this germplasm collection was classified into 46 defined cultivars and four genetically heterogeneous ones (Khadari et al., 2004). Many synonyms (several denominations of the same genotype) and homonyms (several genotypes under the same denomination) were identified (Oukabli and Khadari, 2005). However, this study was limited to a small number (six) of microsatellite loci based on a combination of two types of

markers, codominant (microsatellites) and dominant (ISSR) that may be statistically linked. This study did not seek to optimize molecular techniques for efficient cultivar identification with a suitable cost analysis.

In the present study, we characterized the fig germplasm collection in Morocco using 17 SSR loci and we selected the most efficient markers to propose a molecular key for Moroccan fig identification. Our main objective was to provide efficient molecular tools to characterize fig germplasm in Morocco and to propose a useful approach for other fruit species.

Materials and Methods

Plant material

The Moroccan fig collection in the experimental domain of Aïn Taoujdate (INRA, Meknes) includes 75 accessions corresponding to 51 local common fig accessions, eight local caprifig (male fig) and 11 foreign accessions, and five accessions of unknown origin (Table 1).

Molecular analysis

DNA PREPARATION. DNA was extracted from 200 mg of young leaves according to the DNeasy Plant Mini Kit (QIAGEN, Courtaboeuf, France) with the modification of adding 1% polyvinylpyrrolidone (PVP 40.000) to buffer AP1.

MOLECULAR MARKERS. Among the developed SSR markers (Ahmed et al., 2007; Giraldo et al., 2005; Khadari et al., 2001; B. Khadari, I. Hochu, and S. Santoni, unpublished data), we selected 17 loci based on their polymorphism and ease of scoring following the screening of 16 distinct Mediterranean cultivars (B. Khadari, H. Achtak, and F. Kjellberg, unpublished data).

POLYMERASE CHAIN REACTION AND SIMPLE SEQUENCE REPEAT GENOTYPING. Polymerase chain reaction (PCR) amplification was performed in a total volume of 20 µL with a PCR buffer [10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100 (Perkin Elmer, Milan, Italy) and 0.02% gelatin], 2 mM MgCl₂, 0.2 mM of each dNTP, 1 to 4 pmol for dye-labeled primers [fluorescent phosphoramidites FAM, HEX (MWG Biotech, Courtaboeuf, France) or NED (Applied Biosystems, Courtaboeuf, France) at the 5' position], and 2 to 8 pmol for unlabeled, 1 U of Taq DNA polymerase (Sigma-Aldrich, Lyon, France), and 20 ng of fig genomic DNA. The PCR was carried out using a PTC 100 thermocycler (MJ Research, Waltham, MA). After 5 min at 94 °C, 35 cycles were performed with 30 s at 94 °C; 1 min at 50, 55, or 60 °C according to primer pair; 1 min at 72 °C; and a final extension step of 7 min at 72 °C. PCR products were diluted 1:20 and 2 µL was mixed with ROX standard formamide mixture [7.9 formamide + 0.1 µL Gensize 400HD (Rox Size Standard; Applied Biosystems)] and separated using capillary electrophoresis on an ABI prism 3710 XL automatic DNA sequencer (Applied Biosystems). Fragment size was determined automatically using the GeneMapper 3.7 software (Applied Biosystems). The allele sizes were transferred to an Excel spreadsheet (Microsoft Inc., Redmond, WA) for storage and genetic analysis.

Data analysis

LOCUS DIVERSITY. SSR data were scored as several alleles per locus distinguished by their size. Based on the identified genotypes, we computed the genetic diversity parameters as

Table 1. List of analyzed fig accessions maintained as ex situ collection in the Institut National de la Recherche Agronomique (INRA), Meknes, Morocco.^z

SSR profile	Accession	Skin color	SSR profile	Accession	Skin color
G01	Abiarous (65-3015) (tree 9-8) ^{uw}	Green	G28	El Khal (84-2283) (tree 11-3) ^t	Purple black
G01	Chbaa Ou Rgoud (10-2249)	Green	G29	El quoti Lebied (49-2263)	Light green
G02	Abiarous (65-3015) (tree 9-9) ^w	—	G30	El quoti Lezreq (62-2883)	Dark green
G03	Aboucharchaou (71-2395) ^w	Green	G31	Embar Lebied (7-2240)	Green
G04	Abrouki (90-2221) ^x	Purple	G32	Fassi (33-2267) ^v	Purple
G05	Ahra (63-2870) ^x	Green	G32	Noukali (31-2254) ^v	Purple
G06	Aicha Moussa (57-2208) ^x	Light green	G33	Ferquouch Jmel (13-2226)	Dark green
G07	Amtalaa Aarch (87-2210)	Purple black	G34	Filalia (34-2211)	Purple
G08	Aoud Elmaa (51-2217)	Dark green	G35	Ghadar El Arch (60-2213)	Dark green
G09	Aounq El Hmam (94-2876) ^u	Dark green	G36	Hafer El Brhel (44)	Dark green
G09	Rhoudane (24-2223) ^t	Purple black	G37	Hafer Jmel (58-2253)	Green
G10	Arouchi (85-2220)	Green	G38	Ham Rham (91-2866) ^{xv}	Purple black
G11	Assel (92-2890)	Yellow	G38	Jebli (8-2288) ^v	Purple black
G12	Azendjar (97-2113) ^w	Purple black	G39	Hamra (22-2225) ^t	Purple black
G13	Azougouar (102-2116) ^w	Dark green	G40	Hamra (35-2588) ^t	Purple black
G14	Beida (11-2256)	Yellow green	G41	Hayoul (83-2265) ^x	Dark green
G14	Embar El Khal (21-2247)	Brown	G42	Hmidi (52-2250)	Yellow
G15	Bioudi (1-2222) ^u	Purple black	G43	Kahoulia (32-2251)	Purple black
G15	Bioudi (64-2218) ^u	Purple black	G44	Mendar (56-2891)	Dark green
G15	Ferzaoui (93-2289) ^v	Purple black	G45	Mhadeq (36-2239)	Purple black
G15	Jeld Elhmar (89-2215) ^v	Purple black	G46	Mtioui (50-2893)	Dark green
G15	Ournakssi (5-2282) ^u	Purple black	G47	Nabout (42-2898)	Green
G16	Bioudi (53-2878) ^t	Yellow green	G48	Ournakssi (3-2280) ^t	Green
G17	Bioudi (61-2255) ^t	Green	G49	Reggoudi (16-2895)	Dark green
G18	Bioudi (66-2258) ^{tv}	Green	G50	Rhazzali (46-2884) ^w	Yellow green
G18	Tameriout (67-2400) ^{vw}	Green	G51	Rhoudane (25-2227) ^t	Purple
G19	Bouankirh (98-2397) (tree 2-4) ^{uw}	Purple	G52	Rhouli (82-2216)	Purple black
G20	Bouankirh (98-2397) (tree 2-5) ^w	Light green	G53	Sebti (4-2898)	Dark green
G21	Bourqui (48-2219)	Green	G54	Taranimt (72-2399) ^w	Yellow green
G22	Bousbati (2-2880) ^v	Green	G55	Amzin ^y	—
G22	Ournakssi (6-2214) ^{tv}	Green	G56	Doukar Tardif (5) ^y	—
G23	Chaari (95-2581) ^u	Purple	G57	Tchellah (3) ^y	—
G24	Chaari (96-2587)	Green	G58	Front deloued (3) ^y	—
G25	El Har (59-2261) ^v	Green	G59	Front deloued (4) ^y	—
G25	Tarlit (68-2398) ^{vw}	Green	G60	Kasbat Skhirat ^y	—
G26	El Hmiri (88-2224)	Purple	G61	Ouzidane (18) ^y	—
G27	El Khal (84-2283) (tree 11-1) ^{tv}	Purple black	G62	Titent Scourt (17) ^y	—
G27	Hamra (86-2252) ^v	Purple black			

^zEach accession is defined by its simple sequence repeat (SSR) profile number and characterized by fruit skin color.

^yCaprifig.

^xUnknown origin.

^wForeign accession.

^vSynonyms noted in the present study.

^uMislabelings noted in the present study.

^tHomonyms noted in the present study. The SSR profiles G16, G17, and G18 under ‘Bioudi’ denomination were distinct by eight to 20 alleles; G27 and G28 under ‘El Khal’ denomination were distinct by 16 alleles; G27, G39, and G40 under ‘Hamra’ denomination were distinct by 12 to 14 alleles; G22 and G48 under ‘Ournakssi’ denomination were distinct by two alleles; and G09 and G51 under ‘Rhoudane’ denomination were distinct by one allele.

allelic frequency (p_i), number of alleles per locus or allelic richness (N), observed (H_o), and expected heterozygosity under Hardy-Weinberg equilibrium [H_e (Nei, 1978)] using the software Genetix Version 4.05 (Belkhir et al., 2004). The Wright inbreeding coefficient (F_{is}) was computed according to Weir and Cockerham (1984) using GENEPOP 3.1 (Raymond and Rousset, 1995).

We computed the discriminating power of each SSR locus D as defined by Tessier et al. (1999): $D_j = \sum p_i [(Np_i - 1)/(N - 1)]$

where p_i is the frequency of the i -th molecular pattern revealed by locus j and N is the number of genotypes. The probability of two individuals sharing the same genetic profile by chance or probability of identity [PI (Paetkau et al., 1995)] was computed using IDENTITY 4.0 (Wagner and Sefc, 1999).

ESTABLISHMENT OF A CULTIVAR IDENTIFICATION KEY. To determine the number of microsatellite loci sufficient to distinguish each individual, we tested the pairwise linkage disequilibrium (LD) among the previously selected 17 loci by

computing the square of the correlation coefficient of allele pairs between two loci (r^2) using the Tassel software Version 1.9.0 (Bradbury et al., 2007). LD significance was assessed using a permutation test ($n = 1000$). Then, we resampled loci using a plot of genotypic diversity versus the number of loci in Multilocus Version 2.1.1 (Agapow and Burt, 2000). Finally, based on the predefined number of markers, we selected the best combination of SSR loci that were not in disequilibrium linkage and discriminated all genotypes. We built a dendrogram using the best combination of SSR loci and a proposed discrimination scheme based on shared allele proportion using the software MICROSAT (Minch, 1997) and the unweighted pair group method with arithmetic mean (UPGMA) algorithm using the software Clustering Calculator (Brzustowski, 2002). Based on the selected SSR combination and a dendrogram, we identified SSR alleles that characterized cultivars or closely related cultivars to establish a molecular identification key.

Results

SIMPLE SEQUENCE REPEAT POLYMORPHISM. The analysis of the 75 fig accessions using 17 SSR loci revealed 62 different genotypes and 85 alleles. The number of alleles ranged from two to nine alleles per locus with an average of five alleles. The highest number of alleles (nine alleles) was detected at the LMFC 30 locus, whereas the lowest number (two alleles) was obtained for the FSYC04 locus. Allele size varied from 104 bp at the locus MFC3 to 310 at the LMFC19 locus (Table 2). Allele frequencies ranged from 0.60 to 91.3 with an average of 20 (data not shown). Among the 85 alleles revealed by the use of

17 SSR loci, 18 were considered rare alleles because they were observed in only one or two genotypes. They were confirmed by examining their occurrence in the cultivated and spontaneous Moroccan populations (H. Achtak, M. Ater, and B. Khadari, unpublished data) and by reanalyzing the individuals displaying these rare alleles.

The expected heterozygosity (H_e) ranged from 0.11 at the locus LMFC32 to 0.76 at the locus MFC3. The observed heterozygosity (H_o) varied between 0.12 at the locus LMFC32 to 0.86 at the locus LMFC30 with a mean of 0.54 (Table 2). For the two loci MFC3 and LMFC19, the F_{is} value was positive with a significant probability of heterozygote deficiency (Table 2). The highest value of identity probability (0.73) was observed at the locus LMFC32 and the lowest (0.09) at the locus LMFC30 (Table 2). Based on the combination of 17 SSR loci, the cumulative identity of probability was 4.78×10^{-10} .

FIG CHARACTERIZATION. The 62 identified genotypes were classified into 54 female figs corresponding to 67 accessions and 54 denominations and eight caprifigs corresponding to seven denominations (Table 1). Each of the 39 cultivars was identified by a distinct and single genotype, whereas five cultivars were genetically heterogeneous. The most separated genotypes were distinguished by 31 alleles as the two accessions 'Abrouki' (90-2221) and 'Bourqui' (48-2219). The most similar genotypes were distinct by only a single allele as in the case of the following pairs of accessions: 'Embar El Khal' (21-2247)/'Embar Lebied' (7-2240); 'Fassi' (33-2267)/'Hamra' (22-2225); 'Mendar' (56-2891)/'Ferquouch Jmel' (13-2226); and 'Bioudi' (64-2218)/'Kahoulia' (32-2251). Of 1891 pairwise comparisons, only 35 were distinguished by one

Table 2. Locus name, fluorescent dye-labeled (HEX, FAM, and NED fluorochromes), size range of amplified fragments, genetic parameters in the Moroccan fig collection, number of profile defined by each locus (Profile), probability of identity (PI) and discriminating power (D).^z

Locus	Fluorescent dye	Size range	Genetic parameters					Profile	PI	D
			N	H_o	H_e	F_{is}	P			
LMFC30 (A) ^x _{ts}	HEX	231–261	9	0.86	0.74	–0.158	0.001	19	0.09	0.97
MFC2 (B) ^{wt} _s	HEX	154–170	6	0.66	0.64	–0.028	0.010	13	0.16	0.88
MFC3 (C) ^{wt} _s	FAM	104–138	8	0.72	0.76	0.050 ^a	0.000	13	0.10	0.85
FSYC01 (D) ^y _{ts}	FAM	117–169	7	0.54	0.52	–0.028	0.641	12	0.24	0.85
MFC1 ^w	HEX	177–195	4	0.66	0.65	–0.002	0.027	7	0.19	0.84
MFC9 (E) ^y _{ts}	FAM	190–211	6	0.52	0.59	0.113	0.161	9	0.24	0.78
LMFC19 ^x	HEX	296–310	7	0.32	0.38	0.161 ^a	0.010	11	0.40	0.78
MFC12 ^v	FAM	152–170	5	0.61	0.59	–0.024	0.006	7	0.24	0.74
LMFC28 ^x	NED	192–203	5	0.61	0.59	–0.020	0.935	11	0.23	0.73
MFC4 ^w	NED	218–224	3	0.50	0.46	–0.086	0.723	5	0.38	0.65
LMFC24 ^x	FAM	272–276	3	0.55	0.43	–0.276	0.060	5	0.39	0.64
LMFC32 ^x	FAM	197–223	5	0.12	0.11	–0.036	1.000	7	0.73	0.62
MFC8 ^v	HEX	167–177	4	0.66	0.47	–0.384	0.007	6	0.37	0.61
FSYC04 ^y	NED	181–183	2	0.57	0.50	–0.145	0.304	4	0.37	0.59
MFC11(F) ^y _{ts}	NED	181–203	5	0.49	0.51	0.054	0.049	9	0.28	0.58
LMFC34 ^x	HEX	224–247	3	0.63	0.51	–0.249	0.063	6	0.36	0.52
LMFC26 ^x	NED	224–236	3	0.15	0.14	–0.053	1.000	4	0.70	0.50

^zGenetic parameters include number of alleles (N), observed heterozygosity (H_o), expected heterozygosity (H_e), within population fixation index (F_{is}), Hardy-Weinberg probability test (P).

^yAhmed et al. (2007).

^xGiraldo et al. (2005).

^wKhadari et al. (2001).

^vB. Khadari, I. Hochu, and S. Santoni, unpublished data.

^aSignificant heterozygote deficiency.

^xCode of selected simple sequence repeat (SSR) loci for identification key.

^sSSR loci selected for the molecular identification key (Fig. 1).

to six alleles and of these 26 by one to three alleles (data not shown).

Cases of mislabeling were detected as 'Bioudi' (64-2218) and 'Ournakssi' (5-2282; Table 1). The vernacular name 'Bioudi' signified white fig, whereas the accessions analyzed were purple. 'Ournakssi' (5-2282) accession (purple color and spherical shape) is different from the two 'Ournakssi' accessions (green skin and pyriform shape) supposedly originating from cuttings from a single tree.

Synonyms were observed for seven genotypes (Table 1) with two denominations per genotype. For each case, fig accessions have similar pomological traits as 'Ferzaoui' (93-2289) and 'Jeld Elhmar' (89-2215) with a purple black skin and spherical fig shape. Five cases of homonymy were observed for which differences were limited in two cases to a single allele [Table 1; 'Rhoudane' (24-2223)/'Rhoudane' (25-2227) and 'Embar El Khal' (21-2247)/'Embar Lebied' (7-2240)]. Other cases were distinguished by two 'Ournakssi' (6-2214)/'Ournakssi' (3-2280) to 20 alleles 'Bioudi' (66-2258) and 'Bioudi' (61-2255). For each of the three accessions, 'Bouankirh' (98-

2397), 'El Khal' (84-2283), and 'Abiarous' (65-3015), two replicate trees were genetically different showing the occurrence of mislabelings (Table 1). Except for the cultivars Embar El Khal (21-2247), Beida (11-2256), and 'Embar Lebied' (7-2240), each phenotype as defined by fruit skin color was characterized by a specific SSR profile indicating a concordance between one phenotype—one genotype (Table 1). To classify fig cultivars, we removed caprifigs and mislabelings and constructed a dendrogram based on 52 distinct genotypes corresponding to 60 fig accessions and 53 denominations (Fig. 1; Table 1). This dendrogram gave a global image on fig cultivar classification, including homonyms and synonyms and also presumed somaclonal variants.

RESAMPLING OF MICROSATELLITE MARKERS AND KEY IDENTIFICATION BASED ON THE EFFICIENT COMBINATION OF SIMPLE SEQUENCE REPEAT MARKERS. The square of the correlation coefficient of allele pairs between two loci (r^2) ranged from 0.004 to 0.118 with an average of 0.023 (data not shown). Among the pairwise disequilibrium linkage, 95% gave an $r^2 < 0.05$ indicating that all microsatellite markers were associated

Fig. 1. Simple sequence repeat (SSR) identification key based on 52 fig genotypes corresponding to 60 accessions and 53 denominations maintained as an ex situ collection in the Institut National de la Recherche Agronomique (INRA), Meknes, Morocco, based on six microsatellite loci. A, B, C, D, E, and F indicate code of SSR loci (Table 2) followed by alleles. ^zsynonym, ^yhomonym, ^xputative somaclonal variant.

at random because no significant disequilibrium linkage was observed. To test whether the 17 loci were informative to distinguish all fig accessions, statistical resampling showed that six microsatellite loci were sufficient to ensure identification (data not shown). According to the discriminating power value for each locus, we tested different combinations starting with the most discriminating and adding one locus at each step. Two optimal combinations of primers were obtained to discriminate among all accessions. The first combination (LMFC30 + MFC2 + MFC3 + FSYC01 + MFC9) allowed discriminating all genotypes except one pair. Adding the locus MFC11 to the previous combination, we defined a second combination (LMFC30 + MFC2 + MFC3 + FSYC01 + MFC9 + MFC11) for distinguishing all genotypes (Table 3). Hence, the primer MFC11 is necessary to discriminate between the last pair of accessions 'Embar El Khal' (21-2247)/'Embar Lebied' (7-2240). Using this second combination, we observed a low probability of identity ($PI = 2.3 \times 10^{-4}$; Table 3).

Based on the selected SSR combination and UPGMA dendrogram of 52 fig genotypes, we established a molecular identification key by identifying SSR alleles that characterized cultivars or closely related cultivars (Table 1; Fig. 1). For instance, only allele MFC9-198 (E198) and allele MFC3-122 (C-122) are sufficient to distinguish between pairs of accessions 'Fassi' (33-2267)/'Hamra' (22-2225) and 'Bouankirh' (98-2397-tree 2-4)/'Mtioui' (50-2893), respectively. Using one of these two loci is sufficient to discriminate between these two pairs of accessions because they are genetically distinct (Fig. 1).

Discussion

The 17 SSR loci used in our study were selected following three molecular and genetic criteria: 1) clear florescent peaks; 2) nonambiguous scoring data; and 3) efficient detection of alleles in a large gene pool.

As a result of the phenotypic variation and the interaction between genotype and environmental conditions, morphological descriptors as proposed by the International Plant Genetic Resources Institute and Center International des Hautes Etudes Agronomiques Méditerranéennes (2003) are not sufficient to identify all fig cultivars, especially the genetically closely related ones. Furthermore, morphological descriptors cannot be used to control the genetic identity of young cutting plants produced by nurseries because most discriminate phenotypic traits are related to the fig fruit. Molecular identification of fig cultivars has been carried out using isozyme markers (Cabrita et al., 2001), RAPD (Cabrita et al., 2001; Galderisi et al. 1999; Khadari et al., 1995), and AFLP markers (Cabrita et al., 2001). Microsatellite markers are now widely used for molecular characterization in fruit trees: grape [*Vitis vinifera* L. (Tessier

et al., 1999)], peach [*Prunus persica* L. (Sosinski et al., 2000)], apricot [*Prunus armeniaca* L. (Hormaza, 2002)], cacao (Zhang et al., 2006), and olive (Charafi et al., 2008). A comparative study of RAPD, ISSR, and microsatellite markers for characterization of fig cultivation showed that ISSR and SSR markers are more informative than RAPD markers, but ISSR markers are less reproducible (Khadari et al., 2003a). These studies have confirmed the efficiency of SSR markers for fruit cultivar identification. They have been widely used for cultivar characterization and for genetic diversity studies of fig (Giraldo et al., 2008; Khadari et al., 2004; Saddoud et al., 2007).

Moroccan fig germplasm maintained as an ex situ collection presents a genetic diversity similar to that of other analyzed fig germplasm. For instance, the Mediterranean ex situ collection in the Porquerolles island, southern France, displays an average of six alleles per locus and 0.54 of observed heterozygosity (B. Khadari, H. Achak, and J.P. Roger, unpublished data). Giraldo et al. (2008) observed a low polymorphism within an ex situ Spanish collection with 3.9 alleles per SSR locus and a relatively low genetic diversity ($H_e = 0.38$). Analysis of European and Asian fig cultivars showed a similar level of diversity (number of alleles = 5.2 and observed heterozygosity = 0.44; Ikegami et al., 2009) as the Moroccan fig germplasm.

We noted accessions that were different by only one allele presenting similar pomological traits but different for skin fruit color as 'Embar El Khal' (21-2247) with brown color and 'Embar Lebied' (7-2240) with green color. Similar observations were noted in a Mediterranean ex situ collection with 'Col de Dame blanche' (white), 'Col de Dame grise' (gray), and 'Col de Dame noire' (black) (Khadari et al., 1995) and in Slovenian fig germplasm with 'Green Matalon' and 'Black Matalon' (Bandelj et al., 2008). Skin color distinction can result from somatic mutation arising during intensive propagation by cuttings as shown in the grape cultivars Pinot noir and Pinot blanc (Hocquigny et al., 2004). We also observed two homonyms with closely related genotypes displaying similar phenotypic traits as the 'Rhoudane' (24-2223)/'Rhoudane' (25-2227). Because these genotypes do not differ sufficiently to result from sexual reproduction, we suggest that somatic mutation on a microsatellite locus is the explanation for such closely related genotypes.

Synonyms and homonyms among fruit trees have been widely reported (Condit, 1955; Giraldo et al., 2008; Khadari et al., 2004; Saddoud et al., 2007). We noted several synonyms and homonyms in the ex situ Moroccan collection making it difficult to identify the reference of fig cultivars. How to define this is a crucial concern for the use of local genetic resources. Until we solve this problem, we can adopt the approach proposed by Khadari et al. (2003b) for olive: the genotype reference of a given cultivar corresponds to the one obtained for

Table 3. Efficiency of the optimal combination of simple sequence repeat (SSR) markers to discriminate all fig cultivars.^z

Combination (no.)	Locus combination	Genotypes not distinguished (no.)
1	LMFC30	51
2	LMFC30 + MFC2	27
3	LMFC30 + MFC2 + MFC3	10
4	LMFC30 + MFC2 + MFC3 + FSYC01	4
5	LMFC30 + MFC2 + MFC3 + FSYC01 + MFC9	1
6	LMFC30 + MFC2 + MFC3 + FSYC01 + MFC9 + MFC11	0

^zThe probability of identity PI for the SSR combination no. 6 is 2.3×10^{-4} .

several trees of different origins (collections, nurseries, orchards, prospecting and so on) presenting the same molecular profile and similar morphological characters. This approach is based on the complementarity between morphological and molecular information.

In the present study, the use of SSR loci and fruit skin color as a phenotypic trait allowed us to detect mislabelings and also somaclonal variants like 'Embar El Khal' (21-2247) and 'Embar Lebied' (7-2240) (Table 1). A complementary approach between molecular and morphological traits is essential to ensure plant material identity (Oukabli and Khadari, 2005).

To efficiently identify all local fig accessions, we proposed a set of only six SSR loci. This set was selected mainly based on the discriminating power of individual SSR markers. First, we verified the equilibrium linkage between markers and selected the most discriminating five loci. Finally, we added a sixth SSR locus to discriminate the few nondistinguishable pairs of genotypes. Because alleles of one locus are not linked to the alleles of other loci, the six SSR loci may potentially discriminate up to 492 genotypes, which is equivalent to eight times the Moroccan collection size. Using these six selected SSRs to characterize the Mediterranean ex situ collection in the Porquerolles island (France), we identified 269 genotypes out of 318 previously defined [$\approx 85\%$ of discrimination (B. Khadari, H. Achtaq, and J.P. Roger, unpublished data)]. Considering this potential for identification with a probability of identity by chance of 2.3×10^{-4} , the set of six SSR loci can be proposed as an efficient tool for identification of fig Moroccan collection and other larger fig gene pools.

Among the six proposed SSR loci, four were used to characterize other fig germplasm (Giraldo et al., 2008) indicating the clearness of the obtained pattern and the facility of scoring. Two multiplexed PCR amplifications are sufficient to analyze fig accessions using the proposed set of SSR loci. Taking into account the annealing temperature of primers and size alleles, we propose two triplex (FSYC01, LMFC30, MFC9 and MFC2, MFC3, MFC11) for PCR and genotyping with an automated sequencer. The efficiency of identification keys using microsatellites was reported in several work and plant species such as olive (Pasqualone et al., 2004) and apricot (Krichen et al., 2006), but also in fig cultivated in Tunisia (Saddoud et al., 2007). The latter study was based only on the screening of six SSR loci, whereas in the present study, we tested 17 SSR loci and selected the most six efficient markers.

What new insights does the present study give us into fig cultivar characterization? Previously, Khadari et al. (2004) characterized the same Moroccan fig collection using six SSR loci and eight ISSR primers and proposed the first molecular database for fig germplasm management. However, this study was limited to a small number of SSR loci, which were selected from a limited set of microsatellites developed in fig (Khadari et al., 2001) and the authors did not test the linkage disequilibrium between SSR markers. In the present study, we proposed a molecular identification key following several steps of marker selection and validation. First, using molecular and genetic criteria, we selected 17 SSR loci from a large set of primers (40 SSRs) developed by several research teams (Ahmed et al., 2007; Giraldo et al., 2005; Khadari et al., 2001; B. Khadari, I. Hochu, and S. Santoni, unpublished data). Second, based on the discriminating power of individual SSR markers and the equilibrium linkage between markers, we selected the best combination of SSR loci for fig cultivar

identification. Third, we noted a concordance between SSR profiles and distinct phenotypes as defined by fruit skin color. Finally, we showed the molecular characterization efficiency of the selected SSR loci combination by identifying 269 genotypes out of 318 previously defined in the Mediterranean fig collection in the Porquerolles island (B. Khadari, H. Achtaq, and J.P. Roger, unpublished data).

Eventually, the molecular identification key we propose may serve as an efficient tool to characterize fig cultivars and to verify the identity of plant material proposed by nurseries. It could also serve as a useful tool for the management of fig genetic resources.

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