Molecular Characterization of Moroccan Fig Germplasm Using Intersimple Sequence Repeat and Simple Sequence Repeat Markers To Establish A Reference Collection

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Abstract. A study was conducted to identify genotypes present in a Moroccan fig germplasm collection and provide the first database for a reference collection in northern Morocco. In total, 75 fig samples were analyzed using 8 intersimple sequence repeat primers and 6 simple sequence repeat loci. From these samples, we identified 72 fig genotypes. In genetically heterogeneous cultivars, genotypes under the same denomination were distinguished by both molecular markers and pomological traits. Molecular analysis was used to classify the germplasm into 46 well-defined cultivars and 6 caprifig trees. The remaining genotypes were not clearly identified due to three cases of mislabeling and four cases of homonymy. No evidence was found for the occurrence of geographically widespread genotypes.

Common fig, Ficus carica L., is one of the most ancient cultivated Mediterranean fruit (Zohary and Hopf, 2000). In northern Morocco, fig cultivars are highly diversified and provide a large array of genotypes (Oukabli et al., 2003). However, due to numerous cases of synonymy (several denominations for the same genotype) and homonymy (several genotypes under the same denomination), pomological characterization is insufficient to establish reference genotypes for fig breeding programs in the region. Molecular markers are needed to accurately identify fig genotypes and define a complete reference collection.

Literature Cited

De Masi et al., 2003; Elisiario et al., 1998; Khadari et al., 1995), but because of the use of arbitrary short sequences as primers and the relatively low annealing temperature, these markers cannot be exchanged among laboratories according to standardized protocols (Jones et al., 1997). To overcome this limitation, we previously compared data obtained by RAPD, intersimple sequence repeat (ISSR), and SSR analyses of 30 fig cultivars and showed that SSR and ISSR markers are complementary tools for a reliable fig characterization (Khadari et al., 2003c).

In the present study, ISSR and SSR markers were used to characterize local accessions in a Moroccan fig collection and provide a molecular database for fig breeding.

Plant material. A Moroccan fig collection located at the Ain Taoujdate experimental station (INRA Meknès, Morocco) was used for study. The collection contained 72 local accessions and consisted of 54 cultivars and 8 caprifig (male) trees collected from northern Morocco and Algeria (Table 1). We defined an accession as one or several fig trees under the same denomination and registration number. In some cases, several accessions were classified under the same denomination (e.g., ‘Bioudi’ denomination in Table 1). Molecular characterization was performed on one tree per accession except for three accessions, ‘Abiarous’ (65-3015), ‘Bouankirh’ (98-2397) and ‘El Khal’ (84-2283), in which two trees were analysed because distinct pomological traits were noted among trees within these accessions.

Molecular analyses. DNA was extracted from 100 mg of frozen leaf material using the Dneasy Plant Mini Kit (Qiagen) following manufacturer recommendations with the exception that 1% polyvinylpyrolidone (PVP 40 000) was added to the AP1 buffer.

For ISSR analysis, four previously selected primers (IMA5, IMA8, IMA9, and IMA12; Table 2; Khadari et al., 2003c) and four new primers were used (IMA303, IMA834, UBC818, and UBC841; Table 2). Following a test for polymorphism and for consistent and reproducible DNA amplification on five fig cultivars, these new primers were selected from seven primers obtained from MGW. Reproducibility in ISSR patterns was verified by three different DNA amplifications from a set of 10 accessions. The PCR amplification was performed in 10 µL Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl2, 0.1% TritonX100, 0.02% gelatin, with 0.2 µM of each dNTP, 10 pmol of primer, 2 units of Taq DNA polymerase (Appligene-Onco), and 50 ng of DNA in a final volume of 25 µL. Amplifications were carried out in a 96-well thermocycler (model PTC100,MJ Research) using the following conditions: initial denaturation at 94 °C for 5
Table 1. List of fig accessions analyzed.

| Denomination | Accession |
|--------------|-----------|
| Abbarous*    | 65-3013 (trees 9-8 and 9-9) |
| Abouacharhou*| 71-2305    |
| Abrouki*     | 90-2221   |
| Ahra*        | 63-2870   |
| Aicha Moussa*| 57-2208   |
| Antalaa Aarch*| 87-2210 |
| Aoud Elmaa*  | 51-2217   |
| Aounq El Hmam*| 85-2220 |
| Aounq El Hmam*| 94-2876 |
| Arouchi*     | 85-2220   |
| Assel*       | 92-2251   |
| Azendarj*    | 97-2113   |
| Azougouar*   | 102-2116  |
| Bioudi       | 1-2222, 53-2878, 61-2225, 64-2218, 66-2258 |
| Beida*       | 11-2256   |
| Bouankirh**  | 98-2397 (trees 2-4 and 2-5) |
| Bouquetir*   | 48-2219   |
| Boussabi*    | 2-2880    |
| Chaaari*     | 95-2881, 96-2587 |
| Chbaa Ou Rgoud*| 10-2249 |
| El Har*      | 59-2261   |
| El Hmiri*    | 85-2220   |
| El Khal*     | 84-2283 (trees 11-1 and 11-3) |
| El quoti Lebed*| 49-2263 |
| El quoti Lezreq*| 62-2883 |
| Embark El Khal*| 21-2247 |
| Embark Lebed*| 7-2240    |
| Fassi*       | 33-2267   |
| Ferquouch Jmel*| 13-2226 |
| Fertaaou*    | 93-2289   |
| Filalia*     | 34-2211   |
| Ghadar El Arch*| 60-2213 |
| Hafer El Brhel*| 44-     |

Notes:
- *Two trees were analyzed.
- †Local cultivar clone.
- ‡North African cultivars.
- §Well-defined cultivars and caprifi gs.
- ¶Local caprifi g clone.

Results

Polymorphism and genomic diversity. In total, 38 alleles were obtained using 6 SSR loci. The number of alleles per locus varied from 3 (loci MFC4 and MFC7) to 14 (MFC6 locus) with an average of 6.3 alleles per locus (Table 2). Observed heterozygosity ranged from 0.175 for the MFC7 locus to 0.900 for the MFC6 locus with an average for all loci of 0.557. Compared to the expected heterozygosity value, a significant heterozygote deficiency was observed for MFC1, MFC2, MFC3 and MFC7 loci, probably due to a selection effect that would have occurred within a limited gene pool (Table 2). The 8 ISSR primers selected for polymorphism and for clear bands revealed a total of 37 markers ranging from 2 (primer IMA303) to 8 (primer IMA5), with a mean value of 4.6 markers per primer (Table 2). The highest frequencies were obtained for the UBC841-350 ISSR marker (97%) and for the MFC4-219 SSR allele (99%). The lowest frequencies (about 1%) were observed for 7 alleles belonging to the MFC2, MFC3, and MFC6 SSR loci and for the IMA303-900 ISSR marker.

Analysis of 75 reactions using 75 polymorphic bands (38 SSR alleles and 37 ISSR bands) revealed 72 different molecular patterns. Of the genotype pairs, 98% were differentiated by 10 to 33 polymorphic bands, among 2,586 pairwise comparisons, only 5,6 and 7 molecular profile pairs were distinguished by 1, 2, and 3 polymorphic bands, respectively (Fig. 1). The distribution of genetic differences in pairwise comparisons showed a bimodal distribution with a main mode centered at about 21 to 22 differences and a minor mode centered at about 2 to 3 differences (Fig. 1). Under the hypothesis of non-linkage between markers, the probability of obtaining a given molecular pattern was very low and ranged from 1.03 x 10^-12 for ‘Filalia’ (34-2211) to 2.87 x 10^-5 for ‘Amzin’ (caprifi g 18-6). The use of ISSR markers alone enabled us to identify 72 molecular profiles, although the probability of obtaining a given pattern was much higher than when using all markers (ranging from 3.90 x 10^-5 to 0.060). In contrast, 18 genotype types were not distinguished using SSR markers alone.

The discriminating power of each ISSR primer or SSR locus used in this study varied from 1.2 to 0.944 (Table 2). The value obtained for the ISSR primer IMA303 was close to null because the markers were detected in only three genotypes. Discriminating power variation among primers or loci was due to either the number of markers per primer or the number of alleles per locus, and also to marker frequencies. Primer IMA5 was more discriminating than the MFC6 and MFC3 loci because the Fst of IMA5 markers varied from 10% to 86% while most of the MFC6 and MFC3 alleles (14 among 22 alleles) were <6%. Seven combinations of primers and/or loci were tested according to their discriminating power in order to identify the most efficient combination for genotype discrimination. Starting with the most discriminating, one or several primers were added for each successive combination. Combination n° 5 (IMA5 + MFC6 + IMA9 + UBC841 + MFC3) discriminated all genotypes except two (IMA8-350 for ‘Bioudi’ (64-2218)/’Ourakssi’ (5-2282) and IMA12-580 for ‘Bosbati’ (2-2880)/’Ourakssi’ (6-2214)) with the probability of obtaining a given genome <10^-5.

Identity of cultivars. In three cases, ‘Abiarous’ (65-3015), ‘Bouankirh’ (98-2397), and ‘El Khal’ (84-2283), two replicate trees of the same accession had different genotypes, which indicates they were mislabeled (Table 3). For ‘Abiarous’ (65-3015), the genotype of one of the trees corresponded to the accession labeled ‘Chbaa Ou Rgoud’ (10-2249). In two other cases, different accessions with the same...
denomination had different genotypes, and one of the genotypes (i.e., ‘Bioudi’ 1-2222 and ‘Ournakssi’ 5-2280) was identical to that of another accession (i.e., ‘Jeld Elhmar’ and ‘Bousbati’, respectively).

Several cases of homonymy were identified in ‘Bioudi’, ‘Chaari’, ‘Hamra’, ‘Ournakssi’, and ‘Front d’eloued’ (Tables 1 and 3). Pairwise comparisons showed that genotype pairs from the same denomination were differentiated by 12-28 markers, except for three pairs distinguished by only one or two ISSR markers and no SSR markers (Table 3). There were also 11 accession pairs from the same SSR genotype distinguished by only 1 to 3 ISSR markers (Table 3).

SSR and ISSR analyses enabled us to classify the germplasm into 52 molecular profiles corresponding to 46 well-defined cultivars and 6 caprifigs (Table 1). Variety assignment was unclear in the 20 remaining due to either mislabelling or homonymy.

Relationships among fig genotypes. Pairwise similarity among the 72 genotypes varied from 0.114 to 0.967 with an average of 0.415 ± 0.108. Phenetic analysis showed four clusters, A, B, C, and D, which included 4, 4, 2, and 62 genotypes, respectively (see Fig. 2 at the following website: http://cbnm.free.fr/Docs_divers/figuiers.doc). Pair-wise similarity among the 8 caprifig clones (in cluster D) varied from 0.357, 0.344, and 0.379, respectively. Accessions ‘Chbaa Ou Rgoud’ (10-2249), ‘El Hmiri’ (88-2224), and ‘Tameriout’ (67-2400) (in clusters B and C) contained specific markers. Some accessions under the same denomination were very similar, including ‘Bioudi’ (64-2218)/’Bioudi’ (1-2222), ‘Ournakssi’ (3-2280)/’Ournakssi’ (6-2214), and ‘Rhoudate’ (24-2223)/’Rhoudate’ (25-2227) (Table 3; also see Fig. 2 at http://cbnm.free.fr/Docs_divers/figuiers.doc).

Discussion

SSR and ISSR markers are reliable and complementary tools for fig characterization (Khadari et al., 2003c). Using these markers, five cases of mislabelling were found among 72 Moroccan fig accessions; three occurred within accessions ‘Abiourou’ (65-3015), ‘Bouankirh’ (98-2397), and ‘El Khal’ (84-2283) in which three cases of mislabelling were also identified as different genotypes (Khadari et al., 2003c). Using these markers, distinct fig genotypes under the same denomination were clearly distinguished in the present study. For example, four of five accessions with ‘Bioudi’ denomination were differentiated by 12 to 28 markers and belonged to different genetic clusters. These four accessions were also identified as different genotypes using pomological descriptors such as skin color, fruit and fruit stalk shape, ostiole size, and skin cracks (Oukabli et al., 2003). In contrast, no pomological differences were observed among the following three pairs of accessions distinguished by only 1 to 2 ISSR markers: ‘Bioudi’ (64-2218)/’Bioudi’ (1-2222), ‘Ournakssi’ (6-2214)/’Ournakssi’ (3-2280), and ‘Rhoudate’ (24-2223)/’Rhoudate’ (25-2227) (Table 3). Validity of discriminating ISSR markers, which were present in several other fig genotypes, was verified by repeating the amplifications and molecular analysis three times. These results strongly suggest that each of these pairs corresponds to a single original clone and that 1 or 2 distinct

![Fig. 1. Frequency distribution of genetic dissimilarity for all pairwise combinations among 72 Moroccan fig genotypes.](http://cbnm.free.fr/Docs_divers/figuiers.doc)
ISSR bands correspond to somatic mutations, which may indicate ancient cultivars. Indeed, the minor mode observed in the distribution of genetic differences in pair-wise comparisons would also indicate somatic variation within clones (Fig. 1). This explanation may also apply to the 11 accession pairs distinguished by only 1 to 3 ISSR bands, which led to misclassification and display the same molecular pattern, they should be considered as a reference genotype for the cultivar. This rule was proposed by Khadari et al. (2003a) to establish reference genotypes for olive germplasm in France. Conversely, choosing one or several genotypes as reference for a genetically heterogeneous cultivar should also take into account discriminative pomological traits, as well as the relative importance of cultivation among the clones.

The present study provides the first molecular database for Moroccan fig germplasm management. Among 50 fig cultivars examined (46 well-defined and four genetically heterogeneous), the germplasm displayed diverse plant material and appears to represent a major component of the Moroccan fig genetic resources. Future collection and introductions must include molecular characterization and genetic diversity evaluation of the fig collection in CNBMP. Acta Hort. 605:77–86.

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