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ACTA BIOLOGICA CRACOVIENSIA Series Botanica 53/2: 7–14, 2011
DOI: 10.2478/v10182-011-0019-y

USING MORPHOLOGICAL CHARACTERS AND SIMPLE SEQUENCE REPEAT (SSR) MARKERS TO CHARACTERIZE TUNISIAN FIG (*FICUS CARICA* L.) CULTIVARS

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Received February 11, 2011; revision accepted October 30, 2011

We used simple sequence repeat markers and 25 morphological characters to characterize 18 Tunisian fig (*Ficus carica* L.) cultivars. Morphological traits suggested a high level of variation in the germplasm. Principal component analysis (PCA) differentiated the studied cultivars. In the derived dendrogram the cultivars clustered independently of their geographical origin and sex of trees. Simple sequence repeat (SSR) markers were used to compare genetic polymorphism with the observed phenotypic variation. Using six microsatellite primers, 39 alleles and 59 genotypes were identified. The high values of polymorphism information content (PIC), ranging from 0.67 to 0.85, confirmed the effectiveness of microsatellite analysis for determining molecular polymorphism and characterizing the studied cultivars. Multilocus genotyping unambiguously distinguished all the cultivars. The ability of each type of feature to differentiate cultivars of this crop is discussed.

Key words: Cultivars, *Ficus carica* L., morphological characters, microsatellites, Tunisia, polymorphism.

INTRODUCTION

The common fig (*Ficus carica* L.) is a gynodioecious species with bisexual trees (functional male caprifigs and unisexual female trees) with 2n = 26 chromosomes (Storey, 1977). It is probably the oldest domesticated crop; we know that it was grown 11,400 to 11,200 years ago at Gilgal I, an early Neolithic village in the lower Jordan Valley (Kislev et al., 2006). It is clonally propagated by cuttings or sexually reproduced from seeds. Sexual reproduction is characterized by mutualism between the caprifig and the fig wasp (*Blastophaga psenes*), which pollinates the female fig trees (Kjellberg et al., 1987). Due to its high adaptability to severe conditions of soil and climate, this crop is well developed in Mediterranean Basin countries. In Tunisia, where the fig is ubiquitous, a large number of local cultivars have been identified recently (Rhouma, 1996; Mars et al., 1998). These are designated by farmers mainly on the basis of fruit color and flavor. The local germplasm is therefore subject to problems of homonymy and synonymy. In addition, severe genetic erosion due to biotic and abiotic stresses is threatening this crop. Strategies to preserve the local fig germplasm need to be elaborated. For this purpose and to identity useful genotypes for breeding programs, variation within and between accessions needs to be assessed. Evaluation of genetic variation within cultivated crop species is central to plant breeding strategies and genetic resource conservation (Dean et al., 1999; Simioniuc et al., 2002). Morphological and agronomic characters are useful in surveys of plant species diversity, but these characters are highly influenced by environmental conditions. To overcome this, a large array of molecular markers is increasingly used to assess genetic polymorphism. Among these, microsatellites are attractive markers which have proved reliable in genetic diversity studies and for fingerprinting (Gupta and Varshney, 2000). Together, morphological and molecular data give a more precise picture of diversity in animals or plants (Cregan et al., 1999; Tanaka et al., 1999; Goulão et al., 2001). Such a combination offers a tool for assessment of germplasm resources to use in future breeding programs for Tunisian fig cultivars.

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PL ISSN 0001-5296

TABLE 1. Names and origin of the 18 Tunisian fig cultivars used in this study

Origin	Cultivar
◈ Testour	Bither arbi
	Soltani
	Zidi
❖ Utique	Marsaoui
	Zidi
● Raf Raf	Bither souri
	Bither arbi
▶ Kerkennah	Abiadh
	Dhokkar*
	Baghli
▪ Gafsa	Dhokkar*
	Sawoudi
	Assal boudchiche
	Soltani
	Gaa Zir
	Khadhouri
	Mlouki
	Bither abiadh

Here we report the use of morphological characters and molecular markers (SSRs) to characterize a set of fig ecotypes in the Tunisian germplasm.

MATERIAL AND METHODS

PLANT MATERIAL

The 18 cultivars used in this study (Tab. 1) were sampled in orchards in northern Tunisia (Testour, Utique, Raf Raf) and the Kerkennah Islands, or were provided from the collection of Commissariat Régional au Développement Agricole (CRDA) Gafsa.

MORPHOLOGICAL CHARACTERS

The morphological characters we used (Tab. 2) are standard fig descriptors (IPGRI and CIHEAM, 2003; Chatti et al., 2004a). The collected data were subjected to principal component analysis (PCA) and canonical discriminant analysis (CDA) using SAS software (Statistical Analysis System, ver. 6.07; SAS, 1990), in order to examine the structure of morphological variation and to estimate distance between cultivars. A UPGMA dendrogram based on Mahalanobis distances was drawn with TreeView software (Page, 1998).

DNA EXTRACTION AND SSR ASSAYS

The plant material consisted of young leaves sampled from adult trees and frozen in nitrogen liquid until use. DNA was extracted from 1 g plant material according to Dellaporta et al. (1983), yielding 100–150 µg.

We used six microsatellite primers (MFC2, MFC3, MFC5, MFC6, MFC7 and MFC8) identified by Khadari et al. (2001) in *Ficus carica*. PCR amplifications were performed as described by Saddoud et al. (2005). The amplified banding patterns were first checked on 2% agarose gels visualized with ethidium bromide staining under UV light. SSRs were then resolved on non-denaturing polyacrylamide gels (10%) and revealed by ethidium bromide staining according to Sambrook et al. (1989).

Genetic polymorphism in each region was evaluated by the mean number of alleles, allele frequencies, and observed (H_{obs}) and expected heterozygosity (H_{exp}) (Nei, 1978) using Genetix ver. 4.04 (Belkhir, 2001). Polymorphism information content

TABLE 2. Morphological parameters chosen from descriptors for fig *Ficus carica* L. (Ipgri and Ciheam, 2003; Chatti et al., 2004a)

Parameter referring to	Abbreviation	Character
Growth descriptors (13)	Lbg	Terminal bud length
	Dbg	Terminal bud width
	Rbg	Terminal bud length/width ratio
	Lr2	shoot length 2005
	L21	shoot first internode length 2005
	D21	shoot first internode width 2005
	L22	shoot second internode length 2005
	D22	shoot second internode width 2005
	Lr3	shoot length 2006
	L31	shoot first internode length 2006
	D31	shoot first internode width 2006
	L32	shoot second internode length 2006
	D32	shoot second internode width 2006
Leaf descriptors (12)	Lc	length of central lobe
	Sb	length of lateral sinus
	L/W	length / width
	Lat1	length of lateral lobe 1
	Lat2	length of lateral lobe 2
	Lat3	length of lateral lobe 3
	Lat4	length of lateral lobe 4
	LaF	Leaf width
	LoF	Leaf length
	Lpf	Petiole length
	Dp	Petiole width
	Rp/f	Length of stalk/ length of leaf

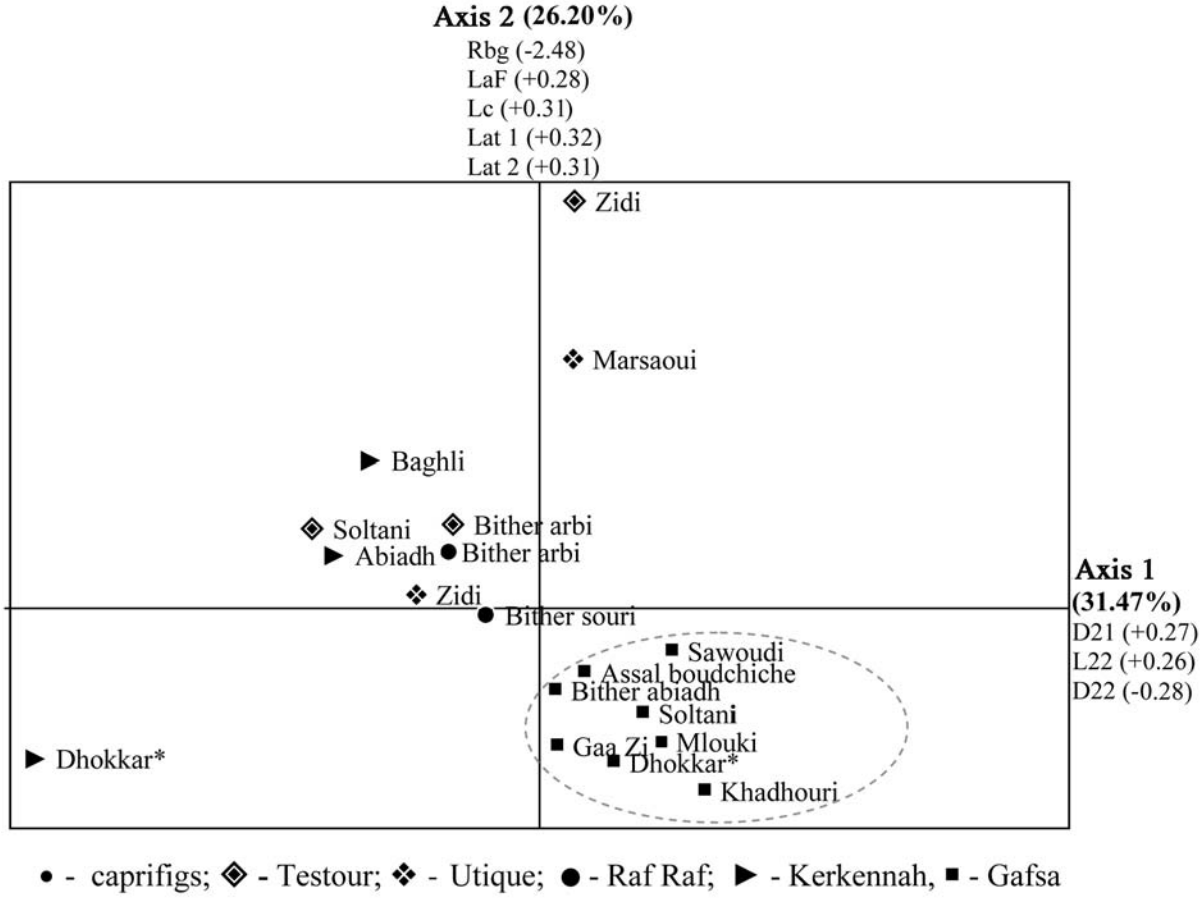

Fig. 1. PCA scatterplot of 18 fig cultivars (caprifigs and edible figs), based on morphological parameters (growth and leaf descriptors).

(PIC) was calculated according to Powell et al. (1996):

$$PIC = 1 - \sum_{i=1}^{k} fi^2$$

where k is the total number of alleles detected for a given marker locus and *f*i is the frequency of the allele in the set of genotypes investigated. Genetic distance between cultivars as well as between defined geographic groups was estimated according to Cavalli-Sforza Edwards (1967). Dendrograms were constructed by the unweighted pair group method with arithmetic averaging (UPGMA). Cluster analysis used PHYLIP ver. 3.57c (Felsenstein, 1995) and the dendrograms were drawn with TreeView ver. 1.5 (Page, 1998). To evaluate the correlation between morphological characters and molecular markers the Mantel test was performed using Pearson coefficients (Mantel, 1967).

RESULTS AND DISCUSSION

PHENOTYPIC CHARACTERISTICS

Morphological variation among the fig accessions was estimated by principal component analysis of the characters described above. PCA showed that 71.07% of total variation was explained by the first three axes. This result is in agreement with Chatti et al. (2004a), who measured fewer morphological parameters and found high variation of Tunisian fig germplasm. The first axis explained 31.47% of total variation and was positively correlated with shoot first internode width 2005 [D21 (+ 0.27)], shoot second internode length 2005 [L22 (+ 0.26)] and shoot second internode width 2005 [D22 (- 0.28)]. The second axis, explaining 26.20% of total variation, was highly correlated with terminal bud length/width ratio [Rbg (-2.48)], leaf width [LaF (+0.28)], length of central lobe [Lc (+0.31)] and length of lateral lobe 1 [Lat 1 (+0.32)]. The PCA scatterplot showed morphological divergence of the Gafsa cultivars, which clustered together (Fig. 1).

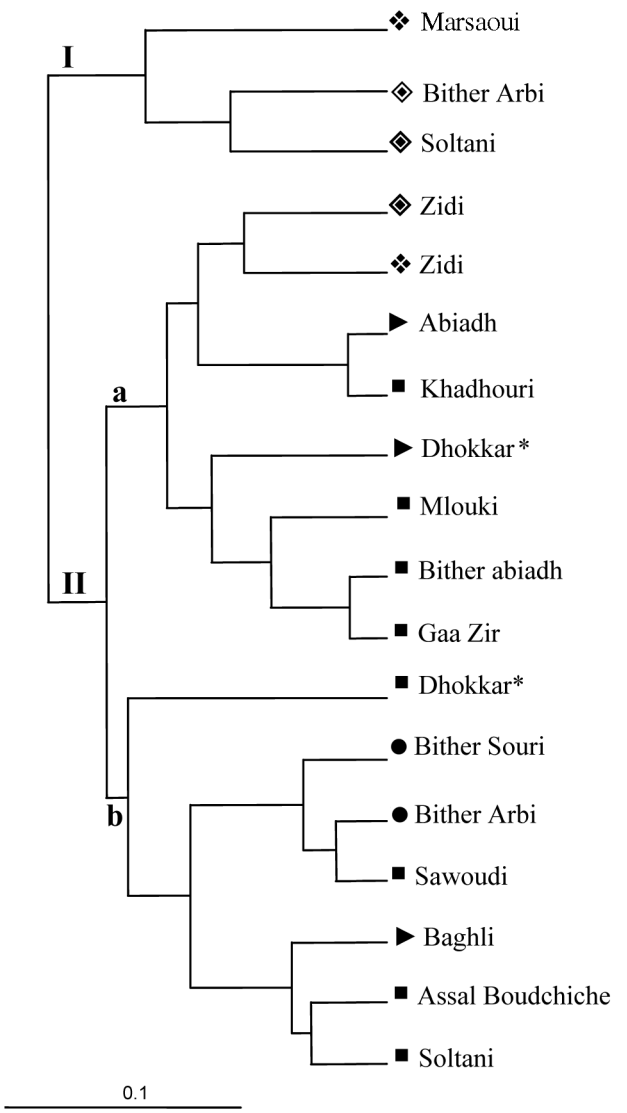

Fig. 2. Dendrogram generated by UPGMA cluster analysis showing the relationships among 18 accessions, based on Mahalanobis distance.

They had the highest values of shoot first internode width 2005, shoot second internode length 2005, leaf width, length of central lobe and length of lateral lobe 1, and the lowest values of terminal bud length/width ratio and shoot second internode width 2005. Two caprifigs were separated by the first axis. Dhokkar from Kerkennah differed from the remaining cultivars by shoot second internode width 2005. The second axis separated two additional cultivars: Zidi 5 (Testour) and Marsaoui (Utique) were characterized by leaf width, length of central lobe and length of lateral lobe 1. Except for Abiadh (Kerkennah) and Bither Souri (Raf Raf), the cultivars from the north were separated from the cultivars from the south on the scatterplot. The

TABLE 3. Polymorphic information content, number of alleles, number of genotypes, length of alleles and expected (H_{exp}) and observed (H_{obs}) heterozygosity for the six microsatellite loci examined to study genetic diversity among Tunisian fig cultivars

Locus	MFC2	MFC3	MFC5	MFC6	MFC7	MFC8	Mean
Allele number	7	7	6	5	6	8	6.5
PIC	0.80	0.83	0.79	0.67	0.84	0.85	0.79
Hexp	0.78	0.80	0.77	0.66	0.82	0.81	0.77
Hobs	0.76	0.41	0.64	0.70	0.76	0.64	0.65

clustering of cultivars on the scatterplot clearly demonstrates the influence of geographic origin on morphology. Chatti et al. (2004a) could not arrive at such a finding because the cultivars they studied belonged to one region of Tunisia, the Sahel. Zidi is one of the most valued varieties in Tunisia, but the Zidi cultivars from different regions were clearly separated by the first axis. Soltani (Gafsa) and Soltani (Testour) were clearly separated by both the first and second axes. This last result suggests homonymy, since the two cultivars bear the same name but are morphologically differentiated. Chatti et al. (2004b, 2007) pointed out the problem of synonymy for the common cultivars Soltani and Kahli. They present similar RAPD, ISSR and RAMPO banding profiles. Generally, homonymy and synonymy is a problem in fig culture, affecting germplasm conservation and complicating the task of listing.

To assess the relationships between the studied cultivars, Mahalanobis distances based on growth characters and leaf parameters were estimated and converted to a dendrogram (Fig. 2). The obtained matrix exhibits distances ranging from 0.02 to 0.92 (mean 0.17). These distances indicate high morphological variation within the local fig germplasm. The shortest distance (0.02) was for cultivars of southern Tunisia (Kerkennah and Gafsa). The most closely related accessions were Gaa Zir (Gafsa) and Abiadh (Kerkennah), Gaa Zir (Gafsa) and Bither Abiadh (Gafsa), Khadhouri (Gafsa) and Bither Abiadh (Gafsa), and Khadhouri (Gafsa) and Abiadh (Kerkennah). These cultivars present similar phenotypic traits. Khadhouri (Gafsa) and Soltani (Gafsa) were the most distant (0.92). The analyzed accessions grouped into two major clusters. The first contained Marsaoui, Bither arbi and Soltani. In the second group the cultivars fell into two subgroups (a, b). The Gafsa cultivars were grouped in the second cluster (Fig. 2). Cultivars from the North were clustered together except for the two Zidi accessions.

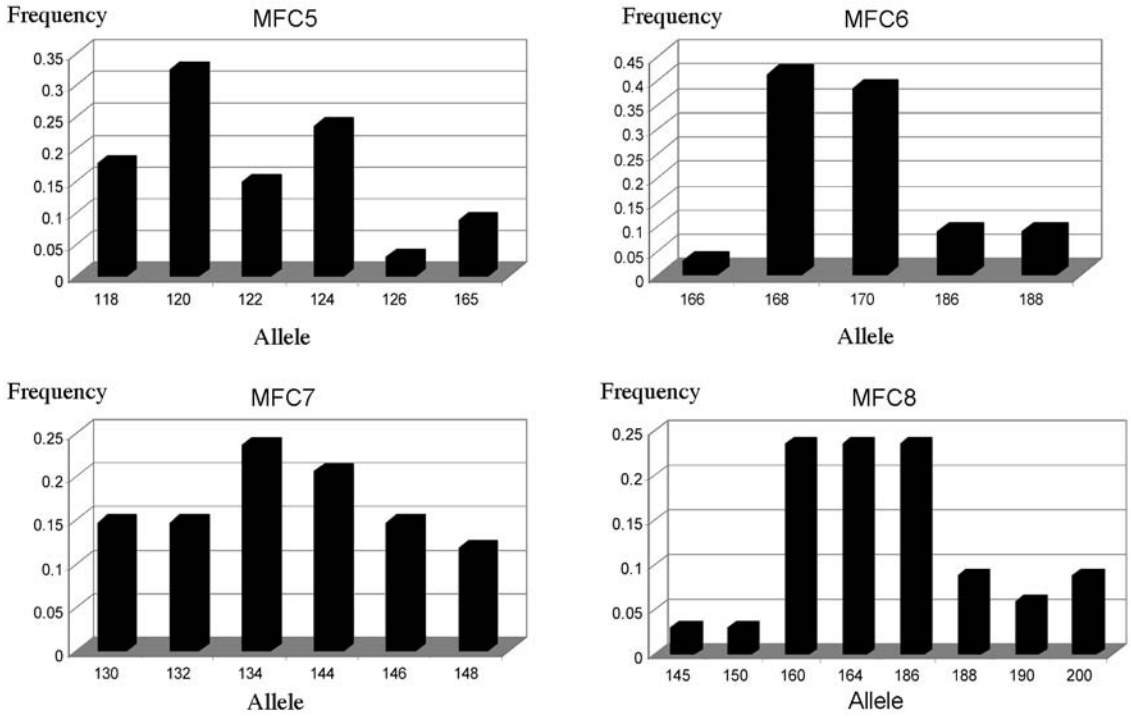

Fig. 3. Distribution of allele frequencies for the six microsatellites studied in fig cultivars.

POLYMORPHISM AND SSR PATTERNS

We used microsatellite markers to improve the diversity assessment. A total of 39 alleles were detected at 6 SSR loci (average 6.5 alleles per locus). The number of amplification products per primer pair varied from 5 for locus MFC6 to 8 for locus MFC8. The size of the amplified fragments ranged from 118 to 228 bp. The six SSR markers generated a total of 57 genotypes. The PIC values ranged from 0.67 for MFC6 to 0.85 for MFC8 (Tab. 3). The H_{obs} values scored for all loci ranged from 0.41 (MFC3) to 0.76 (MFC2, MFC7), with an average of 0.65, suggesting that the Tunisian germplasm is characterized by a high level of polymorphism at the DNA level (Tab. 3). Heterozygosity expected from Hardy-Weinberg equilibrium was higher than observed heterozygosity. Multilocus heterozygosity was also lower than expected(H_{obs} = 0.65, H_{exp} = 0.77) (Tab. 3). This is in agreement with microsatellite marker results for other Tunisian cultivars (Saddoud et al., 2007).

All markers showed high polymorphism information content, demonstrating the usefulness of microsatellite markers for assessing genetic polymorphism in fig cultivars. Allele frequencies ranged from 0.029 for locus MFC2 allele 170, to 0.41 for locus MC6 allele 168 (Fig. 3). Allele 166 (MFC6) is specific to Dhokkar (Kerkennah). Alleles 170 (MFC2) and 126 (MFC5) were amplified only in Baghli (Kerkennah).

Cavalli-Sforza Edward's genetic distances were estimated and used to draw a dendrogram representing clusters of fig cultivars (Fig. 4). Two groups were observed: the first was represented by Soltani (Testour) and the second grouped the remaining cultivars. The tree clustered the cultivars independently of the origin and sex of the trees. This suggests typically continuous variation of the fig germplasm, and seems in line with the monoecious origin of *Ficus*, which has evolved into two gynodioecious forms (Machado et al., 2001). Similar data have been reported for Tunisian figs in studies using other molecular markers such as RAPD, ISSR, RAMPO and AFLP (Chatti et al., 2004b; Salhi-Hannachi et al., 2006, 2007; Baraket et al., 2009).

Based on the multilocus genotype of fig cultivars, we constructed an identification key for precise discrimination of fig cultivars (Fig. 5). It should prove useful in describing, registering and certifying plant material, as well as for managing conservation of the Tunisian fig germplasm.

CORRELATION BETWEEN GENETIC MATRICES (MANTEL TEST)

The Mantel test confirmed the disparity between morphological variation and genetic polymorphism of these cultivars. A negative and not significant correlation between morphological characters and

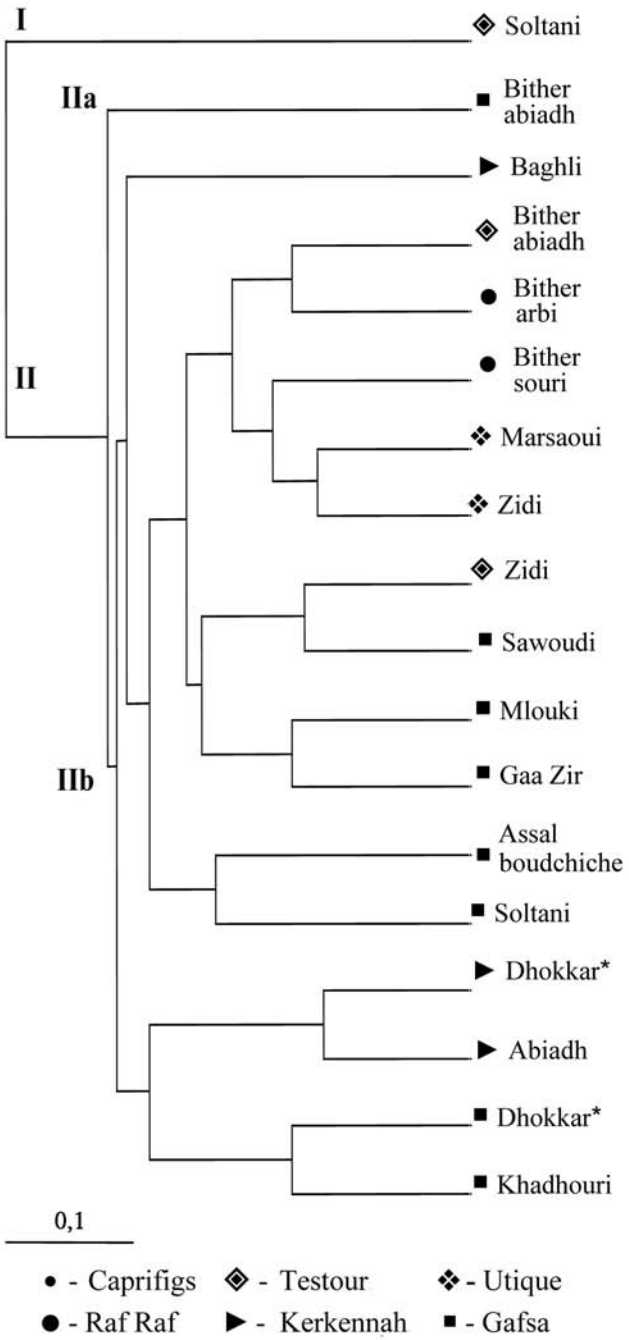

Fig. 4. Dendrogram obtained from microsatellite data for the 18 fig cultivars.

molecular markers (-0.031, p = 0.7) was found. In line with this, the grouping of fig cultivars differed between the dendrogram based on morphology and the one based on microsatellite genetic distance. Morphological characters are highly influenced by environmental conditions. Microsatellites are environment-neutral; they clustered the cultivars on the basis of divergence between genotypes.

We used morphological features and molecular markers to assess the diversity of Tunisian fig cultivars sampled from north to south. The two types of features contribute differently to diversity evaluation. Fig germplasm is easy to characterize morphologically, but microsatellite markers allow precise genotyping and give a picture of genetic diversity independent of geographic origin. Both morphological characters and SSR markers provide important information about the diversity of fig germplasm. Together they provide a powerful tool for future agricultural and conservation tasks.

ACKNOWLEDGEMENTS

This research was supported by grants from the Ministère de l'Enseignement Supérieur, de la Recherche Scientifique et de la Technologie

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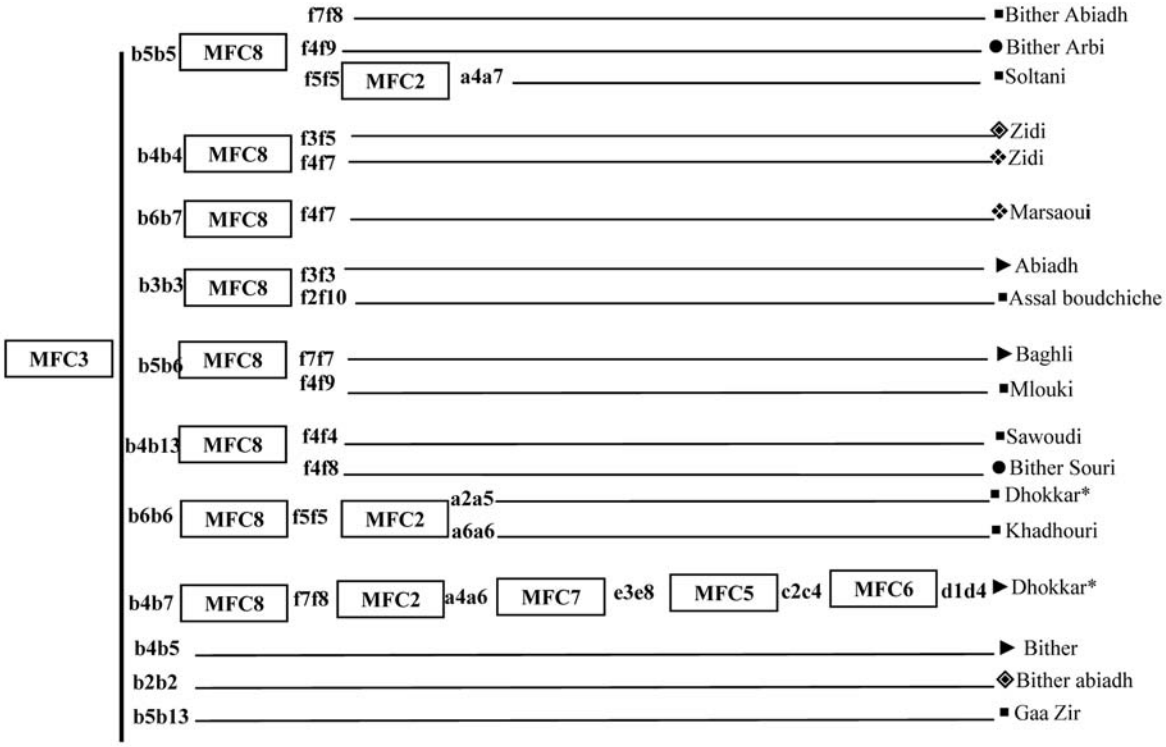

Fig. 5. Identification key for 18 fig cultivars based on multilocus genotypes.

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