Genetic diversity of different Tunisian fig (*Ficus carica* L.) collections revealed by RAPD fingerprints

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The genetic diversity in Tunisian fig (*Ficus carica* L.) was studied using RAPD markers. Thirtyfive fig cultivars originating from diverse geographical areas and belonging to three collections were analysed. Random decamer primers were screened to assess their ability to detect polymorphisms in this crop. Fortyfour RAPD markers were revealed and used to survey the genetic diversity and to detect cases of mislabelling. As a result, considerable genetic diversity was detected among the studied *F. carica* accessions. The relationships among the 35 varieties were studied by cluster analysis. The dendrogram showed two main groups composed of cultivars with similar geographic origin. Moreover, the male accessions (caprifigs) were clustered indistinctively within the female ones, suggesting a narrow genetic diversity among these accessions. Our data proved that RAPD markers are useful for germplasm discrimination as well as for investigation of patterns of variation in fig. Since this designed procedure has permitted to establish a molecular database of the reference collections, the opportunity of this study is discussed in relation to the improvement and rational management of fig germplasm.

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Fig (*Ficus carica* L.) is a fruit crop species that is particularly well suited for the different environmental conditions of the Mediterranean basin countries, and more 600 cultivars are locally grown and called varieties (Condit 1955). These consists of individuals selected for their edible fruits and the trees are clonally propagated through cuttings. Fig genotypes are preserved in the Mediterranean basin countries since many centuries for many different purposes. For instance, figs are consumed either fresh or dried or used for jam or spirit beverage production. In Tunisia, the fig groves cover all areas throughout the country. These are located in sites with contrasting climates and soils (plain, seacoast, oases and moist areas of high altitude). However, for several decades, the cultivated area has significantly decreased due to: first, the fig tree is a marginal fruit crop and it is vulnerable to biotic and abiotic stresses; second, the reduction of the number of genotypes selected and maintained since ancient time; and third, the intensive urbanisation. As a consequence of these trends, severe genetic erosion is threatening the local germplasm. Moreover, the actual number of cultivars is at present difficult to estimate since synonymy constitutes error sources in cultivar identification. Hence, it is imperative to establish a research program aiming at the preservation and the evaluation of the Tunisian germplasm. Recently, prospecting and collecting actions have been initiated and led to identification of more than 50 ecotypes (Rhouma 1996; Mars 1998, 2003). These are ex situ maintained in four collections at the Institut des Régions Arides of Medenine, the Centre de Recherches Phoénicicoles of Degache, the Commissariat Régional du Développement Agricole of Gafsa and the Ecole Supérieure d'Horticulture et d'Elevage of Chott Mariem. On the other hand, data based on the use of morphological traits, particularly those concerning the fruits, have been reported and involved the main cultivars that are locally predominant (Valdeyron 1967; Ben Salah et al. 1995; Hedfi et al. 2003; Salhi-Hannachi et al. 2003; Chatti et al. 2004). However, these characters are either sensitive to the environmental conditions or limited to the fruit production season. In addition, surveys of molecular diversity have been reported by Hedfi et al. (2003) and Salhi-Hannachi et al. (2004a, 2004b) using isozymes and inter simple sequence repeat markers (ISSRs). These studies have proven some benefit since they allowed discrete markers and recommendation reliable to rationally manage this important genetic resource. However, the involved ecotypes were limited in number and concerned the

predominant ones in the southern areas. Consequently, investigations including a large number of Tunisian fig genotypes is imperative to survey the genetic diversity and to have a deeper insight of the genetic organisation in this germplasm. This could be made possible throughout the analysis of the polymorphisms in fig accessions preserved in the main collections. Many methods for the detection of plant DNA polymorphism have been reported. One of the most widely tested techniques seems to be the random amplified polymorphic DNA (RAPD) method of WILLIAMS et al. (1990). Due to its high sensitivity, this procedure constitutes a powerful method to distinguish genotypic variants (SANTONI et al. 2000; TRIFI et al. 2000; AMADOU et al. 2001; AL-KHALIFA and ASKARI 2003; KHADARI et al. 2003a; RAJORA et al. 2003; Onguso et al. 2004). The use of RAPD technology has been reported in fig (KHADARI et al. 1995; SALHI-HANNACHI et al. 2004a, 2004b) for identification of cultivars. The aim of this investigation was to produce suitable markers to study the DNA polymorphism in Tunisian fig ecotypes and to further reveal the genetic diversity and ecotype identification.

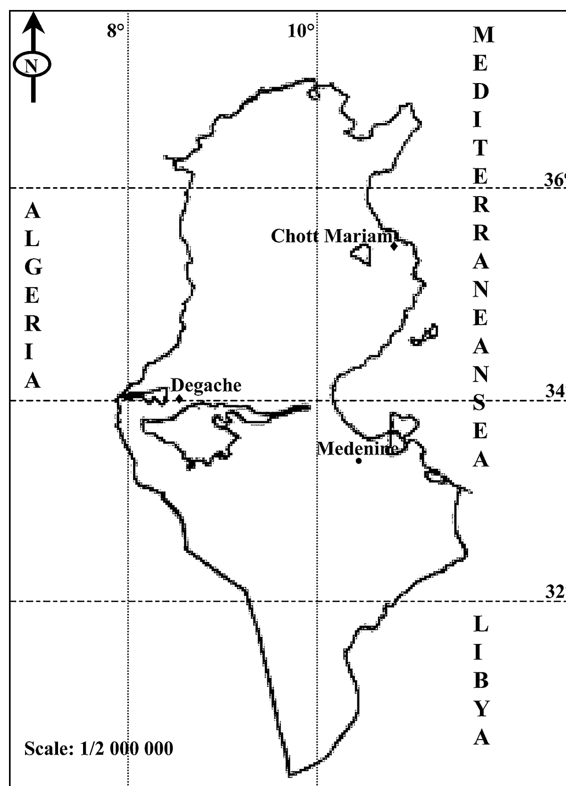

Fig. 1. Map of Tunisia fig collection sites.

MATERIAL AND METHODS

Plant material

We used a set of 35 Tunisian fig ecotypes preserved in three main collections established in the ESHE Chott Mariem, IRA Médenine and CRPh Degache (Fig. 1). These, listed in Table 1, consisted of 31 common fig genotypes and 4 caprifigs corresponding to the main cultivated ecotypes in Tunisia.

DNA extraction

DNA was extracted from frozen young leaves sampled from adult trees according to DELLAPORTA et al. (1983). After purification, the DNA concentration was estimated spectrophotometrically. DNA integrity was performed by analytic agarose gel electrophoresis (SAMBROOK et al. 1989).

Primers and PCR assays

Universal decamer oligonucleotides purchased from Operon Technologies Inc. (Alameda, USA) were used for the amplification of random DNA banding patterns (Table 2).

PCR reactions were performed in a 25 µl volume reaction mixture containing: 20 ng of total cellular DNA (1.5 µl), 50 pM of primer (1 µl), 2.5 µl of Taq DNA polymerase buffer, 1.5 U of Taq DNA polymerase (QBIOgène, France), 200 mM of each dNTP (DNA polymerization mix, Pharmacia). The reaction mix was overlaid with drop of mineral oil to avoid evaporation during the cycling. PCR was performed in a DNA thermocycler (Crocodile III QBIOgène, France). Samples were first heated at 94°C for 5 min and subjected to 35 repeats of the following cycle: 30 seconds at 94°C, 1 min at 35°C, 1 min at 72°C. A final step of five min at 72°C was always run.

To reduce the possibility of cross contamination and variation in the amplification reactions, master mixes of the reaction constituents were always used. A negative control (reaction mix without any DNA or without any enzyme) was also included.

Amplification products were electrophoresed in 1.5% agarose gels in TBE (0.5×) buffer and detected after ethidium bromide staining according to SAMBROOK et al. (1989). Amplifications were performed at least twice and only reproducible products were taken into account for further data analysis.

Data analysis

For each primer, the number of bands and the polymorphic ones were calculated. The ability of the most informative primers to differentiate between accessions was assessed by the estimation of their resolving power (Rp) (PREVOST and WILKINSON 1999). The Rp has been described to correlate strongly

Table 1. *Tunisian Ficus carica L. ecotypes studied with their localities of origin.*

Collection site	Accession name	Label	Botanical variety	Locality origin
ESHE Chott Mariem*	Soltani	01*	Common type	Ouardanine
	Soltani	02*	" "	Kalaa Kebira
	Khahli	03*	" "	Kalaa Kebira
	Khahli	04*	" "	Enfidha
	Hemri	05*	" "	Enfidha
	Hemri	06*	" "	Ghadhabna
	Bither Abiadh	07*	" "	Mesjed Aissa
	Bither Abiadh	08*	" "	Chott Mariem
	Bither Abiadh	09*	" "	Khamara
	Bidhi	10*	" "	Kalaa Kebira
	Bidhi	11*	" "	Khamara
	Baghali	12*	" "	Mesjed Aissa
	Zidi	13*	" "	Mesjed Aissa
	Besbassi	14*	" "	Mesjed Aissa
	Goutti	15*	" "	Chott Mariem
	Jrani	16*	Caprifig	Ghadhabna
	Assafri	17*	Caprifig	Ghadhabna
IRA Médenine**	Bither Abiadh	18**	Common type	Tataouine
	Dchiche Assal	19**	" "	Ghadhabna
	Dhokkar Zarzis	20**	Caprifig	Zarzis
	Hammouri	21**	Common type	Beni Khedache
	Kahli	22**	" "	Enfidha
	Makhbech	23**	" "	Zarzis
	Rogaby	24**	" "	Beni Khedache
	Sawoudi	25**	" "	Beni Khedache
	Tayouri Asfar	26**	" "	Douiret
	Widlani	27**	" "	Beni Khedache
	Zaghoubi	28**	" "	Beni Khedache
	Zidi	29**	" "	Ghadhabna
CRPh Degache***	Dhokkar	30***	Caprifig	Tozeur
	Grichy	31***	Common type	Tozeur
	Hamri	32***	" "	Tozeur
	Khalt	33***	" "	Tozeur
	Khzami	34***	" "	Tozeur
	Tounsi	35***	" "	Tozeur

with the ability to distinguish between accessions according to the following Gilbert et al. formula (1999):

$Rp = \Sigma\ Ib$, where $Ib = 1 - (2 \times |0.5 - p|)$ where p is the accessions' proportion containing the I band.

In addition, for each DNA sample, the presence of a reproducible polymorphic DNA band at a particular position on the gels was scored 1, while its absence was denoted 0. RAPD bands were thus transformed into a binary character matrix. Data were computed

Table 2. *Primers and RAPD banding patterns.*

Primer	Sequence (5′– 3′)	RAPD bands		PPB	Rp
		Total	Polymorphic		
OPH02	TCGGACGTGA	Smear	-	-	-
OPT10	CCTTCGGAAG	" "	-	-	-
OPT20	GACCAATGCC	" "	-	-	-
OPA01	CAGGCCCTTC	8	6	75.0	2.914
OPA02	TGCCGAGCTG	14	8	57.1	3.428
OPA05	AGGGGTCTTG	10	7	70.0	3.543
OPA11	CAATCGCCGT	13	7	53.8	2.857
OPA16	AGCCAGCGAA	10	9	90.0	5.657
OPA18	AGGTGACCGT	8	7	87.5	3.371
Total		63	44	72.23	21.771

with the GeneDist program (version 3.572c) to produce a genetic distance matrix using the formula of NEI and LI (1979), which assesses the similarity between any two populations on the basis of the number of generated bands. The resultant matrix was computed with the Neighbour program to produce a tree-file using the unweighted pair group method with the arithmetic averaging (UPGMA) algorithm (SNEATH and SOKAL 1973). The TreeView program (Win32, version 1.5.2) was used to draw phylogenetic diagrams from the resultant tree-file. All these analyses were carried out using appropriate programs in PHYLIP (phylogeny inference package, version 3.5c) gently provided by FELSENSTEIN (1993) (Dept of Genetics, Univ. of Washington, Seattle, WA, USA) and the Page's TreeView software (PAGE 1996).

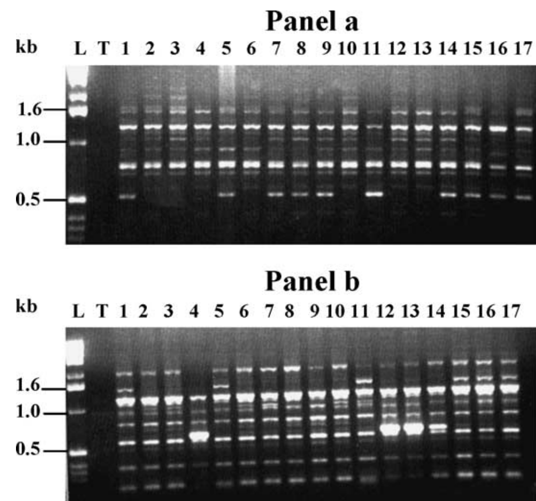

Fig. 2. Example of RAPD banding patterns generated in Tunisian *F. carica* L. ecotypes using OPA11 (panel a) and OPA05 (panel b). L: standard molecular size (1kb ladder, Gibco-BRL), T: negative control, lanes 1–17: ecotypes tested.

RESULTS

Primers and resolving power

A total of nine primers were screened for their ability to generate consistently amplified band patterns and to assess polymorphism in the tested varieties. Among these primers, only six have revealed unambiguously scorable polymorphic bands. These are identified as OPA01, OPA02, OPA05, OPA11, OPA16 and OPA18. In fact, these mentioned primers generated multiple banding profiles with six to nine polymorphic amplified DNA bands ranging in size from 100 to 2500 bp. A total of 44 out of 63 were polymorphic (72.23%) with a mean of 7.33 bands per primer. As shown in Fig. 2, RAPD banding patterns were typically generated from the included ecotypes. In this case, the OPA11 (panel a) and OPA05 (panel b) primers were used to generate banding profiles from the accessions studied. A minimum of six and a maximum of nine DNA fragments were obtained using (OPA1) and (OPA16) respectively (2). Hence, we may assume that a large genetic diversity at the DNA level characterises the Tunisian fig germplasm.

Estimation of the Rp values exhibited a collective rate of 21.771 and varied from 2.857 for the (OPA11) primer to 5.657 for the (OPA16) one with a mean of 3.628 (Table 2). In addition, the (OPA16), (OPA02), (OPA05) and (OPA18) primers seem to be the most efficient to assess the genetic diversity since they have presented relatively high Rp rates.

Genetic distances and ecotypes relationships

A between accessions genetic distance matrix shows an average distance range from 0.000 to 0.78 with a mean of 0.39 (Table 3). Thus, the accessions tested in this study are highly divergent at the DNA level. The smallest distance value of 0.00 was observed between Soltani [02*] and Kahli [03*] cultivars, which seem to be nearly similar. The maximum distance value of 0.78, suggesting great dissimilarities, was observed between either Hemri [05*] and Khzami [34***] or Dchiche Assal [19**] and Rogaby [24**]. All the remaining ones display different intermediate levels of similarity and are grouped with the other ones.

The UPGMA analysis confirmed the genetic divergence described above (Fig. 3). In fact, the distinctiveness of the clusters identified in this UPGMA derived dendrogram exhibits two main clusters. The first group labelled (a), is composed of three genotypes maintained in the Medenine collection and identified as Bither Abiadh [18**], Dchiche Assal [19**] and Hammouri [21**]. All the remaining accessions housed in the three collections are ranged in the second cluster labelled (b). Note that in this last group, ecotypes are organised in the two subgroups labelled (c) and (d) respectively. The subgroup (c) is composed only of genotypes maintained in the ESHE collection with a significant divergence of the Hemri [05*] genotype from the others. However, the subgroup (d) contains the Medenine collection ecotypes ranged together with the remaining ones from Degache.

In addition, a significant clustering divergence was scored in the case of ecotypes similarly named and originating from different localities. This is well exemplified in the case of Zidi cultivars labelled [13*] and [29**]; the Hemri ([05*] and [06*]); the Bither Abiadh ([07*], [08*] and [09*]) and Bidhi

Table 3. *Genetic distances matrix among 35 Tunisian fig ecotypes based on RAPD data and computed using the Nei and Li's formula.*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
1	0.00																																		
2	0.22	0.00																																	
3	0.22	0.00	0.00																																
4	0.22	0.25	0.25	0.00																															
5	0.25	0.48	0.48	0.42	0.00																														
6	0.25	0.17	0.17	0.22	0.31	0.00																													
7	0.14	0.22	0.22	0.35	0.45	0.31	0.00																												
8	0.25	0.48	0.48	0.35	0.38	0.31	0.31	0.00																											
9	0.22	0.38	0.38	0.20	0.48	0.35	0.35	0.12	0.00																										
10	0.22	0.09	0.09	0.25	0.56	0.17	0.22	0.48	0.38	0.00																									
11	0.28	0.31	0.31	0.38	0.35	0.28	0.28	0.48	0.38	0.20	0.00																								
12	0.25	0.17	0.17	0.12	0.45	0.25	0.25	0.31	0.17	0.22	0.35	0.00																							
13	0.31	0.28	0.28	0.17	0.38	0.20	0.31	0.25	0.22	0.28	0.41	0.09	0.00																						
14	0.20	0.35	0.35	0.17	0.45	0.31	0.25	0.14	0.07	0.35	0.35	0.14	0.20	0.00																					
15	0.12	0.25	0.25	0.25	0.22	0.28	0.28	0.35	0.25	0.31	0.25	0.22	0.35	0.28	0.00																				
16	0.35	0.31	0.31	0.45	0.56	0.48	0.28	0.41	0.38	0.38	0.38	0.28	0.41	0.35	0.25	0.00																			
17	0.31	0.41	0.41	0.41	0.60	0.52	0.25	0.31	0.35	0.41	0.48	0.31	0.38	0.25	0.41	0.17	0.00																		
18	0.56	0.60	0.60	0.69	0.64	0.64	0.56	0.64	0.60	0.69	0.69	0.64	0.73	0.64	0.60	0.45	0.56	0.00																	
19	0.48	0.60	0.60	0.69	0.56	0.73	0.56	0.56	0.60	0.52	0.45	0.73	0.83	0.56	0.60	0.60	0.48	0.52	0.00																
20	0.38	0.28	0.28	0.48	0.69	0.45	0.38	0.38	0.35	0.35	0.42	0.38	0.45	0.31	0.48	0.35	0.25	0.48	0.48	0.00															
21	0.48	0.52	0.52	0.60	0.56	0.64	0.35	0.56	0.52	0.60	0.38	0.56	0.64	0.48	0.52	0.38	0.41	0.31	0.45	0.48	0.00														
22	0.35	0.52	0.52	0.52	0.56	0.56	0.28	0.41	0.38	0.45	0.31	0.41	0.48	0.35	0.45	0.31	0.28	0.45	0.60	0.35	0.38	0.00													
23	0.35	0.45	0.45	0.69	0.64	0.64	0.41	0.41	0.38	0.38	0.38	0.56	0.64	0.41	0.45	0.52	0.41	0.60	0.31	0.35	0.52	0.31	0.00												
24	0.48	0.52	0.52	0.60	0.73	0.56	0.48	0.35	0.38	0.60	0.69	0.64	0.64	0.41	0.60	0.52	0.41	0.52	0.78	0.22	0.60	0.38	0.45	0.00											
25	0.41	0.25	0.25	0.52	0.64	0.35	0.28	0.48	0.52	0.31	0.60	0.41	0.41	0.48	0.60	0.52	0.48	0.45	0.69	0.35	0.45	0.45	0.45	0.38	0.00										
26	0.45	0.48	0.48	0.64	0.45	0.52	0.45	0.31	0.35	0.56	0.48	0.52	0.52	0.38	0.48	0.41	0.38	0.48	0.41	0.25	0.41	0.35	0.41	0.28	0.35	0.00									
27	0.28	0.45	0.45	0.52	0.41	0.56	0.41	0.35	0.31	0.52	0.52	0.56	0.64	0.35	0.38	0.45	0.41	0.31	0.31	0.35	0.45	0.38	0.31	0.31	0.38	0.28	0.00								
28	0.35	0.45	0.45	0.52	0.48	0.56	0.41	0.35	0.31	0.52	0.69	0.48	0.56	0.35	0.45	0.42	0.35	0.45	0.52	0.28	0.45	0.38	0.38	0.25	0.31	0.22	0.20	0.00							
29	0.35	0.60	0.60	0.45	0.56	0.64	0.41	0.41	0.31	0.60	0.60	0.48	0.56	0.41	0.38	0.45	0.35	0.52	0.69	0.48	0.45	0.38	0.60	0.38	0.60	0.48	0.31	0.25	0.00						
30	0.60	0.64	0.64	0.64	0.69	0.60	0.60	0.31	0.41	0.56	0.56	0.60	0.52	0.52	0.73	0.48	0.45	0.48	0.48	0.45	0.48	0.41	0.48	0.41	0.64	0.31	0.48	0.48	0.56	0.00					
31	0.23	0.41	0.41	0.41	0.52	0.60	0.31	0.31	0.28	0.48	0.56	0.38	0.45	0.35	0.41	0.41	0.25	0.56	0.56	0.25	0.41	0.28	0.35	0.28	0.35	0.25	0.28	0.22	0.28	0.31	0.00				
32	0.35	0.52	0.52	0.45	0.73	0.64	0.28	0.28	0.31	0.52	0.52	0.41	0.48	0.28	0.52	0.31	0.22	0.52	0.69	0.35	0.38	0.20	0.38	0.38	0.52	0.41	0.45	0.45	0.38	0.28	0.17	0.00			
33	0.35	0.31	0.31	0.31	0.73	0.48	0.41	0.41	0.31	0.38	0.45	0.35	0.41	0.35	0.45	0.38	0.35	0.52	0.60	0.28	0.45	0.31	0.38	0.38	0.38	0.41	0.31	0.45	0.38	0.35	0.17	0.20	0.00		
34	0.45	0.48	0.48	0.48	0.78	0.60	0.38	0.38	0.35	0.41	0.41	0.45	0.52	0.31	0.56	0.56	0.45	0.73	0.56	0.38	0.48	0.28	0.22	0.35	0.35	0.45	0.41	0.35	0.56	0.38	0.25	0.28	0.22	0.00	
35	0.35	0.41	0.41	0.45	0.48	0.48	0.48	0.28	0.20	0.58	0.45	0.41	0.48	0.28	0.31	0.38	0.35	0.45	0.38	0.28	0.38	0.45	0.31	0.38	0.45	0.28	0.25	0.25	0.38	0.48	0.28	0.45	0.38	0.41	0.00

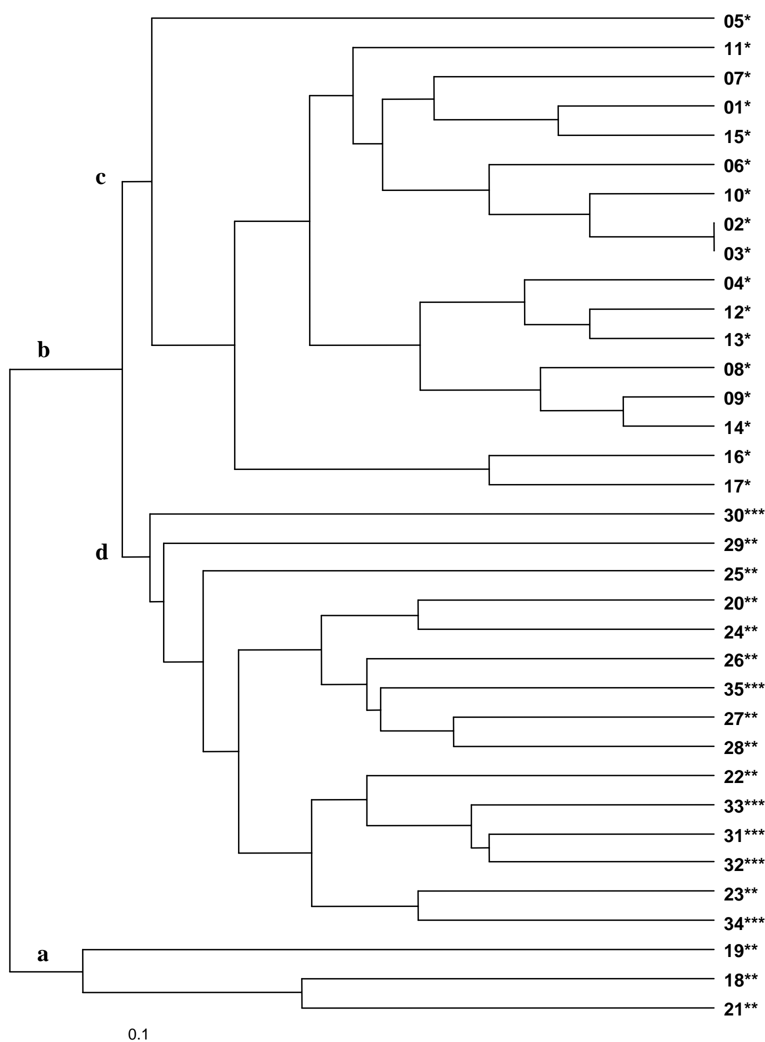

Fig. 3. Dendrogram of 35 Tunisian fig ecotypes constructed by UPGMA and based on RAPD banding patterns (§ table I for ecotypes' labels).

([10*] and [11*]). The opposite situation is observed in the case of Soltani [02*] and Khahli [03*] that are nearly similar. The hypothesis of synonymy could be forwarded to explain these particularities.

On the other hand, the dendrogram illustrates ecotype clustering made independently from the sex of the trees since the male accessions labelled [16*], [17*] and [30***] did not significantly diverge from the female ones. This result suggests the presence of a narrow genetic diversity in the accessions studied.

DISCUSSION AND CONCLUSION

Fingerprinting of the Tunisian fig was carried out using RAPD in order to obtain molecular data of the national gene pool. The present study shows the reliability of RAPD analysis to detect DNA polymorphisms in this crop. In fact, the tested primers are characterised by relatively high collective Rp rate of 21.771. Similar Rp values have been reported in Tunisian figs using either RAPD or ISSR methods (Salhi-Hannachi et al. 2004b). The primers generated 44 polymorphic out of 63 bands with a mean of 7.33. This is significantly higher than reported for fig varieties originating from the French CBNM of Porquerolles island (Khadari et al. 1995). Using nine universal primers tested in 30 fig varieties, we registered a mean of 2.2 markers per primer. Thus, we may assume that the Tunisian fig germplasm is characterised by a relatively high genetic diversity at the DNA level. This assumption is strongly supported with regard to the scored genetic distances among the ecotypes studied (0.00 to 0.78).H

The UPGMA cluster analysis divided the genotypes studied into two main groups that are consistently in agreement, in major part, with their geographic origin.

Hence, we may assume that the present study has permitted to precise the genetic diversity organisation in three main collections. It is worth noting that different clusterings have been reported in Tunisian figs using RAPD and/or ISSR makers (SALHI-HANNACHI et al. 2004a,b). In fact these molecular markers have permitted to cluster the Medenine and Degache ecotypes independently from their geographic origin and shown a genetic diversity consistently structured according to a continuous model, described by KHADARI et al. (1995) for French cultivars.

The RAPD method also made it possible to sort out the mislabelling of different ecotypes based on the obtained banding pattern (homonymy and synonymy). The technique has been used in the same manner previously, to discriminate fig genotypes (ELISARIO et al. 1998; GALDERISI et al. 1999; DE MASI et al. 2003; KHADARI et al. 2003b). The RAPD procedure can easily be used for a large number of samples and ecotypes and/or universal primers would generate fingerprints to identify the genetic background of the plants-a knowledge which is necessary for rational management of this important fruit crop. Work is currently in progress to provide a large number of valid RAPDs as well as co-dominant molecular markers such as microsatellites (KHADARI et al. 2003b). Moreover, the genetic diversity analysis in Tunisian figs shows, in comparison with related sub spontaneous fig tree growing in situ, how important this technique is in the establishment of a national fig reference collection and also how to molecularly assist the selection for improvement of this crop.

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