Using Biochemical and Simple Sequence Repeats (SSR) Markers to Characterize (*Ficus carica* L.) Cultivars

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Abstract: The common fig (*Ficus carica* L.) trees are one of the earliest cultivated fruit-bearing trees. It is a moderately important world crop, with an estimated annual fruit production of 1,027,194. The aim of the study was to select the most efficient markers to propose a molecular key for seven fig cultivars identification. Genetic similarity matrix was generated on the basis of Nei and Li's coefficients. The coefficients were used to make clusters using un-weighted pair group method of arithmetic means (UPGMA) which separated the studied seven fig cultivars into two main groups depending on results PPO, POD isozymes and SSR-PCR combination. The first group I included Katoda cultivar (similarity range 0.43 to 0.92). However, the second group (II), the cultivars fell into three sub-groups closely related (a, b and c). Sub-group (a) (similarity range 0.36 to 0.81) contained on Sultany and Black Mission. Sub-group (b) (similarity range 0.41 to 0.75) included White Acdy, Aboudi and Conadria. Sub-group (c) consisted of Gizy (similarity range 0.36 to 0.81). Consequently, this study suggests the PPO and POD markers are suitable, but SSR marker is the most efficient for genetic variability and fig cultivars fingerprinting. In addition, genetic diversity could be used for rational design of breeding programs, conservation of local germplasm and management of fig genetic resources.

Key words: *Ficus carica* L. · Molecular marker · Peroxides · Polyphenol oxidase

INTRODUCTION

The common fig (*Ficus carica* L.) trees are one of the earliest cultivated fruit-bearing trees. *F. carica* with (2n = 2x = 26 chromosomes) belongs to family *Moraceae*, with over 1400 species classified into about 40 genera [1]. The *Ficus* species are gynodioecious but functionally dioecious. According to reproductive organs, there are two main types of trees: the Caprifig (male tree) and the female tree [2]. This group includes three types Common fig, Smyrna fig and San Pedro. Today, the fig is a moderately important world crop, with an estimated annual fruit production of 1,027,194 [3]. In Egypt, where the fig is ubiquitous, a large number of local cultivars have been identified recently [4]. 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 need to be assessed. Evaluation of genetic variation within cultivated crop species is central to plant breeding strategies and genetic resource conservation [5, 6]. Morphological and agronomic characters are useful in surveys of plant species diversity, but these characters are highly influenced by

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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 [7]. Biochemical and molecular markers such as isozymes, random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR), randomly amplified microsatellite polymorphism (RAMPO), simple sequence repeat (SSR), amplified fragment length polymorphism (AFLP) and internal transcribed spacer (ITS) (rDNA) have also been applied. Results of such studies provided evidence of a significant but unstructured genetic diversity [8-14]. Molecular identification of fig cultivars has been carried out using isozyme markers, RAPD and AFLP markers [15]. 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 [16]. 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 [17, 18].

In the present study, we characterized seven fig cultivars in Egypt using PPO and POD isozymes as biochemical and molecular markers using six SSR loci and we selected the most efficient markers to propose a molecular key for fig identification. In addition, information can be used for rational design of breeding programs, conservation of local germplasm and management of fig genetic resources.

MATERIALS AND METHODS

Plant Material: The seven fig cultivars used in this study were sampled different areas (private farm in north coast of Egypt (White Adcy), Faculty of Agriculture Farm, University of Alexandria (Sultany), Desert Research Center, America region (Aboudi, Black Mission, Conadria and Katoda) and Agricultural Research Center, Giza (Gizy). Four of these cultivars represented local cultivars (Sultany, Aboudi, White Adcy and Gizy). Meanwhile, (Black Mission, Conadria and Katoda) were as imported fig cultivars.

Isozymes Electrophoresis: Native-polyacrylamide gel electrophoresis (Native-PAGE) was conducted according to Stegemann *et al.* [19] to identify isozyme variations between seven fig cultivars. Two isozymes systems; peroxidase and polyphenol oxidase were analyzed.

Table 1: The six SSR-PCR primers used in the study and their sequences.

MFC1	F: CTAGACTGAAAAAACATTGC
	R:TGAGATTGAAAGGAAACGAG
MFC2	F: GCTTCCGATGCTGCTCTTA
	R:TCGGAGACTTTTGTTCAAT
MFC3	F:GATATTTTCATGTTTAGTTTG
	R:GAGGATAGACCAACAACAAC
MFC6	F:AGGCTACTTCAGTGCTACA
	R:GCCATAAGTAATAAAAACC
MFC7	F:CACAATCAAAATAGTTACCG
	R:AGCGAAGACAGTTACAAAGC
MFC8	F:GTGGCGTCGTCTCTAATAAT
	R:TATTCTATGCTGTCTTATGTCA

After electrophoresis, gels were stained according to their enzyme systems with the appropriate substrate and chemical solutions and then incubated at room temperature in dark for complete staining. For peroxidase, benzidine-dihydrochloride HCl of 0.125 g and 2 ml glacial acetic acid and was completed with distilled water up to 50 ml. Gel was placed into this solution and 5 drops of hydrogen peroxide was added. The gel was incubated at room temperature until bands appear [20]. For polyphenol oxidase, 100 ml of sodium phosphate buffer 0.1 M at pH 6.8, 15 mg cathecol and 50 mg sulfanilic acid were used. The Gel was placed into this solution and incubated at 30°C for 30 min until bands appeared.

SSR Assays: Six microsatellite primers (MFC1, MFC2, MFC3, MFC6, MFC7 and MFC8) were used to determine biodiversity among studied fig cultivars identified by Khadari *et al.* [21] in *Ficus carica* (Table 1). PCR amplifications were performed as described by Saddoud *et al.* [22]. The amplified banding patterns were first checked on 2% agarose gels visualized with ethidium bromide staining under UV light.

Data Analysis: A matrix for isozymes, SSR-PCR combined was generated by scoring reproducible bands as 1 for their presence and as 0 for their absence across the lines. Genetic similarity coefficients were computed according to Nei and Li [23] as follow:

$$\text{Similarity} = \frac{2N_{xy}}{N_x + N_y}$$

Where 'Nx' and 'Ny' are the number of bands present in genotypes 'x' and 'y', respectively; and Nxy are the number of bands shared by the cultivar 'x' and 'y'. The data were subsequently used to construct a dendrogram using the un-weighted pair group method of arithmetic averages (UPGMA) [24] employing sequential, agglomerative

hierarchic and non-overlapping clustering (SAHN). All the computations were carried out using the software NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System), version 2.1 [25]. Correlation coefficients were calculated using similarity coefficients obtained from combined isozymes and SSR-PCR analysis.

RESULTS AND DISCUSSION

Polyphenol Oxidase (PPO): Polyphenol oxidase patterns of the seven fig cultivars exhibited a maximum number of seven bands, which are not necessarily present in all samples; five of them show polymorphic (71.43%). The relative mobility (*Rf*) of the bands ranged from 0.199 to 0.955 in seven fig cultivars. The largest number of bands was found in Gizy and White Adcy cultivars (five bands). The lowest number of bands was found in Aboudi, Conadria, Katoda cultivars (two bands). The two bands were found as common bands with *Rf* 0.744 and 0.833. Black Mission and White Adcy cultivars scored one a new band with *Rf* 0.429 and 0.199, respectively (Fig. 1). These bands could be used as markers for these cultivars. In addition, one isoform band with *Rf* 0.295 appeared in three cultivars Black Mission, Gizy and Sultany and disappeared in the other cultivars. On the other hand, two bands with *Rf* 0.513 and 0.955 were detected only in Gizy and White Adcy cultivars (Fig. 1).

Peroxidse (POD): The isozyme banding patterns of the seven fig cultivars are presented in Fig. 2. The peroxidase patterns exhibited a maximum number of six bands; four of them present polymorphic (66.67%). The highest number of bands was found in Conadria cultivar (five bands). The minimum number of bands was found in the Black Mission cultivar (two bands). On the other hand, Conadria cultivar has a new peroxidase band at the *Rf* 0.101 which could be used as marker for this cultivar. Moreover, the cultivar Katoda, exhibited one isozyme marker with *Rf* 0.221. Consequently, these bands could be considered as biochemical markers to characterize the cultivars. Furthermore, one band with *Rf* 0.317 was detected in Sultany, White Adcy and Conadria cultivars and disappeared in the remaining cultivars. Also, Gizy, Aboudi and Conadria showed one isoform band with *Rf* 0.538. Phenetic analysis of the genetic polymorphism carried out by means of the UPGMA clustering algorithm helped to clarify the genetic relationships among seven fig cultivars based on PPO and POD isozymes combination. The phenogram showed the presence of two main clusters, the first cluster included the White Adcy
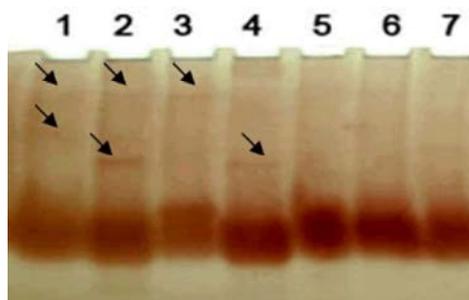

Fig. 1: Isozyme of banding pattern polyphenol oxidase for the seven fig cultivars from different locations. Lane 1: Black Mission, lane 2: Gizy, lane 3: Sultany, lane 4: White Adcy, lane 5: Aboudi, lane 6: Conadria and lane 7: Katoda.
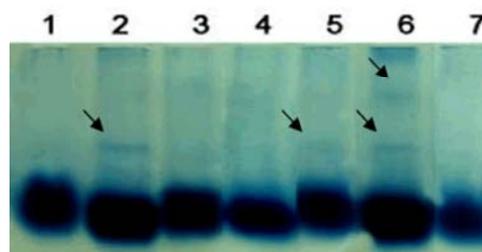

Fig. 2: Isozyme of banding pattern peroxidase for the seven fig cultivars from different locations. Lane 1: Black Mission, lane 2: Gizy, lane 3: Sultany, lane 4: White Adcy, lane 5: Aboudi, lane 6: Conadria and lane 7: Katoda.

and Gizy, while the second cluster was subdivided to three groups a, b and c (Fig. 3): Group (a) composed of Black Mission and Sultany. Group (b) contains on Katoda and group (c) includes Aboudi and Conadria. The resulted profiles showed different patterns indicating variability among cultivars. These results were in an agreement with those obtained by El-Fiky *et al*. [26], who showed that peroxidase and polyphenol oxidase, revealed high levels of polymorphism (or specific markers). El-Sharkawy *et al*. [27] confirmed that the cultivars could be identified by peroxidase and esterase isozyme banding patterns. Mukhlesur *et al.* [28] noted that isozyme analysis (Native-PAGE) offers a rapid and more reliable means for producing genetic profiles (fingerprints) and elucidation of genetic relationships within and different taxa. These techniques are efficient tools for genetic, systematic and breeding studies. Tanksley and Orton [29] mentioned that isozymes were the first markers used for plant genotype characterization. However, the number of these markers is very limited and they display low
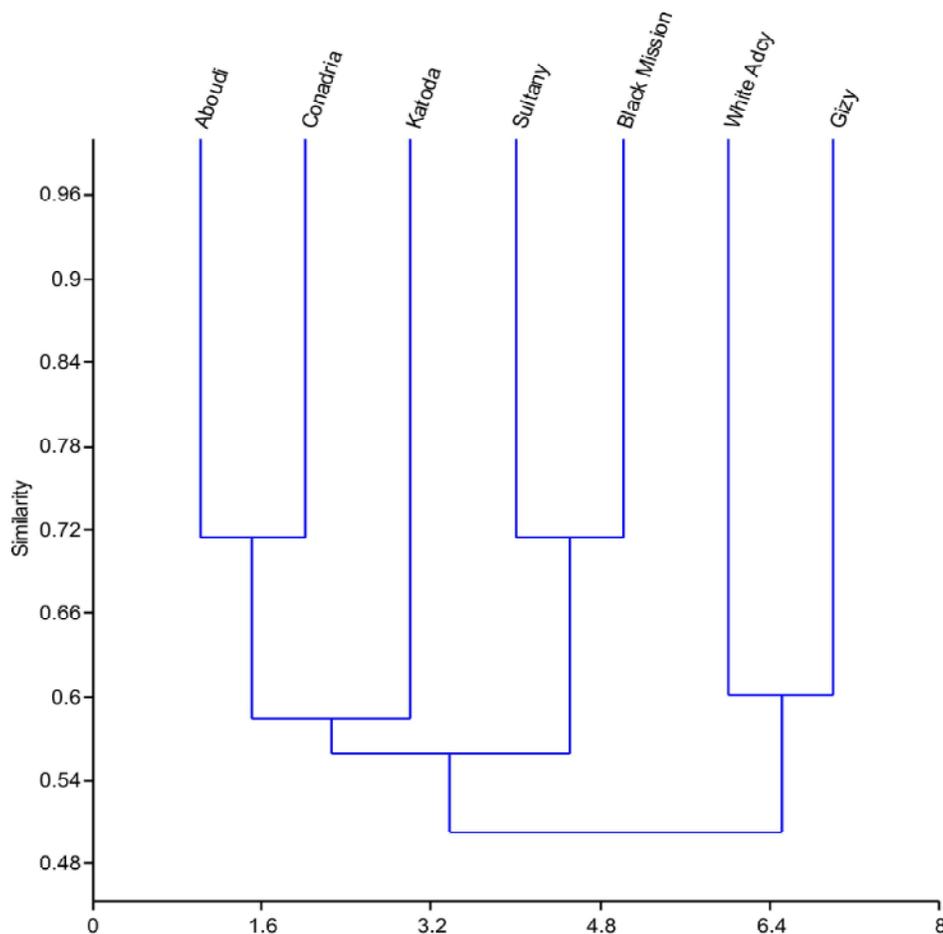
Fig. 3: Dendrogram revealing the genetic distance among seven fig cultivars using PPO and POD isozymes combination by UPGMA algorithm using Jaccard's similarity coefficient.

Table 2: Polymorphism of the SSR-PCR primers among seven fig cultivars.

Primer Code No.	Size range of the scorable bands (bp)	Total bands	No. of monomorphic bands	No. of polymorphic bands	% Polymorphism	Unique bands	Molecular size of unique markers
MFC1	50-500	10	2	8	80.00	3	100, 360, 450
MFC2	50-480	9	3	6	66.67	2	65, 300
MFC3	40-670	10	4	6	60.00	2	320, 350
MFC6	40-740	15	2	13	86.67	3	80, 150, 520
MFC7	45-180	2	2	0	0.00	0	0
MFC8	45-300	5	2	3	60.00	3	130, 220, 300
Total	40- 740	51	15	36	70.59	13	25.49

polymorphism (few alleles per locus). Furthermore, in some cases, isozyme polymorphism is influenced by environmental conditions. In general, it can be concluded that isozyme patterns obtained from the two systems, polyphenyl oxidase and peroxidase, are sufficient to detect phylogenetic relationships among the seven fig cultivars used in the present study.

SSR Patterns: Six SSR primers were used to identify the studied seven cultivars of fig as shown in (Fig. 4 and Table 2). A total number of 51 scorable amplified DNA fragments ranging from 40 to 740 bp were observed using the six SSR primers, whereas 36 fragments were polymorphic and the other 15 amplified were monomorphic bands. The polymorphic percentage was
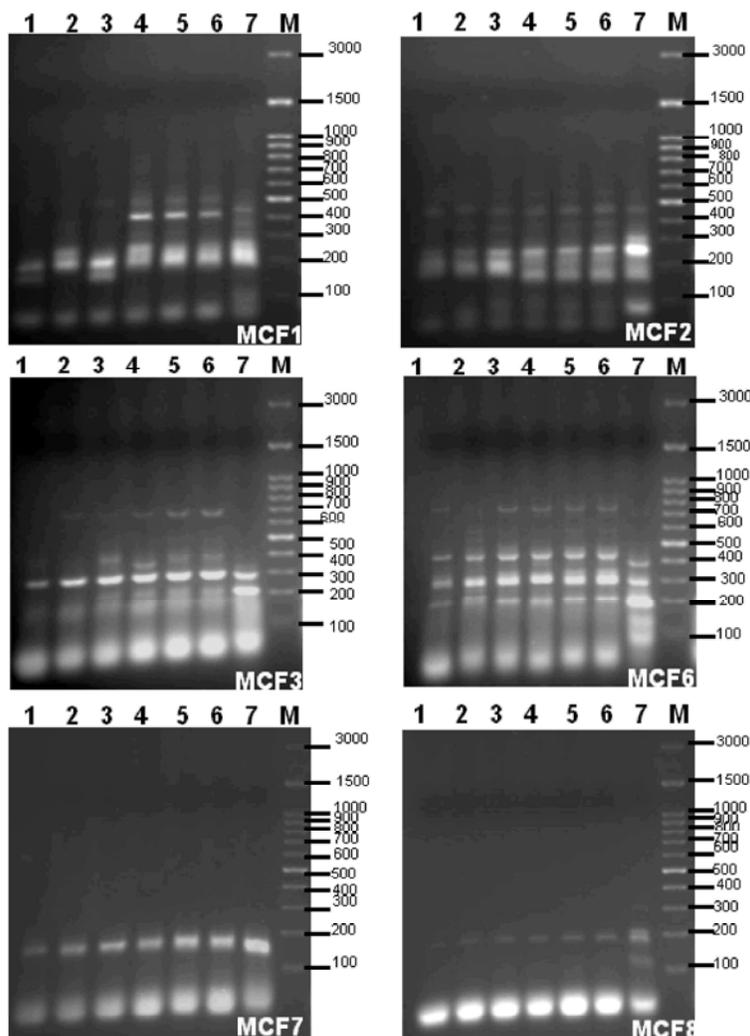
Fig. 4: SSR analysis of seven fig cultivars with six microsatellite primers (MFC1, MFC2, MFC3, MFC6, MFC7 and MFC8). Lane M = 100 bp DNA ladder. Lane 1: Black Mission, lane 2: Gizy, lane 3: Sultany, lane 4: White Adcy, lane 5: Aboudi, lane 6: Conadria and lane 7: Katoda.

70.59%. The number of amplification products per SSR primer varied from 2 for locus MFC7 to 15 for locus MFC6 (Table 2). Specific markers for fig cultivars detected by using SSR-PCR analysis are listed in (Table 2). Among the 36 polymorphic bands, 13 bands were unique markers with a total average of 25.49%. There was a marked difference in the unique number of dominant SSR bands observed among the seven fig cultivars. Katoda cultivar scored ten markers, Gizy had the two unique bands, while White Acdy appeared one specific band. On the other hand, primers MFC1, MFC6 and MFC8 gave the highest number of molecular markers (three) bands, followed by primers MFC2 and MFC3 (two) specific bands. In addition, the primer MFC7 did not display any bands (Table 2).

The cluster analysis using UPGMA based on genetic distances from SSR marker analysis revealed that the seven fig cultivars can be divided into two main groups (Fig. 5). The first group (I) included Katoda cultivar. The second group (II) contained three sub-groups. Sub-group (a) consisted of Gizy, sub-group (b) included Aboudi, Conadria, White Adcy, sub-group (c) included Black Mission and Slutany cultivars (Fig. 5). The dendrogram resulted from the combination between the banding patterns of PPO and POD isozymes and SSR-PCR (Table 3 and Fig. 6) revealed that seven fig cultivars clustered in two major groups. The first group 1 included Katoda cultivar (similarity range 0.43 to 0.92). However, the second group (II), the cultivars fell into
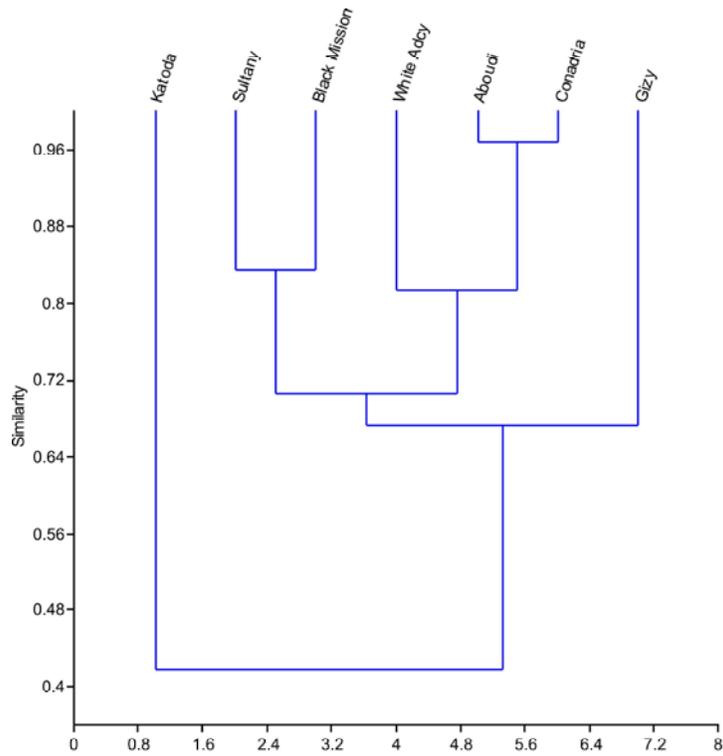
Fig. 5: Dendrogram revealing the genetic distance among seven fig cultivars using SSR by UPGMA algorithm using Jaccard's similarity coefficient.
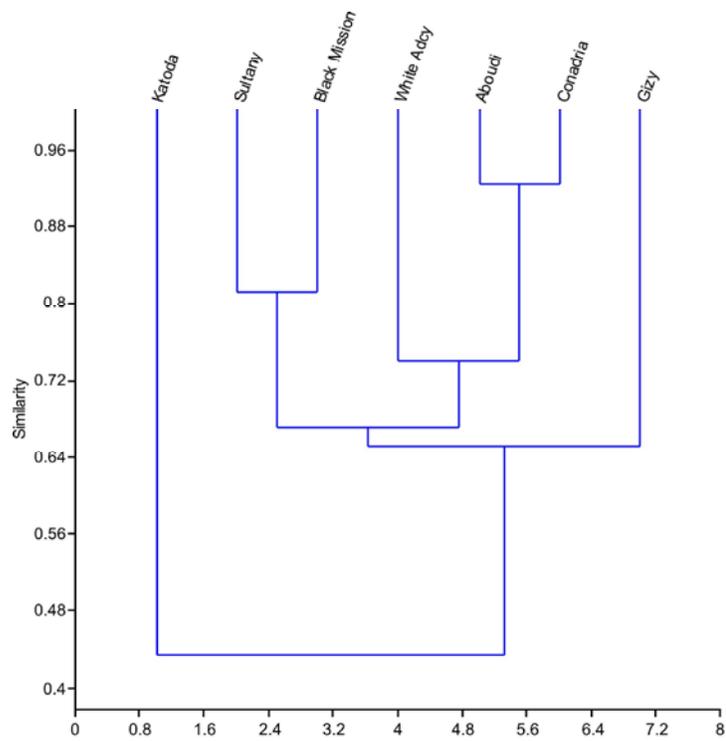

Fig. 6: Dendrogram of seven fig cultivar based on PPO, POD isozymes and SSR-PCR combination by UPGMA algorithm using Jaccard's similarity coefficient.

Table 3: Similarity indices among the seven fig cultivars as estimated using PPO, POD and SSR-PCR combination.

Cultivars	Gizy	White Adcy	Sultany	Conadria	Katoda	Aboudi	Black Mission
Gizy	1.00						
White Adcy	0.67	1.00					
Sultany	0.81	0.67	1.00				
Conadria	0.58	0.61	0.68	1.00			
Katoda	0.64	0.67	0.76	0.73	1.00		
Aboudi	0.63	0.63	0.74	0.75	0.92	1.00	
Black Mission	0.36	0.41	0.39	0.47	0.43	0.42	1.00

three sub-groups closely related (a, b and c). Sub-group (a) (similarity range 0.36 to 0.81) contained on Sultany, Black Mission, sub-group (b) (similarity range 0.41 to 0.75) included White Acdy, Aboudi and Conadria. Subgroup (c) consisted of Gizy (similarity range 0.36 to 0.81). These results were in an agreement with those obtained by Saddoud *et al*. [30] used simple sequence repeat (SSR) markers to characterize 18 Tunisian fig (*Ficus carica* L.) cultivars 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. Khadari *et al*. [21] designed 20 microsatellites to fig characterization; eight primer pairs produced amplification products that were both interpretable and polymorphic in 14 fig cultivars and two French wild-growing populations of *F. carica*. Number of alleles per locus ranged from three to six. Except for one microsatellite locus, the observed heterozygosity was higher than the expected value. The *F. carica* microsatellites gave amplification products in 17 other *Ficus* species in 86% of the cases. Caliskan *et al*. [31] evaluated the genetic variabilities of 76 fig accessions from Hatay province of Turkey by analysis of ten SSR loci. The number of alleles revealed by SSR analysis ranged from 3 to 12 alleles per locus with a mean value of 6.8. A total of 68 alleles were detected by SSR and the average heterozygosity was higher than the expected one. Achtak *et al*. [32] proposed six SSR loci as a sufficient tool to characterize fig germplasm in Morocco maintained in an ex situ collection. A set of 17 microsatellite loci was used to characterize 75 accessions representing eight Capri figs, 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 may be resulted from somatic mutation.

Based on genetic criteria, including linkage disequilibrium, discrimination power and molecular criteria as polymerase chain reaction conditions of loci multiplexing, he proposed a key identification set using six microsatellite markers to discriminate all genotypes present in the *ex situ* collection. Selected SSR loci set can be used for larger genetic studies of fig germplasm and a similar approach could be adopted for other fruit species. Saddoud *et al*. [22] applied only four microsatellites to unambiguously identifying the fig cultivars studied. The remaining ones would provide multilocus genotypes suitable for discriminating between any additional common fig cultivars grown in Tunisia and in any areas of the world. Thus, they assume that the microsatellite markers constitute an efficient tool in the identification of fig cultivars according to Bailey criteria [33, 34]. Our results showed that SSR or isozyme markers are powerful tools to identify genetic diversity among fig cultivars, while SSRs analysis is the most efficient. In addition, we have found that this rich genetic diversity would be useful for development of table figs in regional and national fig breeding programs.

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