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Genetic diversity and relationships among wild and cultivated *Ficus carica* L.: Usefulness of RGA markers

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Abstract

Disease resistance and the maintenance of genetic diversity in wild and cultivated populations are very important challenges to implement breeding program and markers assisted selection of *Ficus carica* L. facing climate change and its consequences. Resistance gene analogs (RGA) markers were used for variety discrimination and assessment of genetic structure and diversity of wild and cultivated *Ficus carica* L. species in Tunisia. The RGA markers were efficient and reliable markers for discriminating wild and cultivated fig. The high level of polymorphism (95.65) detected suggests the effectiveness of RGAs for both genetic fingerprinting and relationships assessment in wild and cultivated fig. The detected markers may represent candidate genes for disease resistance and could be further used to facilitate the identification of candidate genes and accelerate the genetic improvement of disease resistance in breeding programs of *Ficus carica* species.

1. INTRODUCTION

The fig, *Ficus carica* L., (Moraceae) is one of the oldest traditional crops and sacred fruit tree widely present in the world and well adapted to Mediterranean environmental conditions (Kislev et al. 2006; Khadivi et al. 2018). The world fig production was about 1.1 million tons while Tunisia is the ninth fig fruit producer in the worldwide with 27400 tones (FAOSTAT 2019). *Ficus carica* L. (2n = 2x = 26) is a gynodioecious species with two sexual forms: male trees (Caprifig) and female trees (edible fig), belongs to the Moraceae family. The edible fig is known to be one of the first plants cultivated by humans (Kislev et al. 2006). It is known to have been domesticated from a group of diverse spontaneous figs occurring in the south and east of the Mediterranean region sometime in the Early Neolithic period (Aradhya et al. 2010). Thus, wild fig trees are abundant, which propagate exclusively by seeds, (Falistocco 2016) and mostly present between rocks along the riverbanks and in steep-sloped valleys where seeds are easily disseminated by birds (Ben Abdelkrim et al. 2015; Falistocco 2020). In Tunisia, the wild fig is mainly found in the northern and central regions such as in the islands of Galite and Kerkennah, Djebba, El Haouaria and Kesra and is widely spread over all the climatic stages (Aljane et al. 2005). The Tunisian fig germplasm is rich. It includes accessions grown under different ecological conditions, representing an important reservoir of genes linked to biotic and abiotic stresses (Aljane and Ferchichi 2010). The genetic diversity of cultivated figs has been widely evaluated, in contrast to wild figs remain to investigate (Haffar et al. 2022). As alternatives to conventional techniques, new molecular markers such as Resistance Gene Analogues (RGAs or RGAP) are functional markers

amplifying conserved DNA sequences of resistance genes (R) and related sequences present in plants, that are the most likely to be target genes for disease resistance (Dong et al. 2009). The main objectives of this work were to determine structure and genetic diversity of wild and cultivated Tunisian fig trees using the conserved RGA primers as resistance gene base markers.

2. MATERIAL AND METHODS

2.1. Plant material and DNA extraction

A total of 62 Tunisian fig accessions (29 wild and 33 of cultivars) were collected from six regions from Tunisia (Table 1). Total genomic DNA was isolated from young leaves sampled from adult trees following Dellaporta et al. (1983) method. The quality of the DNA was checked on a 0.8% agarose gel electrophoresis and the concentrations were quantified using a QubitR fluorometer (Invitrogen). The final DNA concentration was adjusted to 40 ng/mL stored at – 20 °C.

2.2. RGA analysis

Five pairs of RGAP primers designed by Dong et al. (2009) were used to characterize wild and cultivated fig trees (Table 2). PCR was carried out in a 20 µL reaction volume containing 40 ng/mL genomic DNA, 0.5 unit of Taq DNA polymerase, 10 mM $MgCl_2$, 0.2 mM of each dNTP, 10 pmol of each primer pair (forward and reverse), 1x PCR buffer. A thermal gradient cycler TProfessional TRIO Thermocycler (Biometra, Germany) was programmed as follows: an initial denaturation step at 94 °C for 3 min, 40 cycles of denaturation for 30 s at 94 °C, annealing for 45 s at 45-65 °C (depending on the nucleotide content of the primers), extension for 1 min at 72 °C and a final extension for 7 min at 72 °C. The thermal cycler was programmed to hold the product at 4 °C. The amplified products and a 100 bp DNA ladder were separated by electrophoresis using 2% agarose gels in 1x TBE buffer for approximately 1:30 h at 90 V. The PCR products were visualized under UV light and photographed using a Gel Documentation System (Bio-Rad Gel Doc 2000, USA).

2.3. Statistical analysis

Only reproducible and clearly amplified bands were scored for the construction of the binary data matrices of each RGAP marker by scoring (1) for presence and (0) for absence of individual allele as described for conserved gene-based markers (Poczai et al. 2013). To

Table 1. Name, type (wild or cultivar) and geographic origin of *Ficus carica* L. accessions.

Code	Cultivar Name/ Label	Locality	Region
Cultivated fig group			
1	Dhokkar	Bizerte	North East
2	Hargui	Bizerte	
3	Bikri	Kerkenah	Center East
4	Akhel Bou Aounk	Kerkenah	
5	Kahla Cherki	Kerkenah	
6	Khédhri Horr	Kesra	Northwest
7	Tirri Horr	Kesra	
8	Safri Guaress	Kesra	
9	Dam Froukh	Kesra	
10	Soltani	Germplasm collection of the High Agronomic Institute (I.S.A) of Chott-Mariem	East (Sahel)
11	Bigh Beghal		
12	Zidi		
13	Besbessi		
14	Goutti		
15	Bidhi		
16	Bither AB		
17	Hemri		
18	taganimt	'Centre Régional de Recherches en Agriculture Oasienne (CRRAO)' Degache	South West
19	Jrani		
20	Assafri		
21	Hobbi Abyath		
22	Boumarra		
23	Zidi jamrou		
24	Bsis assal		
25	Khlit Jbéni		
26	Basoul Elkhadem		
27	Hammi		
28	Aounk Ehmém		
29	khzéfi		
30	Limi		
31	Caprifig F207	Nefta souani	
32	Caprifig F208	Nefta souani	
33	Caprifig F209	Nefta souani	
Wild fig group			
34	FAS 11	El Mida	North-West (Cap Bon)
35	FAS 12	El Mida	
36	FAS 13	El Mida	
37	FAS 14	El Mida	
38	FAS 22	Haouaria	
39	FAS 23	Haouaria	
40	FAS 25	Haouaria	
41	FAS 27	Haouaria	
42	FAS 37GE	Ghar el milh	North-East
43	FAS 45	Ghar el milh	
44	FAS 46	Ghar el milh	
45	FAS 47	Rafraf	
46	FAS 49	Rafraf	
47	FAS 55	Rafraf	
48	P4 Zag tir	El Alia	
49	P5	Ras jebal	
50	P10	Bejjou	
51	P11	Bejjou	
52	P12	Bejjou	
53	FAS 28	Kerkenah	Center East
54	FAS 30	Kerkenah	
55	FAS 34TN	Kerkenah	
56	FAS 35TN	Kerkenah	
57	FAS 63	Siliana	North-West
58	FAS 65	Siliana	
59	FAS 88	Kesra	
60	FAS 90	Kesra	
61	FAS 93	Kesra	
62	FAS 96	Kesra	

assess the efficiency of each RGAP primer to detect polymorphic loci among the accessions, the total number of amplified bands (TNB) and the number of polymorphic bands (NPB) were estimated using the data generated. The ability of the most informative primers to differentiate between fig accessions was evaluated by calculating their resolving power (Rp) (Prevost and Wilkinson 1999) and the ability of a marker to detect polymorphisms, was estimated by polymorphic information content (PIC) according to Smith et al. (1997). A similarity matrix using the Ochiai coefficient (Ochiai 1957) was calculated to construct an Unweighted Pair Group Method with Arithmetic Means (UPGMA) dendrogram and Principal Coordinate Analysis (PCoA) with the Paleontological Statistics (PAST) software Version 3.25 (Hammer et al. 2001).

3. RESULTS AND DISCUSSION

3.1. Polymorphism analysis

Five RGAP primers were screened to study genetic diversity among 62 Tunisian fig genotypes. All the primers produced distinct scorable fragments. A total of 23 bands were generated, out of which 22 were polymorphic. The number of polymorphic bands varies from 2 for the RGA-RLRR and RGA-XLRR primer pairs to 7 for the RGA-NLRR primer pair with an average of 4.4 polymorphic bands. This agrees with a previous study on common beans (Nemli et al. 2015) where the highest number of polymorphic bands for RGA-NLRR primer pair was 4 bands. The RGA markers showed a high percentage of polymorphism (95.65>90%). This proves the effectiveness of these primers in highlighting molecular polymorphism in fig trees. Four primer pairs showed an extreme level of polymorphism (100%). These are primer pairs designed according to the leucine-rich repeats (LRR) domain (RGA-RLRR, RGA-XLRR-INV, RGA-NLRR and RGA-XLRR). Consequently, all the primers used participate in the discrimination between the 62 fig trees studied.

The PIC values ranged from 0.446 (RGA-XLRR) to (The primer RGA-NLRR display the highest PIC value (0.807) indicating that these loci were very informative due to its higher PIC values.

The resolving power (Rp) varied from 1.097 for the RGA-RLRR primer to 4.258 for RGA-NLRR which is the best marker for discriminating between genotypes (Table 2).

3.2. Cluster analysis

The dendrogram shown in Fig. 1 was constructed using the UPGMA method to evaluate relationships between cultivated and wild figs. The UPGMA dendrogram revealed that the studied fig accessions clustered into two main clusters at a similarity value of 0.525. Cluster I comprised 29 genotypes all of them are wild fig while cluster II included cultivated fig accessions. The clustering analysis based on the similarity matrix of the RGAP markers showed evident, grouping of genotypes according to fig type (wild or cultivated). This result suggests the existence of a broad genetic base structured into two well-differentiated gene pools. Similar results were reported by Ali-Shtayeh et al. (2014) by examining the genetic diversity of fig trees by RAPD markers. The separation observed between the cultivated and wild

Table 2. Description of the polymorphism, PIC and RP values of molecular markers used in the genetic diversity analysis of 62 fig genotypes.

Primer name	Gene	Primer paire	TNB	NPB	PPB (%)	PIC	Rp
RGA-RLRR	*RPS2, N*	RLRR for	2	2	100	0.447	1.097
	RPS2, N	RLRR rev					
RGA-XLRR-INV	*Xa21*	XLRR-INV1 for	6	6	100	0.782	3.096
	Xa21	XLRR-INV2 rev					
RGA-Pto kin	*Pto*	Pto kin3 for	6	5	83.33	0.733	2.516
	Pto	Pto kin4 rev					
RGA-NLRR	*N, Cf9*	NLRR for	7	7	100	0.807	4.258
	N, Cf9	NLRR rev					
RGA-XLRR	*N, Cf9*	XLRR for	2	2	100	0.446	1.355
	N, Cf9	XLRR rev					
		Total	23	22	-	-	12.32
		Mean	4.6	4.4	95.65	0.643	2.465

TNB: total number of bands, NPB: number of polymorphic bands, PPB: percentage of polymorphic bands, Rp: resolving power, PIC: polymorphism information content.

compartment could be due to the accumulation, during domestication, of different RGA loci which should be linked to adaptive traits such as resistance to various microbial and non-microbial pathogens (Satya et al. 2014). Thus, cultivated and wild fig trees show differences at the genome level due to the mode of reproduction and traits related to the domestication syndrome. These mechanisms have helped accumulate different combinations of alleles for adaptive loci like RGAs in wild populations and in cultivars of *Ficus carica* L.. We also noted that individuals 'Fas88' and 'Fas90' from Kesra and 'Fas45' and 'Fas46' from Rafraf (cluster I) showed the same RGAP profiles and could not be differentiated, and they also (Fig. 1). The RGA markers amplified by the degenerate RGA primers generated a low number of polymorphic bands that could not discriminate between several genotypes, especially closely related ones. The decrease in the number of polymorphic bands for RGA markers may result from the use of a limited number of RGAP primer pairs and its gene region targeting nature. Indeed, the RGA primers are designed based on coding conserved regions of the R genes (Nemli et al. 2015).

3.3. Principal coordinate analysis

The Ochiai similarity matrix generated from RGA markers data was used for Principal Coordinate Analysis (PCoA) to get an alternative view of the relationships and distance between the 62 *Ficus carica* L. genotypes. The first two coordinates accounted for 14% and 16.65% of the total variation (30.65%) (Fig. 2). The projection of the

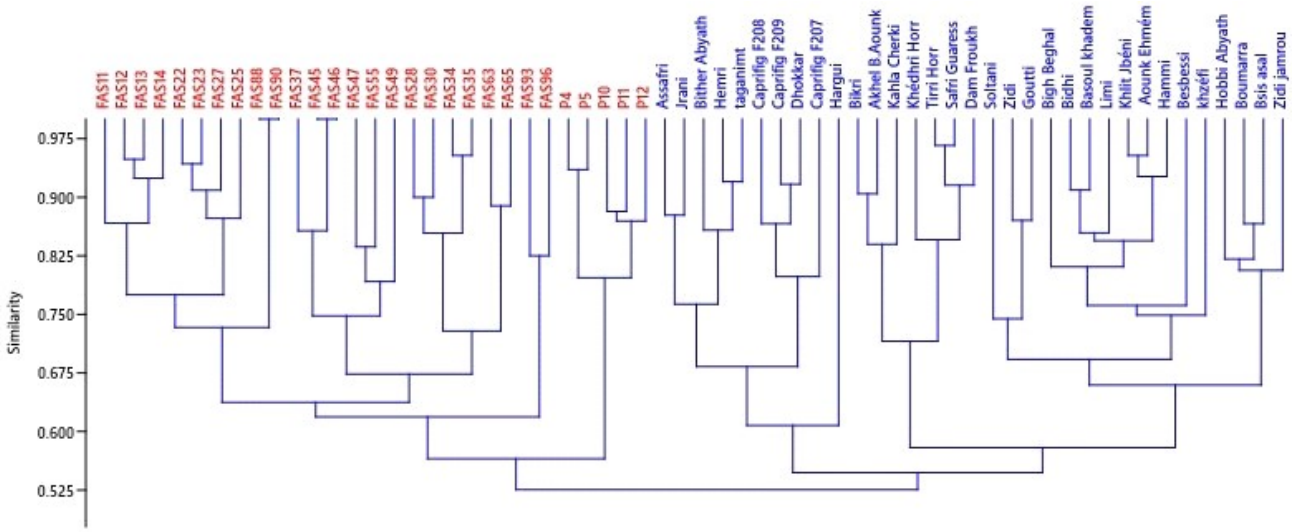

Fig. 1. UPGMA dendrogram illustrating genetic relationships among 62 Tunisian fig accessions constructed based on similarity matrix from 23 RGAP markers.

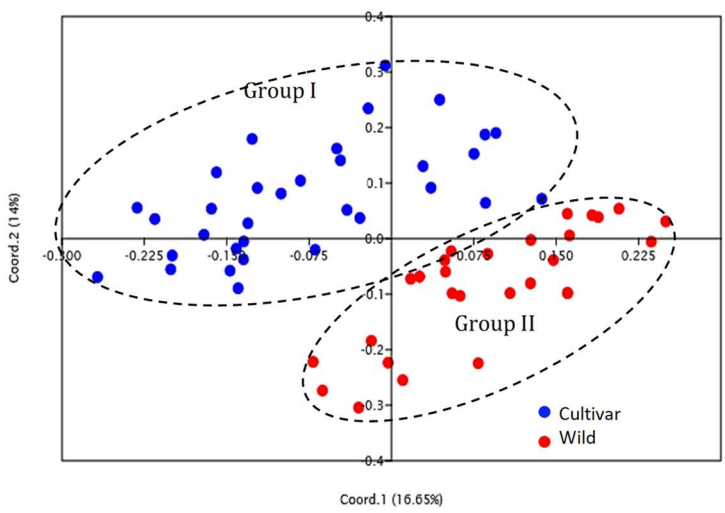

Fig. 2. PCoA analysis of fig accesions based on RGA data matrix

62 fig trees on the two-dimensional plane of the PCoA shows that fig genotypes were separated into two groups that correspond to cultivated and wild figs. This pattern is similar to UPGMA clustering. Besides, PCoA showed a slight overlap indicating a gene flow between the two groups. PCoA support UPGMA clustering regarding the genetic differentiation among cultivated and wild figs based on RGAP markers. It is obvious that the RGAP markers allowed the amplification of different conserved patterns of resistance genes that differentiate the fig trees studied according to their wild or cultivated nature. These results argue in favor of the hypothesis of the existence of two independent genetic bases that differentiate between cultivated and wild Tunisian fig trees based on amplified RGAP markers and the different genes targeted by the RGAP technique.

4. CONCLUSION

In this study, RGAP as a gene-based molecular marker proved to be a potential genetic marker system in *Ficus carica* L. species that could be useful in many applications including genetic diversity analysis, resistance gene identification, germplasm conservation and molecular breeding for resistance against diseases.

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