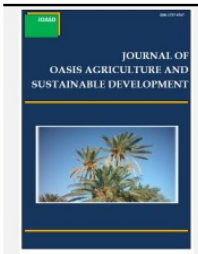

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Molecular characterization of some Tunisian biferous fig accessions (*Ficus carica L.*) using RAPD and ISSR markers

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Abstract

This work studied the evaluation of the genetic diversity of twelve biferous Tunisian fig accessions ('Bither Akhal,' 'Bither Ahmer1', 'Bither4', 'Bither Ahmer2', 'Bither2', 'Bither3', 'Bither1', 'Besbessi,' 'Wahchi,' 'Khenziri,' 'Garghi' and 'Bouholi') using molecular markers (RAPD and ISSR) and to determine the relatedness between accessions. Among the 31 DNA loci, 29 were polymorphic (93.61 %). With primers OPL11 and OPW04, DNA fragments produced a minimum of 8 and a maximum of 11 polymorphic bands. Primers had a collective resolving power value (Rp) of 13. Regarding ISSR markers, 47 DNA loci were identified, with 41 loci (87.24 %) being polymorphic. Polymorphic DNA fragment bands ranged from 8 (AM5) to 14 (AM6). Primers revealed 14.8 collective resolving power values (Rp). For RAPD and ISSR markers, the 'Bither3' and 'Bither4' accessions showed the highest genetic distances. UPGMA generated independent dendrograms based on RAPD and ISSR banding patterns. This study revealed significant genetic diversity among local fig accessions, indicating their potential as sources for breeding programs in fig selection.

1. INTRODUCTION

The fig (Ficus carica L.) is one of the world's most commonly cultivated fruit trees, particularly in the Mediterranean regions. The global fig production is approximately 1 152 799 tons per year, with 90 % being produced in Mediterranean countries (FAOstat, 2019). In Tunisia, the Fig is an essential fruit species, mainly cultivated throughout the country (Aljane, 2011), with a production of around 27 400 tons in 2018 (GIFruits, 2018).

Fig cultivars are divided into three groups: Smyrna (Ficus carica var. Smyrna Shinn.), Common Fig (Ficus carica var. hortensias Shinn.), and San Pedro (Ficus carica var. intermedia Shinn.) (IBPGR, 1986; Aljane et al., 2022). The Smyrna type (uniferous) necessitates pollination with pollen from caprifigs and mature fruit with viable seeds. Without pollination, fruits will fall from the tree before maturation. The most extensively grown Tunisian cultivar of this kind is 'Zidi' (Singh et al., 2015). The standard type (biferous and uniferous) produces figs without caprification. Some cultivars have a first crop, known as breba, followed by a second excellent crop, referred to as the main crop. Many cultivars in Tunisia, including 'Bayoudhi,' belong to this type (Singh et al., 2015; Aljane, 2016). The San Pedro type (Biferous) produces the first crop as a breba crop that persists to maturity (parthenocarpic) and does not require any flower fertilization. Instead, pollination is essential for the second (main crop) to mature. The main cultivars of this type are 'Bither' and 'Bouholi' (Singh et al., 2015; Aljane, 2016).

Numerous Fig cultivars exist, and genotypes are misidentified or given different local denominations. Many cultivars represent it; among these, Minangoin (1931) studied 65 cultivars using morphological traits. Twenty-two cultivars were identified by Valdeyron and Crossa-Raynaud (1950), 28 cultivars by Lahbib (1984), 22 cultivars by Mars et al. (1998), and 6 cultivars by Ben Salah (2004). Additionally, more than 100 female and 20 male cultivars have been characterized by Aljane (2004), Aljane and

Ferchichi (2010), Aljane et al. (2012); Essid et al. (2015a), Essid et al. (2015b); Essid et al. (2017); Essid et al. (2021). The morphological studies can often yield ambiguous results due to high plasticity for many characters, as well as phenotypic modification caused by environmental conditions and farming practices (Chatti et al., 2004; Çalişkan and Polat, 2008; Polat and Caliskan, 2008; Aljane and Ferchichi, 2010; Podgornik et al., 2010; Giraldo et al., 2010). This is why it is necessary to use morphological characterization in conjunction with DNA molecular markers to pursue genetic diversity studies.

The primary objective of this study is to characterize and analyze the genetic diversity of 12 Tunisian biferous fig accessions maintained in an ex-situ germplasm collection using RAPD and ISSR molecular markers. In Fig, molecular markers (SSR, ISSR, and RAPD) are widely used for cultivar characterization and identification (Papadopoulou et al., 2002; Salhi-Hannachi et al., 2003; Saddoud et al., 2008; Agarwal et al., 2008; Almajali et al., 2012; Essid et al., 2015; Essid et al., 2021). These markers offer numerous advantages because they are stable and detectable in all tissues and are not affected by environmental factors.

2. MATERIAL AND METHODS

2.1. Plant Material and DNA Extraction

The study was conducted on 12 biferous (San Pedro type) fig accessions belonging to three traditional fig-growing geographic regions (Table 1). These are maintained at the ex-situ germplasm collection established in El Gordhab, Tataouine, in Southeastern Tunisia. Hardwood cuttings propagated fig plant materials.

The DNA was extracted from 30 mg of young leaves using the CTAB technique, as described by Doyle and Doyle (1990) and Aras et al. (1990), with modifications by Rout and Aparigita (2009). The purity and DNA concentration were determined by spectrophotometry at 260/280 nm and by running 2% agarose gel electrophoresis.

2.2. RAPD and ISSR primers and PCR reactions temperate

A set of seven RAPD (OPL11, OPWO2, and OPWO4) and ISSR (AM 3, AM 5, AM 6, and AM 9) primers were used in a total volume of 20 µl reactions containing 35 ng genomic DNA, 200 mM dNTPs, 1.5 mM MGCl2, 0.4 µM each primer, 1 U Taq Polymerase, and 1 x Taq Buffer.

Amplification was carried out in a thermocycler Gen-Amp PCR System 9700 Thermal Cycler (Applied Biosystems, USA) using the following temperature profile: an initial cycle of 5 min at 94° C, the denaturing step at 94° C for 60 seconds, Followed by hybridization (45 cycles) of 52° C for 60 seconds, and a final extension at 72 ° C for 7 min. Amplified PCR products were separated by electrophoresis on a 2% agarose gel using 100 bp markers and then stained with ethidium bromide. The generated bands were visualized using a Gel Doc system and digitally photographed with software (Bio-Rad, France).

2.3. Data Analysis

For each primer, different parameters of genetic diversity were estimated, including total bands, polymorphic bands (PPB), monomorphic bands (MPB), and the percentage of polymorphic markers (% PPB). A genetic distance matrix was estimated based on Jaccard's DICE similarity coefficient (DICE, 1945). The ability of the most informative primers to differentiate within

Table 1. Studied Tunisian fig accessions along with their respective localities of origin.

Population	N°	Accession name	Label	Geographic origin
Southeast	1	Bither Akhal	BAK	Zarzis- Médenine
	2	Bither Ahmer1	BAH1	Beni Ghezeil- Médenine
	3	Bither4	BTH4	Toujen- Gabès
	4	Bither Ahmer2	BAH2	Matmata- Gabès
Center east	5	Bither2	BTH2	Kerkennah Islands- Sfax
	6	Bither3	BTH3	Kerkennah Islands- Sfax
	7	Bither1	BTH1	Ghadhabna – Mahdia
	8	Besbessi	BES	Maissjed Aissa- Sousse
Northwest	9	Wahchi	WAH	Djébba- Béjà
	10	Khenziri	KZR	Djébba- Béjà
	11	Garghi	GAG	Djébba- Béjà
	12	Bouholi	BHL	Djébba- Béjà

accessions was assessed by calculating their resolving power (Rp), defined as Rp = Σ Ib, where Ib = 1 – [2 × (0.5 – p)] (Prévost and Wilkinson, 1999; Gilbert et al., 1999). Reproducible fragments were scored 1 or 0 for each sample: 1 was assigned for the presence of an amplicon and 0 for its absence.

Molecular variance (AMOVA) was performed using GenAlEx 6.41. (Peakall and Smouse, 2012). Genetic relationships within the accessions studied were calculated using the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) cluster analysis of the similarity matrix. Principal Component Analysis (PCA) was performed using IBM SPSS Statistics 20 (IBM SPSS Statistics, 2020) and XLSTAT Pro 7.5.3 (XLSTAT, 2005).

3. RESULTS

3.1. Molecular results

3.1.1. Genetic Polymorphism and RAPD Patterns

A set of 31 DNA fragments (loci) were amplified. Polymorphic bands were found in all three RAPD primers tested. The bands' sizes ranged from 150 to 1200 bp. Regarding polymorphic markers, RAPD showed that 93.61% of the total bands were polymorphic. Furthermore, the maximum percentage of polymorphic markers was 100 % in the OPWO4 primer, and the lowest was 88.89% in OPL11. Resolving power (Rp) values revealed variation within the three primers. OPW04 RAPD primers showed the highest Rp value of 14.83 (Table 2).

3.1.2. Genetic Polymorphism and ISSR Patterns

Results showed that the four ISSR primers tested have polymorphic bands. The band size varied from 150 to 1000 bp, and 47 DNA fragments were amplified, with a percentage of polymorphic markers accounting for 87.24% of the total bands. Two ISSR primers (AM3 and AM6) showed high Rp values of 17.83 and 16.17, respectively (Table 3). AM6 has the highest rate of polymorphic markers (93.33%).

Molecular RAPD and ISSR results showed that one RAPD primer (OPW04) and two ISSR primers (AM3 and AM6) appeared to be the most valuable for assessing the biferous fig accessions. Furthermore, markers with high Rp values are more suitable for analyzing genetic diversity. According to these results, there is high genetic diversity among 12 local biferous fig accessions

Table 2. Degree of polymorphism of the selected RAPD primers and the resolving power (Rp) obtained among 12 local Tunisian biferous fig accessions.

Primer Code	Size (Pb)	Total Bands	Polymorphic Bands	Monomorphic Bands	Percentage of Polymorphic Markers	Resolving Power (Rp)
OPL11	400 -1100	9	8	1	88,89	12
OPWO2	220 - 1000	12	11	1	91,67	12,17
OPWO4	150 - 1200	10	10	0	100	14,83
Total	150 - 1200	31	29	2	-	39
Polymorphism average	-	10,33	9,67	0,66	93,61	13

Table 3. Degree of polymorphism of the selected ISSR primers and the resolving power (Rp) among 12 local Tunisian biferous fig accessions.

Primer Code	Size (Pb)	Total Bands	Polymorphic Bands	Monomorphic Bands	Percentage of Polymorphic Markers	Resolving Power (Rp)
AM3	650 - 150	12	10	2	83.33	17.83
AM5	200 - 700	10	8	2	80	11.17
AM6	240 - 1000	15	14	1	93.33	16.17
AM9	300 - 900	10	9	1	90	14
Total	200 - 1000	47	41	6	-	59,17
Polymorphism average	-	11,75	10,25	1,5	87,24	14,8

from the three fig-growing regions (Southeast, Central East, and Northwest) of Tunisia.

3.2. Molecular Variance (AMOVA) Analysis

We estimated the variance components to assess which contributes more to genetic diversity: within-group or among-group variance (Table 4). The respective percentages of variation were 1% and 11% for RAPD and ISSR, indicating that the genetic background attributable to geographical origin contributes slightly to genetic diversity in both types of polymorphism. The among-group variance components were low.

3.3. Cluster and PCA Analysis

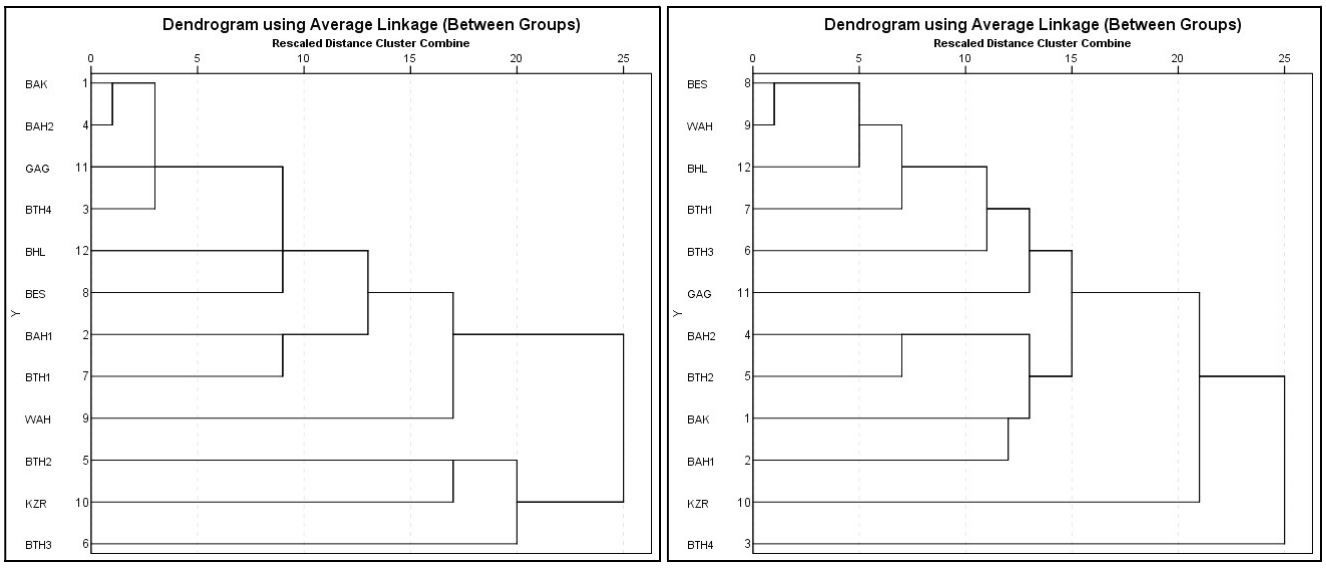
The clustering pattern obtained with two types of markers (RAPD and ISSR) data showed almost mutually independent results (Fig. 1). However, some common groupings were observed. The dendrogram based on RAPD markers was clustered into three major clusters and two individuals.

The first cluster (BAK, BTH2, GAG, BTH4) was divided into two sets; the binomial BAK and BTH2 exhibited the highest similarity and are closely related accessions. The second group consisted of four accessions (BHL, BES, BAH1, and BTH1), which were characterized by low similarity. The three groups contained an individual accession (BTH2 and KZR) and exhibited very low genetic relations. Based on the clustering method among accessions, as illustrated by the dendrogram, the following accessions (WAH and BTH3) are supported by their low genetic relationships with other accessions. They may be considered distinct accessions (Fig. 1).

Furthermore, some common groupings were observed in ISSR-based dendrograms. The first cluster (BES, WAH, BHL, BTH1, BTH3, GAGA) revealed a subgroup (BES, WAH) that was the most closely related to the accessions, and a branch (GAG) was the most divergent from the other accessions in this group. The second cluster

Table 4. AMOVA for the 12 Tunisian biferous fig accessions based on RAPD and ISSR markers.

Source of variation	DF	SCE	CM	% Variation	Estimated variation
			RAPD		
Among group	2	10.833	5.417	1	0.063
Within group	9	46.500	5.167	99	5.167
Total	11	57.333		100	5.229
			ISSR		
Among group	2	20.667	10.333	11	0.840
Within group	9	62.750	6.972	89	6.972
Total	11	83.417		100	7.813



a **b**

Fig. 1. UPGMA phenogram of the genetic relationships among 12 Tunisian biferous fig accessions constructed using an estimated simple matching genetic distance based on (a) RAPD and (b) ISSR markers.

(BAH2, BTH2, BAK, BAH1) was divided into two binomials. Additionally, BAH2 and BTH2 showed strong genetic similarities, suggesting that they are likely to be closely related.

The other accessions (KZR and BTH4) were classified separately, as evidenced by their low similarities to different studied accessions. The two dendrograms resulted in two breeding lines, BTH3 and BTH4, which originated from the Kerkennah Islands (Sfax) and Toujen (Gabès), respectively, and are distinct from all the other accessions. Using cluster analysis, the PCA analysis from RAPD and ISSR data produced similar results (Fig. 2).

4. DISCUSSION

These findings demonstrate that the deployment of RAPD and ISSR markers is valuable and informative for assessing fig genetic diversity. The most useful RAPD primer was OPWO4, which generated ten banding patterns with an Rp of 14.83. In contrast, the most useful ISSR primers were AM3 and AM, yielding 12 and 15 banding patterns with RPs of 17.83 and 16.17, respectively. AM6 was the most effective ISSR primer in detecting polymorphism in 93.33% of bands.

Among these markers, the RAPD marker was considered to generate the most significant number of markers (47). However, the numbers of polymorphisms (Rp 13 in 3 ISSR primers; Rp 14.8 in 4 RAPD primers) were lower overall than those described for foreign figs (Rp 29.65 in 4 ISSR primers: Salhi-Hannachi et al. (2004); Rp 62.28 in 7 RAPD primers: Salhi-Hannachi et al. (2005). These results indicate low genetic diversity in these three fig populations, which may be attributed to the adaptation of breeding accessions and the arbitrary introduction and exchange of gene fig materials within Tunisian

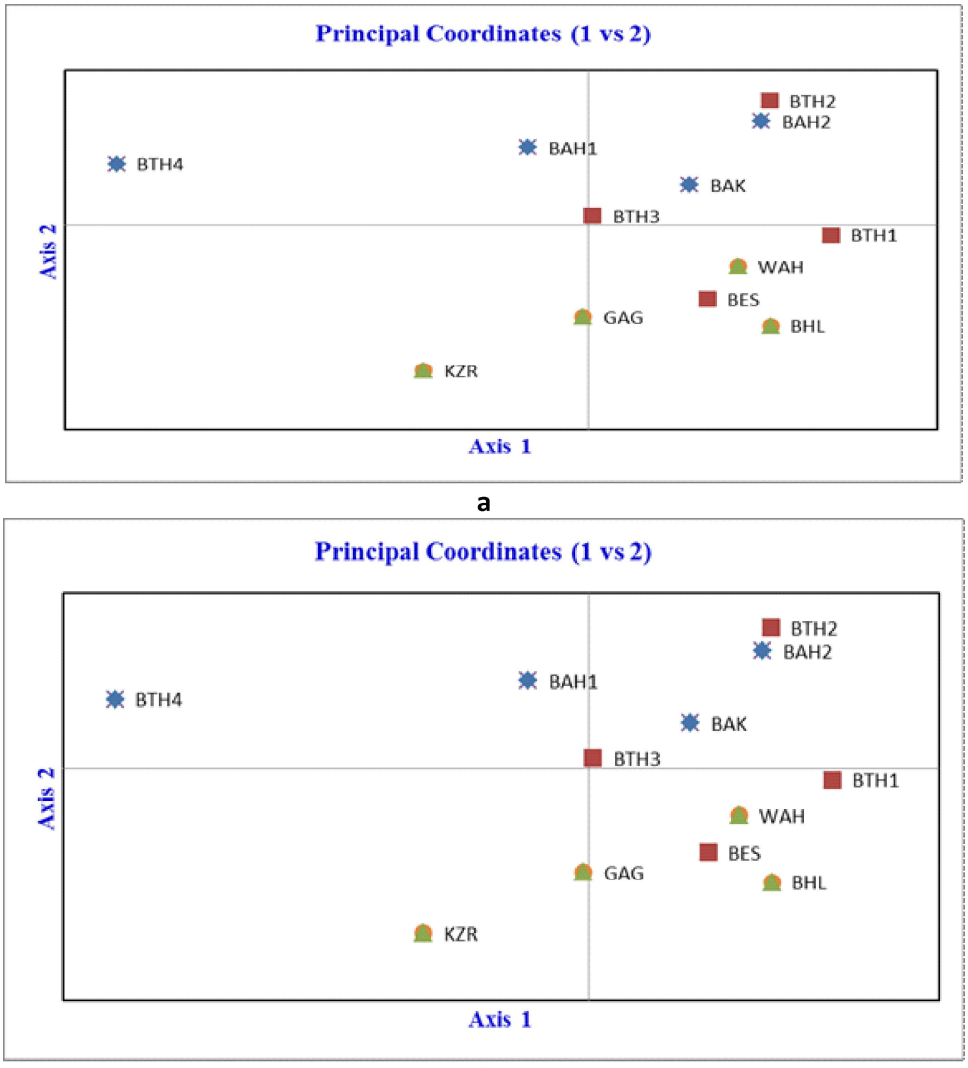


Fig. 2. PCA of the 12 fig accessions based on RAPD (a) and ISSR (b) markers. The contributions of PC1, PC2 and PC3 were74,93% and 77,47 % %, respectively.

regions. In addition, AMOVA analysis indicated that more than 99% and 89% of the total genetic diversity, as determined by RAPD and ISSR, respectively, is distributed within groups. However, only 1 % of the diversity is attributed to differences between regions in RAPD markers. Similarly, other studies in Tunisia have also reported this low variability between regions (Salhi-Hannachi et al., 2005).

On the other hand, ISSR accounts for 11% of the group variation, indicating that ISSR reflects slightly more variation across geographical regions. According to Salhi-Hannachi et al. (2005), the low divergence between collections or groups can be explained by the occurrence of gene flow or the common origin of the populations.

Different dendrograms were obtained; some conclusive common grouping was detected in two markers. The low correlation coefficient supported this result among RAPD and ISSR markers and various degrees of fit (Fig. 1). These results imply that the manner of polymorphism varies due to marker specificity. In addition, the relation is assumed to depend on the genome coverage and sequence type recognized by each marker system (Powell et al. 1996; Sehgal and Raina 2005). These topologies showed no correlation with the geographical origin, even though twelve fig beferous accessions are weakly structured in the combined data, which is explained by the fact that each marker represents only a part of the genome, contributing to dendrogram incongruence.

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Data Clustering and PCA analyses revealed distinct groups. BTH3, BTH4, and KZR from Kerkennah Islands- Sfax, Toujen- Gabès, and Djébba-Béjà stand out from the others accessions. This result shows that these accessions, which have different origins, are the oldest varieties in Tunisia. On the other hand, KZR was clustered with BTH2 in a separate group (Fig. 2(a)), and KZR was separated in Fig. 2(b). These findings lend credence to supposing that KZR was introduced to Southeast Tunisia independently of other foreign varieties.

5. CONCLUSION

This study found that (1) the genetic diversity of this fig population was lower than in other areas, and (2) the use of multiple markers, particularly RAPD and ISSR markers, can be crucial for the estimation of the relatedness of Fig at the variety level because they engender different classification results based on their respective characters. Furthermore, the results support that (3) Tunisian fig genetic materials were frequently exchanged between geographic regions over centuries. Fig's cultivation in Tunisia can be characterized in more detail by adding some germplasm that was not used in this research.

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AUTHOR CONTRIBUTION

Fateh Aljane, Mohamed Hichem Neily, Awatef Essid, and Hajer Abou El Farah were actively involved in the research investigation, laboratory analysis, and documentation process. Fateh Aljane drafted the manuscript, and all authors conceptualized the research work, reviewed the manuscript, and approved the manuscript for publication.

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