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Biochemical and Molecular Markers Differentiate *Ficus* Species from Four Locations in Saudi Arabia

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HEREIN, the biochemical and molecular genetic variability among some *Ficus* species collected from four locations (Shafa, Baha, Hawia, and Hada) in Saudi Arabia were studied using protein, isozyme profiles, and random amplified polymorphic DNA from PCR (RAPD) analysis. Results revealed significant variations among *Ficus* species. A significant level of polymorphism (96.5%) was recorded at the protein level. For all isozymes, high polymorphism, between 67% and 100%, was detected. The dendrogram, generated using pooled data from SDS-PAGE and isozyme studies, divided species into a main cluster including all accessions except a single species, *F. ingens* (Shafa), found in another cluster forming a separate operational taxonomic unit (OTU). The main cluster was subdivided into subclusters reflecting the high variability among *Ficus* species or accessions collected from various locations. The RAPD-PCR fingerprints for four *Ficus* species were created using five primers. Among the 35 RAPD bands detected, 18 were polymorphic and 17 unique bands. The dendrogram resulting from unweighted pair group method with arithmetic average analysis clustered the four species into one primary cluster except *Ficus ingens* that agreed with the previous dendrogram. SDS-PAGE, isozymes, and RAPD-PCR techniques helped revealing geographical effects on the genetic diversity of *Ficus* species. The preliminary information here generated can be used for taxonomical grouping, breeding, genetic improvement, local germplasm conservation, and management of Saudi fig genetic resources.

.**Keywords:** *Ficus,* Genetic diversity, SDS-PAGE, Isozyme, RAPD.

Introduction

The Kingdom of Saudi Arabia is known for having a variety of natural sites with high biological diversity and production; these sites are vital to the ecosystems' synergy (Abuzinada et al., 2005). Changes in water resources, climate conditions, edaphic variables, and anthropogenic pressures along the elevation gradient may have influenced the vegetational distribution in different parts of the country. Saudi Arabia's climate can be described as arid (Hegazy et al., 2007). There are diverse medicinal plants in the Kingdom (Rahman et al., 2004), and their use to treat a variety of human maladies dates back thousands of years. They are still available among tribal, local people, and traditional healers.

Ficus L., also known as 'Fig,' is a keystone plant in tropical rain forests because its fruits are eaten by birds, insects, animals and human throughout the year, making it an important role of the ecosystem. It is one of the largest angiosperm genera with over 750 species. The genus is distributed throughout the

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world especially in tropical and subtropical regions (Berg & Corner, 2005). Their species concentrated in Asian-Australian region with about 500 species which is about 66% of the world species. Although, the great majority of the species grow in lowlands but some of them reach up to about 2,000m altitudes (Chaudhary et al., 2012). Several *Ficus* species, including *F. glabrata, F. racemosa, F. platyphylla, F. bengalensis, F. insipid, F. sycomorus, F. asperifolia, F. capensis, F. glomerata, F. bengalensis, F. religiosa, F. exasperate, F. hirta, F. chlamydocarpa,* and *F. gnaphalocarpa* have been shown to exhibit biological functions (Hubert et al., 2011). *F. cordata* Thunb is a 10 m tall savana tree native to Senegal, Angola, South Africa, and Cameroon (Sabatie, 1985). Its leaves are used to treat hyperesthesia, ataxia, muscle tremor, and padding motions in heifers, and they can kill them 48 h after intake (Poumale et al., 2008). Additional ethnopharmacological research revealed that some western Cameroonian traditional healers utilize its stem bark to cure jaundice and to treat some liver diseases. The fig (*Ficus carica* L.) is one of the Mediterranean's oldest cultivated fruit crops. Figs have recently garnered interest for culinary purposes due to their nutritional, pharmacological, and decorative characteristics (Flaishman et al., 2008), and become widely distributed over the world. Mulberry (genus: *Morus*) is an economically important plant in sericulture as it is the only food supply for the domesticated silkworm Bombyxmori. Morus can be found in Asia, Europe, North and South America, and Africa, and it is commonly cultivated for silk production in East, Central, and South Asia. Mulberries are also valued for their edible fruit (*M. alba* and *M. laevigata*) and wood (*M. laevigata* and *M. serrata*) (Weiguo et al., 2005).

For years, morphological markers were used to identify and characterize genotypes. Caliskan & Polat (2012), Mohamed et al. (2017), and Abdelsalam et al. (2019) reported high variations based on leaf morphological characters of 21 accessions of fig collected from Turkey, Libya, and Egypt. Isozymes are helpful in studying genetic variation within and among plant and animal populations. Isozymes were used in genetics, taxonomy, ecological and evolutionary, research, as well as cultivar and line identification (Peirce & Brewbaker, 1973; Mondini et al., 2009). Isozyme electrophoresis has been utilized to detect clones and investigate the clonal organization of plant populations with great effectiveness (Johanson et al., 1996; Lehmann, 1997).

Due to the arbitrary primer sequence, random amplified polymorphic DNA from PCR (RAPD) is relatively quick and easy to use as DNA-based identifier (Welsh & McClelland, 1990; Williams et al., 1990). Thus, RAPD has been widely applied to study genetic relations between diverse accessions of various plant species (Khan et al., 2009). In 2019, Ahmed and Fadl used morphological characterization and DNA barcoding to differentiate between *Fircus carica, Ficus cordata* subsp. *Salicifolia* and *Ficus palmata* in Taif highlands (Saudi Arabia). Mostafa et al. (2020) distinguished among 17 *Ficus* species based on leaf morphological and AFLP markers in Egypt. However, the limitations of phenotype-based genetic markers led to the creation of DNA-based markers, which are now widely used, due to advantages such as being detectable and stable in all tissues regardless of the cell's growth, differentiation, development, or defensive status. Furthermore, DNA markers are unaffected by environmental, pleiotropic, or epistatic effects (Agarwal et al., 2008). The diversity and genetic relations has been examined in the figs (Khadari et al., 1995; Achtak et al., 2009, Abou-Ellail et al., 2014; Akin et al., 2020).

The present work aims at studying the relationship between geography and biochemical genetic variability of the *Ficus* species found in four locations in Saudi Arabia, with biochemical (isozyme and protein electrophoresis) and molecular markers (RAPD analysis).

Materials and Methods

The study area

Saudi Arabia extends over approximately 16° of latitude, from 16° 22′ at the Yemen border in the south; to 32° 14′ at the Jordanian border in the north, and from 34° 29′ in the Red Sea to 55° 40′ E in the East (Fig. 1). The Taif region is located in the middle of the western mountains at an altitude of up to 2500m. Many wild species, including medicinal plants, disappeared as vast tracts of virgin lands were converted to agricultural regions. The climate in the area is tropical and arid with a total monthly mean of 23.2°C±5.1°C and a yearly amount of rainfall from 83.3mm yr^{-1} to 3312mm yr^{-1} (Al-Sodany et al., 2016). Four locations: Shafa, Baha, Hawia, and Hada were selected for this study (Fig. 1).

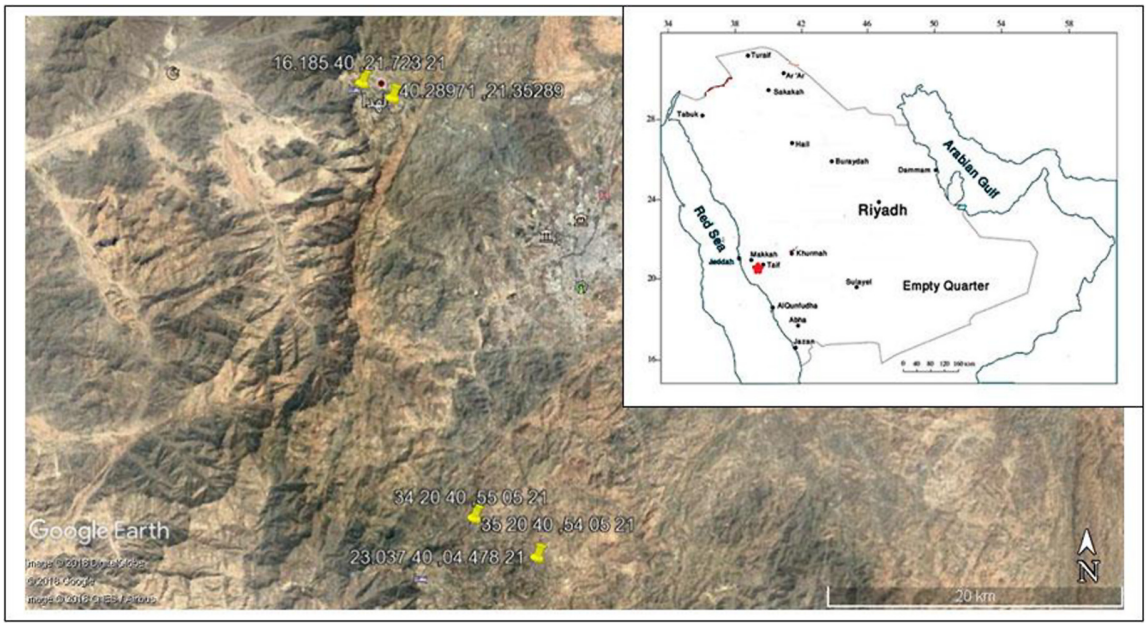


Fig. 1. Map showing the study area and sampled sites

Sample collection

Seeds and fresh leaves (young and matured) of 32 individuals belonging to the genus *Ficus*, family Moraceae, ranging from 2 to 4, were collected from the four sites. Table 1 shows how the gathered natural materials were recognized as indicated by Collenette (1999) and Chaudhary (2001).

Protein electrophoresis

Protein extraction was conducted by homogenizing 1 g fresh leaves in 1mL extraction buffer (0.5M Tris buffer pH 6.8) using a mortar and pestle. The extract was then transferred into clean Eppendorf tube and centrifuged for 5min at 10000rpm. The supernatants were moved to clean Eppendorf tube and stored at −20°C until use. The Laemmli method was used to perform SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). Gel analyzer 3 software was used to photograph, scan, and analyze the results.

TABLE 1. Names and sources of the studied species of the family Moraceae

Species	Chorotype	Source	Height (m)	Leaf shape (Long x wide cm)	Fruit
Ficus cordata subsp. *salicifolia*	TR	Shafa, Hada	8-15	Lanceolate; (5-20 x 1-6)	Orange to red when ripe
F. ingens	TR	Shafa	8-10	Ovate or lanceolate, (12-16 x 5-8)	White at first, becoming pink, red or purple when ripe
F. palmata	SZ	Shafa, Baha, Hawia, Hada	6-10	Broad ovate, (13-27 x 14-24)	Syconoid, varying from deep violet to black
F. carica	ME+IT	Baha, Hawia, Hada	3-9	Cordate, (11-27 x 9-23)	Yellow to green to purple to bronze on the outside
F. palmata var. *virgata*	SZ	Baha	1-3	Broadly ovate, cordate at base, (5-12.5 × 5-12)	Glabrous at maturity, yellow or purple when ripe

Isozyme electrophoresis

To find isozyme differences across *Ficus* species, native-polyacrylamide gel electrophoresis (Native-PAGE) was used. Acid phosphatase (Acph), alcohol dehydrogenase (Adh), aldehyde oxidase (Ao), a, b- Esterase (EST), malate dehydrogenase (Mdh), malic enzyme (Mal), and peroxidase (Px) were the isozymes selected for analysis. According to the protocol, these isozymes were separated on 10% polyacrylamide gel electrophoresis (Stegemann et al., 1985). Acph gels were stained using Wendell & Weeden (1989) procedures, while Adh gels, Mdh, Mal, and Ao were stained following Jonathan & Wendell (1990). Scandalios (1964) method was used for a, b-EST, and Heldt (2005) for Px.

RAPD analysis

Genomic DNA was extracted from fresh and young leaf tissues of four species as indicated in Table 6 according to Dellaporta et al. (1983). For RAPD analysis, five primers (Table 5) were added to a 25µL reaction volume containing 2.5µL from the primer, 2.3µL genomic DNA, 12.5µL 2 x Ready Mix RED *Taq* PCR Reaction mix., and 7.7µL ddH_2O. The Perkin Elmer Gene Amp PCR thermocycler 2400 was utilized for amplification. The ideal PCR amplification settings were as follows: a denaturation step for 2min at 94°C, followed by 40 cycles of 2min at 94°C, 1.5min at 36.5°C, and 2min at 72°C, with an extension step for 12min at 72°C. DNA was visualized using 12µL PCR product on 1.5% agarose in TBE buffer with ethidium bromide at 100 V for 1 h and photographed by the Gel Documentation system (GelDoc BioRad 2000) under UV transillumination.

Data analysis

The relationship between the analyzed accessions was quantified using protein, isozyme, and RAPD fingerprinting differences in 12 samples and in 4 samples for RAPD fingerprinting. The presence or absence of each protein, isozyme, and RAPD band was treated as a binary character in a data matrix to detect genetic similarities and generate a dendrogram among the investigated samples (coded 1 and 0, respectively). Isozymes data, SDS-PAGE, and RAPD-PCR data were utilized to generate a binary matrix for cluster analysis. The genetic similarities between species were calculated according to the Dice similarity coefficient (Dice, 1945), and used for generating a dendrogram using the unweighted pair group method with arithmetic average (UPGMA) in the SPSS-20 program (SPSS, 2011).

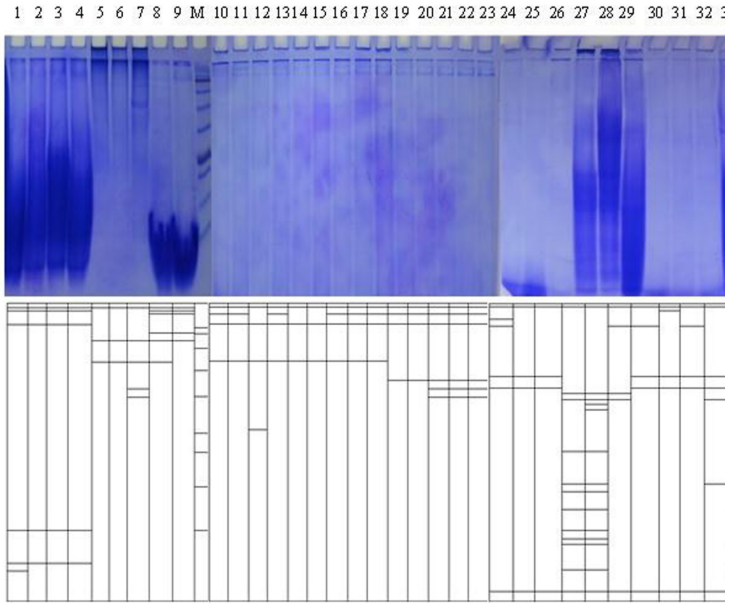
Results

Protein analysis

In the protein profile of leaves of *Ficus* species, a significant level of polymorphism (96.5%) was recorded (Table 2, Fig. 2). Only one monomorphic band of 125.2kDa was detected and 28 polymorphic bands, among which 10 were unique, were identified; one in *Ficus cordata* subsp. *Salicifolia* (Hada) at about 51.6kDa. A band at about 66.7kDa was recorded only in *F. palmata,* and *F. carica* collected from Hada. Moreover, three bands at 19.8, 16.4, and 15.2kDa distinguished *Ficus cordata* subsp. *Salicifolia* collected from Hada.

TABLE 2. Number and types of bands as well as the percentage of the polymorphism generated by the SDS-PAGE and isozymes in 12 accessions of the family Moraceae

Parameter	Monomorphic bands	Polymorphic bands		Total bands	Polymorphism %
		Non-unique bands	Unique bands		
SDS-PAGE	1	18	10	29	96.5
Acp	-	4	3	7	100
Adh	-	-	3	3	100
Ao	-	1	1	2	100
α-Est	1	4	2	7	86
β-Est	-	4	2	6	100
Mdh	1	1	1	3	67
Mal	-	5	2	7	100
Px	-	5	4	9	100
Total	3	42	28	73	96



Lane No.	Species	Source
1-4	*F. cordata subsp. salicifolia*	Shafa
5-7	*F. ingens*	
8-9	*F. palmata*	
10	*F. carica*	
11-13	*F. carica*	Baha
14-16	*F. palmata*	
17-19	*F. palmata var. virgata*	
20-21	*F. palmata*	Hawia
22-24	*F. carica*	
25-27	*F. palmata*	
27-29	*F. cordata subsp. salicifolia*	Hada
30-32	*F. carica*	

Fig. 2. Photograph (A) and diagram (B) representing SDS-protein banding pattern of accessions of the genus Ficus [M= Marker]

Isozyme analysis

For all isozymes, significant levels of polymorphism were detected, ranging between 67% and 100%, with 18 unique bands in all enzymes as indicated in Table 2. The absence/ presence of isoforms for all isozymes is shown in Table 3 and Fig. 3.

Acid phosphatase

Seven bands were scored and considered as polymorphic (100% polymorphism percentage). The isoforms at Rf of 0.201 and 0.352 discriminated *F. cordata* subsp. *Salicifolia* collected from Shafa and seemed as specific bands (positive marker). The band at Rf of 0.061 was present in all species but for *F. ingens* collected from Shafa (negative marker for this species). The band at Rf of 0.106 disappeared in all Shafa species (negative marker for this location) and appeared in the other three locations.

Alcohol dehydrogenase

The analysis revealed three polymorphic bands (100% polymorphism percentage). As a negative marker, the band at Rf of 0.054 was absent in *F. ingens* but detected in all other species. The bands at Rfs of 0.142 and 0.372 discriminated *F. cordata* subsp. *Salicifolia* and *F. palmata* collected from Shafa, respectively; and were considered specific bands.

Aldehyde oxidase

The Ao enzyme was found as two isoforms. One negative marker at Rf of 0.054 was found for *F. ingens*, whereas a band at Rf of 0.14 discriminated *F. palmate* and *F. carica* from Hawia from other species; it can be considered a marker for this location.

α- Esterase

The α- EST profile showed seven isoforms with two unique bands and 86% polymorphism (Tables 2 and 3). As a common marker for this family, the band at Rf of 0.041was found in all accessions. The band at Rf of 0.232 was absent in *F. ingens* and considered a negative marker. In contrast, bands at 0.635 and 0.896 Rf were expressed in *F. palmate* and *F. cordata* subsp. *Salicifolia* collected from Hada and considered species-specific bands.

β- Esterase

The β-EST pattern showed the presence of six polymorphic bands (100% polymorphism percentage). The band at an Rf of 0.033 was scored in all accessions and absent in *F. ingens*; thus, it was considered a negative marker for that species. A band at an Rf of 0.065 was absent in all accessions of Shafa (negative marker) and present in those of other locations. The band at 0.907 discriminated *F. cordata* subsp. *Salicifolia* from Hada from other accessions and was considered a species-specific band.

TABLE 3. The presence (+) and absence (-) of bands in 8 enzymes in accessions of genus Ficus.

Rf	*F. Cordata* subsp. *salicifolia* (Sh)				*F. ingens* (Sh)			*F. palmata* (Sh)		*F. carica* (Ba)			*F. Palmata* (Ba)			*F. p. var. virgata* (Ba)			*F. pal.* (Hw)			*F. carica* (Hw)			*F. palmata* (Hd)			*F. Cordata* subsp. *salicifolia* (Hd)			*F. carica* (Hd)		
	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
Acid phosphatase																																
0.061	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.106	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.201	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.274	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	-	-	-	-	-	-	-
0.352	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.637	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
0.860	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-
Alcohol dehydrogenase																																
0.054	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.142	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.372	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aldehyde oxidase																																
0.054	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.140	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-
α-esterase																																
0.041	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.104	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.232	-	-	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.519	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-
0.589	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	-	-	+	-	-
0.635	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	+
0.896	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-
β-esterase																																
0.033	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.065	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.158	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+
0.437	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	+	+
0.628	-	+	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	-	-
0.907	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-

TABLE 3 cont.

Rf	*F. Cordata* subsp. *salicifolia* (Sh)				*F. ingens* (Sh)			*F. palmata* (Sh)		*F. carica*(Ba)			*F. Palmata* (Ba)			*F. p. var. virgata* (Ba)			*F. pal.* (Hw)		*F. carica*(Hw)			*F. palmata* (Hd)			*F. Cordata* subsp. *salicifolia* (Hd)			*F. carica* (Hd)		
	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
Malate dehydrogenase																																
0.046	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.080	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-
0.291	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Malic enzyme																																
0.034	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.061	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	+	-	+	+	+
0.108	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-
0.189	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
0.277	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	+	+	+
0.351	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-
0.669	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+
Peroxidase																																
0.028	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
0.050	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
0.083	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.166	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.293	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.392	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.492	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
0.702	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.862	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	+	+	+

(Sh): Shafa (Ba):Baha (Hw): Hawia (Hd):Hada

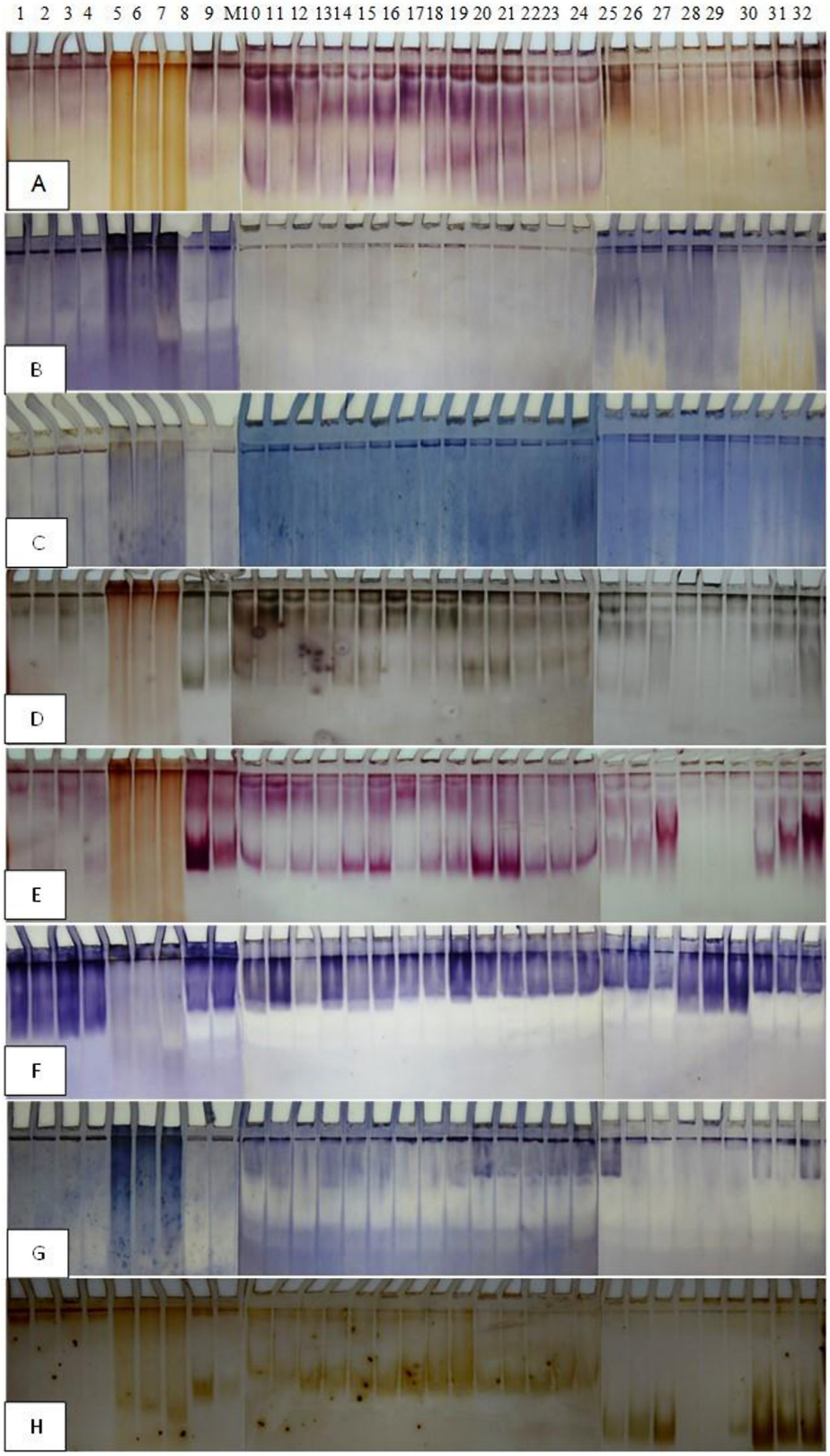


Fig. 3. Photographs; (A) Acid phosphatase, (B) Alcohol dehydrogenase and (C) Aldehyde oxidase, (D) α-esterase, (E) β-esterase, (F) Malate dehydrogenase, (G) Malic enzyme and (H) Peroxidase, representing banding patterns of accessions of the genus *Ficus* [Lanes number indicated in Fig. 2]

Malate dehydrogenase

The pattern showed one monomorphic band at 0.046 Rf and two polymorphic ones (67% polymorphism percentage). As with α-EST, a common marker for this family was scored at Rf of 0.046 in all accessions. The *F. cordata* subsp. *Salicifolia* from Hada was discriminated by a band at 0.080; therefore, it could be a specific band for this species**.**

Malic enzyme

Table 3 illustrates the absence and presence of Mal isoforms with seven polymorphic bands (100% polymorphism percentage). Two markers were detected; one negative at Rf of 0.034 in *F. ingens* and another positive at Rf of 0.189 in *F. palmata* from Hada. The band at Rf of 0.061 was only present in accessions from Hada (positive marker for this location).

Peroxidase

The pattern revealed the highest number (9) of polymorphic bands (100% polymorphism percentage). The band at 0.028 distinguished the three species of the *Ficus* genus from Hada and *F. ingens* was discriminated by an apparently specific band at an Rf of 0.702.

Genetic similarity and UPGMA tree

The genetic distance among the 12 accessions of the genus *Ficus* family based on SDS-PAGE and isozymes markers is indicated in Fig. 4 by an UPGMA tree. The dendrogram grouped accessions into a main cluster including all accessions except a single species, *F. ingens* (Shafa), found in another cluster forming a separate operational taxonomic unit (OTU) at a distance 25 (Fig. 4). The main cluster was subdivided into two subclusters at distance 16, the first included two accessions from Hada (*F. palmata* and *F. carica*). In the second subcluster, *F. cordata* subsp. *salicifolia* from Shafa split at distance 11, *F. carica* (Hawia) and *F. cordata* subsp. *salicifolia* (Hada) were differentiated. Whereas at a distance 6, *F. palmata* from Shafa was separated as single unit. *F. palmata* var. *virgata* (Baha) and *F. palmata* (Hawia) were differentiated at a distance about 3. *F. carica* (Shafa), *F. carica* (Baha), and *F. palmata* (Baha) were near one another.

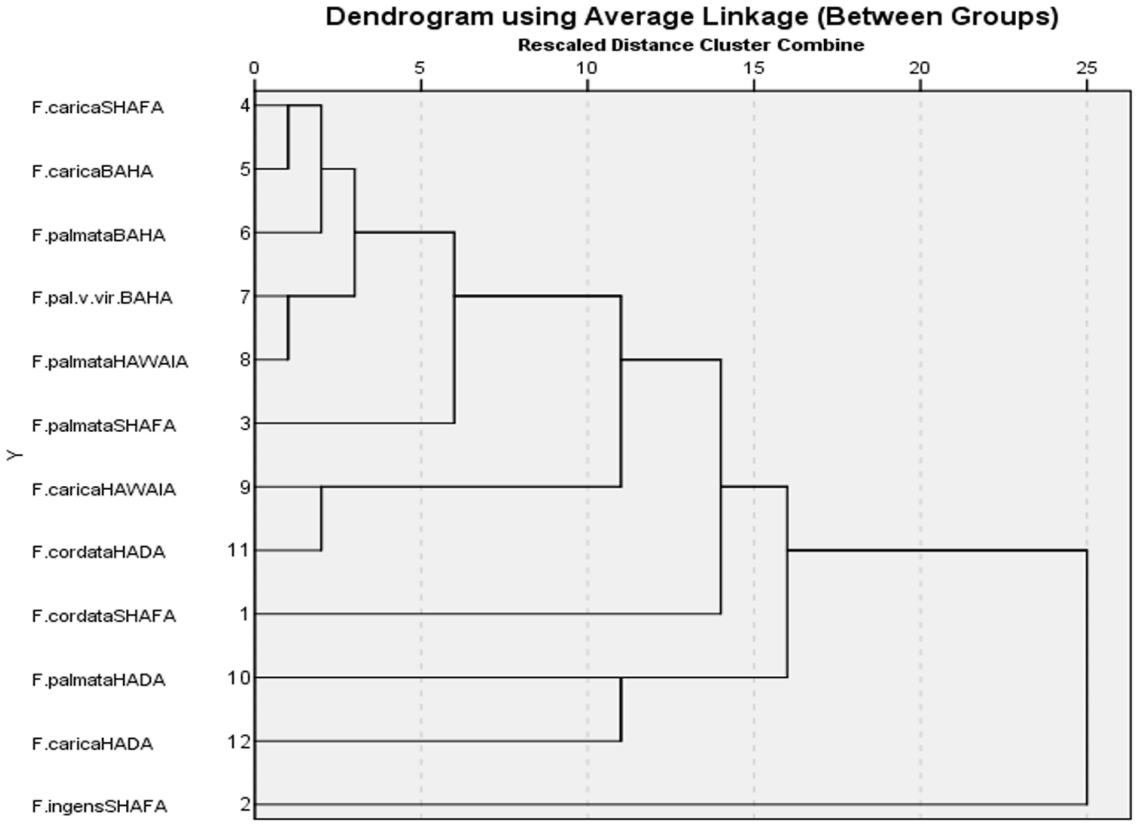


Fig. 4. UPGMA- phenogram based on pooled data obtained from SDS-PAGE and isozymes profiles of 12 accessions of the genus *Ficus*

RAPD fingerprinting

Four species' RAPD-PCR fingerprints were created using five primers (Table 4). Thirty-five RAPD bands were revealed, among which 18 polymorphic bands were recorded; 17 were unique. The highest percentage of polymorphism (60%) was recorded by two primers (OPO-8 and OPO-10), while the lowest (40%) was recorded by the primer OPO-11. Figure 5 demonstrates the RAPD-PCR profiles of the studied species. The amplified fragment size ranged from about 1666.9 to 150bp. Four monomorphic and 6 polymorphic bands were detected by the primer OPO-08. Five of these are unique and all present in *F. cordata* subsp. *Salicifolia* (Table 5 and Fig. 5-A); two are positive at 479.711 and 170.6bp and three are negative at 428.4, 180.2 and 155.7 bp. These unique bands appeared to be specific markers for *F. cordata* subsp. *Salicifolia*. The primer OPO-09 produced three monomorphic bands of 577.9, 512.5 and 370.3bp and three unique bands. One positive marker at 428.0bp was recorded in *F. cordata* subsp. *Salicifolia* and two negative and positive markers at 709.4, and 343.9bp, respectively, were recorded in *F. ingens*. The lowest number of amplified bands (5) was recorded by primers OPO-10 and OPO-11. The primer OPO-10 generated three negative markers at 860.8, 772.8, 669.2bp specific for *F. ingens*. Three monomorphic and two polymorphic bands were revealed in OPO-11's primer profile (Fig. 5-D). Two negative markers at 975.0 and 843.7 bp were revealed in *F. ingens*. In the RAPD profile produced by the primer OPO-13, five monomorphic and four polymorphic bands were detected including one positive unique band at 579.3 bp was considered species-specific for *F. cordata* subsp. *Salicifolia*. Three negative markers were revealed at 922.1, 636.5, and 430.2bp in *F. ingens*.

Genetic similarity and UPGMA tree

The dice similarity index value was used to calculate the genetic similarity of four species based on RAPD-PCR markers. *F. palmata* and *F. carica* had the highest genetic closeness (1.000), whereas *F. ingens* and *F. cordata* subsp. *Salicifolia* had the lowest similarity (0.667; Table 6). The UPGMA method was performed to assess evolutionary relationships among species using RAPD fingerprinting (Fig. 6). A dendrogram based on UPGMA analysis revealed that all *Ficus* species were included in a main cluster except *F. ingens that agreed with SDS-PAGE and isozymes dendrogram*. The closest species in the tree were *F. palmata* and *F. carica*.

Discussion

The present results revealed significant differences in all studied characters among and within *Ficus* species collected from four locations in Saudi Arabia. In the current study, SDS-PAGE revealed a significant level of polymorphism (96.5%) in the protein banding leaf profiles of different *Ficus* species, in agreement with Hirano (1982) who used 2D-gel electrophoresis to show varietal differences in the leaf protein profiles of 17 *Morus* spp. Despite only eight isozymes being investigated and the fact that isozyme research can only reveal genetic alterations in coding regions resulting in an altered amino–acid sequence, our research could detect variability among species of the *Ficus* in various sites in Saudi Arabia, namely levels of polymorphism ranging from 67% to 100%. These findings may be explained by adaptations to local environmental conditions linked to selection processes passed down through generations, or by mutational events affecting the performance of genes encoding certain isozymes. This suggestion is in accordance with Kannenberg & Gross (1999) who used 10 isozyme systems to detect genetic variation in Norway spruce populations from central and southeastern Europe spruce regions versus those from northern and north eastern Europe. Moreover, Hassan et al. (2002) revealed extensive genetic polymorphism by isozymes (e.g. peroxidase and esterase) in 10 accessions representing five *Populus* species from various locations; in addition, high variations were observed among samples of the same species from different locations. Among 73 polymorphic bands, 28 were unique bands and seemed to be useful as genetic markers. Ten were recognized in the protein pattern and 18 among isozyme patterns. These results disagree with those of Elisiàrio et al. (1998) and Cabrita et al. (2001) who found identical zymograms for the MDH, IDH, PGM, and PGI isozyme systems in all fig clones (Sarilop and Sarizeybek), however, all Sarilop clones had a monomorphic pattern for GOT enzymes, as opposed to the heterozygous pattern (three bands) demonstrated by the other cultivar Sarizeybek.

TABLE 4. The five primers and their sequence used in RAPD fingerprinting, the number and type of the amplified bands, and the percentage of polymorphism in four species of genus *Ficus*

Primer code	Sequences	Mono-morphic bands	Polymorphic bands		Total bands	Polymorphism %
			Non-unique bands	Unique bands		
OPO-08	5-CCTCCAGTGT-3	4	1	5	10	60
OPO-09	5-TCCCACGCAA-3	3	-	3	6	50
OPO-10	5-TCAGAGCGCC-3	2	-	3	5	60
OPO-11	5-GACAGGAGGT-3	3	-	2	5	40
OPO-13	5-GTCAGAGTCC-3	5	-	4	9	44.4
Total		17	1	17	35	51.4

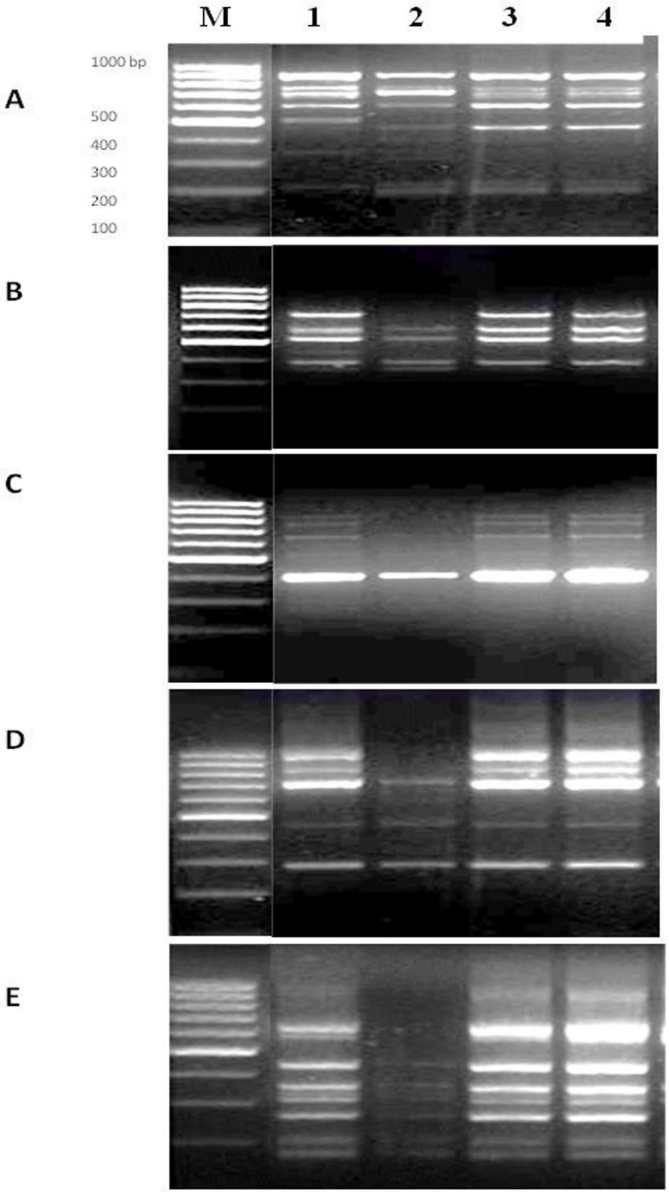


Fig. 5. RAPD fingerprinting in four species of the genus *Ficus*; 1: *F. cordata* subsp. *salicifolia*, 2: *F. ingens*, 3: *F. palmata*, 4: *F. carica*; A: OPO-08, B: OPO-09, C: OPO-10, D: OPO-11, E: OPO-13 [M= Marker]

TABLE 5. Number of positive (+) and negative (-) unique markers produced by RAPD primers in four species of the genus *Ficus*

Genotype	OPO-08		OPO-09		OPO-10		OPO-11		OPO-13	
	+	-	+	-	+	-	+	-	+	-
F. cordata subsp. *salicifolia*	2	3	1	0	0	0	0	0	1	0
F. ingens	0	0	1	1	0	3	0	2	0	3
F. palmata	0	0	0	0	0	0	0	0	0	0
F. carica	0	0	0	0	0	0	0	0	0	0

TABLE 6. Matrix of the genetic similarity estimates of RAPD analysis among four species of the genus *Ficus*.

Genotype	Matrix			
	F. cordata* subsp. *salicifolia	***F. ingens***	***F. palmata***	***F. carica***
F. cordata subsp. *salicifolia*	1.000			
F. ingens	.667	1.000		
F. palmata	.867	.784	1.000	
F. carica	.867	.784	1.000	1.000

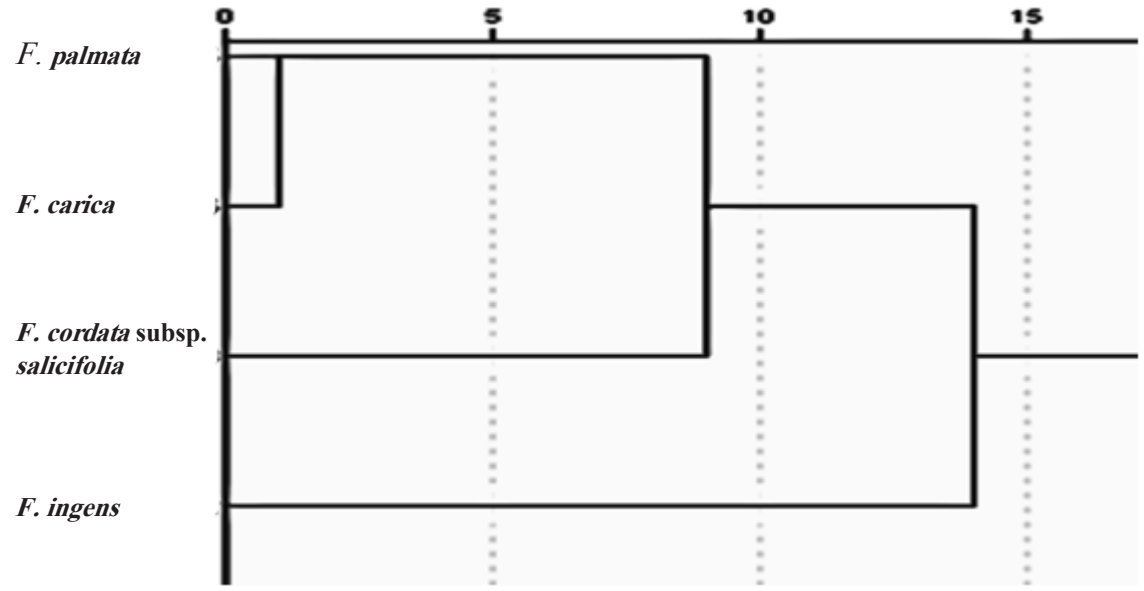


Fig. 6. UPGMA- phenogram based on data obtained from RAPD analysis of four species of the genus *Ficus*

The dendrogram, generated from pooled data derived from SDS-protein and isozymes analyses belonging to *Ficus* species, separated the studied species into one main cluster, while a single accession, *F. ingens* (Shafa), formed a separate OTU. The main cluster was divided into several subclusters reflecting the high variability among *Ficus* species and accessions of the same species sampled from different sites, except *F. carica* (Shafa) that showed the maximum genetic similarity (0.943) with *F. carica* (Baha). This result was similar to that of Abou-Ellail et al. (2014) who performed UPGMA clustering depending on PPO, POD isozymes, and SSR-PCR combination and proposed a molecular key for identification of seven fig cultivars. Dalkılıç et al. (2011) placed 43 different male *Ficus carica caprificus* accessions in nine different groups in a dendrogram drawn from a similarity matrix. Although, Teoman et al. (2017) showed that fig genotypes were not grouped based on their origin, Caliskan & Polat (2012) and Simsek et al. (2020)

reported that caprifig accessions from Turkey were clustered to their geographic origin based on SSR fingerprinting. Moreover, Basheer-Salimia et al. (2012) created a dendrogram for nine fig genotypes cultivated in Palestine's Northern West Bank. The dendrogram looks to be at odds with the morphological descriptors, particularly for the Swadi and Biadi genotypes (which are genetically similar but physically distinct), which could be owing to phenotypic alterations brought about by environmental variances between locales.

RAPD analysis allows identifying relevant genetic markers in both coding and non-coding portions of the genome at various loci (Williams et al., 1990). Thus, the RAPD technique allows the examination of fine scale genetic differences among individuals. The dominant character of RAPD markers requires sampling of more individuals and loci compared to co-dominant markers like isozymes. Therefore, a combined approach including both isozymes and RAPD may be more appropriate for estimating connections and genetic diversity than either method alone (Chan & Sun, 1997). Five primers were successful in generating polymorphic and repeatable amplification products in the present study. A significant level of polymorphism ranging from 60% in primer OPO-13 to 100% in primer OPO-10 was detected in the RAPD profiles of six species of the genus *Ficus*. Similar results were reported by Srivastava et al. (2004) who employed 10 RAPD primers to study the genetic link between 11 genotypes of mulberry collected from Japan, India, and Italy, and found 60.75% DNA polymorphism among these genotypes. Chatti et al. (2010) could detect considerable genetic diversity among Tunisian fig tree cultivars using RAPD (60), ISSR (48), RAMPO (63), and SSR (34) markers. Zolfaghari et al. (2019) reported infraspecific genetic variations among 14 populations of *F. carica* in Iran using RAPD fingerprinting. Basheer-Salimia et al. (2012) employed the PCR-RAPD method to investigate the genetic diversity and relatedness of nine fig genotypes cultivated in Palestine's Northern West Bank. In the present study only 9 of the 28 primers examined for the RAPD method provided acceptable amplification results, with 70.2% of polymorphism. The high percentages of differences obtained by the five primers in the current study could be due to most variation being found in repetitive DNA instead of coding DNA within the studied *Ficus* species. Accordingly, Irwin et al. (1998) reported that a great number of *Colocasia esculenta* varieties might derive from crosses or selection and propagation of mutant accessions. RAPD markers also found in areas with many repetitive sequences, and thus in non-coding regions, which are more sensitive to alterations than coding parts, according to those authors. These results agree with Arnau et al. (1994) and N'Goran et al. (1994), who suggested that repeated sequences might be preferentially amplified by RAPD analysis.

The five primers developed 17 unique bands for *Ficus* species; they could be used as molecular markers. *F. ingens* was differentiated by nine negative and one positive marker and *F. cordata* subsp. *Salicifolia* was differentiated by 4 positive and 3 negative marker compared with other *Ficus* species. The relevance of RAPD markers in elucidating in part identification difficulties and relationships between *Ficus* species was demonstrated by these findings. The present results are similar to those of Orhan et al. (2007), Saleh (2013), and Abou-Ellail et al. (2014) that detected 101 DNA markers by 16 RAPD primers in 15 selected white mulberry species (*Morus alba* L.). These RAPD markers revealed distinct mean frequency patterns among white mulberry species. Furthermore, Cabrita et al. (2001) generated 14 polymorphic markers from 10 RAPD primers, which distinguished between two fig clones (Sarizeybek and Sarilop). Furthermore, one of these 10 primers, OPN 10, identified two RAPD markers (OPN 10-650 and OPN 10-450) that distinguish between Sarilop clones, splitting them into two groups based on their similarities. Using RAPD, Mohammed et al. (2014) discovered significant genetic variability between 22 landrace (*Ficus carica* L. *sativa*) and two wild form (*Ficus carica* L. *caprificus*) fig accessions in Palestine.

RAPD-PCR markers could distinguish all *Ficus* species through clustering. The accessions were classified into the main cluster based on UPGMA analysis, and a single accession, *F. ingens* in was differentiated in a subcluster away from species of the genus *Ficus*. In the second cluster that agreed with that found with SDS and isozyme analysis, *F. carica* recorded high genetic similarity with *F. palmata* (1.0). These results agree with those of Ahmed & Fadl (2019) who used matK, ITS markers to separate *F. palmata* and *F. carica,* collected from the Taif highlands (Saudi Arabia), together in one group and *F.*

cordata subsp. *Salicifolia* in a separate group; however, the rbcL marker placed the three species in one group and the trnH-psbA marker separated *F. carica* alone. Seventeen *Ficus* species grown in Egypt were successfully distinguished using AFLP fingerprinting (Mostafa et al., 2020). Using EST and ISSR-PCR, Abdelsalam et al. (2019) discovered a wide diversity across 21 cultivated fig (*F. carica* L.) accessions in Egypt and Libya. Recently, 34 fig varieties from different countries were differentiated using ISSR molecular markers (Zhang et al., 2020). ISSR, RAPD, and SSR markers demonstrated limited genetic diversity of 19 fig varieties and lines from Europe and Asia (Ikegami et al., 2009).

Isozymes, RAPD, and SDS-PAGE markers could genetically distinguish *Ficus* species with high accuracy. However, the discriminative power for each type molecular marker was variable. Using SDS-PAGE and isozymes to differentiate between fig accessions has been explained. Furthermore, using the RAPD method, we discovered polymorphic markers discriminating across species belonging to the same genus. In a breeding program, knowing the genetic similarity (distance) between genotypes, individuals, or populations allows more efficient germplasm organization and genotype sampling (Hussein et al., 2001; Prasad et al., 2000).

Conclusion

In conclusion, using SDS-PAGE, isozymes, and RAPD-PCR techniques, we established biochemical genetic and molecular markers to characterize the geographical effect on genetic diversity of fig species in the studied area in Saudi Arabia. This study's early findings can be helpful for taxonomic categorization, breeding, genetic improvement, local germplasm conservation, and the management of Saudi fig genetic resources. To accurately analyze differences and interactions among fig species, extensive sampling, and detection of new molecular markers are necessary.

Competing interests: The authors report no conflicts of interest regarding this work.

Authors' contributions: Proposed the idea of this study, and designed the experimental work and made the measurements. SMA, YMA and ASE analyzed and interpreted the data and wrote the manuscript., SMA, ASE and ML performed the calculations and statistical analysis, participated with YMS in the analysis and interpretation of the data, revised the manuscript, checked the manuscript for plagiarism, and acted as the corresponding author. All authors participated in the drafting of the manuscript and have read and approved the final draft.

Ethics approval: Not applicable.

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استخدام العلامات البيوكيميائية والجزيئية لتمييزأنواع التين من أربعة مواقع في المملكة العربية السعودية

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تم دراسة التباين الوراثي الكيميائي والجزيئي بين بعض أنواع التين التي تم جمعها من أربعة مواقع (الشفا، الباحة، الحوية، والهدا) في المملكة العربية السعودية باستخدام التفريد الكهربى للبروتينات، والنظائر الانزيمية المختلفة، وقطع DNA المكثرة عشوائيا من خلال تحليل التكبير العشوائى متعدد الاشكال (RAPD). أظهرت النتائج وجود اختلافات معنوية بين أنواع التين حيث تم تسجيل اختلاف معنوي على مستوى البروتين (96.5%) ولجميع الايزوزيم المستخدمة حيث تراوحت نسب التباين بين 67% و100%. كما تم إنشاء شجرة القرابة المستندة على حزم التفريد الكهربى للبروتين ودراسات الإيزوزيم حيث ميزت الأنواع إلى مجموعة رئيسية باستثناء نوع واحد وهو *F. ingens* الذى شكل وحدة تصنيف تشغيلية منفصلة (OTU). وتم تقسيم المجموعة الرئيسية إلى مجموعات فرعية تعكس التباين الكبير بين أنواع *Ficus* أو المدخلات التي تم جمعها من مواقع مختلفة. كما ساعدت بصمات RAPD-PCR لأربعة أنواع من التين باستخدام خمسة بادئات والتى انتجت 35 حزمة من RAPD وكان 18 منها متعدد الأشكال و17 نطاقًا فريدًا وجمعت الشجرة المستمدة من بيانات الدنا الأنواع الأربعة في مجموعة أولية واحدة باستثناء *F. ingen* مما يتفق مع الشجرة السابقة. ساعدت تقنيات SDS-PAGE و isozymes و RAPD-PCR في الكشف عن التأثيرات الجغرافية على التنوع الجيني لأنواع *Ficus*. ومن الممكن استخدام المعلومات الأولية الناتجة هنا في المجموعات التصنيفية، والتربية، والتحسين الوراثي، والحفاظ على الأصول الوراثية المحلية، وإدارة الموارد الوراثية للتين السعودي.