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GENETIC DIVERSITY ASSESSMENT AMONG SOME *FICUS* SPECIES USING MORPHOLOGICAL CHARACTERS AND AFLPS

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Abstract

In the present investigation seventeen *Ficus* species grown in Egypt, collected from two botanical gardens, were studied using twenty leaf morphological characters based on the Fig (*Ficus carica* L.) IPGRI descriptor, and seven AFLP primer combinations. The morphological characters, included four measured and sixteen descriptive characteristics. The one-way ANOVA test for the measured traits showed significant differences among the seventeen *Ficus* species. *F. microcarpa* Hawai showed the lowest value for both leaf length and leaf width, while the highest leaf length and leaf width were revealed by *F. hispida* and *F. carica*, respectively. Moreover, *Ficus carica* possessed the distinctive unique descriptive character of leaf lobation, as it was the only species with five latate lobed leaf, while the other species had entirely unlobed blades. *Ficus hispida* and *F. elastica* Decora showed the largest leaf area (400-550 cm^2). Also, they were the only two species with serrate dentate leaf margin. On the other hand, the seven AFLP primer combinations generated a total of 662 amplicons across the seventeen species and 652 amplicons were polymorphic with a polymorphism rate of 98.49%. AFLP markers characterized each species with unique positive and/or negative markers. *F. hispida* amplified the highest number of unique markers (11positive markers) while, *F. microcarpa* Hawai revealed only 1 unique positive marker. The similarity index value ranged from 0.29 to 0.66 in species (*F. microcarpa* Hawai and *F. carica*) and (*F. microcarpa* Hawai and *F. benjamina*), respectively. The dendrograms constructed from UPGMA clustering algorithm for the morphological and AFLP datasets revealed different topologies. Nevertheless, these dendrograms showed some similarities, for example, the grouping *F. lutea* and *F. afzelii*. Also, the grouping of *F. virens* and *F. trijuja* based on morphological and AFLP markers.

Keywords: *Ficus species*, AFLP, morphological characterization, dendrogram, genetic diversity.

Introduction

The genus *Ficus* is one of the largest and diverse genera in *Moraceae*. It comprises about 750 species with distinctive morphological characters, with trees, shrubs, and climber, most notably by specialized inflorescence (syconium) and pollinator mutualism (fig wasps) (Weiblen, 2000; Loutfy *et al*., 2005; Esmaiel *et al*., 2014 and Bruun-Lund *et al*., 2017). *Ficus* is distributed worldwide, especially, in warm tropical and subtropical regions. Where, the most diverse region is in Asia, Malesia, and Australia (Weiblen, 2000). Most *Ficus* species are diploids (2n=26), harboring a basic chromosome number of x=13, with the exception of *F. elastica* Decora which has a 2n=39 (Condit, 1964; Ohri and Khoshoo 1987 and Fang *et al*., 2007). Corner (1965) classified the *Ficus* species into four subgenera based on the breeding system, one dioecious subgenus (subgen. *Ficus*) and three monoecious subgenera (*Urostigma*, *Sycomorus*, and *Pharmacosycea*). Recently, Berg and Corner (2005) divided the *Ficus* genus into six subgenera. In Egypt no species was recorded belonging to subgenus *Pharmacosycea* (Sharawy, 2004 and Tantawy *et al*., 2014). Boulous (1999) recorded three species in the Egyptian flora; *Ficus cordata* subsp *salicifolia*, *F. palmate* and *F. carica*. *Ficus* species could be used for different purposes. In general, *F. elastica* Decora, *F. benjamina* and *F. retusa* are used for landscaping or indoor decoration, while, *F. carica* and *F. sycomorus* are consumed as edible fruits. In addition, different parts of some *Ficus* species (leaves, barks, roots and fruits) are employed in folk medicine for their therapeutic actions (Elansary and El-Ansary, 2013 and Mohamed *et al*., 2018).

Plant genetic resources (PGR) represent the natural gene pool for adaptation to environmental stresses, disease and pests resistance. In addition, they are the resources for food security especially in developing countries, as they provide the genetic raw materials for farmers and breeders. In recent years the massive increase in human population, in addition to the global climate change posed increased risk of extinction of valuable genetic resources. This raised the global awareness for conservation of germplasm resources (Rao, 2004; Ogwu *et al*., 2014 and Bhandari *et al*., 2017). PGR conservation activities are based on collection, conservation, and identification of genetic materials, by characterization and evaluation techniques (Chandrakant *et al*., 2017). One key role of gene banks is to maintain and safeguard genetic variation in case of loss, to be accessible for futuristic demand. PGR are conserved in gene banks in different forms such as seeds, vegetative parts, cuttings, pollen, or DNA, which reveal unique forms of diversity (McCouch *et al*., 2012). Effective conservation of plant genetic resources requires accurate characterization to describe the identity of the germplasm. Conventionally, this was carried out by characterization of morphological variation, especially agro-morphological characteristics, based on specified descriptors. Morphological characters represent the easiest and cheapest markers to characterize, as they are based on visual observation (Govindaraj *et al*., 2015; Prajapati *et al*., 2018 and Roughani *et al*., 2018).

Morphological characterization is the preliminary step in the description and identification of species (Weerakoon and Somaratne, 2010). Morphological markers reflect the expressed regions of the genome, but they are highly affected by environment and plant developmental stage (Esmaiel *et al*., 2014).

The advances in molecular techniques resulted in the development of molecular markers which provides accurate tools for genetic resources characterization and resolution of evolutionary relationships among species. The identification of species is important for biodiversity conservation and management (Mosa *et al*., 2019). Identification of molecular variation is crucial in utilization, conservation, maintenance

and management of PGR in gene banks. Molecular markers possess many advantages over phenotypic traits, as they are stable and detectable in all tissues, besides, they are not affected by environmental effects (Mondini *et al*., 2009 and Sonnino, 2017). To date, various molecular markers platforms are available with variable properties, such as ISSR, SSR, SCoT, RAPD, and AFLP (Arif *et al*., 2010; Abdel-Lateif and Hewedy, 2018 and Al-Soudy *et al*., 2018). AFLP is a PCR-based fingerprinting method first described by Vos *et al*. (1995). It possesses many advantages, including no previous genomic sequences background required, and sensitivity, especially in automated detecting methods. AFLP is vastly used in plant diversity studies to estimate the genetic diversity between and within closely related species (Blears *et al*., 1998; Santos and Simon, 2002; Rinehart, 2004; Paun and Schönswetter, 2012 and Mosa *et al*., 2019). Nowadays, molecular markers are used as complementary tools for morphological characterization.

The present study has been carried out with the main objective to characterize seventeen different *Ficus* species grown in Egypt based on the leaf morphological descriptor and AFLP technology. The genetic diversity among the seventeen species has been assessed and a fingerprint has been developed for each species. In addition, a comparison between the efficiency of the leaf morphological markers and AFLP markers has been discussed.

Materials and Methods

Plant materials

The plant material consisted of seventeen different *Ficus* species. Eleven species were collected from Orman Botanical Garden, Giza, Egypt, and the other five species were grown in the National Gene Bank Botanical Garden (Table 1). The species collected from Orman Botanical Garden were previously collected, propagated, and conserved in the National Gene Bank greenhouses to be accessible for further studies. *F. carica* is the only dioecious species, while the other sixteen species are monoecious.

Table 1 : *Ficus* species, subgenus, and source.

No	Species	Subgenus	Source
1	*F. afzelii* Hort. Berol. ex Kunth and Bouche.	*Urostigma*	Orman Botanical Garden
2	*F. asperrima* Roxb. (*F. exasperate* Vahl.)	*Urostigma*	Orman Botanical Garden
3	*F. benghalensis* L.	*Urostigma*	Orman Botanical Garden
4	*F. hispida* L.f.	*Urostigma*	Orman Botanical Garden
5	*F. lutea* Vahl.	*Urostigma*	Orman Botanical Garden
6	*F. microcarpa* L.f. 'Hawai'	*Urostigma*	Orman Botanical Garden
7	*F. platypoda* A. Cunn. ex Miq (*Urostigma platypodum* Miq. Hook.)	*Urostigma*	Orman Botanical Garden
8	*F. pyriformis* Hook and Arn.	*Urostigma*	Orman Botanical Garden
9	*F. racemosa* L. (*F. glomerata* Roxb.)	*Sycomorus*	Orman Botanical Garden
10	*F. spragueana* Mildr and Burret (*F. thonningii* Bl.)	*Urostigma*	Orman Botanical Garden
11	*F. sycomorus* L.	*Sycomorus*	National Gene Bank Botanical Garden
12	*F. trijuja* L.	*Urostigma*	Orman Botanical Garden
13	*F. virens* Aiton. (*F. infectoria* Roxb.)	*Urostigma*	Orman Botanical Garden
14	*F. carica* L.	*Ficus*	National Gene Bank Botanical Garden
15	*F. retusa* L. (*F. nitida* Thunb.)	*Urostigma*	National Gene Bank Botanical Garden
16	*F. elastica* Roxb. ex Hornem. 'Decora'	*Urostigma*	National Gene Bank Botanical Garden
17	*F. benjamina* L.	*Urostigma*	National Gene Bank Botanical Garden

Morphological Analysis

Leaf morphological characterization was conducted according to the Fig (*Ficus carica* L.) descriptor of the International Plant Genetic Resources Institute (IPGRI and CIHEAM, 2003). Leaf characters were recorded over two consecutive years (2016 and 2017), based on 40 leaves of each species. For each tree sample, four measured characters in addition to sixteen descriptive characters were recorded. The measured characters were leaf length, leaf width, petiole length/leaf length, and petiole thickness. While, the descriptive characters included leaf shape, number of lobes, shape of lobes, location of little lateral lobes, length of central lobe/leaf length, shape of leaf base, leaf area, leaf margin dentation, leaf margin, density of hairs on leaf upper surface, density of hairs on lower surface, leaf venation, leaf color, petiole length, petiole cross-section and petiole color.

Molecular Analysis

AFLP analysis was conducted on the genomic DNA of the 17 *Ficus* species, based on the method described by Vos *et al.* (1995). The AFLP analysis was performed using the Invitrogen AFLP core reagent kit (Cat. No. 1082 – 016). Total DNA was extracted from young leaves using Qiagen DNeasy plant mini kit (Cat No. 69104). DNA was quantified and adjusted to 250 ng/µl and digested with 1 µl mixture of *EcoRI/Mse*I (1.25 U/µl) at 37°C for 3 h. Digested DNA was ligated to *EcoRI/Mse*I adaptors using 1.5 µl (1 U/µl) T4 DNA Ligase at 20°C for 3 h. The diluted adaptor-ligated DNA was amplified with a mixture of 2.5 µl DNA of the ligation reaction, composed of 20 µl pre-amp mix, 2.5 µl 10X PCR buffer plus Mg++ and 1 µl Taq DNA polymerase (5U/µl). The PCR reactions were performed on a MyCyler – BioRad ® thermo cycler. The pre-selective PCR thermo cycling profile consisted of 20 cycles set at 94°C for 30 sec, 56°C for 1 min and 72 °C for 1 min. The pre-amplification products were diluted 1:5 in sterilized de – ionized H_2O, and subjected to selective amplification using seven primer combinations (Table 5). The selective amplification temperature profile consisted of one cycle at 94°C for 30 sec, 65°C for 30 sec and 72°C for 1 min. This was followed by a

decrease in the annealing temperature at each cycle by 0.7 °C for 12 cycles that gave a touch down phase of 13 cycles. Then, 23 cycles were performed at 94 °C for 30 sec, 56 °C for 30 sec and 72°C for 1 min. The product of selective amplification was electrophoresed on 6% denatured polyacrylamide gel in 1X TBE buffer. The gel was preheated to 50°C. The samples were denatured at 95°C for 10 min and placed directly on ice. Electrophoresis was performed at 60 W, 50°C for 2h. The gel was fixed in 10% acetic acid for 30 min, and developed using silver nitrate staining.

Statistical Analysis

For the morphological measured characters, the One-way ANOVA test was performed according to Snedecor and Cochran (1980) using the Duncan's multiple range (Duncan, 1955) method at 0.05% significance level. UPGMA morphological characters based dendrogram and AFLP were developed according to Nei's (1972). AFLP banding patterns were compared to determine the genetic relationships among the different species, using Phortix nonlinear dynamics (UK) software Version 10.

Results and Discussion

1- Morphological Characterization of *Ficus* species:

Morphological characterization represents the phenotypic data of the species, thus facilitating to distinguish and discriminate between species. Accordingly, this information is essential for proper management, effective conservation and use of germplasm (Bioversity International, 2007).

A- Leaf measured morphological characteristics:

The mean values of the four measured characters for the seventeen *Ficus* species are presented in Table (2). The one-way ANOVA test for the measured traits showed significant differences ($p < 0.05$) among the seventeen *Ficus* species. Leaf length ranged from 4.29 cm in *F. microcarpa* Hawai to 30.09 cm in *F. hispida.* Leaf width exhibited an average ranging from 2.07 cm in *F. microcarpa* Hawai to 18.23 cm in *F. carica.* Petiole length/leaf length ratio ranged from 0.09 to 0.53 in *F. pyriformis* and *F. sycomorus,* respectively. Petiole thickness ranged from 0.10 mm in *F. benjamina* to 0.57 mm in *F. elastica* Decora.

Table 2 : Mean values of leaf measured characters of the seventeen *Ficus* species.

Characters	*Ficus* species															
	F. afzelii	*F. asperrima*	*F. benghalensis*	*F. hispida*	*F. lutea*	*F. microcarpa* Hawai	*F. platypoda*	*F. pyriformis*	*F. racemosa*	*F. spragueana*	*F. sycomorus*	*F. trijuja*	*F. virens*	*F. carica*	*F. retusa*	*F. elastica* Decora	*F. benjamina*
Leaf length (cm)	19.77 d	15.47 e	14.81 ef	30.09 a	22.84 c	4.29 k	10.76 h	11.62 gh	15.85 e	13.04 fg	9.68 hi	12.92 fg	9.81 hi	22.14 c	8.13 ij	27.35 b	7.47 j
Leaf width (cm)	7.25 ef	4.267 ij	8.92 d	16.09 b	11.33 c	2.07 l	5.24 ghi	4.34 ij	8.25 de	5.08 hi	6.31 fg	5.95 gh	6.06 gh	18.23 a	3.44 jk	15.29 b	3.12 k
Petiole length /leaf length	0.17 fg	0.26 cd	0.23 de	0.17 efg	0.26 cd	0.22 def	0.29 c	0.09 h	0.13 gh	0.14 gh	0.53 a	0.20 def	0.43 b	0.44 b	0.16 fg	0.21 def	0.18 efg
Petiole thickness (mm)	0.41 cd	0.24 ef	0.36 d	0.45 bc	0.47 b	0.13 hi	0.28 e	0.19 fg	0.45 bc	0.21 fg	0.21 fg	0.21 fg	0.17 gh	0.49 b	0.19 fg	0.57 a	0.10 i

Figures followed by different letters are significantly different according to Duncan`s Multiple Range Test ($P < 0.05$).

Our results demonstrate that *F. microcarpa* Hawai had the lowest value for both leaf length and leaf width. Esmaiel *et al*. (2014) studied only eight of the species included in the present study, and revealed similar findings for *F. microcarpa* Hawai. However, the mean values recorded for all the other species were different. In addition, the values of the two species *F. lutea* and *F. spragueana* were higher than those reported by Sonibare *et al*. (2004).

B- Leaf descriptive characteristics:

The descriptor-based records for the sixteen descriptive leaf traits are presented in Tables (3 and 4). *Ficus carica* possessed the distinctive unique character of the leaf lobation, as it was the only species with five lobed blades, while the other species had unlobed blades. *Ficus hispida* and *F. elastica* Decora showed the largest leaf area (400-550 cm^2).

The leaf shape character revealed 5 shapes. Six species were distinguished by a lanceolate leaf shape, 5 species were ovate, three species had an oblong shape, two species possessed cordate, and only one species (*F. asperima*) revealed elliptical leaf shape. The leaf lobes character showed only two types. *F. carica* was marked by lobed bladed leaves with five latate lobes. While, the remaining 16 species showed entirely undivided blades. Four leaf base shapes were scored across the seventeen species, eight species were obtuse, and 7 species were acute. While, truncate and cordate shape were each expressed by only one species (*F. virens* and *F. carica*, respectively). The leaf area was either small (<250cm^2) which was scored in 13 species or medium (250-400cm^2) or large (400-500cm^2) each presented in two species (*F. lutea*, *F. carica* and *F. hispida*, *F. elastica* Decora, respectively). *F. carica* and *F. hispida* were the only two species with serrate dentate leaf margin. While, the other 15 species possessed undented margin. Also, these two species revealed sparse density of hairs on both leaf sides, while the other 15 species had no hairs on any side. The leaf veins were marked as slightly apparent in only 3 species (*F. asperima*, *F. platypoda*, and *F. microcarpa* Hawai), while 14 species revealed apparent veins on the lower leaf side. Eleven species expressed dark green leaves, 5 species were green colored, and only (*F. microcarpa*

Hawai) revealed light green leaves (yellow-green color category). *F. carica* was the only species with long length petiole (>80mm), while 3 species had medium (50-80mm) and 13 species showed short petioles (<50mm). The petiole cross-section was round in only two species (*F. carica* and *F. retusa*), while it was flattened in 15 species. *F. afzelii* and *F. racemosa* were the only two species which expressed brown colored petiole, eight species revealed green and 7 species were with light green petioles.

Table 3 : Descriptive morphological characteristics of *Ficus* species.

Characterization	*F. afzelii*	*F. asperrima*	*F. benghalensis*	*F. hispida*	*F. lutea*	*F. microcarpa* Hawai	*F. platypoda*	*F. pyryformis*	*F. racemosa*	*F. sprugeana*	*F. sycomorus*	*F. trigyna*	*F. virens*	*F.carica*	*F. retusa*	*F. elastic* Decora	*F. benjamina*
Leaf shape	lanceolate	Elliptical	Ovate	Ovate	Oblong	Lanceolate	Lanceolatee	Lanceolate	Ovate	Lanceolate	Ovate	Oblong	Cordate	Cordate	Lanceolate	Oblong	Ovate
Number of lobes	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
Shape of lobes	0	0	0	0	0	0	0	0	0	0	0	0	0	latate	0	0	0
location of little lateral lobes	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Length of central lobe/leaf length	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0
Shape of leaf base	Acute	Obtuse	Obtuse	Acute	Obtuse	Acute	Obtuse	Acute	Obtuse	Acute	Obtuse	Obtuse	Truncate	Cordate	Acute	Acute	Obtuse
Leaf area	1	1	1	3	2	1	1	1	1	1	1	1	1	2	1	3	1
Presence of teeth	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0
Leaf margin	0	0	0	2	0	0	0	0	0	0	0	0	0	2	0	0	0
Density of hairs on upper surface	0	0	0	3	0	0	0	0	0	0	0	0	0	3	0	0	0
Density of hairs on lower surface	3	0	0	3	0	0	0	0	0	0	0	0	0	3	0	0	0
Leaf venation	3	2	3	3	3	2	2	3	3	3	3	3	3	3	3	3	3
Leaf color	2	3	2	3	2	1	3	3	3	3	3	2	3	3	3	3	2
Petiole length	1	1	1	1	2	1	1	1	1	1	2	1	1	3	1	2	1
Petiole cross section	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	2	2
Petiole color	4	1	2	2	2	1	1	2	4	2	2	2	1	2	1	1	1

Figures refer to the Fig descriptor of the International Plant Genetic Resources Institute (IPGRI) and CIHEAM (2003).
Number of lobes: (0) undivided, (2) five lobes. Shape of lobes: (0) unlobed, (latate) wider lobes. Location of little lateral lobes: (0) unlobed, (1) in central lobe. Length of central lobe/leaf length: (0) no leaf lobation, (3) marked lobation. Leaf area (leaf length x leaf width): (1) small <250 cm^2, (2) medium=250-400 cm^2, (3) large=400-500 cm^2. Presence of teeth: (0) no dentation, (1) only upper margins dented. Leaf margin: (0) undented, (3) serrate. Density of hairs on upper surface: (0) none, (3) sparse. Density of hairs on lower surface: (0) none, (3) sparse. Leaf venation: (2) slightly apparent, (3) apparent. Leaf color: (1) light green, (2) green, (3) dark green. Petiole length: (1) short <50mm, (2) medium=50-80 mm, (3) long >80mm. Petiole cross section: (1) round, (2) flattened. Petiole color: (1) light green, (2) green, (4) brown.

Table 4: Summary of the morphological characters for each of the seventeen *Ficus* species.

No	Species	Morphological characters
1	*F. afzelii*	Leaves lanceolate, unlobed blade, acute base shape, small leaf area (<250 cm^2), no teeth on margin, undented margin, no hairs on upper leaf side, spare hairs on lower leaf side, apparent leaf venation on lower leaf side, green colored leaf, short length petiole (<50 mm), flattened petiole cross section, and brown petiole.
2	*F. asperrima*	Leaves elliptical, unlobed blade, obtuse base shape, small leaf area (<250 cm^2), no teeth on margin, undented margin, no hairs on both leaf sides, slightly apparent leaf venation on lower leaf side, dark-green colored leaf, short length petiole (<50 mm), flattened petiole cross section, and light-green petiole.
3	*F. benghalensis*	Leaves ovate, unlobed blade, obtuse base shape, small leaf area (<250 cm^2), no teeth on margin, undented margin, no hairs on both leaf sides, apparent leaf venation on lower leaf side, green colored leaf, short length petiole (<50 mm), flattened petiole cross section, and green petiole.
4	*F. hispida*	Leaves ovate, unlobed blade, acute base shape, medium leaf area (250-400 cm^2), teeth on only upper margins, dentate margin, spare hairs on both leaf sides, apparent leaf venation on lower leaf side, dark-green colored leaf, short length petiole (<50 mm), flattened petiole cross section, and green petiole.
5	*F. lutea*	Leaves oblong, unlobed blade, obtuse base shape, medium leaf area (250-400 cm^2), no teeth on margin, undented margin, no hairs on both leaf sides, apparent leaf venation on lower leaf side, green colored leaf, medium length petiole (50-80 mm), flattened petiole cross section, and green petiole.
6	*F. microcarpa Hawai*	Leaves lanceolate, unlobed blade, acute base shape, small leaf area (<250 cm^2), no teeth on margin, undented margin, no hairs on both leaf sides, slightly apparent leaf venation on lower leaf side, light-green colored leaf, short length petiole (<50 mm), flattened petiole cross section, and light-green petiole.
7	*F. platypoda*	Leaves lanceolate, unlobed blade, obtuse base shape, small leaf area (<250 cm^2), no teeth on margin, undented margin, no hairs on both leaf sides, slightly apparent leaf venation on lower leaf side, dark-green colored leaf, short length petiole (<50 mm), flattened petiole cross section, and light-green petiole.
8	*F. pyriformis*	Leaves lanceolate, unlobed blade, acute base shape, small leaf area (<250 cm^2), no teeth on margin, undented margin, no hairs on both leaf sides, apparent leaf venation on lower leaf side, dark-green colored leaf, short length petiole (<50 mm), flattened petiole cross section, and green petiole.

9 *F. racemosa*	Leaves ovate, unlobed blade, obtuse base shape, small leaf area (<250 cm^2), no teeth on margin, undented margin, no hairs on both leaf sides, apparent leaf venation on lower leaf side, dark-green colored leaf, short length petiole (<50 mm), flattened petiole cross section, and brown petiole.
10 *F. sprague ana*	Leaves lanceolate, unlobed blade, acute base shape, small leaf area (<250 cm^2), no teeth on margin, undented margin, no hairs on both leaf sides, apparent leaf venation on lower leaf side, dark-green colored leaf, short length petiole (<50 mm), flattened petiole cross section, and green petiole.
11 *F. sycomorus*	Leaves ovate, unlobed blade, obtuse base shape, small leaf area (<250 cm^2), no teeth on margin, undented margin, no hairs on both leaf sides, apparent leaf venation on lower leaf side, dark-green colored leaf, medium length petiole (50-80 mm), flattened petiole cross section, and green petiole.
12 *F. trijuja*	Leaves oblong, unlobed blade, obtuse base shape, small leaf area (<250 cm^2), no teeth on margin, undented margin, no hairs on both leaf sides, apparent leaf venation on lower leaf side, green colored leaf, short length petiole (<50 mm), flattened petiole cross section, and green petiole.
13 *F. virens*	Leaves cordate, unlobed blade, truncate base shape, small leaf area (<250 cm^2), no teeth on margin, undented margin, no hairs on both leaf sides, apparent leaf venation on lower leaf side, dark-green colored leaf, short length petiole (<50 mm), flattened petiole cross section, and light-green petiole.
14 *F. carica*	Leaves cordate, five lobes blade, latate lobe shape, little lateral lobe located in central lobe, length of central lobe/length of leaf was marked lobation, cordate base shape, medium leaf area (250-400 $cm^{2)}$, teeth on only upper margins, dentate lobes, hairs found in spare manner on both leaf sides, apparent leaf venation on lower leaf side, dark-green colored leaf, long length petiole (>80 mm), rounded petiole cross section, medium length petiole (50-80 mm), and light-green petiole.
15 *F. retusa*	Leaves lanceolate, unlobed blade, acute base shape, small leaf area (<250 cm^2), no teeth on margin, undented margin, no hairs on both leaf sides, apparent leaf venation on lower leaf side, dark-green colored leaf, short length petiole (<50 mm), rounded petiole cross section, and light-green petiole.
16 *F. elastica* Decora	Leaves oblong, unlobed blade, acute base shape, large leaf area (400-550 $cm^{2)}$, no teeth on margin, undented margin, no hairs on both leaf sides, apparent leaf venation on lower leaf side, dark-green colored leaf, medium length petiole (50-80 mm), flattened petiole cross section, and light-green petiole.
17 *F. benjamina*	Leaves ovate, unlobed blade, obtuse base shape, small leaf area (<250 cm^2), no teeth on margin, undented margin, no hairs on both leaf sides, slightly apparent leaf venation on lower leaf side, green colored leaf, short length petiole (<50 mm), flattened petiole cross section, and light-green petiole.

Genetic distance among the *Ficus* species

To assess the genetic relationship among the *Ficus* species, UPGMA-Euclidian distances based on measured and descriptive characters were used and converted to a dendrogram (Fig. 1).

The morphology based dendrogram was divided into two main clusters. The first cluster comprised only three species, i.e., *Ficus hispida, F. elastica* Decora, and *F. carica.* Interestingly, these three species showed the highest measured values of leaf length (30.09cm, 27.35cm, and 22.14cm, respectively) and leaf width (18.23cm, 15.29cm, and 16.09cm, respectively). These results are in disagreement with Esmaiel *et al*. (2014), based on their morphological dendrogram, *F. Elastica* Decora*, F. afzelii* and *F. platypoda* were grouped together. While, according to Loutfy *et al*. (2005), the dendrogram splitted *F. carica* apart from all the other taxa.

The second cluster contained 14 species, divided into two main subclusters. The first subcluster included two species, *F. afzelii* which clustered with *F. lutea*. Meanwhile, it was in disagreement with the results of Loutfy *et al*. (2005), as species *F. afzelii* was assigned as closely related to species *F. racemosa* and *F. virens*.

The second subcluster was divided into two groups. The first grouped three species (*F. microcarpa* Hawai, *F. retusa* and *F. benjamina*) with *F. benjamina* and *F. retusa* closer to each other than *F. microcarpa* Hawai. This could be attributed to their low value of petiole thickness (0.13mm 0.19mm, and 0.10mm, respectively), also they shared the lowest values of petiole length and leaf area. This is in partial agreement with Loutfy *et al*. (2005), as they reported that *F. benjamina* and *F. retusa* were clustered in one group.

The second group was divided into two subgroups. The first subgroup contained two species (*F benghalensis* and *F. racemosa*). While, the second subgroup comprised two classes, one containing three species (*F. trijuja*, *F. virens* and *F. sycomorus*) with *F. sycomorus* closer to *F. virens* than to *F. trijuja*. In this respect, Esmaiel *et al*. (2014) mentioned that the *F. sycomorus* and *F. virens* were grouped together. While, the second class contained *F. asperima*, *F. platypoda*, *F. pyriformis* and *F. spragueana*. Similarly, Loutfy *et al*. (2005) showed that *F. spragueana* and *F. pyriformis* clustered together.

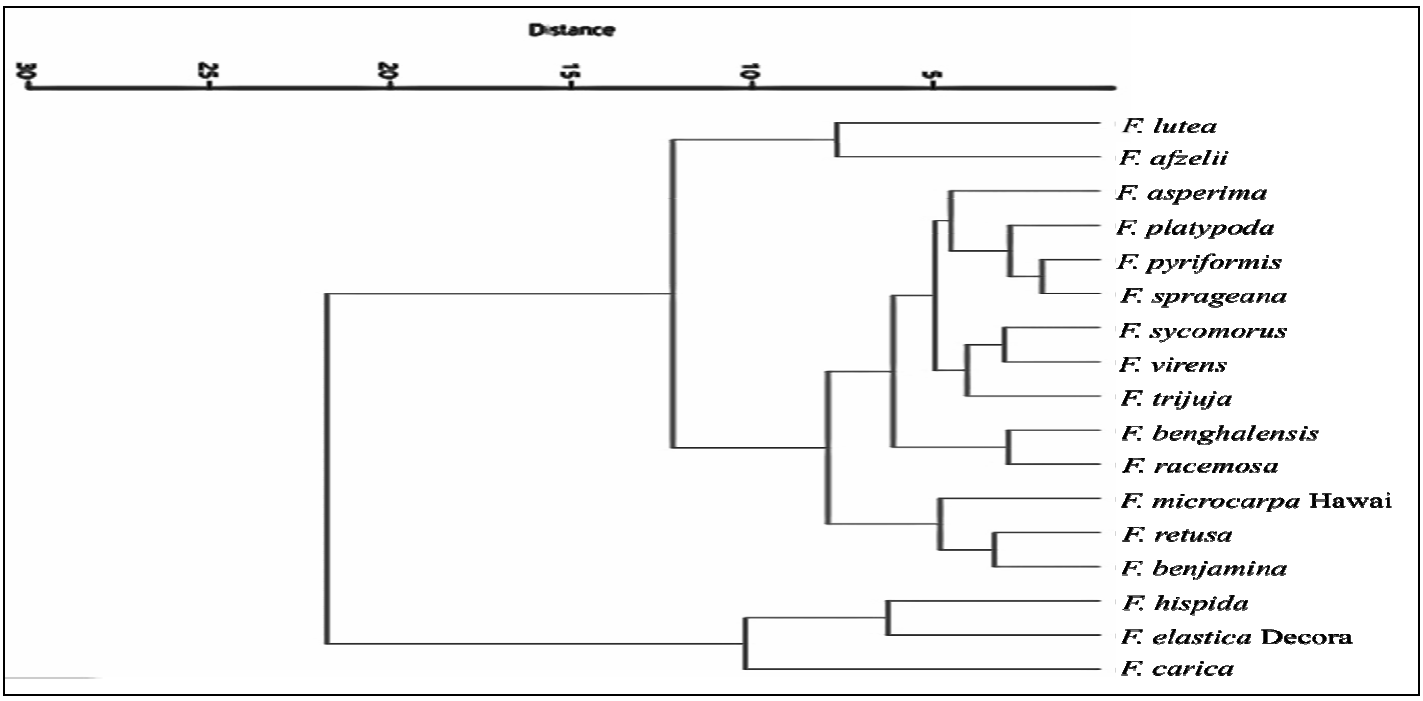

Fig. 1 : UPGMA-Euclidian distances of the morphological characters.

2- Molecular Characterization of the seventeen *Ficus* species:

Seven AFLP primer combinations were used to assess the genetic variation among the seventeen *Ficus* species. As shown in Table (5), the total number of amplicons was 662 with an average of 94.57 amplicons/primer combination. The highest number of amplicons was 120 generated by combination primer E-AAC/M-CTA, and the lowest number was 81 amplicons generated by combination E-AAC/M-CAG. The total number of polymorphic bands was 652 with an average of 93.14/primer combination. Polymorphic amplicons ranged from 80 to 119 amplicons. The percentage of polymorphism ranged from 95.96 % as revealed by primer combination E-AGC/M-CAA, to 100% amplified by primer combination E-ACA/M-CTA.

Table 5 : Primer combination, total number of amplicons, size range (bp), number of monomorphic amplicons, number of polymorphic amplicons and percentage of polymorphism.

No	Primer combination	Total number of amplicons	Size range (bp)	Monomorphic amplicons	Polymorphic amplicons	% Polymorphism
1	E-AAC / M- CTA	120	201-666	1	119	99.17%
2	E-AAC / M-CTA	89	102-683	1	88	98.88%
3	E-ACA / M-CAG	83	100-641	2	81	97.59%
4	E-AAC / M-CAT	94	149-658	1	93	98.94%
5	E-AAC / M-CAG	81	101-607	1	80	98.77%
6	E-AGC / M-CAA	99	103-606	4	95	95.96%
7	E-ACA / M-CTA	96	150-690	0	96	100%
	Total	662		10	652	98.49
	Average	94.57		1.42	93.14	

AFLP unique markers

AFLP analysis successfully characterized each of the seventeen studied *Ficus* species by unique markers (Table 6). The seven primer combinations amplified 100 unique markers (93 unique positive and 7 unique negative markers). *Ficus hispida* was characterized by eleven unique positive markers. Whereas, *F. microcarpa* Hawai was identified by only one unique positive marker. *Ficus trijuja* showed ten unique positive markers, while *F. afzelii* revealed seven unique positive and two unique negative markers. Four species revealed seven unique positive markers, i.e., *F. lutea*, *F. platypoda*, *F. elastica* Decora and *F. retusa*. Meanwhile, *Ficus pyriformis* exibited one unique negative and six unique positive markers. While, two species, i.e., *F. asperima* and *F. benghalensis* were identified by six unique positive markers. *Ficus virens* amplified one unique negative and four unique positive markers, meanwhile two species, i.e., *F. sycomorus* and *F. carica* revealed one unique negative and three unique positive markers. Two species revealed three unique positive markers, i.e., *F. benjamina* and *F. spragueana*, while, *F. racemosa* amplified one unique negative and two unique positive markers.

Table 6 : Species, unique positive markers, and unique negative markers.

No	Species	Unique positive marker	Unique negative marker	Total
1	*F. afzelii*	Combination-2(418, 304 bp), Combination-5(579, 382, 185 bp), Combination-6(176, 132 bp)	Combination-3 (209 bp), Combination-6 (455 bp)	9
2	*F. asperrima*	Combination-1 (378 bp), Combination-2 (580 bp) Combination-5 (580, 393, 377, 197 bp)	---	6
3	*F. benghalensis*	Combination-1 (590, 265 bp), Combination-3 (476, 131 bp), Combination-5 (583, 568 bp)	---	6
4	*F. hispida*	Combination-1 (323, 209 bp), Combination-3 (141, 111, 105, 100 bp), Combination-6 (463, 218, 203, 130 bp), Combination-7 (654 bp)	---	11
5	*F. lutea*	Combination-1 (586 bp), Combination-2 (201 bp) Combination-3 (263, 155, 123 bp), Combination-4 (519 bp), Combination-6 (156 bp)	---	7
6	*F. microcarpa 'Hawai'*	Combination-7 (200 bp)	---	1
7	*F. platypoda*	Combination-1 (585, 248 bp), Combination-3 (302, 125 bp), Combination-5 (607, 424 bp), Combination-7 (299 bp)	---	7
8	*F. pyriformis*	Combination-3 (531, 281 bp), Combination-4 (238 bp), Combination-5 (430, 221 bp), Combination-6 (463 bp)	Combination-6 (410 bp)	7
9	*F. racemosa*	Combination-3 (148, 125 bp)	Combination-4 (210 bp)	3
10	*F. spragueana*	Combination-6 (322 bp), Combination-7 (587, 251 bp)	---	3
11	*F. sycomorus*	Combination-5 (463 bp), Combination-6 (251 bp), Combination-7 (283 bp)	Combination-4 (150 bp)	4
12	*F. trijuja*	Combination-1 (220 bp), Combination-2 (116 bp) Combination-3 (186, 175 bp), Combination-4 (250 bp), Combination-5 (437, 182 bp), Combination-6 (158 bp), Combination-7 (603, 218 bp)	---	10
13	*F. virens*	Combination-2 (120 bp), Combination-6 (253, 157 bp), Combination-7 (271 bp)	Combination-7 (283 bp)	5
14	*F. carica*	Combination-5 (242 bp), Combination-6 (151 bp), Combination-7 (560 bp)	Combination-6 (197 bp)	4
15	*F. retusa*	Combination-3 (321, 224 bp), Combination-4 (257 bp), Combination-5 (270, 261, 234 bp), Combination-6 (222 bp)	---	7
16	*F. elastica* 'Decora'	Combination-3 (524, 422 bp), Combination-5 (266, 214, 211 bp), Combination-6 (457, 125 bp)	---	7
17	*F. benjamina*	Combination-2 (131 bp), Combination-6 (498 bp) Combination-7 (348 bp)	---	3
	Total	93	7	100

AFLP-based genetic distances among the *Ficus* species

The similarity indices among the studied *Ficus* species ranged from 0.29 between *F. microcarpa* Hawai and *F. carica* to 0.66 between *F. microcarpa* Hawai and *F. benjamina.*

The UPGMA-dendrogram based on AFLP markers (Fig 2) classified the seventeen species into two main clusters. Where, *F. sycomorus* was separated from all the other species at 0.30 similarity. The same trend of results was previously reported by Tantawy *et al*. (2014), based on ISSR markers, as their dendrogram splitted *F. sycomorus* in a separate cluster.

The second cluster containing the 16 species, was divided into two main subclusters. One included two species that clustered together (*F. carica* and *F. pyriformis*) at 0.39 similarity index. The second subcluster containing 14 species divided into two groups. The first group consisted of three species *F. lutea, F. afezlii* and *F. hisbida,* whereas, species *F. lutea* was closer to *F. afezlii* than *F.hispida* at 0.52 similarity. It is worth noting, these two species clustered together in the morphological based dendrogram.

The second group was divided into three subgroups, the first one comprised *F. virnes* and *F. trijuja* together as they showed 0.58 similarity. While, the second subgroup contained nine species (*F. asperrima, F. platypoda, F. benghalensis, F. racemosa*, *F. retusa. F. spragueana, F. elastica* Decora, *F. microcarpa* Hawai and *F. benjamina*), where *F. microcarpa* Hawai and *F. benjamina* showed the closest relationship with similarity index of 0.66. This result is in accordance with Anuntalabhochai *et al*. (2008) and Heikal *et al*. (2008) based on HAT-RAPD and ISSR markers, respectively. In contrast, the results of Elansary and El-Ansary (2013) revealed that *F. benjamina* was closer to *F. sycomorus* based on RAPD markers.

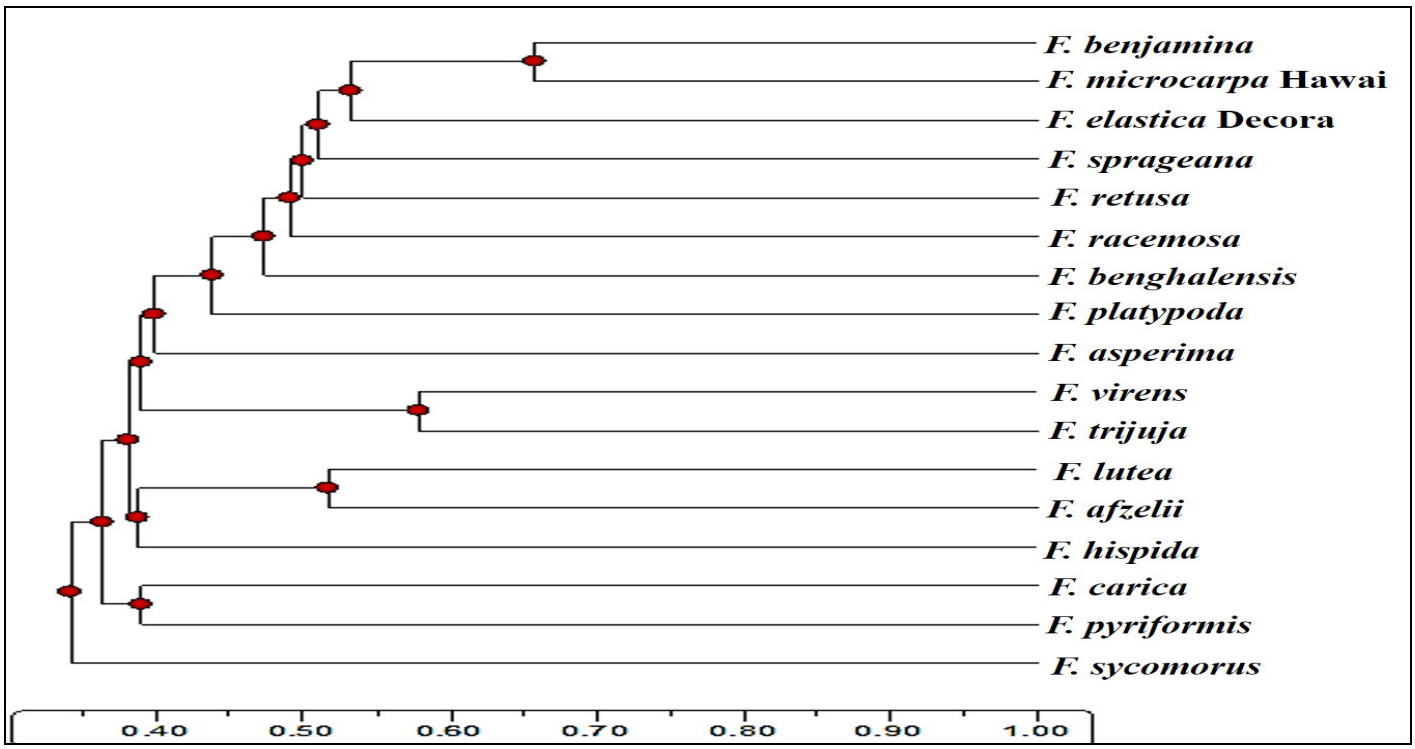

Fig 2 : UPGMA dendrogram of the seventeen *Ficus* species based on AFLPs.

Comparison between the dendrogram based on morphological traits and the AFLP-based dendrogram

The dendrogram generated depending on the leaf traits (Fig. 1) divided the 17 species into two main clusters. The first one was comprised of three species, i.e., *F. carica*, *F. elastica* Decora, and *F. hispida*, where the latter two species were more closely related than *F. carica*. While, in the AFLP based dendrogram, these three species were separated in distinct subgroups. As shown in Tables (2, 3 and 4), the three species exhibited great variation in their leaves, nevertheless they were clustered in one cluster based on their leaf characteristics. This could be attributed to the large leaf length and leaf width in these three species as they exhibited the highest values in these attributes. While, the AFLP analysis distinguished them by specific positive and/or negative markers as *F. hispida* revealed 11 unique positive markers, *F. elastica* Decora expressed 7 unique positive markers, and *F. carica* was distinguished by 3 positive and 1 negative unique marker (Table 6).

The second cluster in the leaf trait-based dendrogram comprised 14 species divided into two main subclusters. One subcluster included *F. lutea* and *F. afzelii*. While, the remaining 12 species constituted the second subcluster. Interestingly, the two species *F. lutea* and *F. afzelii* clustered together in the two dendrograms based on morphological characters and AFLPs, as they share several leaf characteristics including unlobed blade, no teeth on margin, undented margin, no hairs on upper leaf side, apparent leaf venation on lower leaf side, green colored leaf, flattened petiole cross section (Table 4). Apart from the leaf shape (lanceolate *vs.* oblong), and color of the petiole (green in *F. lutea vs.* brown in *F. afzelii*) no clear morphological differences were observed. While, the AFLP analysis was successful in identifying *F. lutea* by 7 unique positive markers and *F. afzelii* by 7 positive and 2 negative markers (Table 6).

Based on morphological dendrogram, the closest species were *F. pyriformis* and *F. sprague*ana as they have similar leaf characteristics (Tables 2, 3 and 4). However, based on AFLPs, these two species were placed in different subclusters where *F. sprague*ana was characterized by 3 unique AFLP positive markers and *F. pyriformis* revealed 6 positive and 1 negative unique AFLP markers (Table 6).

Also, *F. benghalensis* and *F. racemosa* revealed high morphological similarity, the only difference was in the petiole color (green *vs.* brown). Therefore, they were grouped together in the morphological traits-based dendrogram and were also assigned close in the AFLP-based dendrogram. Although, 6 unique positive AFLP markers identified *F. benghalensis*, while, *F. racemosa* expressed 2 positive and 1 negative unique AFLP markers (Table 6).

F. retusa, *F. benjamina* and *F. microcarpa* Hawai were grouped in the dendrogram based on the morphological traits (Fig. 1). While, in the AFLP-based dendrogram (Fig 2) *F. benjamina* and *F. microcarpa* Hawai constituted a subgroup separated from *F. retusa*. It might be due to the morphological attributes of petiole thickness, as they possess the lowest values. Interestingly, *F. retusa* was distinguished from both *F. benjamina* and *F. microcarpa* Hawai by its dark-green colored leaf and rounded petiole cross section. In addition, AFLP identified each of the three species by unique positive markers (1, 7 and 3 for *F. microcarpa* Hawai, *F. retusa* and *F. benghalensis*, respectively).

Based on the leaf traits, *F. sycomorus*, *F. virens* and *F. trijuja* appeared in the same subgroup where *F. sycomorus* was closely related to *F. virnes* than to *F. trijuja*. While, in the AFLP dendrogram *F. virens* and *F. trijuja* clustered in one group. Whereas, *F. sycomorus* was separated from the remaining 16 *Ficus* species under investigation. In addition, the AFLP analysis identified each of these three species by unique AFLP markers, i.e., 3 positive and 1 negative markers for *F. sycomorus*, 10 positive markers for *F. trijuja* and 4 positive and 1 negative markers for *F. virens*. Similarily, Tantawy *et al.* (2014) showed that *F. sycomorus* separated distantly from the other species. This result was also supported by the classification of Corner (1965). Meanwhile, it was in disagreement with Esmaiel *et al.* (2014) according

to their results based on EST-SSR markers, as *F. sycomorus* and *F. pyriformis* clustered together in a subcluster.

Our data demonstrated that the dioecious species *F. carica*, classified as subgenus *Ficus* by Corner (1965), clustered with monoecious species (subgenus *Urostigma*) in the dendrograms based on morphological and AFLP markers. It clustered with *F. hispida* and *F. elastica* Decora based on leaf traits. While, it formed one group with *F. pyriformis* based on AFLPs. Therefore, these results did not comply with the classical taxonomy of Corner (1965), and support the opinion of Weiblen (2000) that the classical taxonomy of *Ficus* species need to be revised in the light of modern molecular techniques.

Species *F. asperima* and *F. platypoda* showed some degree of similarity (0.38) and fell in one subgroup in the dendrogram based on leaf traits. Similarly, in the AFLP-based dendrogram they were closely related. Tables (4 and 5) revealed that the leaf shape (elliptical *vs.* lanceolate) was the unique leaf difference between these two species, while 6 AFLP unique positive markers distinguished *F. asperima* and 7 AFLP unique positive markers characterized *F. platypoda* (Table 6).

Finally, AFLP dendrogram showed that *F. racemosa,* which belongs to subgenus *Sycomorus,* was closer to some species belonging to subgenus *Urostigma* (*F. retusa*, *F. spragueana*, *F. elastica* Decora*, F. microcarpa* Hawai, and *F. benjamina*) than to *F. sycomorus,* which belongs to the same subgenus according to Corner (1965). Therefore, these results contradict the classification of Corner (1965) as he classified both *F. sycomorus* and *F. racemosa* in subgenus *Sycomorus*.

Conclusion

Ficus genus is considered to be the largest genera in *Moraceae*. The classical taxonomy of this genus is ambiguous, as it is based on morphological characters. In the current work we attempted to characterize seventeen species of *Ficus* grown in Egypt, based on leaf morphological and AFLP markers. As the fruits of some species are rarely seen in Egypt, therefore morphological characterization was conducted based on leaf characters only, while, the results could not present a clear characterization for each species. On the other hand, AFLP successfully distinguished each species with different unique markers, which reflects the added value of using the molecular tools and confirms the necessity to supplement the conventional methods by advanced techniques for more accurate characterization of germplasm. However, we believe that more molecular markers are required to elucidate more precisely the pattern of diversity and relationships among the seventeen *Ficus* species.

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