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ARTICLE

Anatomical and Molecular Identification of Ornamental Plant *Ficus* L. Species

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ABSTRACT

This present study includes twelve species that represent the *Ficus* genus, namely; *aspera, carica, tinctoria* subsp. *gibbosa, hirta, hispida, neriifolia, palmata, pumila, racemosa, septica, sur*, and *sycomorus*, belonging to the Moraceae family. The species samples were collected from various locations in Egypt. The study focused on the anatomical and molecular characteristics of mature foliage leaves. Since the identification and classification of taxa are highly dependent on the anatomical features of leaves, the anatomical characteristics were recorded in the form of a comparison between the examined plants in the data matrix. This study aims to contribute to the identification of the studied species based on the anatomical details of the matured leaves. Anatomical characterization includes the variations in upper and lower epidermal layers that are covered by a thin or thick cuticle; the number of palisade and spongy layers; crystals; secretory elements; lithocysts; the midrib zone has parenchyma associated with mechanical tissue, vascular system, and investigation of trichomes; on the other hand, in the current study, the phylogenetic analysis was conducted by using the ITS and 5.8 S sequences. From the analysis of all the available data, it could be stated that there is an overall agreement with the anatomical character dendrogram.

KEYWORDS

ITS; trichomes; leaf anatomy; *Ficus*; moraceae

1 Introduction

Ficus species are rich sources of naturally occurring antioxidants, of which phenolic compounds and flavanoids play a vital role in preventing innumerable health disorders related to oxidative stress including cardiovascular diseases, neurodegenerative diseases, and cancer [1]. Many researchers have successfully determined the antioxidant activity and total phenolic content (TPC) of crude extracts from *F. carica* leaves, Furthermore, the TPC and antioxidant activity of *F. carica* Linn latex from 18 cultivars were investigated, the latex of these three *F. carica* L. cultivars could be a potential source of natural antioxidants and polyphenols [2]. *Ficus* species contain a high polyphenol content and ideal ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) values, which could be an excellent source of antioxidants. Moreover, the polysaccharide has the function of improving immunity, which indicates that it may be beneficial for treating diseases [3]. Chemical constituent and bioactivity investigation showed that the stems and leaves of *F. tikoua* mainly contain antifungal isoflavonoids [4], antioxidant lignans and phenolic compounds [5].

The genus *Ficus* belongs to the family Moraceae. This family includes figs, banyan, breadfruit, jackfruit, and mulberry. More than 845 species are included in the genus *Ficus*, of which the common fig (*F. carica* L.) and sycamore (*F. sycomorus* L.) have been widely recorded since pharaonic times in Egypt for their edible fruit and valuable wood [6]. *Ficus* is a pantropical genus native to the tropics, with a few species extending into the semi-warm temperate zone. It includes trees, shrubs, vines, epiphytes, hemi-epiphytes, and vines occupying various ecological niches. Based on morphological characteristics and distributional patterns, the genus *Ficus* has recently been classified into six subgenera, 19 sections, and 27 Subsections [7]. In addition, Pederneiras (2015) revised nomenclatural, which leads to the displacement of three subgeneric names (*F.* subg. *Terega* Raf., *F.* subg. *Sycomorus* Raf., *F.* subg. *Spherosuke* Raf.), and four sectional names (*F.* sect. Cordifoliae G. Don, *F.* sect. Pogonotrophe (Miq.) Miq., *F.* sect. Platyphyllae Mildbr. & Burret, *F.* sect. Urostigma (Endl.) Griseb.) that have priority over other long-used sub-generic and sectional names [8].

In this study, *Ficus* is represented by twelve species belonging to four subgenera distributed into seven sections. Most Egyptian species fall under the section Sycomorus of the subgenus Sycomorus. Taxonomically, the genus *Ficus* has been considered a problematic taxon. Infraspecific classification of the genus is based on morphological traits of leaves, flowers, and fruits. Syconium contains a minute flower found inside a fleshy receptacle. The syconium represented a challenging structure of the genus [9,10].

Anatomy represents another tool for the infraspecific delimitation of the genus *Ficus* [11–15]. Moreover, leaf indumentum should be a helpful tool for the same purpose [16–21]. Recently, DNA barcoding has been used extensively to resolve the classification of many proplomatic taxa [22–24]. DNA barcoding facilitates fast species identification based on standardized and short DNA sequences from organelle genomes or inter-spacer ribosomal genes [25–27]. Li et al. proposed a combination of several loci as *Ficus* barcodes, including *rbc*L, *mat*K, *psb*K-*psb*I, *trn*H-*psb*A, *atp*F-*atp*H, and ITS [28–30].

The objectives of this contribution were to investigate the leaf anatomical traits and molecular characteristics of the 12 cultivated using previously published ITS sequences. *Ficus* taxa from Egypt.

2 Materials and Methods

2.1 Plant Materials

Fresh samples for each species were collected from three botanical gardens in Giza and Aswan Governorates. All samples were taxonomically revised to confirm the identification using the available literature [9,10,31–33]. An extra confirmatory identification check using the valuable materials of the herbaria of Cairo University Herbarium (CAI) and The Agriculture Museum in Cairo (CAIM) was performed. All collected samples were then kept in the booth mentioned herbaria above (Table 1).

Table 1: List of the twelve *Ficus* taxa collected within their taxonomic groups along with their voucher specimens used in the present study and kept at CAI

Taxonomic groups and taxa	Voucher specimens
Subgenus: *Ficus*	
Section: ***Ficus***	
1 *F. carica* L.	Aswan: Aswan botanic island, 10-8-2021, H. Rofaeel
2 *F. hirta* Vahl	Aswan: Aswan botanic island, 10-8-2021, H. Rofaeel
3 *F. palmata* Forssk.	Giza: Orman botanic garden, 16-8-2021, R. Hamdy
Section: *Erioscycea*	
4 *F. neriifolia* Sm.	Giza: Orman botanic garden 16-8-2021, R. Hamdy
Subgenus: *Terega*	
Section Sycidium	
5 *F. aspera* G. Forst.	Giza: Mazhar botanic garden, 13-8-2021, T. Labib
Section: *Palaeomorphe*	
6 *F. tinctoria* subsp. *gibbosa* Blume Corner	Giza: Orman botanical garden, 16-8-2021, R. Hamdy
Subgenus: *Sycomorus*	
Section: *Sycomorus*	
7 *F. racemosa* L.	Giza: Orman botanic garden, 16-8-2021, R. Hamdy
8 *F. sycomorus* L.	Giza: Orman botanic garden, 16-8-2021, R. Hamdy
Section: *Syocarpus*	
9 *F. hispida* L.f.	Giza: Mazhar botanic garden, 13-8-2021, T. Labib
10 *F. septica* Burm.f.	Giza: Mazhar botanic garden, 13-8-2021, T. Labib
11 *F. sur* Forssk.	Giza: Mazhar botanic garden, 13-8-2021, T. Labib
Subgenus: *Synoecia*	
Section: *Pogonotrophe*	
12 *F. pumila* L. var. *pumila*	Giza: Mazhar botanic garden, 13-8-2021, T. Labib

2.2 Leaf Anatomy

Twelve leaf samples were prepared for anatomical investigation according to the method proposed by Nassar and El-Sahhar [34]. A square centimeter of the leaf was removed, and it was dehydrated in a succession of solutions with ethyl alcohol concentrations ranging from 50% to 100%. The samples were then embedded in paraffin wax [the melting point of paraffin wax range is 58°C–62°C using xylol as a solvent. Sections were cut at a thickness of 15 microns using a rotary microtome and then mounted on slides using egg albumin as an adhesive agent. The slides were subjected to a declining sequence of ethyl alcohol solutions ranging from 100% to 50% ethyl alcohol concentrations.

A double stain composed of safranin and light green was used. Canada balsam was used as a mounting medium. Sections were investigated using a light microscope, Serico XSZ-107BN, at the Department of Botany and Microbiology, Faculty of Science, Cairo University, Egypt. Photomicrographs were taken using a photomicroscope; Optika fitted with a premiere MA88-900 digital camera in the Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Egypt.

All terms used to describe the leaf anatomical traits were after [12,17,19,20]. Forty anatomical traits of the leaf were scored and coded to build up a numerical data matrix. Statistical analysis using PC-ORD (Software, Version 5) was conducted to compare the investigated taxa. To build a data matrix for numerical analysis, a total of 40 comparative anatomical features for the analyzed taxa were scored and coded. Finally, artificial keys were generated using the statistical programme DELTA [35].

2.3 Genomic Data Analysis

To investigate the molecular characteristics of Egyptian *Ficus* species. We retrieved the ITS and 5.8 S sequences from NCBI (Table 2). Next, we used 19 bp upstream and downstream of the sequence of *F. neriifolia* as a potential primer for *in-silico* PCR (Table 3). The Primer-Blast module of NCBI extracted the amplicons from the target sequences. Finally, we used the ClustalW module on Mega7 [36] to execute Multiple Sequence Alignment (MSA) for the amplicons and construct a phylogenetic tree based on a maximum likelihood algorithm. While the maximum likelihood tree shows the most likelihood topology for the taxonomical tree and branches, it also shows a general collinearity agreement with the hierarchical morphological tree.

Table 2: ITS *in-silico* PCR for the studied *Ficus* species

Species	A.N	Sequence length	Forward primer	Reverse primer	Insilico PCR
F. carica	LC375796	820	…….	…..AA……	627
F. palmata	LC375804	820	…….	…….	622
F. hirta	AY730127	788	…….	…….	625
F. neriifolia	KY388571/ JX185787	609/611	…….	……T.	619
F. aspera	EU091660	786	…….	…….	623
F. tinctoria subsp. *gibbosa*	JN117649	713	……C..G…….	…….	625
F. racemosa	AF165405	718	…….	……T……...	631
F. sycomorus	AY063575	700	…T……	……T……...	627
F. hispida	EU091623	748	…..T……G…	……T……...	625
F. sur	AF165411	713	…….	……T……...	626
F. septica	AF165409	715	……C	……T……...	628
F. pumila var. *pumila*	AY063580	695	…….	…….	623

Table 3: Manually designed PCR for ITS sequences

Primers	Sequences	Length
Forward primer	ACCCGCGAACACGTTACAA	19
Reverse primer	TCGCGTTGGAGGCACCCGT	19

3 Results

3.1 Leaf Indumentum

Hairy leaf surfaces were recorded in ten *Ficus* species. The leaves of *F. neriifolia* and *F. septica* were glabrous. The following types of trichomes were recognized on both abaxial and adaxial leaf surfaces (Table 4 and Fig. 1).

3.1.1 Glandular Trichomes

The stalks are composed of 1–2 cells with spherical heads: *F. sur*, *F. hirta*, *F. carica*; *F. aspera*; *F. palmata* and *F. hispida* (Fig. 1A).

3.1.2 Non-Glandular Trichomes

They are categorized into four groups:

a) Unicellular trichome: *F. sycomorus* (Fig. 1B).
b) Unicellular trichomes with prominent bases surrounded by raised epidermal cells: *F. hirta*; *F. carica* (Fig. 1C) and curved end in *F. palmata*, and *F. hispida* (Fig. 1D).
c) Multicellular, slender bristle trichomes, terminating gradually into the tapered end, *F. racemosa, F. sur, F. palmata, F. carica*, *F. aspera* and *F. hispida* (Fig. 1E), while in *F. hirta*, the trichome was found to be slightly curved (Fig. 1F).
d) The adaxial leaf surfaces have trichomes that are mainly described as long clothing hairs: *F. carica,* and *F. sycomorus* (Fig. 1G).

3.1.3 Cystolith

This type of trichome is composed of a rounded, sometimes swollen base sunken into the mesophyll layer of the leaf, with very short mucronate at the apex. Cystolith was found in *F. hirta*, *F. hispida*, and *F. carica* (Fig. 1H). Cytoliths were recorded for all studied taxa, with a large base sunken in mesophyll and a sharp mucronate apex more on the abaxial surface than adaxial; *F. palmata*, *F. aspera*, *F. pumila* and *F. tinctoria* subsp. *gibbosa* (Fig. 1I).

3.2 Leaf Anatomy

Table 4 and Figs. 2 and 3 summarize the anatomical features of the leaf that permit effective discrimination between the studied taxa.

In the transverse section (TS), the *Ficus* leaf is dorsiventral and consists of a central midrib and two wings. The epidermis is covered by a thin cuticle in eleven taxa while it was found in *F. neriifolia*. Cuticular ridges are found on the abaxial surface of the leaf. The cuticle layer was folded in *F. sycomorus* and slightly folded in the other *Ficus* species under investigation.

The convex midrib is found in most studied taxa. It was flat-convex in *F. tinctoria* subsp. *gibbosa*, *F. septica*, and *F. pumila* var. *pumila*, and concave in *F. neriifolia* only. In *F. tinctoria* subsp. *gibbosa*, *F. septica*, and *F. pumila* var. *pumila*, the midrib is abaxially outlined and flat-arched.

Collenchymal tissue circulates the central vascular bundle. It is composed of 5–9 layers in *F. hirta*, *F. neriifolia*, *F. septica*, and *F. pumila* var. *pumila*; 10–15 layered in *F. hispida*; *F. sycomorus*; *F. carica*, *F. tinctoria* subsp. *gibbosa; F. racemosa*, *F. aspera* and *F. palmata*; and 10–15 layers in *F. aspera*, *F. tinctoria* subsp. *gibbosa*, and *F. racemosa*, *F. carica, F. palmata, F. hirta, F. hispida, F. septica*, and *F. pumila* var. *pumila*. Two layers of hypodermis were recorded in *F. neriifolia, F. aspera, F. tinctoria* subsp. *gibbosa, F. racemosa* and *F. sycomorus*, while it is composed of a single layer in *F. hispida*, *F. sycomorus*, *F. carica* and *F. septica*. Sclerenchyma tissue is found as marginal strands in *F. tinctoria* subsp. *gibbosa*, *F. sycomorus*, *F. hispida*, and *F. septica*.

Table 4: Characters list for the numerical analysis of some studied taxa of *Ficus*

Taxa anatomical characters	*F. carica*	*F. palmata*	*F. hirta*	*F. neriifolia*	*F. aspera*	*F. tinctoria* subsp. *gibbosa*	*F. racemosa*	*F. sycomorus*	*F. sur*	*F. hispida*	*F. septica*	*F. pumila* var. *pumila*
1- Cuticle: thin (0), thick (1)	0	0	0	1	0	0	0	0	0	0	0	0
2- Cuticular ridges Abaxially: smooth-slightly folded (0), folded (1)	0	0	0	0	0	0	0	1	0	0	0	0
3- Presence of trichomes: Absent (0), Present (1)	1	1	1	0	1	1	1	1	1	1	0	1
4-Adaxially: simple trichomes: Absent (0), Present (1)	1	1	1	0	1	0	1	1	1	1	0	0
5-Adaxially: glandular trichomes: Absent (0), Present (1)	0	0	1	0	1	0	0	1	1	1	0	0
6- Adaxially: cystolith trichomes: Absent (0), Present (1)	0	0	1	0	0	1	0	0	0	1	0	0
7- Abaxially: simple trichomes: Absent (0), Present (1)	1	1	1	0	1	0	1	1	1	1	0	1
8- Abaxially: glandular trichomes: Absent (0), Present (1)	1	1	1	0	1	0	0	1	0	1	0	0
9- Abaxially: cystolith trichomes: absent (0), present (1)	1	1	1	0	1	1	0	0	0	1	0	1
10- Adaxial epidermis layers: 1(0), 2(1)	0	0	0	1	1	1	1	1	1	0	0	0
11- Abaxial epidermis layers: 1(0), 2(1)	0	0	0	1	0	0	0	0	0	0	0	0
12- No. of palisade layers: 1 (0), 2-3 (1)	1	1	1	1	0	0	1	1	1	1	1	0
13- No. of spongy layers: less than 3 (0), more than 3 (1)	1	1	0	1	1	1	1	1	1	1	1	1
14- Appearance of spongy cells: typical (0), aerenchymatous (1), palisade-like (2),	1	1	0	0	0	1	1	2	1	0	1	0
15- Midrib outline adaxially: convex (0), -flat-convex (1), concave (2)	0	0	0	2	0	1	0	0	0	0	1	1
16- Midrib outline abaxially: arched (0), flat-arched (1)	0	0	0	0	0	1	0	0	0	0	1	1
17- Crystals in adaxial parenchyma: none (0) druses (1)	1	1	0	0	0	0	0	0	0	0	0	0
18- Crystals in abaxial parenchyma: none (0) druses (1)	1	1	1	0	0	1	0	0	0	0	0	0

(Continued)

Table 4 (continued)

Taxa anatomical characters	*F. carica*	*F. palmata*	*F. hirta*	*F. neriifolia*	*F. aspera*	*F. tinctoria* subsp. *gibbosa*	*F. racemosa*	*F. sycomorus*	*F. sur*	*F. hispida*	*F. septica*	*F. pumila* var. *pumila*
19- Crystals in abaxial parenchyma: none (0), prismatic (1)	1	0	0	0	0	0	0	0	0	0	0	0
20- Crystals in mesophyll: none (0), druses (1)	1	1	1	0	1	1	0	0	0	1	1	0
21- Crystals in bundle sheath and midrib parenchyma: none (0), druses (1)	1	1	1	1	1	1	1	1	1	1	1	0
22- Crystals in bundle sheath and midrib parenchyma: none (0), prismatic (1)	1	1	0	0	1	1	1	1	1	0	1	0
23- Secretory elements-non branched laticiferous tubes: absent (0), present (1)	1	1	0	0	0	1	1	1	1	0	0	1
24- Secretory elements-tanniniferous cells adaxially: absent (0), present (1)	0	0	0	0	0	0	1	1	0	0	0	0
25- Secretory elements-tanniniferous cells in mesophyll: absent (0), present (1)	0	0	0	0	0	0	1	1	1	0	1	0
26- Secretory elements-tanniniferous cells abaxially: absent (0), present (1)	0	0	0	0	0	0	1	1	1	0	0	0
27- Secretory elements-gum-resin& mucilage cells: absent (0), present (1)	1	1	1	0	1	1	0	0	0	0	0	0
28- Starch grains in mesophyll, bundle sheath and midrib parenchyma: absent (0), present (1)	0	0	0	1	0	0	0	0	0	0	0	1
29- Annular xylem vessels: absent (0), present (1)	1	1	1	1	0	1	1	1	1	1	1	0
30- Lithocysts: absent (0), present (1)	1	1	0	1	1	1	1	1	1	1	1	1
31- Lithocysts- position adaxially: absent (0), present (1)	0	0	0	1	0	1	0	1	0	0	0	0
32- Lithocysts- position abaxially: absent (0), present (1)	1	1	0	1	1	1	1	1	1	1	1	1
33- Ducts: absent (0), present (1)	0	0	0	0	0	0	1	1	0	1	1	0
34- Marginal schlerenchyma: absent (0), present (1)	1	1	1	1	1	0	1	0	1	0	0	1
35- No. of phloem strands 0 (0), 1-5(1), 6-10(2), 11-25(3)	1	1	1	2	2	1	2	3	2	1	3	0
36- Bundle sheath in lamina: circular (0), vertically transcurrent (1)	1	1	1	0	1	0	1	1	1	1	1	1

(Continued)

Table 4 (continued)

Taxa anatomical characters	*F. carica*	*F. palmata*	*F. hirta*	*F. neriifolia*	*F. aspera*	*F. tinctoria* subsp. *gibbosa*	*F. racemosa*	*F. sycomorus*	*F. sur*	*F. hispida*	*F. septica*	*F. pumila* var. *pumila*
37- No. of collenchyma adaxially 5-9(0), 10-15(1)	1	1	0	0	1	1	1	1	1	1	0	0
38- No. of collenchyma abaxially 5-9(0), 10-15(1)	0	0	0	0	1	1	1	0	0	0	0	0
39- Thickness of midvein/thickness of lamina: 4.7-6.5 µm (1)-7.7-10 µm (2)- 13.7-15.74 µm (3)	1	2	2	1	3	1	2	2	1	3	2	1
40- Thickness of spongy/thickness of palisade : 0.8-1.4 µm (1)-1.5-2 µm (2)-2.65-3.81 µm (3)	2	1	2	1	2	3	1	2	3	1	1	1

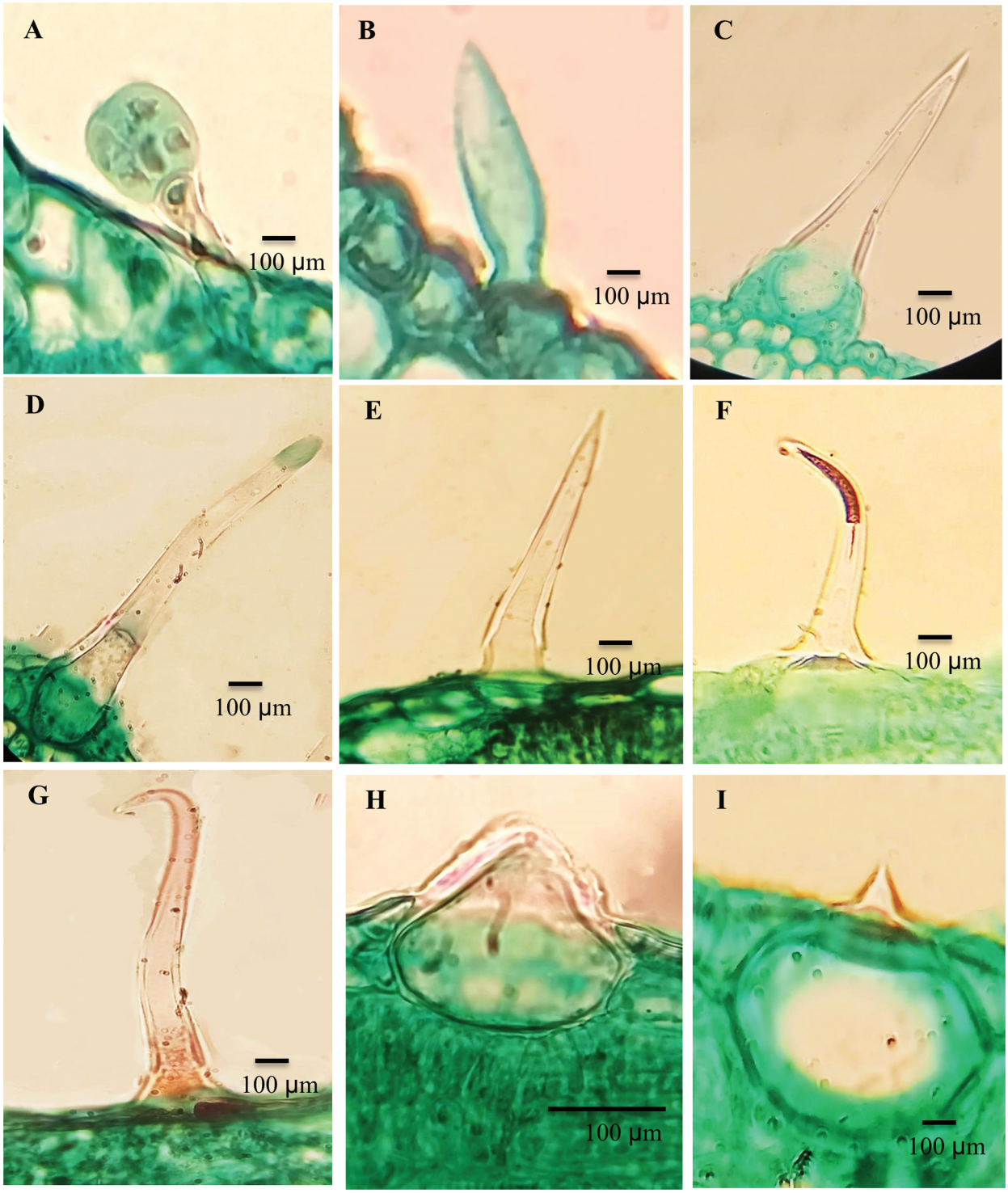

Figure 1: Trichomes of the studied taxa *Ficus*. A. *F. hispida*, B. *F. sycomorus*, C. *F. carica*, D. *F. hispida*, E. *F. carica*, F. *F. hirta*, G. *F. sycomorus*, H. *F. carica* and, I. *F. tinctoria* subsp. *gibbosa*

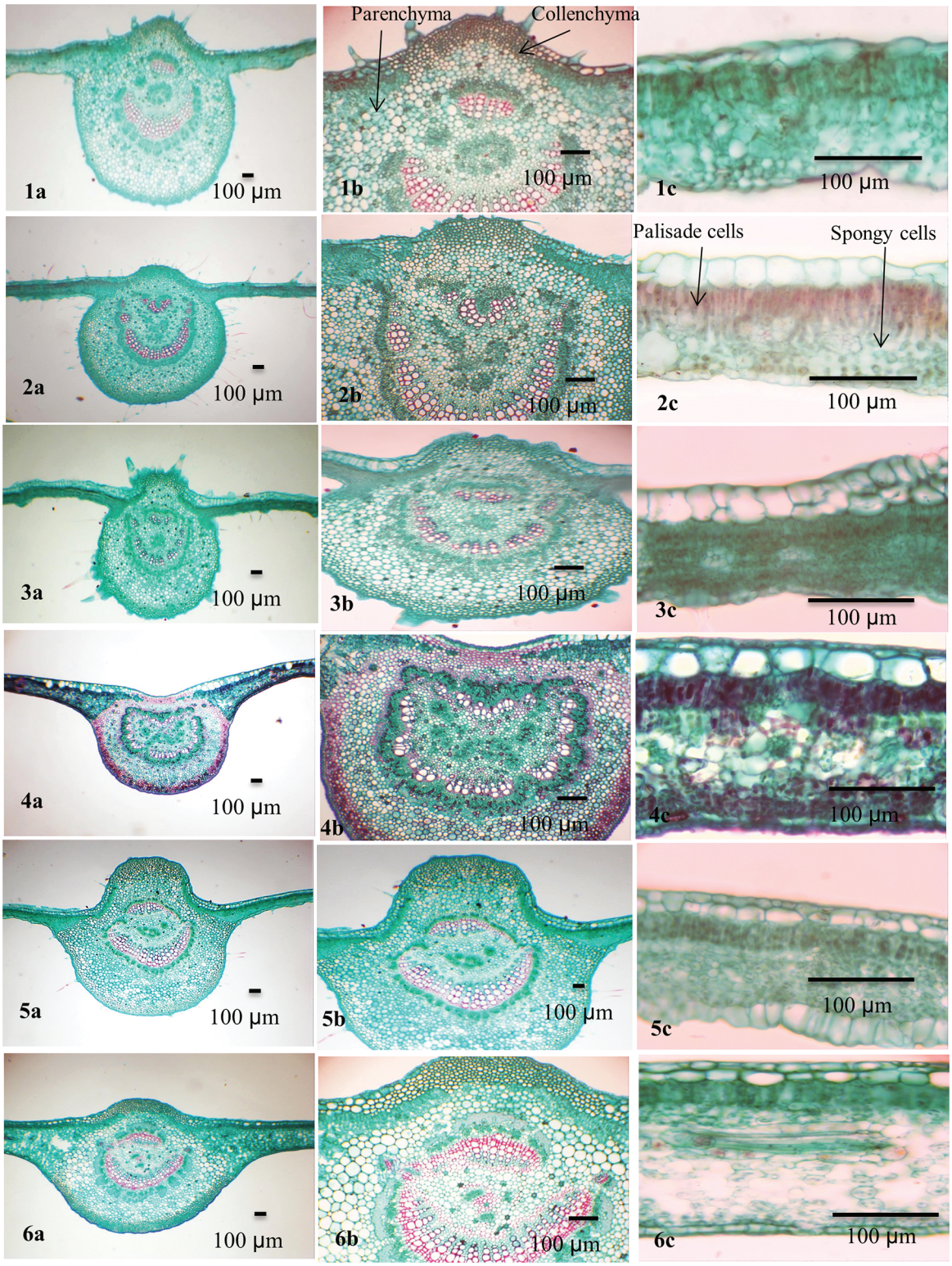

Figure 2: Blade anatomy of the studied taxa of *Ficus*. 1. *F. carica*, 2. *F. palmata*, 3. *F. hirta*, 4. *F. neriifolia*, 5. *F. aspera*, 6. *F. tinctoria* subsp. *gibbosa*

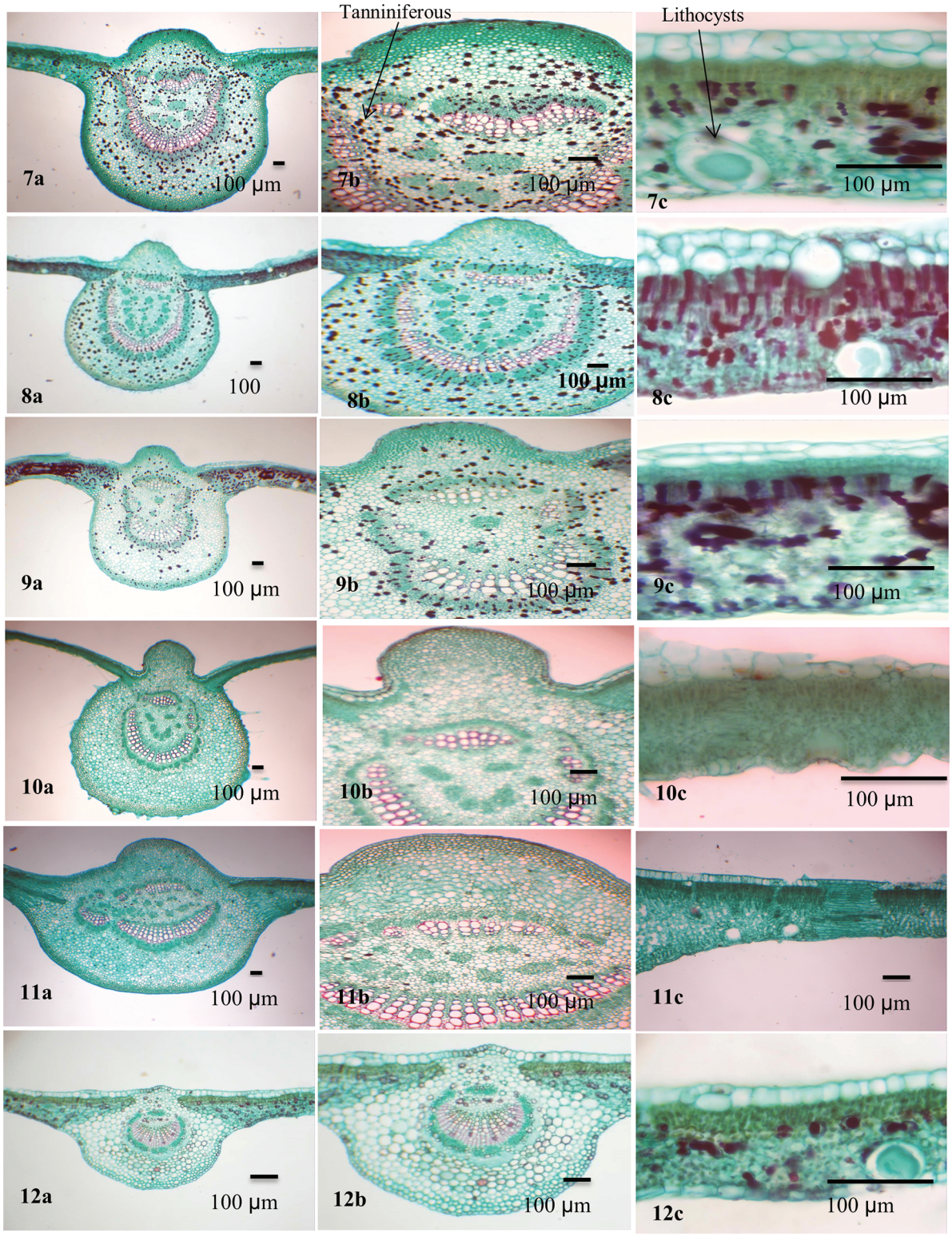

Figure 3: Blade anatomy of the studied taxa of *Ficus*. 7. *F. racemosa*, 8. *F. sycomorus*, 9. *F. sur*, 10. *F. hispida*, 11. *F. septica*, 12. *F. pumila* var. *pumila*

The bundle sheath extends to both abaxial and adaxial epidermal layers (vertically transcurrent) in most of the taxa while closed, not extending to adaxial and abaxial epidermal layers (circular) as in *F. neriifolia* and *F. tinctoria* subsp. *gibbosa*. Annular xylem vessels are present in all studied taxa except *F. aspera* and *F. pumila* var. *pumila*. Lithocysts occur in both the abaxial and adaxial epidermis and are recorded in most studied taxa but are absent in *F. hirta* only, and they mostly appear on the abaxial epidermis of the lamina.

The mesophyll was dorsiventral with predominantly one to three layers of adaxial palisade cells. Spongy tissue consists of two layers in *F. hirta*; other taxa under investigation have multiple layers of spongy tissue. Parenchyma cells are found in the spongy layer. The parenchyma has various forms and might help in distinguishing among species: typical, palisade-like aerenchyma: *F. hirta*, *F. neriifolia*, *F. aspera*, *F. hispida*, and *F. pumila* var. *pumila*; typical aerenchymatous: *F. carica*, *F. palmata*, *F. tinctoria* subsp. *gibbosa*, *F. racemosa*, *F. sur*, and *F. septica*, whereas they were palisade-like in *F. sycomorus* only.

Two types of crystals are recorded in the leaves of the studied taxa: druses and prismatic crystals. Druse crystals are commonly present in the adaxial and abaxial parenchyma in *F. carica* and *F. palmata*. While present abaxially only in *F. hirta* and *F. tinctoria* subsp. *gibbosa*. Druse crystals are also recorded in mesophyll in *F. carica*, *F. palmata*, *F. hirta*, *F. aspera*, *F. tinctoria* subsp. *gibbosa*, *F. hispida*, and *F. septica*. Prismatic crystals occur in the abaxial parenchyma in *F. carica* only. Both druses and prismatic crystals are mainly found in the bundle sheath and midrib parenchyma in all studied taxa. Secretory element -tanniniferous cells are present adaxially and abaxially in *F. racemosa* and *F. sycomorus*, abaxially only in *F. sur* and absent in the rest. It is found in the mesophyll in *F. racemosa*, *F. sycomorus*, *F. sur*, and *F. septica*. Secretory element-nonbranched laticiferous tubes are present in *F. carica*, *F. palmata*, *F. tinctoria* subsp. *gibbosa*, *F. racemosa*, *F. sycomorus*, *F. sur*, and *F. pumila* var. *pumila*. Secretory elements-tanniniferous secretory elements-gum-resin and mucilage cells are present in *F. carica*, *F. palmata*, *F. hirta*, *F. aspera*, and *F. tinctoria* subsp. *gibbosa*. Starch grains are recorded in the mesophyll, bundle sheath and midrib parenchyma in *F. neriifolia* and *F. pumila*.

3.3 Computer Based Generating Key

In the present study, 12 taxa were used as the Operational Taxonomic Units (OTU). A total of 37 anatomical leaf aspects were examined and recorded. The construction of the key was carried out by using DELTA (Description Language for Taxonomy).

Key 5. Confirmatory characters

Characteristics: 37 in data, 37 included, 20 in key.

Items: 12 in data, 12 included, 12 in key.

Parameters: R base = 1.40, Abase = 2.00, Reuse = 1.01, Varywt = 0.80.

Characters included: 1–37.

Character reliabilities: 1–37, 5.

1. Spongy parenchyma typical….**2**

   Spongy parenchyma aerenchymatous ……..…….**5**

   Appearance of spongy cells palisade-like……***F. sycomorus***

2(1). Midrib outline adaxially convex……**3**

   Midrib outline adaxially flat-convex……..…….. ***F. pumila* var. *pumila***

   Midrib outline adaxially concave…… ***F. neriifolia***

3(2). Epidermis two layered, cystolith trichomes absent in adaxial epidermis; presence of prismatic crystals in bundle sheath and midrib parenchyma, palisade parenchyma uniseriate layered……..……... ***F. aspera***

Epidermis one layered, Cystolith trichomes present in adaxial epidermis; absence of prismatic crystals in bundle sheath and midrib parenchyma, palisade parenchyma 2-3 layered……..**4**

4(3). Spongy layers less than 3; Crystals in abaxial parenchyma druses; Secretory elements-gum-resin and mucilage cells present; Lithocysts absent…….. ***F. hirta***

Spongy layers more than 3; Crystals in abaxial parenchyma none; Secretory elements-gum-resin and mucilage cells absent; Lithocysts present…….. ***F. hispida***

5(1). Cystolith trichomes absent in abaxially epidermis; Crystals in abaxial parenchyma none; Secretory elements-tanniniferous cells in mesophyll present; Secretory elements-gum-resin and mucilage cells absent…… **6**

Cystolith trichomes present in abaxially epidermis; Crystals in abaxial parenchyma druses; Secretory elements-tanniniferous cells in mesophyll absent; Secretory elements-gum-resin and mucilage cells present ……**8**

6(5). Midrib outline adaxially convex; presence of simple trichomes in adaxial and abaxial epidermis ……7

Midrib outline adaxially flat-convex; absence of simple trichomes in adaxial and abaxial epidermis…… ***F. septica***

7(6). Glandular trichomes absent in adaxial epidermis; Secretory elements-tanniniferous cells adaxially present; Ducts present; Number of collenchyma abaxially 10-15…… ***F. racemosa***

Adaxially glandular trichomes present; Secretory elements-tanniniferous cells adaxially absent; Ducts absent; Number of collenchyma abaxially 5-9…….. ***F. sur***

8(5). Adaxially cystolith trichomes absent; Midrib outline adaxially convex; Adaxially simple trichomes present; Abaxially simple trichomes present ……..**9**

Adaxially cystolith trichomes present; Midrib outline adaxially flat-convex; Adaxially simple trichomes absent; Abaxially simple trichomes absent…… ***F. tinctoria*** **subsp.** ***gibbosa***

9(8). Absence of prismatic crystals……... ***F. palmata***

Presence of prismatic crystals…….. ***F. carica***

3.4 Numerical Analysis

Table 4 shows 40 leaf anatomical traits of 12 *Ficus* species. These traits are used for numerical analysis. In Fig. 4, the anatomical data showed that the studied taxa belonged to two main clusters in the dendrogram produced using PCORD. Cluster 1 consisted of three *Ficus* taxa, which were further split into two subgroups; group No. 3 included two taxa, *F. neriifolia* and *F. septica*, while group No. **4** had one taxon, *F. pumila* var. *pumila*. Cluster **2** was divided into two groups. Group No. **5** was divided into two subgroups. Subgroup **7** included *F. carica*, *F. palmata*, *F. hirta*, *F. hispida*, and *F. aspera,* and was separated into a distinct subgroup. Subgroup **8** consisted of the studied taxa of *F. racemosa, F. sycomorus*, and *F. sur*, whereas the second group **6** contained *F. tinctoria* subsp. *gibbosa*.

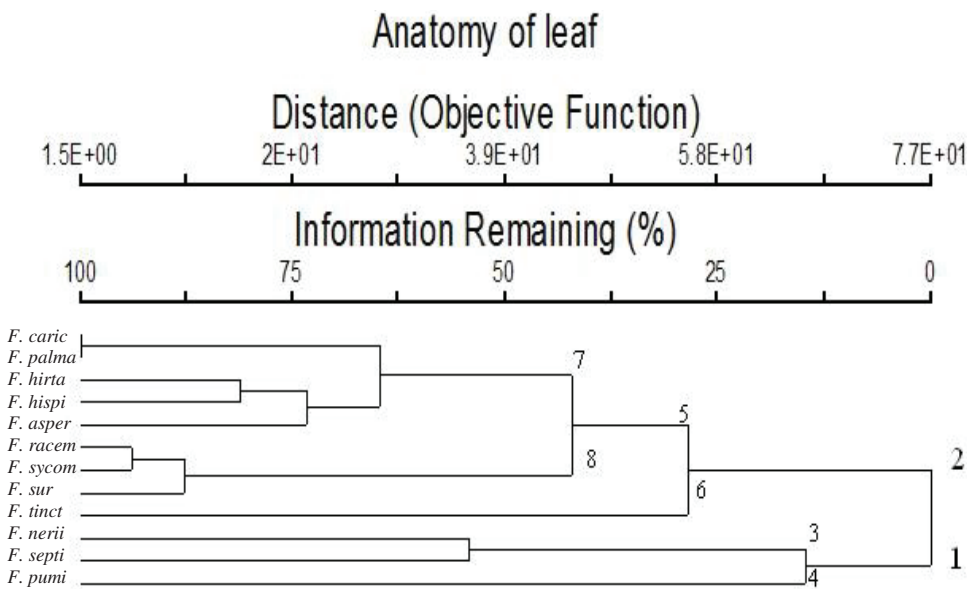

Figure 4: Dendrogram showing the interrelationships between 12 taxa of *Ficus* based on 40 leaf anatomical characters by using the PCORD program

3.5 Genomic Data Analysis

Dendrogram cluster based on data from leaf anatomy was illustrated in Fig. 4, this revealed that studied taxa classified into two main groups. The first group included three taxa (*F. pumi*, *F. septi*, and *F. neni*). The second group included two sub-group; first sub-group contained *F. tinct* and the second sub-group contained other eight studied taxa. The second sub-group divided into two sub-subgroups; first sub-subgroup included (*F. surr*, *F. sycom*, and *F. racem*). the second sub-subgroup included the other five studied taxa.

On the other hand, the molecular characteristics data showed that the studied taxa belonged to two main clusters in the dendrogram produced using the Clustal W module on Mega7 to execute MSA. Cluster **(A)** consisted of two taxa, *F. carica* and *F. palmata*. Cluster **(B)** was divided into two groups. Group No. **(C)** was divided into two subgroups. Subgroup **(E)** includes *F. aspera*, *F. tinctoria* subsp. *gibbosa* and *F. hirta*. Subgroup **(F)** consisted of the studied taxa *F. neriifolia* and *F. pumila* var. *pumila*, whereas the second group **(D)** contains *F. hispida*, *F. septica*, *F. racemosa*, *F. sur* and *F. sycomorus* (Fig. 5).

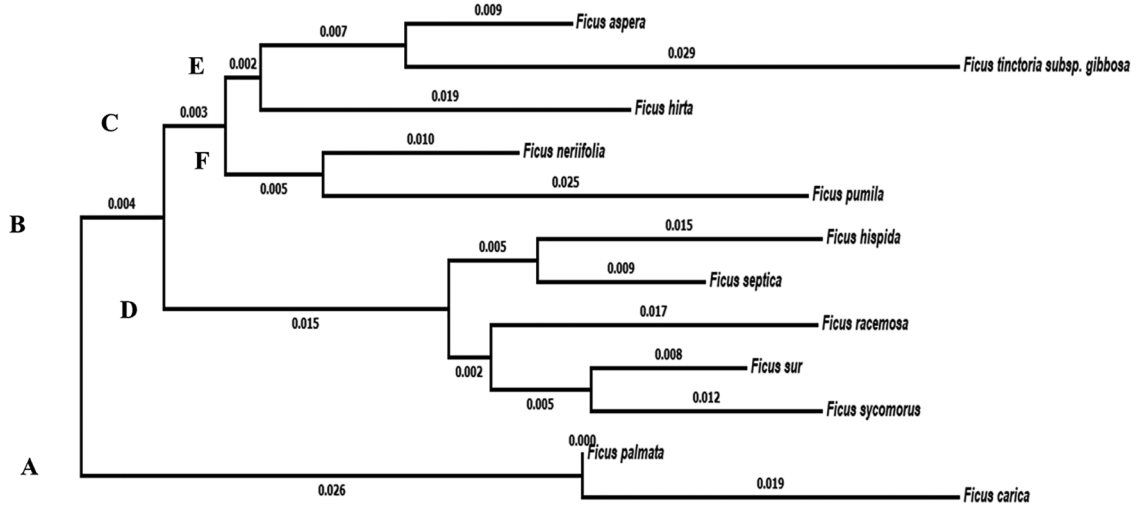

Figure 5: Dendrogram showing the interrelationships between the twelve taxa of *Ficus* based on ITS sequence

4 Discussion

In many branches of biology, species delimitation is crucial. Despite its significance, there is no consensus on the criteria for defining species because different biological disciplines have different points of view. Traditional anatomical and molecular diagnostic characters are the two main categories frequently used to differentiate between species. Since it has been studied for more than a century, traditional morphological taxonomy is a very sophisticated method of identifying species. There still seem to be some differences within the method and disagreements about its efficacy among those who study it, despite a century of continuous improvement. The use of molecular markers as tools for determining the boundaries of species has significantly increased. In principle, all three plant genomes (nuclear, mitochondrial, and chloroplastic) can be used to delimit species. Nuclear and chloroplastic genomes are most frequently utilized in plants. The fundamental idea behind using molecular markers to identify species is that the "species tree" should be deduced from a "gene tree". In other words, one or more DNA pieces can be used to infer the evolutionary history of a phylum.

The twelve *Ficus* taxa showed substantial leaf anatomical variations. These variations would help in the infraspecific classification of the taxa under investigation. The identification and classification of taxa are highly dependent on the anatomical traits of leaves, including cuticle, trichome, crystals, secretory elements, lithocysts, number of layers of palisade and spongy tissues. In the majority of taxa under investigation, the epidermis was covered by a thin or thick cuticle; the midrib has parenchyma associated with mechanical tissue.

Sonibare et al. examined the leaf anatomy of 25 different *Ficus* species from Nigeria and concluded that foliar anatomical traits might be helpful in the infraspecific classification of the genus *Ficus* [15]. According to Berg & Corner's study on *Ficus* leaf anatomy, the microscopic features of leaves can be used to distinguish subgenera, series, and subseries, but at the species level, more data are needed [7]. However, Van Greuning and Grobbelaar observed that the anatomical characteristics of the leaves of *Ficus* are significant for taxonomy even at the species level [17]. Corner classified leaves using a combination of morphological and anatomical traits, such as the position of the lithocysts [37,38]. Chew also combined leaf anatomy, such as lithocysts and hypodermis, with morphology to identify *Ficus* subgenera, sections, and series [39]. Lithocyst and epidermal features were used by Berg and Corner and Berg et al. [7,40].

Calcium oxalate crystals have been used in the taxonomic classification of plants; the appearance and position of calcium oxalate or calcium carbonate crystals (such as cystolith, a crystal associated with the cell wall) can be distinctive and helpful [22,23,41]. In the lower epidermis of the leaf of *F. deltoidea* var. *motleyana*, a cystolith, was found in a cell known as a lithocyst [41].

According to Berg and Corner, in section of subgen. *Ficus*, cystoliths can only be found on the lower side of the leaf lamina [7]. This is contrary to research by Awang et al. [42]. Lithocysts were recorded in the current study on the abaxial side the leaves of *F. palmata* and *F. carica*. A bundle sheath outline is considered a valuable taxonomical trait [17]. Circular vascular bundles were recorded in *F. hispida*, *F. pumila* var. *pumila* and *F. sycomorus*.

The current research showed that the epidermal cells were simple and covered by a thin cuticle layer or thick cuticle layer, druses and sandy crystals, hypodermis and cystolith (calcium carbonate), and a secretory cell ridge with papillae, which distinguishes them from the hypodermis. The results were consistent with cystolith appearance in several mulberry family species (Moraceae), such as those recorded in *F. elastica* [43].

In the Moraceae family, the spongy parenchyma has taxonomic importance. Three different types of spongy parenchyma, including typical spongy parenchyma, palisade-like parenchyma, and aerenchymatous spongy parenchyma tissue, may be recognized in this study, which agrees with

Van-Greuning et al. In their study of leaves from four mulberry cultivars' leaves, they observed that the mesophyll had 8–10 layers and significant air spaces [44].

The trichomes were recorded in three types simple, glandular, and cystolith. All the trichomes on the adaxial and abaxial epidermises of leaves in both studied taxa, except for *F. neriifolia* and *F. septica*, are glabrous. Sonibare et al. revealed that trichomes are distributed differently in upper and lower leaves and that the taxonomic value of the trichomes is a critical-essential diagnostic for classifying *Ficus* species from others [15]. According to Mamoucha et al. [45] and Sosnovsky [20], *F. carica* only has glandular trichomes on the lower surface, but Giordano et al., discovered them on both the upper and lower surfaces [20,21,45], but in this study, the glandular trichomes in *F. carica* are present on the lower surface, agreeing with Mamoucha et al. [45] and Sosnovsky [20].

As one of the most promising DNA barcoding locations, ITS is frequently utilized to shed light on the taxonomical relationships of plants [27,46]. Even though the ITS was very useful for phylogenetic analyses, it was quite challenging to resolve *Ficus* connections. Unfortunately, the sequences were considerably divergent from those of other taxa, and the overlapping indels made the alignment process and phylogenetic construction difficult [47]. Fortunately, the alignment in our experiment was simple, and the ML phylogenetic tree produced exhibits collinearity with the morphological taxonomical approach.

5 Conclusion

We conclude that anatomical characteristics, notably the blade, are important for recognizing and differentiating the species under consideration here. The selected *Ficus* species' differences and affinities have been fairly described by the anatomical characterization used in this study. Additionally, it is crucial to distinguish between taxa based on the presence or absence of crystals, starch grains, lithocysts, bundle sheaths in the lamina, and trichomes. Based on the features examined in this article, a key is offered for the identification of the investigated taxa. The outcome of the numerical analysis demonstrates that anatomical traits are crucial in discriminating between species that belong to the same genus. Additionally, the anatomical character dendrogram and the phylogenetic analysis of the ITS and 5.8 S sequences indicated general concordance. The traits assessed in this study were consistent with previous classifications of the genus *Ficus*.

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