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Cystolith micromorphology in selected taxa of *Ficus* L. (Moraceae) in India and its taxonomic implications

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Abstract

Cystoliths are microscopic calcium deposits found remarkably in some genera and is of key importance to understand its taxonomic value. Leaf samples of 19 species belonging to 7 sections of Indian *Ficus* were processed for anatomical studies and surface micromorphological characteristics of cystoliths. The morphology, distribution and sculpture patterns of cystolith found varying among species. Four types of basic sculpturing patterns have been observed in this study: aculeate, colliculate, verrucate, and tuberculate. The aculeate sculpturing pattern is the most common type observed in twelve species of section *Cordifoliae*, but further division of this pattern into three subtypes i.e. broad, moderate and reduced aculeate along with the length and width of cystoliths appendages and the mode of stalk fixation provides more informative insight into sub-sectional classification. Principal component analysis (PCA) provides a supporting evidence for earlier classification at sectional/subsectional level. Though in few cases it has not support the decision taken by molecular studies. This study suggests that cystolith micromorphological characters could be utilized in the re-classification of *Ficus* taxa at sub-sectional level.

Keywords: Cystolith sculpture patterns, Indian *Ficus*, Leaf anatomy, SEM, Taxonomic status

Introduction

The genus *Ficus* L. (Moraceae) usually known as 'Fig' is one of the largest genera of angiosperms with almost 850 species distributed globally in the tropical and warm regions (Mabberley 2017). It is considered as one of the most diversified genera due to its different types of lifeforms (Berg 2004). *Ficus* species are considered keystone resources and have enormous ecological, cultural and commercial importance (Kumar *et al.* 2011, Woode *et al.* 2011, Kuaraksa *et al.* 2012, Tiwari *et al.* 2015).

Globally, the infrageneric classification and nomenclature of the genus *Ficus* have been variously dealt by several authors (Berg 2004, Chantarasuwan *et al.* 2013). Recently, Pederneiras *et al.* (2015), have made major changes in the sub-generic and sectional names to the classification given by Berg (2004). The subgenera *Sycidium* (Miq.) Mildbr. & Burret and *Urostigma* (Endl..) Miq. have been replaced by *Terega* Raf. and *Spherosuke* Raf. and the "sections" *Rhizocladus* Endl., *Urostigma* Endl. and *Stilpnophyllum* Endl. have been replaced by *Pogonotrophe* (Miq.) Miq., *Cordifoliae* G.Don and *Urostigma* (Endl.) Griseb.. This classification provides several new combinations and typification.

Chaudhary *et al.* (2012) reported 89 species and 26 infraspecific taxa of *Ficus* from India. They have identified 44 species belonging to the section *Cordifoliae* (as "section" *Urostigma*). In which 21 species belonging to "subsection *Urostigma*" and 23 species belonging to subsection *Conosycea*. *Cordifoliae* is a complex section in the subgenus *Spherosuke*. Species identification and its systematic classification is a persistent problem in this section. Some species in *Cordifoliae* are varying greatly in their morphological characters. Traditional identification based on morphological characteristics is not easy in *F. virens* Aiton and *F. amplissima* Sm. which vary greatly in their leaf morphology. The systematic classification of the species within and between *Cordifoliae* species is difficult, as evident in the case of *F. amplissima*, *F. arnottiana* (Miq.) Miq. and *F. elastica* Roxb. ex Hornem. (Rønsted *et al.* 2008b, Chantarasuwan *et al.* 2014).

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Worldwide, several strategies have been followed to resolve the taxonomical status of section *Cordifoliae*. The notable approaches include: morphology and geographic distribution (Dixon 2003, Berg 2004, Chantarasuwan *et al.* 2013, Pederneiras *et al.* 2018), genetic diversity using ISSR markers (Rout & Aparajita 2009), molecular sequences (Weiblen 2000, Rønsted *et al.* 2008b, Li *et al.* 2012a,b, Kusumi *et al.* 2012, Olivar *et al.* 2014, Chantarasuwan *et al.* 2015, Pederneiras *et al.* 2018), leaf and wood anatomical studies (Ogunkunle *et al*. 2008, Ogunkunle *et al.* 2014, Chantarasuwan *et al.* 2014), leaf epidermal studies (Klimko & Truchan 2006) and mutualism between figs and their pollinating wasps studies (Ramirez 1977, Rønsted *et al.* 2008a).

Ficus species are recognized for the presence of cystoliths; intracellular amorphous calcium crystals around cellulose skeleton with silicified stalk. They are distributed in the adaxial (upper) and/or abaxial (lower) layers of the leaf lamina within the large epidermal cells known as lithocysts (Evert 2006). The shape, size and distribution pattern of cystolith varies from species to species (Rao *et al.* 1988). The morphology of cystoliths in *Ficus* have been investigated however, their suitability as a marker for taxonomic classification has not been thoroughly explored. Lithocyst, cystolith, surface of cystolith and stalk shape, along with leaf characters have been suggested to be important species differentiating characters in the thirty-three taxa of *Ficus* (Sharaway 2004). Ummu-Hani & Noraini (2013) studied the structure of cystoliths in fifteen species of *Ficus* and suggested that these features may give an additional diagnostic character for the identification of species. To date, there is no report addressing the significance of cystolith micromorphology in the systematics of *Ficus*.

This study was carried out to evaluate the quantitative and qualitative characters of cystoliths micromorphology, distribution and sculpturing pattern in selected *Ficus* species, with an aim to understand their value as species differentiating markers.

Material and Methods

Specimen collection

A total of nineteen taxa of *Ficus* representing five subgenera and seven sections from West Coast and Western Ghats of India were collected and, the coordinates were recorded using handheld GPS receiver (Garmin MONTANA 650) (Table 1). The species were identified based on their morphological characters and keys (Rao 1986, Naithani *et al.* 1997, Datar & Lakshminarasimhan 2013), the samples were processed for preparation of herbarium as described by Fosberg & Sachet (1965). Voucher specimens with collection number were deposited in the Herbarium at Department of Botany, Goa University.

Anatomy and SEM micromorphology

Lamina part (1 X 1 cm) was cut with a fresh blade from mid leaf portion adjacent to the midvein of freshly collected leaf samples. Free hand sections were taken from these cut portions, stained with safranin 'O', mounted on the slides with a drop of 60% glycerin (Johansen 1940) and were observed under light microscope to see the distribution of cystoliths in the lithocyst cells. For scanning electron microscopic study, fresh and cleaned matured leaves were cut into small pieces, blended with a blender in absolute ethanol for 1–2 minutes and then the homogenate mixture was filtered through muslin cloth. The heavier cystoliths were separated from the lighter leaf fragments by a series of decantations in ethanol as per the protocol developed by Arnott (1980). The extracted cystoliths were preserved in ethanol at -20°C for further use. Extracted cystoliths were observed under light microscope to confirm its purity. Cystoliths were mounted on a slide and allowed to dry in the room temperature for 1 minute. The dried specimens were mounted directly on stubs using double-side adhesive tape and sputter-coated with gold and palladium alloy (80% gold + 20% palladium). Images of the cystoliths with different magnifications were taken by VEGA3 (Tescan) or CARL-ZEISS scanning electron microscope.

Data collection and analysis

The physical characteristics of cystolith such as length, width, sculpturing, shape, stalk length, appendage length and width, holes on cystolith, lithocyst length and width were documented. The terminology used by Lee (2009) for seed sculpture pattern has been adapted for describing the cystolith sculpture pattern in this work. The measurements were made on 30–40 cystoliths per species and the range of cystolith measurements were presented in box plot graphics. Principal component analysis (PCA) was performed based on the qualitative and quantitative data using SPSS statistics v20 software (SPSS Inc.).

TABLE 1. Details of *Ficus* specimens collected from Goa, India.

Sl. No.	Taxa	Taxonomy			Collection numbers
		Subgenus	Section	Subsection	
1	*Ficus pumila* L.	*Synoecia*	*Pogonotrophe*	*Plagiostigma*	AM06
2	*Ficus exasperata* Vahl	*Terega*	*Sycidium*	*NA*	AM05
3	*Ficus tinctoria* G. Forst. subsp. *gibbosa* (Blume) Corner*	*Terega*	*Palaeomorphe*	*NA*	TE-AM22 EP-AM42
4	*Ficus racemosa* L.	*Sycomorus*	*Sycomorus*	*Sycomorus*	AM44
5	*Ficus auriculata* Lour.	*Sycomorus*	*Sycomorus*	*Neomorphe*	AM36
6	*Ficus hispida* L. f.	*Sycomorus*	*Sycocarpus*	*Sycocarpus*	AM46
7	*Ficus callosa* Willd.	*Pharmacosycea*	*Oreosycea*	*Pedunculatae*	AM12
8	*Ficus amplissima* Sm.	*Spherosuke*	*Cordifoliae*	"*Urostigma*"	AM17
9	*Ficus arnottiana* (Miq.) Miq.	*Spherosuke*	*Cordifoliae*	"*Urostigma*"	AM50
10	*Ficus religiosa* L.	*Spherosuke*	*Cordifoliae*	"*Urostigma*"	AM47
11	*Ficus tsjakela* Burm. f.	*Spherosuke*	*Cordifoliae*	"*Urostigma*"	AM32
12	*Ficus virens* Aiton	*Spherosuke*	*Cordifoliae*	"*Urostigma*"	AM35
13	*Ficus benghalensis* L.	*Spherosuke*	*Cordifoliae*	*Conosycea*	AM01
14	*Ficus benjamina* L.	*Spherosuke*	*Cordifoliae*	*Conosycea*	AM04
15	*Ficus costata* Aiton	*Spherosuke*	*Cordifoliae*	*Conosycea*	AM38
16	*Ficus drupacea* Thunb. var. *pubescens* (Roth) Corner	*Spherosuke*	*Cordifoliae*	*Conosycea*	AM10
17	*Ficus elastica* Roxb. ex Hornem.	*Spherosuke*	*Cordifoliae*	*Conosycea*	AM03
18	*Ficus microcarpa* L. f.	*Spherosuke*	*Cordifoliae*	*Conosycea*	AM02
19	*Ficus talbotii* King	*Spherosuke*	*Cordifoliae*	*Conosycea*	AM40

* = Cystoliths are absent in both the surfaces

Ficus tinctoria subsp. *gibbosa* collected from terrestrial (TE) and epiphytic (EP) conditions.

All the collections were made by Abhipsa Mohapatra

Results

The cystolith microscopic characters of all investigated taxa are listed in Table 2. Cystoliths are completely absent in *F. tinctoria* G. Forst. subsp. *gibbosa* (Blume) Corner or present only on adaxial (seven species), only on abaxial (eight species) and both (three species) the epidermal layers. The shape varies from spherical to conical, ovoid, obovoid and oblongoid. The average cystolith length ranges from 44.71 µm (*F. virens*) to 175.30 µm (*F. elastica*) and 31.08 µm (*F. exasperata* Vahl) to 85.88 µm (*F. elastica*) in width. The stalk length ranges from 2.80 µm (*F. pumila* L.) to 81 µm (*F. elastica*). *Ficus elastica* has the highest cystolith length, width and stalk length. SEM micrographs of cystoliths shows the cystolith surface sculpture pattern, and can be broadly classified into four types; namely aculeate, colliculate, verrucate and tuberculate (Figure 1 and 2). The most common type is the aculeate type and it is divided into three subtypes; broad, moderate and reduced aculeate. Aculeate type of sculpturing is found in 13 species (2-broad, 5-moderate and 6-reduced) followed by colliculate (2 species), tuberculate (2 species) and verrucate (1 species). Dimorphic cystoliths are observed in *F. microcarpa* L. f. which vary in their shape and sculpture pattern (Figure 2G). Leaf anatomy images are illustrated in Figure 3 and 4. Species of subgenera *Synoecia*, *Terega, Sycomorus* and *Pharmacosycea* possess uniseriate epidermal layer on the adaxial epidermis while *F. racemosa* L. has a multiseriate epidermal layers. Similarly, species of subgenera *Spherosuke* possess multiseriate epidermal layers on adaxial epidermis except in *F. religiosa* L., *F. tsjakela* Burm. f., *F. virens* and *F. talbotii* King where it is uniseriate layer. *F. microcarpa, F. benjamina* L. and *F. elastica* possess cystoliths on both the epidermal layers. The cystoliths in adaxial layer are comparatively larger than those found in abaxial layer in each of the species where they are found in both the layers. Development of two cystoliths in a lithocyst cell are occasionally observed in the adaxial epidermal layer of *F. elastica* (Figure 4F). *F. exasperata* possess conical shaped cystoliths projecting into the multicellular trichomes thus associating with them. The length ranges considerably within the species as shown in Figure 5. Among the studied species, *F. exasperata*, *F. callosa* Willd. and *F. amplissima* have extreme variation in size, whereas it is minimal in *F. pumila* and *F. arnottiana.*

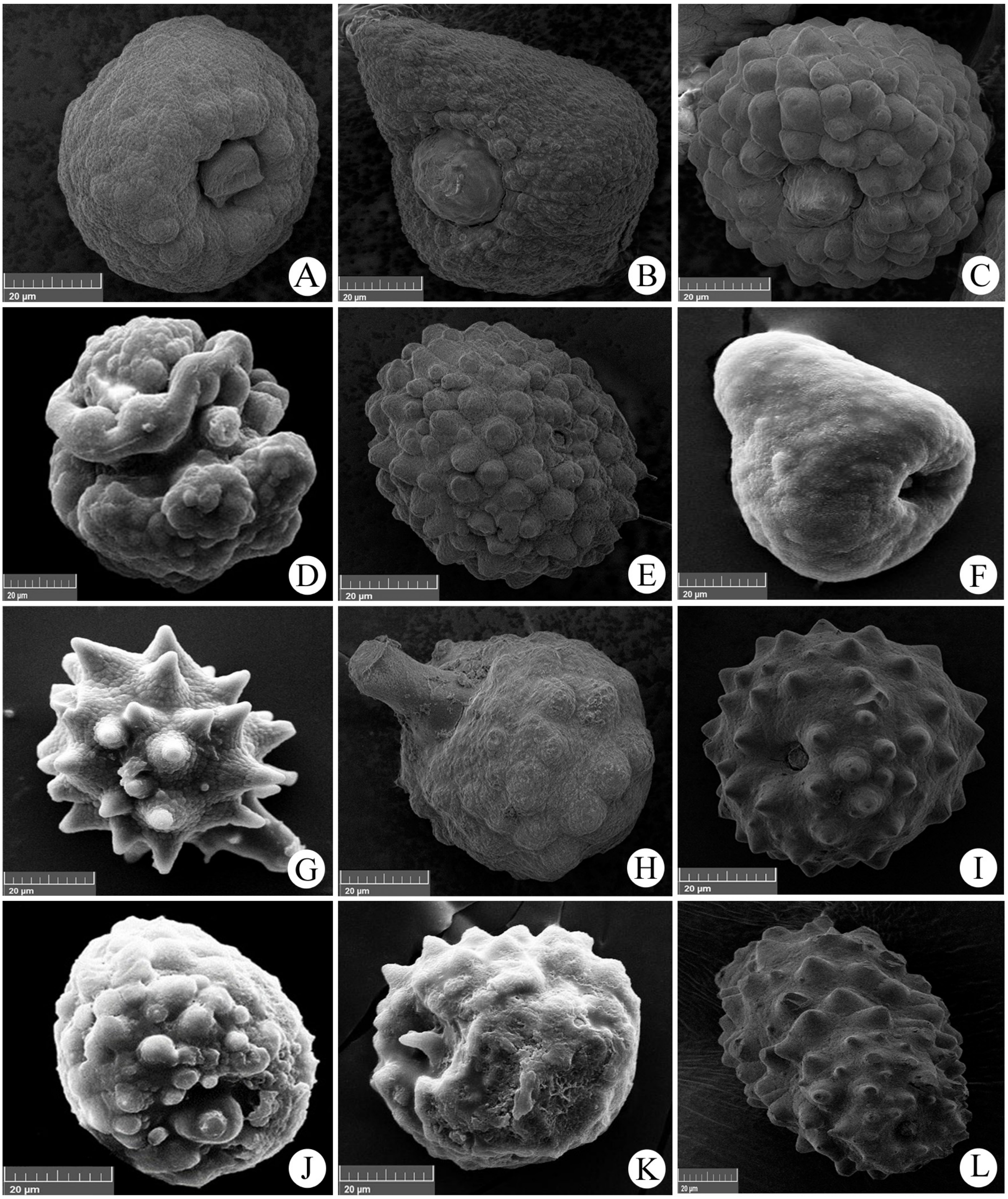

FIGURE 1. Sculpture patterns of cystoliths in *Ficus* species (SEM micrographs): **A.** *F. pumila*; with colliculate pattern (abaxial surface); **B**. *F. exasperata*; with verrucate pattern (adaxial surface); **C.** *F. racemosa*; with tuberculate pattern (abaxial surface); **D.** *F. auriculata*; with moderate aculeate pattern (abaxial surface); **E.** *F. hispida*; with tuberculate pattern (abaxial surface); **F.** *F. callosa*; with colliculate pattern (abaxial surface); **G.** *F. amplissima*; with broad aculeate pattern (adaxial surface); **H.** *F. arnottiana*; with reduced aculeate pattern (adaxial surface); **I.** *F. religiosa*; with moderate aculeate pattern (abaxial surface); **J.** *F. tsjakela*; with reduced aculeate pattern (abaxial surface); **K.** *F. virens*; with reduced aculeate pattern (abaxial surface); **L.** *F. benghalensis*; with moderate aculeate pattern (adaxial surface).

TABLE 2. Quantitative and qualitative details of cystoliths present on the epidermal layer of *Ficus* species from Goa, India. (AD = Cystoliths are seen on adaxial surface, AB = Cystoliths are seen on abaxial surface, * = Cystoliths are absent on both surfaces).

Species investigated	Adaxial Epidermal Layer	Lithocyst Size (µm) L × W	Cystolith Shape	Cystolith Size (µm) L × W	Stalk (µm)	Surface sculpturing	Appendages Size (µm) L × W	Holes on cystolith
Ficus pumila (AB)	Uniseriate	60.00±07.90 x 58.06±06.10	Spherical	52.15±03.32 x 53.10±04.93	02.80±00.56	Colliculate	0.00	Absent
Ficus exasperata (AD)	Uniseriate	82.00±17.88 x 81.00±19.49	Conical	62.45±17.12 x 31.08±05.74	10.49±02.63	Verrucate	0.00	Absent
Ficus tinctoria subsp. *gibbosa**	Multiseriate	0.00	-	0.00	0.00	-	0.00	-
Ficus racemosa (AB)	Multiseriate	92.00±10.36 x 93.00±12.04	Spherical	63.82±10.25 x 78.66±11.30	13.45±03.71	Tuberculate	09.48±00.29 x 06.36±00.31	Present
Ficus auriculata (AB)	Uniseriate	83.21±08.80 x 76.23±09.54	Spherical	63.43±12.67 x 58.68±09.39	27.35±06.30	Moderate Aculeate	10.56±01.74 x 09.90±01.24	Present
Ficus hispida (AB)	Uniseriate	69.80±06.30 x 70.80±04.26	Spherical	63.09±12.07 x 63.74±06.40	12.60±02.30	Tuberculate	09.21±00.54 x 06.13±00.49	Present
Ficus callosa (AB)	Uniseriate	71.66±04.58 x 60.43±12.77	Ovoid	67.82±14.35 x 53.22±06.81	14.01±03.44	Colliculate	0.00	Absent
Ficus amplissima (AD)	Multiseriate	123.43±12.01 x 105.61±16.10	Ovoid	82.75±12.23 x 60.28±09.72	21.79±04.99	Broad Aculeate	13.21±02.08 x 06.56±00.51	Present
Ficus arnottiana (AD)	Multiseriate	79.20±06.53 x 69.00±04.95	Spherical	69.51±02.14 x 63.88±04.53	21.57±03.26	Reduced Aculeate	07.13±01.62 x 06.39±01.05	Present
Ficus religiosa (AB)	Uniseriate	73.80±04.81 x 64.00±09.61	Ovoid	63.43±08.59 x 43.42±01.68	28.80±04.81	Moderate Aculeate	09.88±00.90 x 07.41±00.41	Present
Ficus tsjakela (AB)	Uniseriate	72.58±08.92 x 63.37±07.22	Ovoid	56.21±04.82 x 50.52±04.29	17.16±02.69	Reduced Aculeate	05.44±01.24 x 04.87±1.00	Present
Ficus virens (AB)	Uniseriate	64.91±10.57 x 65.83±10.21	Spherical	44.71±04.05 x 44.32±04.45	09.94±02.60	Reduced Aculeate	04.86±00.86 x 04.46±00.77	Present
Ficus benghalensis (AD)	Multiseriate	110.00±07.90 x 72.00±10.36	Oblongoid	103.00±08.55 x 63.97±02.83	36.90±02.88	Moderate Aculeate	11.48±00.34 x 06.62±00.36	Present
Ficus benjamina (AD)	Multiseriate	108.80±14.04 x 66.80±05.80	Ovoid	106.74±05.09 x 63.93±02.73	20.00±07.90	Reduced Aculeate	05.78±00.39 x 04.13±00.23	Present
Ficus benjamina (AB)	-	76.20±06.38 x 66.60±05.94	Spherical	65.82±03.96 x 58.62±05.95	14.80±03.49	Reduced Aculeate	05.75±00.43 x 05.53±00.38	Present
Ficus costata (AD)	Multiseriate	84.08±07.93 x 61.41±06.55	Oblongoid	71.89±09.55 x 54.52±07.34	21.65±04.84	Moderate Aculeate	13.04±01.70 x 07.27±00.59	Present
Ficus drupacea (AD)	Multiseriate	114.05±14.49 x 76.87±10.62	Ovoid	80.67±10.23 x 53.39±03.78	20.17±06.67	Reduced Aculeate	06.61±01.07 x 05.96±00.92	Present
Ficus elastica (AD)	Multiseriate	372.00±19.23 x 107.00±12.04	Oblongoid	175.30±08.63 x 85.88±04.81	81.00±19.49	Reduced Aculeate	07.36±00.50 x 05.82±00.44	Present
Ficus elastica (AB)	-	102.00±13.50 x 90.00±07.90	Spherical	79.01±04.99 x 75.73±03.49	39.00±05.65	Reduced Aculeate	04.92±00.71 x 04.09±00.24	Present
Ficus microcarpa (AD)	Multiseriate	112.00±10.36 x 66.40±05.59	Ovoid	87.75±08.91 x 58.96±03.88	43.00±12.04	Broad Aculeate	09.96±00.51 x 04.78±00.37	Present
Ficus microcarpa (AB)	-	64.00±12.94 x 63.00±10.36	Spherical	59.58±06.91 x 53.38±02.76	27.40±05.59	Reduced Aculeate	07.79±01.11 x 03.80±00.38	Present
Ficus talbotii (AD)	Uniseriate	97.22±07.99 x 69.59±08.76	Obovoid	50.60±17.21 x 41.70±04.21	15.81±02.88	Moderate Aculeate	10.44±01.65 x 06.92±00.44	Present

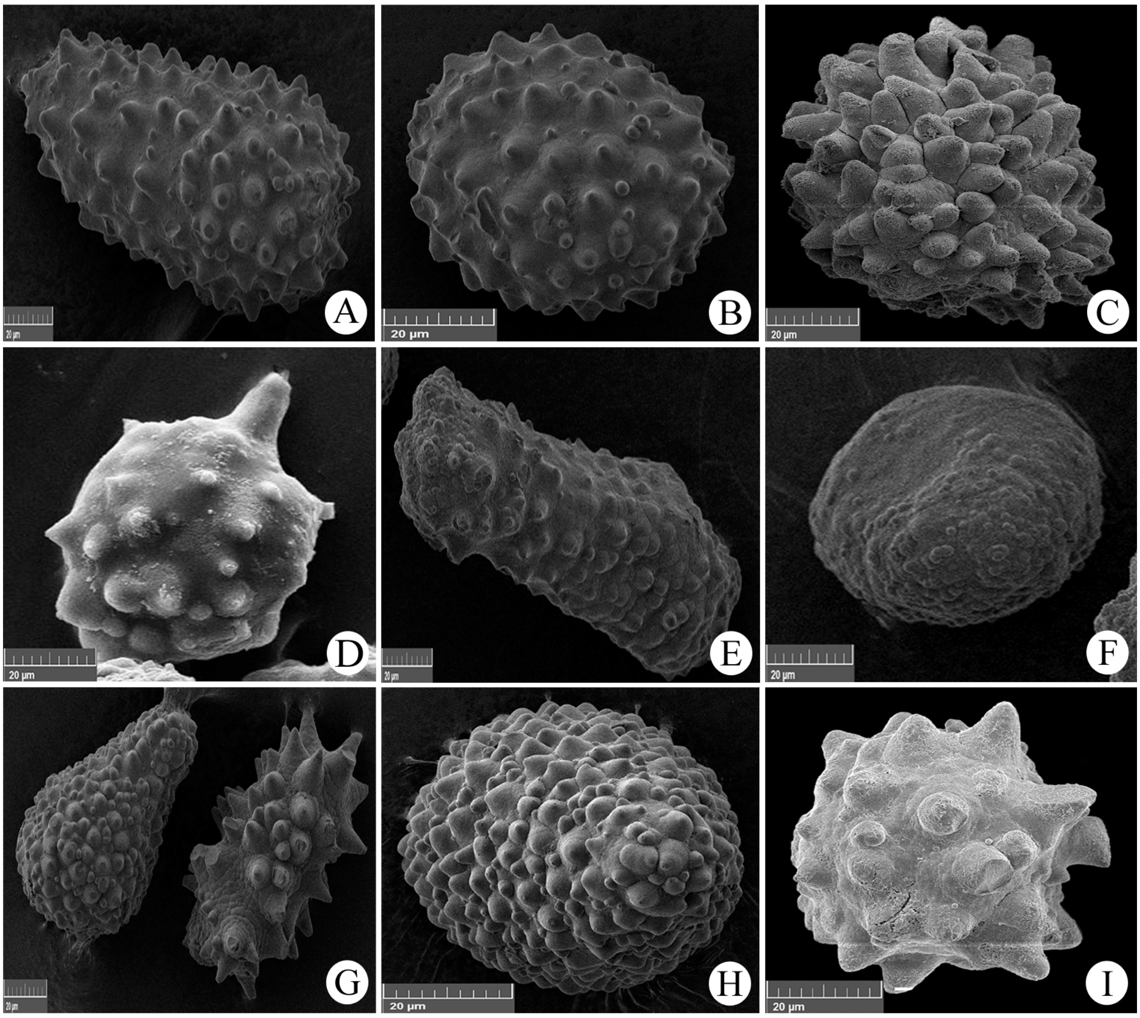

FIGURE 2. Sculpture patterns of cystoliths in *Ficus* species (SEM micrographs): **A.** *F. benjamina*; with reduced aculeate pattern (adaxial layer); **B.** *F. benjamina*; with reduced aculeate pattern (abaxial layer); **C.** *F.* costata; with moderate aculeate pattern (adaxial surface); **D.** *F. drupacea*; with reduced aculeate pattern (adaxial surface); **E.** *F. elastica*; with reduced aculeate pattern (adaxial layer); **F.** *F. elastica*; with reduced aculeate pattern (abaxial layer); **G.** *F. microcarpa*; with broad aculeate pattern (adaxial layer); **H.** *F. microcarpa*; with reduced aculeate pattern (abaxial layer); **I.** *F. talbotii*; with moderate aculeate pattern (adaxial surface).

PCA analysis

In the present study, Principal Component Analysis (PCA) has been performed on cystolith micromorphology and leaf anatomy which allows the species to be clearly segregated into different groups (Figure 6). The species of "subsection *Urostigma*" and section *Sycomorus* form one group each. It is noticed that extreme variation among the species is seen in "subsection *Urostigma*" whereas it is minimal in section *Sycomorus.* The subsection *Conosycea* can be divided into three different groups. The first group of subsections *Conosycea* is the largest where most of the sub-sectional species are grouped together along with *F. amplissima* and *F. arnottiana* of "subsection *Urostigma*". The second group consists of *F. microcarpa* and *F. benjamina* while *F. elastica* forms the third group. Key has been constructed using qualitative and quantitative characters to check the usefulness of identification of species.

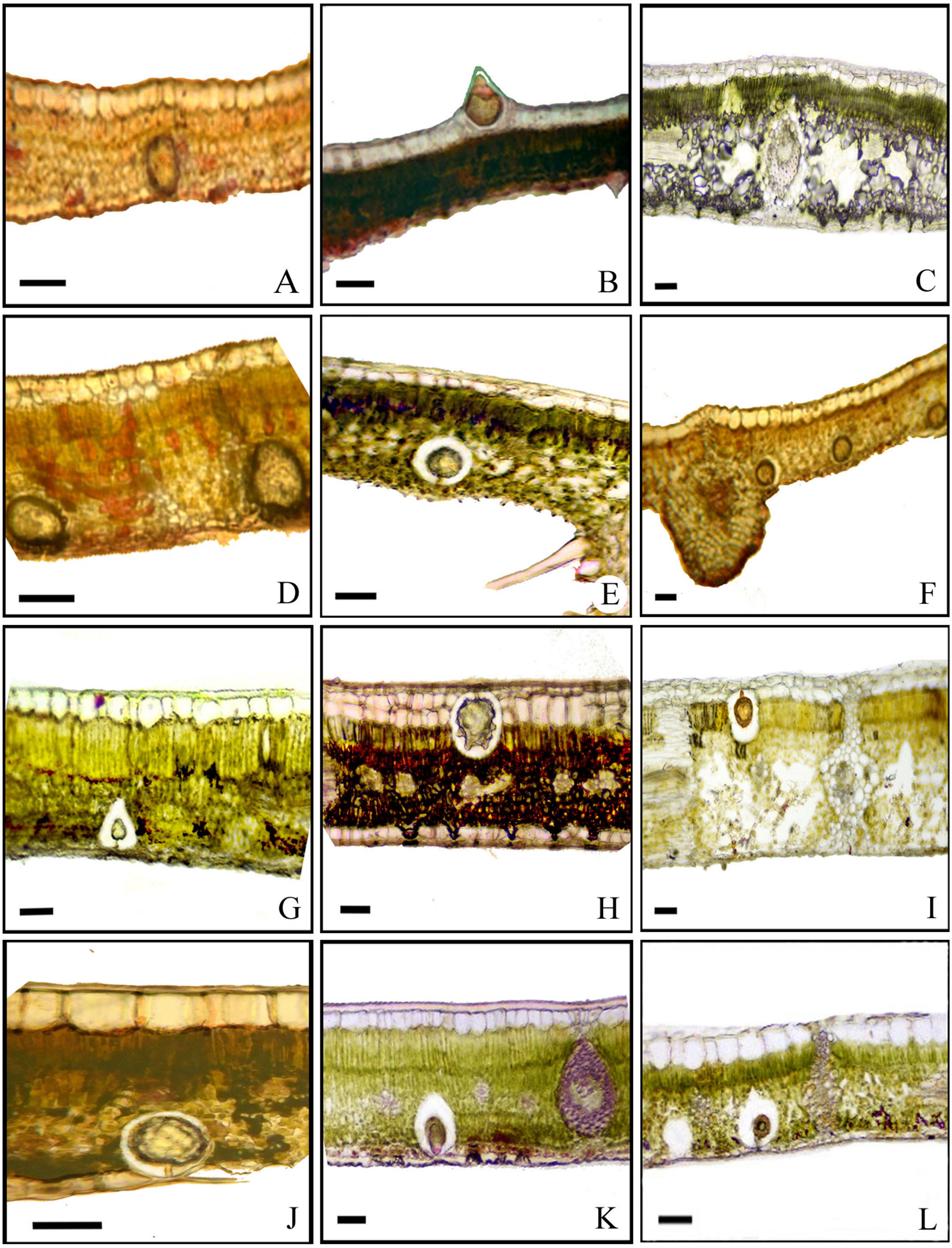

FIGURE 3. Transections of the leaf of *Ficus* species: **A.** *F. pumila*; spherical shaped cystolith (abaxial surface); **B.** *F. exasperata*; conical shaped cystolith develop only in the trichomal lithocyst (adaxial surface); **C.** *F. tinctoria* subsp. *gibbosa*; cystolith are found absent on both the epidermal surfaces; **D.** *F. racemosa*; spherical shaped cystolith (abaxial surface); **E.** *F. auriculata*; spherical shaped cystolith (abaxial surface); **F.** *F. hispida*; spherical shaped cystolith (abaxial surface); **G.** *F. callosa*; ovoid shaped cystolith (abaxial surface); **H.** *F. amplissima*; ovoid shaped cystolith (adaxial surface); **I.** *F. arnottiana*; spherical shaped cystolith (adaxial surface); **J.** *F. religiosa*; ovoid shaped cystolith (abaxial surface); **K.** *F. tsjakela*; ovoid shaped cystolith (abaxial surface); **L.** *F. virens*; spherical shaped cystolith (abaxial surface). Scale bars are 50 µm.

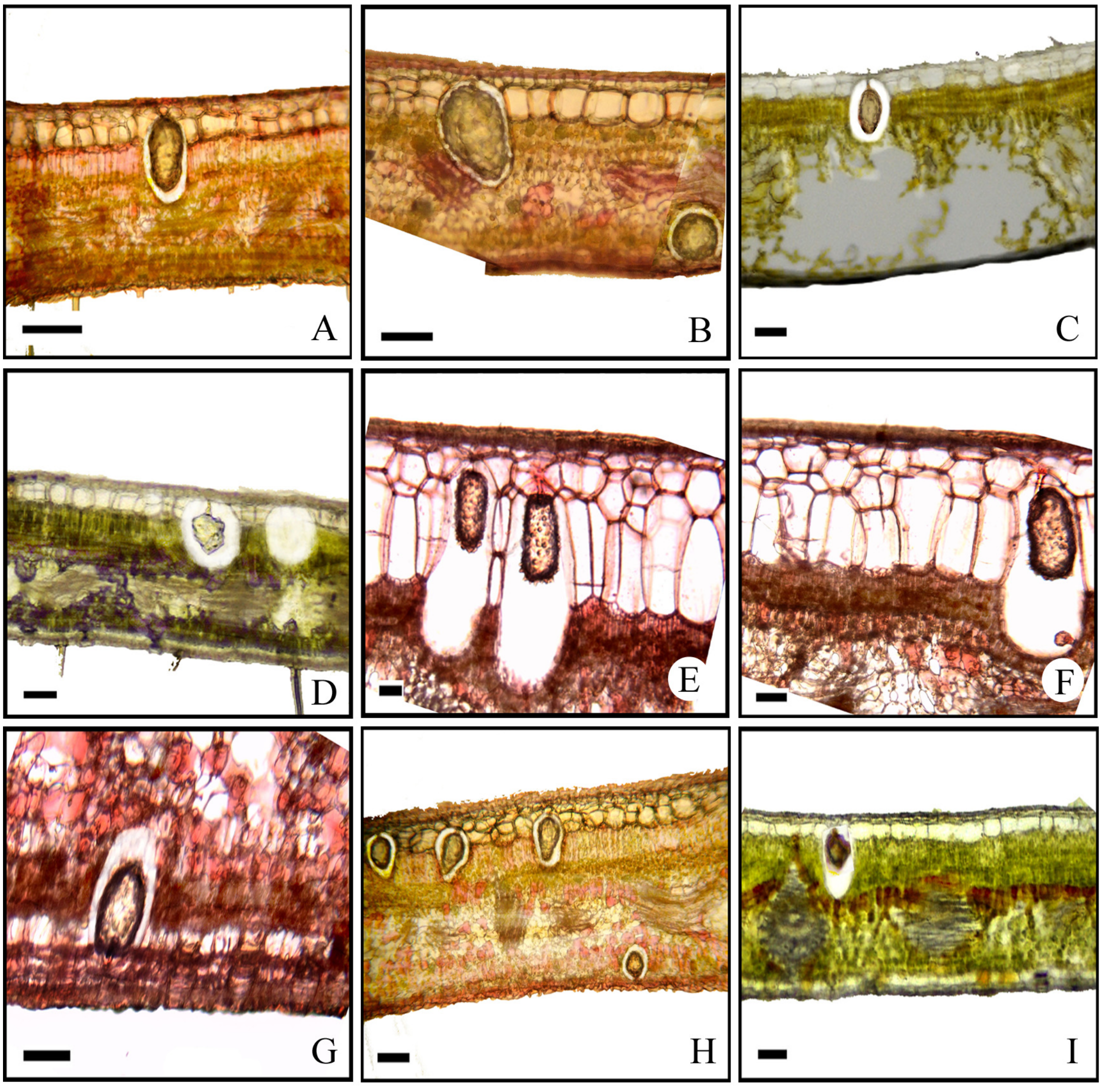

FIGURE 4. Transections of the leaf of *Ficus* species: **A.** *F. benghalensis*; oblongoid shaped cystolith (adaxial surface); **B.** *F. benjamina*; ovoid shaped cystolith (adaxial surface), spherical shaped cystolith (abaxial surface); **C.** *F. costata*; oblongoid shaped cystolith (adaxial surface); **D.** *F. drupacea*; ovoid shaped cystolith (adaxial surface); **E.** *F. elastica*; oblongoid shaped cystolith (adaxial surface); **F.** *F. elastica*; Occurance of two cystoliths in a single lithocyst in adaxial surface; **G.** *F. elastica*; spherical shaped cystolith (abaxial surface); **H.** *F. microcarpa*; ovoid shaped cystolith (adaxial surface), spherical shaped cystolith (abaxial surface); **I.** *F. talbotii*; obovoid shaped cystolith (adaxial surface). Scale bars are 50 µm.

Discussion

The findings of the present study revealed that cystolith shape, size and sculpturing pattern among *Ficus* species have great taxonomic significance. An earlier study (Sharaway 2004) has suggested that the presence of lithocyst, cystolith surface and stalk shape, along with leaf characters are the important features that can be used to segregate the *Ficus* species. Our observations also clearly show that the morphology, distribution and sculpturing pattern of cystolith are valuable. Among the 19 *Ficus* species studied, 12 belong to section *Cordifoliae,* 2 belong to section *Sycomorus* and the remaining 5 belong to sections *Pogonotrophe, Sycidium, Palaeomorphe, Sycocarpus* and *Oreosycea* (Table 1). The results obtained from the present study are discussed below in a comparative context.

Section- *Cordifoliae*

Cordifoliae is one of the largest sections of Indian *Ficus* and represents the most taxonomically diverse group of plants. Berg (2004) divided this section into two subsections: "*Urostigma*" and *Conosycea*, based on morphological characters. A total of twelve species of this section have been studied, of which five belongs to "subsection *Urostigma*" and seven to subsection *Conosycea.* Among the twelve species, six species have cystoliths on the adaxial layer, three on abaxial and three have cystoliths on both the layers.

In section *Cordifoliae*, among different cystolith characters studied, the type of sculpturing has been considered to be taxonomically the most significant one. The aculeate type is the most common type among the studied species, but further division of this pattern into three subtypes, i.e. broad, moderate and reduced aculeate along with the variations in length and width of cystolith appendages and the mode of stalk fixation provide further support for cystolith morphology to distinguish the species at sub-sectional level. For instance, *F. arnottiana* is morphologically quite similar to *F. religiosa,* both showing aculeate type, but can be distinguishable by the mode of stalk fixation and sub patterns of aculeate type, i.e. *F. arnottiana* with reduced aculeate and *F. religiosa* with moderate aculeate (Figure 1H, I). In addition, leaf anatomical characters such as number of epidermal layers, distribution of cystoliths either on adaxial or abaxial surface and their shape (spherical or ovoid) also support these distinctions (Figure 3I, J). The detailed description for each species of this section and the comparisons are discussed below subsection wise.

(i) "Subsection *Urostigma*"

Among the five species studied under "subsection *Urostigma*", three species, i.e. *F. religiosa*, *F. tsjakela* and *F. virens* have cystoliths on the abaxial layer, whereas they are on the adaxial layer in *F. amplissima* and *F. arnottiana* (Figure 3H-L). These two species also possess multiseriate epidermal layers which are also seen in six out of seven species studied under subsection *Conosycea*. (Figure 3H, I). The size of their cystoliths is also in the range of species under subsection *Conosycea* rather than the rest of the species under "subsection *Urostigma*". This is well supported by PCA (Figure 6) wherein *F. amplissima* and *F. arnottiana* appear among the species of subsection *Conosycea.* Chantarasuwan *et al.* (2014) observed enlarged lithocysts on both the epidermal surfaces of *F. amplissima* and *F. arnottiana*, which is considered as typical for subsection *Conosycea* whereas Rao *et al*. (1988) recorded this character only in *F. amplissima*. They recorded that in *F. arnottiana*, cystoliths are present only on the adaxial layer, as observed in the present study. Observation of lithocyst and cystolith only on abaxial layer in *F. religiosa* in the present study supports the earlier findings of Rao *et al*. (1988) and Chantarasuwan *et al.* (2014). However, Klimko & Truchan (2006) observed their presence on both the surfaces and Pierantoni *et al.* (2018) recorded their absence on both the surfaces. Ummu-Hani & Noraini (2013) reported the cigar shaped cystoliths and the absence of stalk of cystoliths in *F. religiosa,* but in the present study, ovoid shaped cystoliths and prominent stalk measuring up to 29 μm (Figure 3J) have been observed. Our observations on cystolith of *F. tsjakela* with respect to its distribution are similar to those reported by Chantarasuwan *et al.* (2014) (Figure 3K). Morphologically *F. virens* is a highly variable species as reported by Chantarasuwan *et al.* (2014) and Sudhakar *et al*. (2017). The distribution of lithocysts and cystoliths in the epidermal surface are also found to be variable in this species. Chantarasuwan *et al.* (2014) reported their distribution on both epidermal layers from the specimens collected from Thailand and Rao *et al*. (1988) observed them only on the adaxial epidermal layer from the specimens collected from India. In the present study, cystoliths are observed only on the abaxial layer (Figure 3L). Whether these variations are due to ecological adaptions or not could not be ascertained at this stage. However, cystolith micromorphological characters can be used unambiguously as highly accurate tools for species recognition in case of *F. virens*.

(ii) Subsection *Conosycea*

The seven species studied in subsection *Conosycea* possess cystoliths either on adaxial layer or in both epidermal layers (Figure 4A-I). Three species, i.e. *F. benjamina*, *F. elastica* and *F. microcarpa* possess cystolith on both the epidermal surfaces (Figure 4B, E, F, G, H), which was also observed by Rao *et al*. (1988) and Klimko & Truchan (2006). Ummu-Hani & Noraini (2013) observed a similar distribution of cystoliths in *F. benjamina* and *F*. *microcarpa* and Pierantoni *et al.* (2018) in *F. elastica* and *F*. *microcarpa.* Taxonomically, *F. elastica* differs from all other species of subsection *Conosycea* by its leaf and stem morphological characters. The enlarged elliptic leaf lamina with connate stipules and circular aperture of the ostiole are some of them (Berg 2004). This is the only *Ficus* species from which the rubber is prepared from the milky latex obtained from the stem and aerial roots. Anatomically this species has large multilayered epidermis on the adaxial surface and enlarged lithocysts with the occasional appearance of 2–3 cystoliths, longer cystolith and stalk size (Figure 4F). These characteristics segregate this species from other members of subsection *Conosycea* which is also reflected in PCA result. Among the species studied, Rao *et al*. (1988) observed that the length of lithocyst in *F. elastica* on the adaxial layer is comparatively greater (116–174 µm) than the other species in the subsection. This is well supported in the present study with a length of almost double (372±19.23 µm) than what they have observed. Highest cystolith length in *F. elastica* (140 µm) has been observed by Pierantoni *et al.* (2018) which is also noted in the present study (175±08.63 µm). Though cystoliths are observed on both the epidermal layers in *F. microcarpa*, dimorphic cystoliths with variation in shapes and sculpture patterns have been observed only on the adaxial epidermal layer (Figure 2G). However, only one morphological type has been reported in earlier studies (Gal *et al.* 2012, Pierantoni *et al.* 2018). In addition, the species shows similarity with its closely allied species *F. benjamina* (Figures 4N, T) in many anatomical characters such as number of epidermal layers, distribution of cystoliths on both surfaces and their shape (ovoid and spherical) (Table 2) and cluster together as a separate group (Figures 6). Ummu-Hani & Noraini (2013) observed oblongoid shape cystoliths in these species which is not observed in the present study. Observations of Bercu (2016), i.e. distribution of cystoliths on both the epidermal layers in *F. benjamina*, is confirmed in the present study. However, oblongoid and spherical shaped cystoliths observed by her on adaxial and abaxial layers are different from present findings wherein they are ovoid and spherical for the adaxial and abaxial layers respectively.

Further, in remaining four species, i.e. *F. benghalensis* L., *F. costata* Aiton, *F. drupacea* Thunb. var. *pubescens* (Roth) Corner and *F. talbotii*, the distribution of cystolith are found to be on the adaxial layer (Figure 4A, C, D, I). This is in agreement with the observations of Rao *et al*. (1988) and Ummu-Hani & Noraini (2013) on *F. benghalensis* though Klimko & Truchan (2006) reported it in both the surfaces. In case of *F. drupacea*, Rao *et al*. (1988) observed it in both the surfaces. In *F. drupacea* and *F. costata,* cystolith lengths are extremely variable, while less variation was observed in *F. talbotii* (Figure 5).

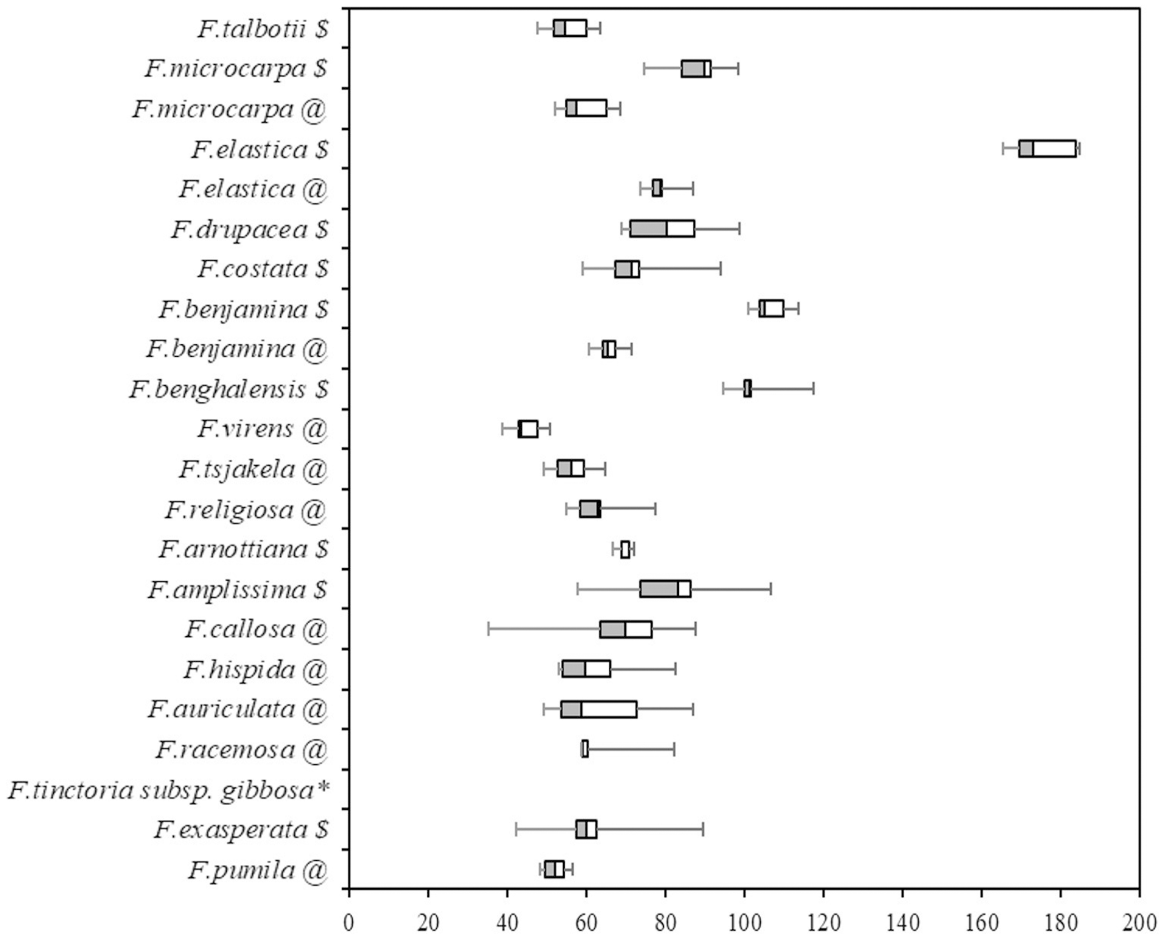

FIGURE 5. Cystolith length (µm) observed in the studied species of *Ficus* ($ = Cystoliths are seen in adaxial surface, @ = Cystoliths are seen in abaxial surface, * = Cystoliths are absent in both surfaces).

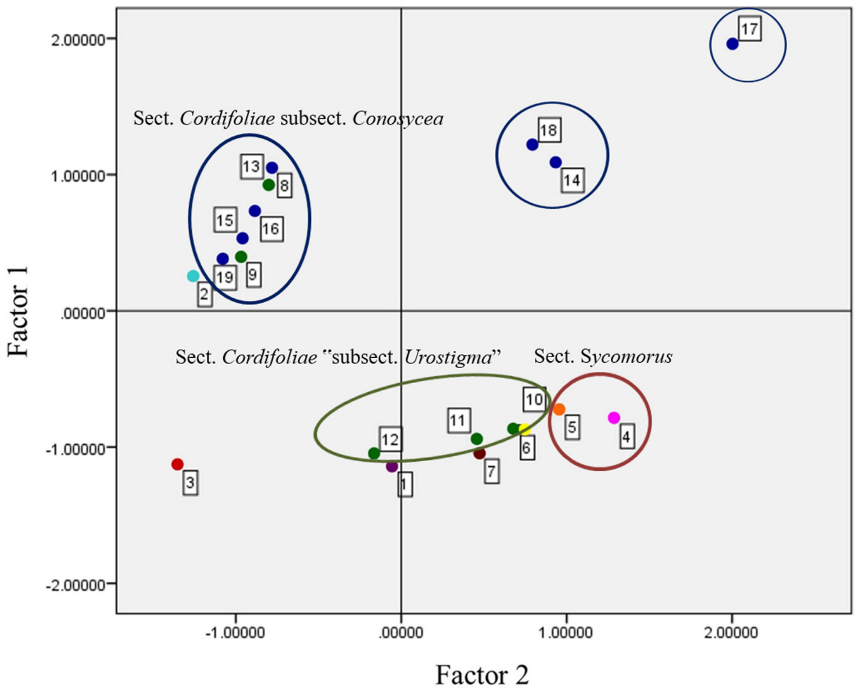

FIGURE 6. Principal component analysis based on *Ficus* cystolith micromorphology. Data numbers represent the species 1–19 (1. *F. pumila*; 2. *F. exasperata*; 3. *F. tinctoria* subsp. *gibbosa*; 4. *F. racemosa*; 5. *F. auriculata*; 6. *F. hispida*; 7. *F. callosa*; 8. *F. amplissima*; 9. *F. arnottiana*; 10. *F. religiosa*; 11. *F. tsjakela*; 12. *F. virens*; 13. *F. benghalensis*; 14. *F. benjamina*; 15. *F. costata*; 16. *F. drupacea*; 17. *F. elastica*; 18. *F. microcarpa*; 19. *F. talbotii*). ([Colour Dots represent the subsections. i.e. Blue-*Conosycea*, Green-"*Urostigma*", Purple-*Plagiostigma*, Maroon-*Pedunculatae*, Yellow-*Sycocarpus*, Orange-*Neomorphe*, Pink-*Sycomorus*, Sky blue & Red- *NA*], [Colour Circles represent the subsections and section i.e. Blue-*Conosycea*, Green-*"Urostigma"*, Brown-*Sycomorus*]).

In general, the cystoliths on the adaxial layer are comparatively larger than those found on the abaxial layer in each of the species where they are found in both the layers. In species such as *F. benjamina*, *F. elastica* and *F. microcarpa*, the cystoliths are found on both surfaces and it is observed that cystoliths on the adaxial surface are oblongoid to ovoid as compared to spherical ones on the abaxial surface in each of these species. This may be due to the periclinal and anticlinal divisions of the epidermis resulting in the formation of lithocyst cells. During asymmetric division of a protodermal cell, the expansion of lithocyst cell in adaxial epidermis is comparatively larger than in abaxial epidermis. Thus, the development of the cystolith body also elongates and increases in diameter together with that of the lithocyst. Hence, larger the lithocyst cells, longer the cystolith length and vice versa (Evert 2006). In addition, several layers of large epidermal cells supported by palisade parenchyma available on the adaxial part as compared to the abaxial side might be supporting larger cystoliths in the former.

Sections- *Pogonotrophe* and *Oreosycea*

In *F. pumila* (section- *Pogonotrophe*) and *F. callosa* (section-*Oreosycea*), cystoliths are present on the abaxial side and sculpture pattern is colliculate. The latter is not observed in other sections studied. Among them they differ in their shape and size, spherical in the former and ovoid in the latter. The presence of lithocyst with cystolith on the abaxial layer and spherical shape in *F. pumila* as observed in the present study are also reported in *F. pumila* var. *awkeotsang* by Kuo-Huang *et al.* (2002). However, the presence of lithocysts with cystoliths in both the surfaces as observed by Klimko & Truchan (2006) in *F. pumila* could not be confirmed (Figure 3A).

Section- *Sycidium*

Pierantoni *et al.* (2018) reported the deposition of the cystolith on both the layers and near the veins of the leaves of *F. exasperata*. They observed a unique mineral deposition of silica in the cone shaped trichomes along with cystoliths on the adaxial layer and all around above the veins of the leaves and named it as silicified trichomes or mineralized spikes. Silica protrudes into the spike completely providing scabrid texture/ rough surface to the leaf surface, probably acting against herbivory. In the present study cystoliths are observed only on the adaxial layer. They are conical shaped cystoliths with verrucate sculpture pattern projecting into the multicellular trichomes (= silicified trichomes).

Among the studied species, this type of peculiar character has been observed only in *F. exasperata* which is also reported in the abaxial leaf epidermis of *F. pygmaea* by Greuning *et al.* (1984) which differentiates it from other species and is placed in the subgenus *Sycidium* (Figure 3B) as that of *F. exasperata*. Thus, it acts as a distinguishing character for section *Sycidium*.

Section- *Palaeomorphe*

Among the studied species, *F. tinctoria* subsp. *gibbosa* (section-*Palaeomorphe*) is the only species, which is devoid of cystoliths. But the presence of bean shaped cystoliths on both the surfaces are recorded by Ummu-Hani & Noraini (2013). This taxon in the study area exists both as an epiphyte and terrestrial one. Several leaf samples from both conditions have been studied to confirm the presence of cystoliths (Figure 3C). However, evidence of their presence could not be obtained.

Sections- *Sycomorus* and *Sycocarpus*

In *F. racemosa*, *F. auriculata* Lour. (section-*Sycomorus*) and *F. hispida* L. f. (section-*Sycocarpus*) cystoliths are present only on the abaxial side and spherical shaped (Figure 3D-F). This is in agreement with the observations of Ummu-Hani & Noraini (2013) on *F. hispida*. The sculpture pattern in *F. racemosa* and *F. hispida* is tuberculate whereas moderate aculeate in *F. auriculata*.

PCA

PCA derived on the basis of cystolith micromorphological and leaf anatomical characters, segregates the species into five distinct groups. The species of subsection *Conosycea* (Berg 2004) are not forming a distinct group in PCA but segregated into three clusters. Majority of species from subsection *Conosycea* formed a large cluster along with *F. amplissima* and *F. arnottiana* of "subsection *Urostigma*". The second group consists of *F. microcarpa* and *F. benjamina* while *F. elastica* alone forms the third group which is far away from these two groups. The species of "subsection *Urostigma*" form another group that shows extreme variation between the species. Section *Sycomorus* form another group and rest of the species are close to "subsection *Urostigma*".

F. arnottiana and *F. amplissima* are formerly included in the "subsection *Urostigma*", based on morphological characters (Chaudhary *et al.* 2012). Chantarasuwan *et al.* (2014) studied leaf anatomical characters of the species in "subsection *Urostigma*" and placed *F. arnottiana* and *F. amplissima* in subsection *Conosycea*. However, in a later study based on morphological, leaf anatomical and molecular evidences (ITS, ETS, G3pdh, and ncpGS), Chantarasuwan *et al.* (2015) placed *F. arnottiana* and *F. amplissima* in subsections "*Urostigma*" and *Conosycea* respectively. But our study shows that these two species can be placed in subsection *Conosycea* which is in agreement with Chantarasuwan *et al.* (2014) and Chantarasuwan *et al.* (2015) only for *F. amplissima.*

Berg (2004) recognized monotypic subsection *Stilpnophyllum* of section *Stilpnophyllum* with *F. elastica* as the only species. Though subsequently, it has been treated as part of section *Malvanthera* (presently section *Urostigma* (Endl.) Griseb.). However, based on molecular study (ITS, ETS and G3pdh), Rønsted *et al.* (2008b) shifted *F. elastica* under section *Cordifoliae,* (as "section *Urostigma*") and subsection *Conosycea*, which is not congruent with our results. They observed its closeness with *F. benjamina* and *F. binnendijkii*. The present study shows it is a distinct species warranting a separate group. However, further studies needed to confirm its taxonomic affinity.

Based on the cystolith micromorphology, the following key has been constructed for the identification of the species.

Key based on cystoliths for the identification of *Ficus* species

1. Cystoliths absent .... *F. tinctoria* subsp. *gibbosa*
1. Cystoliths present 2
2. The cystoliths present on both the epidermal layers 3
2. The cystoliths present on only one epidermal layer 5

3. Shape of cystolith on adaxial layer oblongoid with the presence of [2–3 cystoliths in most lithocyst cells] .... *F. elastica*
3. Shape of cystolith on adaxial layer ovoid with the presence of only [one cystolith in the lithocyst cells].......................................4
4. Length of adaxial cystolith < 100 µm with dimorphic cystoliths.. *F. microcarpa*
4. Length of adaxial cystolith > 100 µm with monomorphic cystoliths..*F. benjamina*
5. Cystoliths present only on adaxial layer 6
5. Cystoliths present only on abaxial layer 12
6. Appendages on cystoliths—absent; surface with verrucate sculpturing pattern; presence of silicified trichomes........ *F. exasperata*
6. Appendages on cystoliths—present; surface with aculeate sculpturing pattern; absence of silicified trichomes.............................7
7. Appendages < 8 µm in length..8
7. Appendages > 10 µm in length..9
8. Cystolith spherical in shape; < 75 µm in length...*F. arnottiana*
8. Cystolith ovoid in shape; > 75 µm in length ....*F. drupacea*
9. Stalk < 20 µm in length; epidermal layer uniseriate .... *F. talbotii*
9. Stalk > 20 µm in length; epidermal layer multiseriate 10
10. Shape of cystolith ovoid; surface broad aculeate ....*F. amplissima*
10. Shape of cystolith oblongoid; surface moderate aculeate 11
11. Length of cystolith > 90 µm ....*F. benghalensis*
11. Length of cystolith < 80 µm .... *F. costata*
12. Appendages on cystoliths – absent..13
12. Appendages on cystoliths – present 14
13. Surface colliculate; cystoliths spherical ....*F. pumila*
13. Surface colliculate; cystoliths ovoid... *F. callosa*
14. Surface tuberculate 15
14. Surface aculeate..16
15. Length of lithocyst > 80 µm; epidermis multiseriate .... *F. racemosa*
15. Length of lithocyst < 80 µm; epidermis uniseriate.. *F. hispida*
16. Appendages > 10 µm in length.. *F. auriculata*
16. Appendages < 10 µm in length...17
17. Length of cystolith < 50 µm ....*F. virens*
17. Length of cystolith > 50 µm 18
18. Stalk > 22 µm in length; appendages c.9 µm long ....*F. religiosa*
18. Stalk < 20 µm in length; appendages c.6 µm long .... *F. tsjakela*

Conclusion

Cystolith micromorphological studies from the west coast and western ghats with representative samples from each sections and subsections shows that cystolith shape, size and sculpture pattern are valuable micromorphological characters. The complete absence of cystoliths, or the presence of them in one (either adaxial or abaxial) or in both epidermises is useful in broad identification of the groups or species. In most of the cases, sculpture pattern of cystoliths are useful for species identification. However, further studies at global level need to be required to establish the wider application.

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