

Chapter 3
MATERIALS
&
METHODS

3. Materials & Methods

Materials

Materials were collected from different parts of India. Proper identification of specimens were carried out with the help of relevant literatures (Masters, 1874; Malick, 1993) and even matched with earlier collections deposited at CUH and CAL. Portions of petioles were preserved in FAA [5ml Formaldehyde (40%) : 5ml Glacial Acetic Acid : 90ml Alcohol (70%)] for further detailed anatomical study.

The study deals with 61 species out of 68 species recorded from India. The rest seven species could not be collected because of rarity of their occurrence as well as few of them are known only from type collections.

Dried Herbarium specimens were prepared through conventional methods (Jain & Rao, 1977; Lakshminarasimhan, 2012) and a total of 61 specimens (including duplicates) under 19 genera are deposited at Calcutta University Herbarium (CUH).

Table 2. List of studied species with respective voucher numbers deposited at CUH

Sl. No.	Name of the species	Voucher Nos.
1	<i>Abroma augusta</i> (L.) L. f.	Mitra & Maity 20003; Mitra & Maity 20025; Majumder s.n.
2	<i>Byttneria andamanensis</i> Kurz	Jagdish Ram 139; Mitra s.n.
3	<i>Byttneria aspera</i> Collebr. ex Wall.	Krishna 10012; Das Das 50; Mitra s.n.; Maity s.n.
4	<i>Byttneria herbacea</i> Roxb.	Mitra 20027; Maity 20107; Mitra s.n.
5	<i>Eriolaena candollei</i> Wall.	Mitra 20030; Mitra s.n.; Maity 20112
6	<i>Eriolaena hookeriana</i> Wight & Arn.	Mitra 20053; Mitra 20055; Maity 20056
7	<i>Eriolaena lushingtonii</i> Dunn	Mitra s.n.; Maity 20113; Maity s.n.
8	<i>Eriolaena quinquelocularis</i> (Wight & Arn.) Wight	Mitra 20033; Das Das 58; Mitra s.n.
9	<i>Eriolaena spectabilis</i> Planch. ex Mast.	Das Das 14; Mitra s.n.; Maity s.n.
10	<i>Eriolaena wallichii</i> DC.	Das Das 34; Mitra s.n.; Maity 20115
11	<i>Firmiana colorata</i> (Roxb.) R. Br.	Maiti 20049; Maiti 20050; Biswas 20052; Biswas 20054
12	<i>Firmiana fulgens</i> (Wall. ex Mast.) K. Schum.	Das Das 54; Mitra s.n.; Das Das 19

Sl. No.	Name of the species	Voucher Nos.
13	<i>Guazuma ulmifolia</i> Lam.	<i>Maity s.n.</i> ; <i>Mitra & Maity 20043</i> ; <i>Sen s.n.</i>
14	<i>Helicteres elongata</i> Wall. ex Bojer	<i>Mitra 20035</i> ; <i>Mitra s.n.</i>
15	<i>Helicteres hirsuta</i> Lour.	<i>Maity 20051</i> ; <i>Mitra s.n.</i> ; <i>Maiti s.n.</i>
16	<i>Helicteres isora</i> L.	<i>Mitra & Das Das 20007</i> ; <i>Mitra & Maity 20001</i> ; <i>Rao s.n.</i>
17	<i>Heritiera dubia</i> Wall. ex Kurz	<i>Mitra 20058</i> ; <i>Mitra s.n.</i> ; <i>Maity s.n.</i>
18	<i>Heritiera fomes</i> Buch. – Ham.	<i>Maiti & Maity s.n.</i> ; <i>Mitra & Maity 20019</i>
19	<i>Heritiera littoralis</i> Aiton	<i>Mitra & Das Das 20008</i> ; <i>Jagadish Ram s.n.</i>
20	<i>Heritiera macrophylla</i> Wall. ex Kurz	<i>Maity 20049</i> ; <i>Mitra 20050</i>
21	<i>Heritiera papilio</i> Bedd.	<i>Mitra 20060</i> ; <i>Maity s.n.</i> ; <i>Mitra 20062</i>
22	<i>Kleinhovia hospita</i> L.	<i>Mitra 20002</i> ; <i>Mitra & Maity 20042</i>
23	<i>Leptonychia caudata</i> Burret	<i>Mitra s.n.</i> ; <i>Das Das 144</i> ; <i>Das Das 178</i>
24	<i>Melhania denhamii</i> R.Br.	<i>Mitra s.n.</i> ; <i>Maiti s.n.</i> ; <i>Das Das 41</i> ; <i>Das Das 44</i>
25	<i>Melhania futteyporensis</i> Munro ex Mast.	<i>Maity s.n.</i> ; <i>Mitra s.n.</i> ; <i>Maity 20116</i>
26	<i>Melhania hamiltoniana</i> Wall.	<i>Mitra 20012</i> ; <i>Maity s.n.</i> ; <i>Maiti s.n.</i>
27	<i>Melhania incana</i> B. Heyne ex Wall.	<i>Das Das 45</i> ; <i>Mitra s.n.</i> ; <i>Maity s.n.</i>
28	<i>Melhania magnifolia</i> Blatt. & Hallb.	<i>Das Das 85</i> ; <i>Mitra s.n.</i> ; <i>Maity s.n.</i>
29	<i>Melochia corchorifolia</i> L.	<i>Maity 20058</i> ; <i>Maity 20059</i> ; <i>Mitra 20061</i>
30	<i>Melochia nodiflora</i> Sw.	<i>Maity & Das Das 20015</i> ; <i>Mitra s.n.</i> ; <i>Maity s.n.</i>
31	<i>Melochia umbellata</i> (Houtt.) Stapf	<i>Mitra 20038</i> ; <i>Das Das 23</i> ; <i>Maity s.n.</i>
32	<i>Pentapetes phoenicea</i> L.	<i>Mitra & Maity 20004</i> ; <i>Mitra & Maity 20044</i> ; <i>Campbell s.n.</i>
33	<i>Pterocymbium tinctorium</i> Merr.	<i>Das Das 20095</i> ; <i>Mitra 20099</i> ; <i>Mitra s.n.</i>
34	<i>Pterospermum acerifolium</i> Willd.	<i>Mitra 20021</i> ; <i>Mitra & Maity 20045</i> ; <i>Campbell s.n.</i>
35	<i>Pterospermum aceroides</i> Wall.	<i>Maity & Saha 20122</i> ; <i>Mitra s.n.</i> ; <i>Das Das 66</i>
36	<i>Pterospermum diversifolium</i> Blume	<i>Mitra 20097</i> ; <i>Maity 20156</i> ; <i>Mitra s.n.</i>
37	<i>Pterospermum lanceifolium</i> Roxb.	<i>Mitra & Das Das 20076</i> ; <i>Das Das 77</i> ; <i>Das Das 78</i> ; <i>Mitra s.n.</i>
38	<i>Pterospermum obtusifolium</i> Wight ex, Mast.	<i>Mitra 20078</i> ; <i>Das Das 92</i> ; <i>Maity s.n.</i>
39	<i>Pterospermum reticulatum</i> Wight & Arn.	<i>Mitra 20073</i> ; <i>Maity 20079</i> ; <i>Mitra & Maity 20091</i>

Sl. No.	Name of the species	Voucher Nos.
40	<i>Pterospermum rubiginosum</i> B. Heyne ex Wall.	Maity 20054; Mitra s.n.; Mitra 20057
41	<i>Pterospermum semisagittatum</i> Buch.–Ham. ex Roxb.	Das Das & Maity s.n.; Mitra & Maity s.n.; Mitra & Das Das 20048
42	<i>Pterospermum suberifolium</i> (L.) Willd.	Mitra 20029; Maity 20057; Maity & Mitra s.n.; Mitra 20046
43	<i>Pterospermum xylocarpum</i> (Gaertn.) Santapau & Wagh	Maity & Saha 20022; Mitra s.n.; Maity s.n.
44	<i>Pterygota alata</i> (Roxb.) R. Br.	Mitra & Das Das 20023; Das Das & Maity s.n.; Mitra 20046
45	<i>Reevesia pubescens</i> Mast.	Mitra 20054; Mitra s.n.; Maity s.n.
46	<i>Sterculia balanghas</i> L.	Mitra and Das Das 20051; Mitra 20063; Mitra 20094
47	<i>Sterculia foetida</i> L.	Mitra 20039; Mitra & Maity 20024; Maity s.n.
48	<i>Sterculia guttata</i> Roxb.	Maity & Saha s.n.; Mitra & Maity 20005; Mitra s.n.
49	<i>Sterculia hamiltonii</i> (Kuntze) Adelb.	Mitra 20086; Mitra 20092; Maity 20098
50	<i>Sterculia kayae</i> P.E. Berry	Mitra s.n.; Maity 20171; Mitra and Maity 20089
51	<i>Sterculia kingii</i> Prain	Mitra 20088; Mitra 20090; Maity 20100
52	<i>Sterculia lanceifolia</i> Roxb.	Mitra 20016; Das Das & Mitra s.n.; Mitra 20047
53	<i>Sterculia macrophylla</i> Vent.	Maity 20067; Mitra and Maity 20077; Mitra 20083
54	<i>Sterculia urens</i> Roxb.	Das Das & Mitra 20006; Mitra 20034; Mitra 20046
55	<i>Sterculia versicolor</i> Wall.	Maity 20061; Mitra and Maity 20070; Mitra 20082
56	<i>Sterculia villosa</i> Roxb.	Maity 20147; Mitra and Maity s.n.; Mitra s.n.
57	<i>Waltheria indica</i> L.	Mitra & Das Das 20009; Mitra & Maity 20017; Maiti 45
CULTIVATED SPECIES		
58	<i>Dombeya burgessiae</i> Gerrard ex Harv.	Mitra & Maity 20010; Mitra & Maity s.n.; Iyengar s.n.
59	<i>Dombeya spectabilis</i> Bojer	Mitra & Das Das 20018; Mitra & Maity 20040; Maiti 20037
60	<i>Dombeya wallichii</i> (Lindl.) K. Schum.	Maity 20010; Mitra & Maity s.n.; Mitra 20031
61	<i>Theobroma cacao</i> L.	Maity s.n.; Mitra 20035; Das Das s.n.; Das Das 26

List of specimen worked out (arranged according to the classification of Bentham and Hooker, 1862–1883)

FAMILY–STERCULIACEAE

Tribe I. STERCULIEAE

1. *Firmiana* Marsili

- i) *F. colorata* (Roxb.) R. Br.
- ii) *F. fulgens* (Wall. ex Mast.) K. Schum.

2. *Heritiera* Aiton

- i) *H. dubia* Wall. ex Kurz
- ii) *H. fomes* Buch. – Ham.
- iii) *H. littoralis* Aiton
- iv) *H. macrophylla* Wall. ex Kurz
- v) *H. papilio* Bedd.

3. *Pterocymbium* R. Br.

- i) *P. tinctorium* Merr.

4. *Pterygota* Schott & Endl.

- i) *P. alata* (Roxb.) R. Br.

5. *Sterculia* L.

- i) *S. balanghas* L.
- ii) *S. foetida* L.
- iii) *S. guttata* Roxb.
- iv) *S. hamilloni* (Kuntze) Adelb.
- v) *S. kayae* P.E. Berry
- vi) *S. kingii* Prain
- vii) *S. lanceifolia* Roxb.
- viii) *S. macrophylla* Vent.
- ix) *S. urens* Roxb.
- x) *S. versicolor* Wall.
- xi) *S. villosa* Roxb.

Tribe II. HELICTEREAE

1. *Helicteres*

- i) *H. elongata* Wall. ex Bojer
- ii) *H. hirsuta* Lour.
- iii) *H. isora* L.

2. *Kleinhovia*

- i) *K. hospita* L.

3. *Leptonychia*

- i) *L. caudata* Burret

4. Pterospermum

- i) *P. acerifolium* Willd.
- ii) *P. aceroides* Wall.
- iii) *P. diversifolium* Blume
- iv) *P. lanceifolium* Roxb.
- v) *P. obtusifolium* Wight ex Mast.
- vi) *P. reticulatum* Wight & Arn.
- vii) *P. rubiginosum* B. Heyne ex Wall.
- viii) *P. semisagittatum* Buch.–Ham. ex Roxb.
- ix) *P. suberifolium* (L.) Willd.
- x) *P. xylocarpum* (Gaertn.) Santapau & Wagh

5. Reevesia

- i) *R. pubescens* Mast.

Tribe III. ERIOLAENEAE

1. Eriolaena

- i) *E. candollei* Wall.
- ii) *E. hookeriana* Wight & Arn.
- iii) *E. lushingtonii* Dunn
- iv) *E. quinquelocularis* (Wight & Arn.) Wight
- v) *E. spectabilis* Planch. ex Mast.
- vi) *E. wallichii* DC.

Tribe IV. DOMBEYEAE

1. Dombeya

- i) *D. burgessiae* Gerrard ex Harv.
- ii) *D. spectabilis* Bojer
- iii) *D. wallichii* (Lindl.) K. Schum.

2. Melhania

- i) *M. denhamii* R.Br.
- ii) *M. futteyporensis* Munro ex Mast.
- iii) *M. hamiltoniana* Wall.
- iv) *M. incana* B. Heyne ex Wall.
- v) *M. magnifolia* Blatt. & Hallb.

3. Pentapetes

- i) *P. phoenicea* L.

Tribe V. HERMANNIEAE

1. Melochia

- i) *M. corchorifolia* L.
- ii) *M. nodiflora* Sw.
- iii) *M. umbellata* (Houtt.) Stapf

2. *Waltheria*

- i) *W. indica* L.

Tribe VI. BUETTNERIEAE

1. *Abroma*

- i) *A. augusta* (L.) L. f.

2. *Buettneria*

- i) *B. andamanensis* Kurz
ii) *B. aspera* Collebr. ex Wall.
iii) *B. herbacea* Roxb.

3. *Guazuma*

- i) *G. ulmifolia* Lam.

4. *Theobroma*

- i) *T. cacao* L.

Methods

Leaves were described based on their morphological as well as anatomical characters, including shape, size, margin, surface, texture and the major venation pattern. The following anatomical characters were taken into consideration as histology of node, histology of petiole, foliar architectural patterns, study of trichome, dermal features, other foliar inclusions like oil cells, mucilage cells, fibers, sclereids, crystals, etc.

Morphological study

Leaves of each species had been described. The mature leaves were taken for description of the morphological features as shape of lamina, size of lamina, while lobed, then the lobes were described. The other features were apex, margin, base, surface and venation patterns, etc. including shape and size of petioles and the stipules.

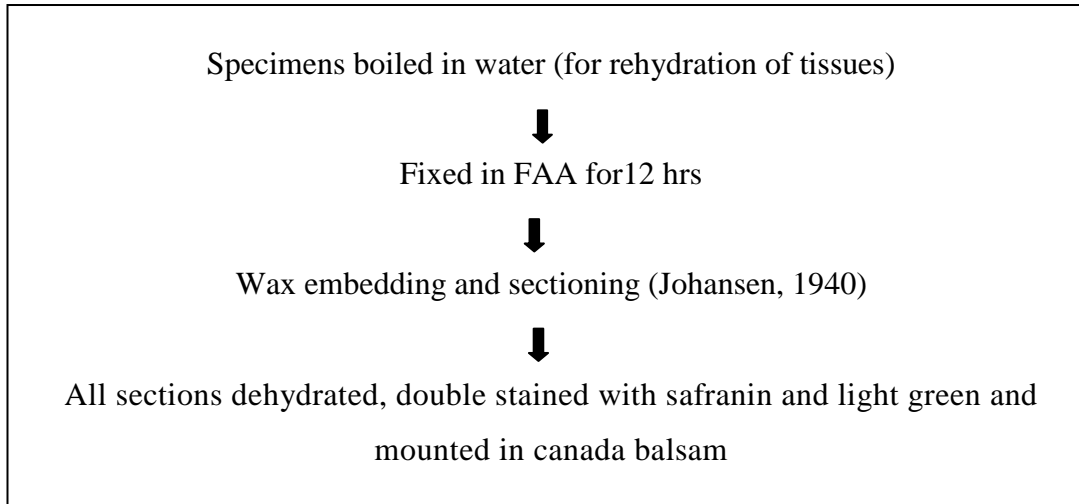
Anatomical study of internodes, nodes and petioles

The anatomical studies of the nodes were done followed by the petiolar anatomy of the same material to get the continuation of vascularization pattern. For the fresh materials free hand transverse sections were made. However, in case of dried specimens and for some fresh materials where free hand transverse sections were not possible to make hand sections were soaked in FAA [Formaldehyde

solutions 5 ml (40%): Glacial Acetic Acid 5 ml: 90 ml of 70% alcohol] for 2–5 days to remove the pigment and other alcohol soluble materials. The materials were transferred to 5% NaOH solution for 12–24 hours depending on the thickness as well as hardness of the specimens. This made the materials soft. These were then boiled for 2–4 hours in the hot water bath whenever required and kept in H₂O₂ for 1 hour to clear other contents of the specimens. Then specimens were washed thoroughly under tap water for several times and mildly heated and again allowed to dehydrate in serial alcoholic grades of 30%, 50% 70%, 80%, 90% and absolute alcohol twice. Then the specimens were ultimately soaked in chloroform by gradual addition with the absolute alcohol. The specimens were kept in paraffin, melting point of 56°–58° C. Two to three times changes of paraffin were done to saturate these materials with the paraffin by completely replacing the chloroform and kept within the hot air oven for 7 to 11 days in 58°C temperature for complete infiltration of paraffin. Paraffin blocks embedding the materials were prepared for microtome sections in the customary procedures and sectioned at 12–13µ. Serial sections were cut at the nodal region, in the proximal end and middle region and distal end of the petiole. Where the length of the petiole was short serial sections were cut for the whole portion, while for the longer petiole two to three more regions were selected to get the petiolar traces and for cutting of the serial sections within span of the middle region of petioles. The prepared microtome sections were dissolved in xylol, then in alcohol grades from absolute to 50% as downward grade. For upper grade both the hand and microtome sections were dehydrated in different alcoholic gradation and stained in 2% safranin dissolved in 70% alcohol and 2% light green dissolved in 90% alcohol. Permanent slides were prepared mounting with Canada balsam.

The diagrammatic drawings of anatomical representation of the nodal features as in transverse sections were drawn under mirror type camera lucida usually at 44–48 times magnification and a few photographs of the typical features of the studied specimens will be given. Although serial transections of the petioles were done from the proximal ends, middle region and distal ends of the petioles but a few essential sections will be selected for proper representation of the nodal and petiolar anatomy. Mirror type camera lucida drawing under 120, 440 magnifications

were also made to present the cellular composition of the stems at the nodal region as well as the petiole whenever different from the stem region. The staining and the preparation of permanent slides were done through conventional methods following Johansen (1940) as stated below.



Lamina

The study of the architectural patterns of lamina were done both from the dried and fresh leaves. The major venation type were described following Hickey (1973, 1979), Melville (1976), Dilchar (1974), Hickey and Doyle (1972), Hickey and Wolfe (1975), and Pole (1991) and for morphological feature and minor venation pattern description were presented under anatomical study following Hickey (1973, 1979) and Dilchar (1974).

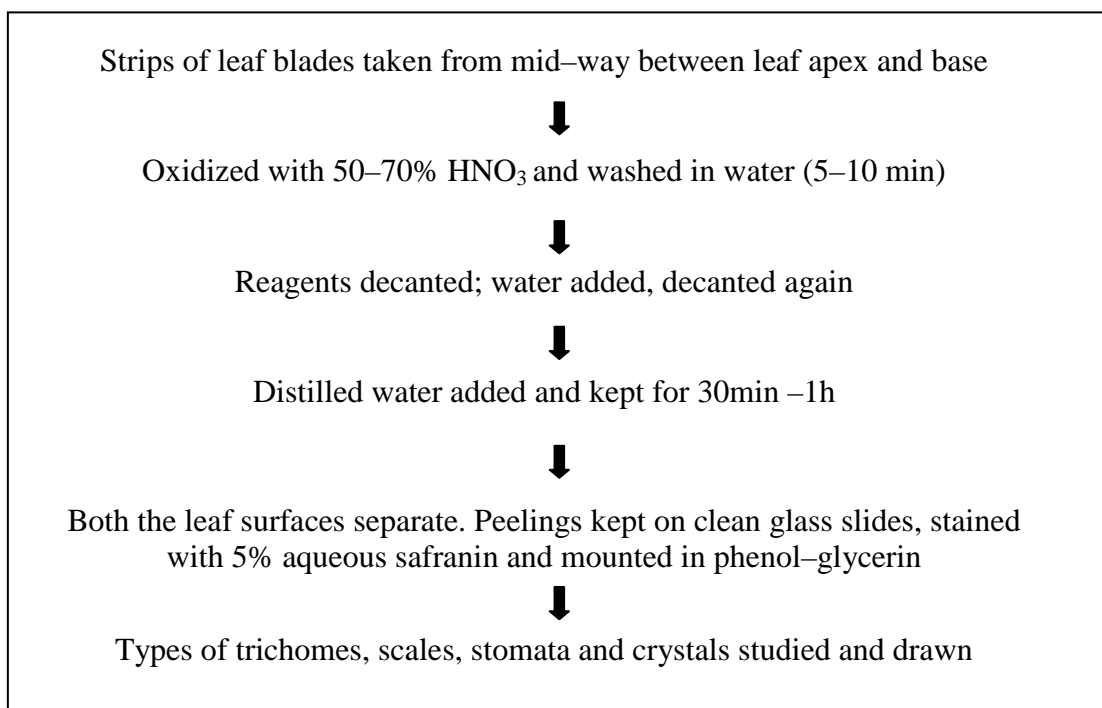
Leaf blades were cut into convenient pieces/sizes mostly selected a portion 1/3 from the apex and 2/3 from the base. Pieces were treated with 2.5% NaOH solution for clearing. The duration of the treatment in NaOH solution for clearing and bleaching depends on the nature of lamina, thickness and the cuticle. The slow process of treatment with least concentration of NaOH solution facilitates better preparation of the materials. After cleaning the pieces were washed in water and transferred into saturated chloral hydrate solution following Arnott (1959) and kept for overnight. After this the materials were then washed thoroughly in water and successively dehydrated and stained in 2% safranin solution dissolved in 70% alcohol. After gradual dehydration the permanent slides were prepared by mounted in Canada balsam. After a thorough study a convenient portion of vasculature was

drawn under mirror type camera lucida with suitable magnification as usually 44–48 times for minor venation pattern and marginal venation under (5x eye piece and 5x objective) extending from 3rd order to the free vein endings and usually 440 times for the free vein endings (5x eye piece and 45x objective). The free vein endings were drawn under higher magnification. From this preparation the trichome and the inclusions, etc. were studied as well as drawn under mirror type camera lucida. A few photographs were taken regarding some typical as well as important features of interests.

Dermal study

Strips of leaf blades were taken mid way between leaf apex and leaf base. Materials were oxidized with 50–70% HNO₃ solution and washed in water for 5–10 minutes and in some cases a pinch of KClO₃ was also added with HNO₃ solution by which the mesophyll tissue gradually disintegrated. The reagent was decanted; water was added, again decanted and kept in distilled water for 30 minutes to 1 hour. Both the surfaces of the leaves were then separated and at that time they were kept on the glass slide with the help of two fine brushes. The two separated surfaces were stained with 5% aqueous safranin, and finally mounted in phenol–glycerine as stated below. Different types of trichomes of each species were studied. Based on the observed features camera lucida (mirror type) drawings were presented for all figures. In addition to this, some photographs was taken from image analyzer. The description of trichomes were followed after Dilcher (1974), Ramayya (1972) and Payne (1978) and also Metcalfe and Chalk (1950).

In study of the anatomical features of stem portion and petiolar parts and their respective tissues the description were based on Metcalfe and Chalk (1950). Regarding the venation pattern of lamina the parameters were taken for description followed after Hickey (1973, 1979), Melville (1976), Dilcher (1974) and Pole (1991).



Morphometric analysis

91 variables (character states), including both morphological and anatomical characters were measured and recorded from 61 Indian species of this family and a matrix of these species was used in multivariate analysis. Cluster analyses using the UPGMA method (unweighted pair-group method with arithmetic averages) were carried out (Sneath and Sokal, 1973), from which a dendrogram representing the phenetic relationships among the taxa was constructed. All analyses were carried out using the software STATISTICA (version 9.0).

Table 3. List of character states used in UPGMA analysis

Sl. no.	Character states
1	Habit of plant
2	Nature of leaves
3	Laminar symmetry
4	Free vein endings associated with sclereids
5	Free vein endings associated with parenchymatous sheath cells
6	Free vein endings associated with both sclereids and parenchymatous sheath
7	Laminar apex
8	Laminar margin

9	Lamina lobation
10	Laminar base
11	Position of pet attachment
12	Nature of petiole
13	Outline shape of T.s. of petiole
14	Primary venation type
15	Presence or absence of crystals
16	Course of midrib
17	Presence or absence of agrophic veins
18	No. of agrophic veins on each sides
19	Secondary (2 ⁰) vein types
20	Secondary (2 ⁰) vein spacing
21	Presence or absence of intersecondary veins
22	Type of tertiary veins pattern
23	Presence or absence of interangular veins
24	Quaternary (4 ⁰) course
25	Marginal ultimate venation
26	Well-developed or ill-developed areolation
27	Areolar shape
28	Type of free vein endings
29	Highest order of veins in minor venation
30	Presence or absence of joining veins
31	Presence or absence of glands
32	Apical termination of marginal teeth
33	No. of principal vein
34	Presence or absence of scales
35	Epidermal cell morphology
36	Presence or absence of trichomes
37	Trichome distribution
38	Nature of petiole processes
39	Unicellular trichomes
40	Multicellular trichomes
41	Presence or absence of colleters
42	Presence or absence of pseudocolleters
43	Stomatal type
44	Cuticular features
45	Presence or absence of nonglandular trichomes
46	Presence or absence of glandular trichomes
47	Presence or absence of stalked glandular trichomes
48	Stalk cell unicellular
49	Stalk cell multicellular
50	Sessile glandular trichomes
51	Presence or absence of unicellular glandular trichomes
52	Presence or absence of multicellular glandular trichomes
53	Glandular trichomes with transverse septa
54	Glandular trichomes with perpendicular septa

55	Glandular trichomes with both transverse and perpendicular septa
56	Presence or absence of acicular trichomes
57	Acicular trichomes curved
58	Presence or absence of stellate trichomes
59	Stellate trichomes with unicellular basal cell
60	Stellate trichomes with multicellular basal cell
61	Stellate trichomes sessile
62	Arms of stellate trichomes stout
63	No. of arms of stellate trichomes
64	Distribution of crystals
65	Fibres within phloem layer in internode and node
66	Position of mucilaginous cavities
67	Arrangement of mucilaginous cavities in pith
68	Arrangement of mucilaginous cavities in cortex
69	Vein end frequency
70	Areole frequency
71	Presence or absence flexuous trichomes
72	Crystal type
73	Presence or absence of secondary growth in petiole
74	Presence or absence of collenchymatous hypodermis
75	Presence or absence of teeth aggregation
76	Presence or absence of sclereids in teeth
77	No. of vascular bundles at proximal end of petiole
78	No. of vascular bundles at middle part of petiole
79	No. of vascular bundles at distal end of petiole
80	Presence or absence of pith at proximal end of petiole
81	Presence or absence of accessory vascular bundles in petiole
82	No. of accessory vascular bundles at proximal end of petiole
83	No. of accessory vascular bundles at middle part of petiole
84	No. of accessory vascular bundles at distal end of petiole
85	Nature of stipules
86	No. of order of teeth
87	Spacing between order of teeth
88	Presence or absence of sclereids in nodal section
89	Arms of stellate trichomes unicellular
90	Arms of stellate trichomes multicellular
91	Presence or absence of hollow pith

Key to the species

Key to the species under each genus and infra specific taxa, whenever there are more than one under a species, based on foliar architecture and anatomical features is provided.