2.1 Introduction

Until detailed genetic evidences are available, the classification of flowering plants was based purely on their morphology and biochemistry, and was typically produced by an individual botanist or by a small group. Existing angiosperm classifications appeared to be ‘outdated’, as 'Angiosperm Phylogeny Group' (APG, 1998; 2003; 2009) re-classified them systematically, primarily based on genetic characteristics. However, the widely accepted natural system of Bentham and Hooker (1876) still stands as the stable point of reference for angiosperm classification even today.

The term ‘Biosystematics’ was derived from ‘Biosystematy’ introduced by Camp and Gilly (1943), attempts to produce a system of classification to express explicit relationships with the application of
experimental, genetic and cytologic approaches at the level of species and infraspecific taxa (Pooja, 2004). Contributions to the systematics from various branches are of considerable value in proper assessment of the status of a taxon and its phylogeny (Singh and Jain, 1989). Though morphological characters have provided the foundation and framework in the classification of plant taxonomy, modern approaches in various disciplines like anatomy, embryology, cytology, palynology, physiology, genetics and ecology have played a significant role for the newer aspects of taxonomy such as biosystematics, cytotaxonomy and chemotaxonomy (Sharma, 2009).

Biosystematics, in the first sense essentially, is an expansion of classical taxonomy (Mustapha, 2009). Before evolutionary conclusions are being drawn, it is important to obtain morphological, molecular and phytochemical data to assist in understanding the relationships within plant groups to resolve complex issues (Alan et al., 2004). Data can be qualitative or quantitative, the former finds more use in distinguishing taxa of specific or higher ranks, and the latter to separate lower taxonomic categories at infraspecific levels (Pandey and Misra, 2008).

**2.1.1 Evidences in relation to systematics**

a) **Morphology in relation to systematics**

Morphology has been traditionally the most important source of information in plant taxonomy; and still the majority of taxonomic groups recognized today are mainly from floral morphology (Sennblad et al., 1998; Singh, 2010). The easily observable and obtainable morphological data provide the basic language for characterization, identification,
classification and relationships (Radford, 1986). Some of the vegetative characters that play a major role in plant nomenclature are: growth habit (herbs, shrubs, trees) and pattern; stem, leaf (phyllotaxy, form, venation, size, shape), petiole, stipule characters, etc. (Sharma, 2009). Even though, both vegetative and floral morphology provides the majority of characters for key identification, the latter being more reliable and practiced widely.

The floral characters are one of the thorough and extensively explored areas in various levels of classification, which include types of inflorescence and flower; perianth, bract, bracteole, pedicel characters, floral symmetry, cohesion and adhesion of floral parts, types of androecium, gynoecium, fruits and seeds (Sharma, 2009).

b) Vegetative anatomy in relation to systematics

Anatomical features have played an increasingly important role in the elucidation of phylogenetic relationships (Singh, 2010). Most useful anatomical characters for identification include stomatal types and their structure (Ranunculaceae, Brassicaceae, Caryophyllaceae, Rubiaceae and Poaceae families are known with their stomatal form), trichomes (Lamiaceae, Malvaceae), epidermal and mesophyll tissues, stelar pattern, types of vascular bundles, rays, shape, size and wall sculpture of xylem and phloem cells, sclereids, ground tissue and parenchyma (Radford, 1986). Wood anatomy, especially tracheid characters and vessel grouping have considerable significance in the identification of Apocynaceae members (Lens et al., 2008; 2009), which would also benefit to plant systematics as a whole.
Moreover, anatomical characters are considered as one of the pillar areas in the field of pharmacognosy to distinguish adulterants and other contaminants when manufacturing of drugs (Lohar, 2007).

c) Embryology in relation to systematics

Embryology, certainly has no claim of supremacy with regard to ranking of taxa at different levels of hierarchy, but, it has been used to solve several disputes in generic and higher levels of classification (Stuessy, 2008). Presence and type of anther tapetum, number and arrangement of anther locules, type of endothecium, partition of microspore mother cells, mature pollen grains, orientation of ovules, etc. are some of the useful characters for embryological analysis (Singh, 2010). Palynology could be regarded as simply one aspect of embryology, because it has much to do with taxonomy than other embryological aspects (Stuessy, 2008).

d) Palynology in relation to systematics

Palynological characters have been used in the repositioning of several disputed genera and related taxonomic problems (Erdtman, 1966; Nair, 1980), and it has been used exclusively for angiosperm classification (Cronquist, 1981). Different types of pollen wall architecture such as psilate, verrucate, foveolate, reticulate, fossulate, echinate, spinulate; aperture characters viz. mono-, bi-, tri-, tetra- or multi-porate or multicellular and inaperturate, are some of the principal characters used in palynology systematics (Singh, 2010).
e) **Phytochemistry in relation to systematics**

Phytochemistry has been yet another promising approachable area for biosystematics. If the genes are similar, the chemical products controlled by them are also similar. The chemical substances, especially secondary metabolites grouped into alkaloids, terpenoids, cardiac glycosides, anthraquinones, phenolic compounds and flavonoids are of wider applications in taxonomic delimitations (Pandey and Misra, 2008). Some special chemical molecules are confined to the members of a particular family, or within certain genera only, a criteria useful for taxon identification.

f) **Molecular evidences in relation to systematics**

The combined analyses of molecular data sequences together with morphological data provide a strong basis for phylogenetic hypotheses, and thus also for classification in plant systematics (Sennblad et al., 1998). As molecular data reflect the gene level changes, its utilization of reflecting true phylogeny is much better, when combined with morphological data. Since evolution is based on genetic changes, use of genetic material for the better understanding of evolutionary relationships has been used during the past 2-3 decades. Closely related species are expected to have greater similarities in their genetic material than the distantly related species (Singh, 2004).

Comparative studies among the morphovariants of *Thevetia* are found absolutely zero at ‘Systematics’ level. Each and every mutant form of the parent, differing minimum in a single character has to be analyzed critically with a nomenclature strategy in order to differentiate the true species. The
combined data from the above mentioned diverse fields encompassing all possible biological parameters can be taken as valid criteria for the systematics of *T. peruviana* under study, which would help to strengthen and to tackle the taxonomic problems at any level of magnification.

### 2.2 Materials and Methods

Fresh twigs of *T. peruviana* (Plate 2.2.1) from three different plants that produce yellow (YFP), orange (OFP) and white flowers (WFP) were collected from different districts of Kerala; and identified with the help of various Regional Flora (Cooke, 1905; Matthew, 1983). The morphological identification was further confirmed with the help of keys, descriptions, illustrations, herbarium specimens and discussions with authoritative taxonomists. The voucher specimens (STHAPC 2458 a-c) are preserved in the Herbarium cabinet of Botany Department, St. Teresa’s College, Ernakulam for future reference.

![Plate 2.2.1 Morphovariants of *Thevetia peruviana*](image)

**Plate 2.2.1 Morphovariants of *Thevetia peruviana***

a) Yellow Flowered Plant (YFP)  
b) Orange Flowered Plant (OFP)  
c) White Flowered Plant (WFP)

Studies on vegetative and floral morphology, anatomy and embryology were carried out systematically using suitable specimens at varying stages of development, from budding to the fruiting stage.
Biosystematics

Micro-preparations (hand sections as well as microtome sections) of vegetative and floral parts were made and images were captured using OLYMPUS MAGNA Trinocular Research Microscope (Germany) equipped with Nikon photographic unit. Physical measurements of various samples were done using graduated scales, graph paper imprints and vernier calipers.

Pollen analysis was carried out following the methodology of Erdtman (1952). Mature flower buds were collected and preparations were made for light microscopy (LM) and scanning electron microscopy (SEM) studies. Acetolysed pollen preparations were dehydrated, loaded on specimen stubs, and coated with gold particles for capturing high resolution images under Scanning Electron Microscope (Make: JEOL Model JSM - 6390LV). Meanwhile, glycerine mounted samples were prepared for LM analysis.

For phytochemical analysis, the procedure of Harborne (1973) was followed. Samples of leaf, flower, fruit wall and seed kernel were extracted using different solvents in the order of increasing polarity. All the extracts were screened for the presence of primary (carbohydrates, starch, proteins) and secondary metabolites (alkaloids, flavonoids, terpenoids, steroids, cardiac glycosides and so on). The results were compared between the same parts of three colour variant plants. A detailed study in this area is presented in Chapter 3.

For inferring a molecular level comparison, genomic DNA was isolated from tender leaves using Sigma Aldrich plant DNA extraction Kit. Its quality was evaluated on 0.8 % Agarose Gel Electrophoresis.
before analysis. From the isolated high molecular weight genomic DNA, Ribulose-1, 5-biphosphate carboxylase \((rbcL)\) gene fragment was amplified by PCR using \(rbcL\)-PCR universal primers.

**Primers used for PCR:**

Forward Primer \((rbcL F): ATGTCACCACAAACAGAGACTAAAGC\)

Reverse Primer \((rbcL R): GTAAAATCAAGTCCACCRCG\)

The PCR amplicon was purified by column purification, and concentration of the purified DNA was determined and subjected to automated DNA sequencing on the ABI3730xl Genetic Analyzer (Applied Biosystems, USA). The edited \(rbcL\) gene sequences were then used for similarity searches using Basic Local Alignment Search Tool (BLAST) program in the NCBI GenBank DNA database for comparing the extent of similarity and diversity among the samples.

### 2.3 Results

#### 2.3.1 Morphology

Both vegetative and floral morphology was evaluated for elucidating the taxonomic relationship (Plates 2.3.1 - 2.3.2)

**a) Yellow Flowered Plant (YFP)**

Distribution: The plant is cultivated in gardens as an ornamental, grown in sandy soil. They can also withstand water scarce areas in summer, with continuous blooming.

Habit: A large, evergreen shrub or a small tree, growing up to 12 - 15 ft height.
Stem: Erect, branched, herbaceous above, woody below, with numerous cracks and fissures on the bark, latex milky, branchlets numerous, slender, leaves densely clustered towards branch tips.

Leaves: Simple, dorsi-ventral, exstipulate, linear to lanceolate; phyllotaxy - alternate; petiole - short, 0.25 - 0.35 cm long, base attenuate; lamina - 10.8 - 16.2 (L) × 0.9 - 1.3 (B) cm, tapering at both ends, adaxial surface lustrous green and glabrous, abaxial surface light green, margin smooth, entire and revolute, apex sub-acute; midrib - prominent, lateral veins inconspicuous and faint.

Inflorescence: Few flowered terminal cymes, 4 - 8 blooms of varying stages at a time, later turns extra axillary due to development of axillary buds; peduncle - light green, 1.2 - 1.4 cm long and narrow.

Flower: Pedicellate, bracteate, large, showy, bright yellow, sweetly fragrant, regular, bisexual, actinomorphic, pentameric, gamopetalous, hypogynous; bracteole - persistent; pedicel - 2.4 - 2.5 cm long, narrow and light green.

Calyx: Sepals 5, unequal, free, glandular, deeply lobed, quincuncial imbricate, base broad, apex pointed, 0.8 - 1.05 cm long, persistent.

Corolla: Petals 5, gamopetalous, lobes sinistrally twisted, corolla tube narrow at the base, 1.4 - 1.5 cm long, broadened above as a throat (0.5 - 0.6 cm) and expanded into 5 lobes (4.5 - 4.8 cm), 5 coronal outgrowths with tuft of hairs at the corolla throat, these 5 appendages give a star shaped appearance from above, corolla tube internally pubescent; two types of hairs, hairs on corona are longer, flexible, appendiculate, oriented horizontally; hairs in between the basal region
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of staminal ridges are straight, smooth, in 2 vertical rows on either side of each outgrowth, inwardly directed, smaller than the upper ones; corolla base green peripherally, colour extend along the margin of petals from base to tip.

Androecium: Stamens 5, epipetalous, inserted at the corolla throat, opposite to petals and situated just below each lobe of densely pubescent corolline corona; filament - short and broad; anther 2-lobed, narrowly oblong, base broad, apex conical, each lobe is 2-loculed; connective - broad at the base, tapers above, prolonged beyond the lobes as an elongated leathery spur, longer than filament, all five extended spur united together in anticlockwise direction, breaks when the flower opens; stamens free from pistil head, but rest on its surface, dehiscence longitudinal and latrose, pollen grains numerous.

Gynoecium: Bicarpellary, basal ¼th part fused and upper ¼th portion free; ovary - superior, each carpel is 2-chambered, one ovule in each chamber, hemianatropous ovules with axile placentation; style - slender, dilated near the apex and the base, completely fused in middle part; stigma - massively thickened and bilobed, level with anthers, dome shaped, apex shortly 2-cleft with numerous single celled glands, middle region contracted, receptive surface 10-lobed and laterally placed with a ring of unicellular hairs; nectar disc - prominent, 5-lobed, light yellow, encircle ovary at the base.

At midday, the receptive stigmatic marginal cleft was observed with numerous germinating pollen grains, having three activated germ pores with pollen tubes of varying lengths.
Fruit: Indehiscent, syncarpous follicle, depressed and bluntly 4-angled, broader, transversely and vertically divided, 4.1 - 4.85 (L) × 3.2 - 4.5 (B) × 3.2 - 4.1 (H) cm; wall three layered, epicarp - outer layer, cuticularised, light green in tender fruits, brownish black in fully matured ones; mesocarp - middle, fleshy and spongy; endocarp - inner, stony, segmented and transversely elongated 3.6 - 3.8 (L) × 1.62 - 1.8 (B) × 1.7 - 1.9 (H) cm, act as the outer shell of seeds after the decay of pericarp, contain 4 well divided locules (mericarp); mostly 2, 3 or 4 seeded, rarely single seeded, fruit stalk 3.6 - 4.0 cm long.

Seed: One per locule, winged and flattened, compressed laterally on lower side and at the distal end, upper surface convex, wings small, on either side of radicle end; radicle short, directed laterally towards fruit margin, cotyledons oily and cream coloured, seed coat leathery.

Fruiting: Flowers most of the year, fruit sets during Sept - May.

Propagation: Usually by seeds; germination - epigeal.

**b) Orange Flowered Plant (OFP)**

Distribution: Cultivated as an ornamental, less common in gardens.

Habit: Large, evergreen, perennial, highly branched, erect shrub or small tree, with milky latex, up to 8 - 12 ft tall, grows up to a height of 18 ft.

Stem: Erect, branched, irregularly ramified, younger twigs green and herbaceous, mature parts woody, bark grayish white, with numerous lenticels.
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Leaves: Simple, petiolate, linear to lanceolate; phyllotaxy - alternate; petiole - short, 0.15 - 0.2 cm; lamina - acute, glabrous, 12.0 - 16.5 (L) × 1.1 - 1.3 (B) cm, slightly leathery, bright green on adaxial surface, pale green abaxially, tapering at both ends, margins revolute; midrib - prominent, lateral nerves faint, venation obscure.

Inflorescence: Terminal peduncled cyme, later turns axillary (sub-terminal) and leaf opposed, 4 - 8 flowered; peduncle - narrow, 1.2 - 1.5 cm long, pale green.

Flower: Bracteate, pedicellate, regular, bisexual, pentamerous, large, showy, peachy orange with mild fragrance, rarely non-fragrant, flower 8.0 - 8.6 cm long; pedicel - pale yellow and 1.8 - 2.2 cm long.

Calyx: Sepals 5, deeply lobed, glandular, acute, spreading, unequal, lanceolate, light green, 0.7 - 0.9 cm long, 0.3 - 0.4 cm broad base, quincuncial imbricate and persistent.

Corolla: Petals 5, gamopetalous, bell shaped, lobes obovate, basal tube cylindrical and narrow, 1.3 - 1.5 cm long, tube widened as a throat (0.4 - 0.5 cm), 5 sinistrally twisted and overlapping lobes above (4.4 - 4.7 cm), throat villous, 5 coronal appendages (corolline corona) present, densely pubescent coronary lobes above the throat opposing with stamens; hairs 2 types - transversely oriented appendiculate ones on coronary lobes, and numerous inwardly directed simple smooth hairs in two vertical rows on either side of projecting base of stamens, light green or purple tinge on the outer surface of corolla, extending along the margins of each petal from base to tip.
Androecium: Stamens 5, small, oblong to cordate, mucronate, completely inserted; filaments - extremely short, nearly sub-sessile; lobes - 2, base broad, apex narrow, each lobe 2-loculed; connective - projects beyond the anther lobes as a short, membranous hairy appendage, all 5 projections jointed together and twisted in anticlockwise direction in buds across the top of the pistil head, appressed, but not adnate to it; dehiscence - longitudinal, latrose; pollen grains - numerous and appear in monads.

Gynoecium: Bicarpellary, partially syncarpous, superior; nectar disc - 5-lobed, encircle the ovary, pale yellow; ovary - pale green, 4-chambered due to the enlargement of placenta, single hemianatropous ovule in each locule, placentation axile; style - narrow and filiform, 1.3 - 1.4 cm long, with a jointed appearance, longitudinally free just below the stigmatic head and above the ovary; stigma - large, broad and massive, apex conical and 2-cleft with numerous unicellular glands, receptive surface 10-lobed, directed sideways, intermingled with numerous unicellular hairs, 10 anther lobes appressed to 10 lobes of stigmatic head in bud condition.

Fruit: Fleshy, follicle, triangular to sub-globose, bluntly 3 - 4 angled, glabrous, broadly turbinate, 4.0 - 4.7 (L) × 3.2 - 4.2 (B) × 3.2 - 4 (H) cm size, compressed laterally, green coloured when young, turns brownish black on ripening, pericarp - three layered; epicarp - outer cuticularised, shining layer; mesocarp - middle, fleshy; endocarp - inner, hard, stony, 3.2 - 3.68 (L) × 1.5 - 1.8 (B) × 1.66 - 1.82 (H) cm size, 2 - 3 seeded, rarely 4 or 1 seeded.
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Seeds: Flattened, protected by stony endocarp, winged, single seed per mericarp, radicle directed sideways, cotyledons thick, fleshy, oily and creamy white, seed coat membraneous.

Fruiting: Flowers major part of the year, fruiting during Sept-May.

Propagation: By seeds; germination - epigeal.

c) **White Flowered Plant (WFP)**

Distribution: Grown as an ornamental, less common in gardens.

Habit: Perennial, large, evergreen shrub or small tree with milky sap, grows up to 16ft.

Stem: Woody, branched, irregularly ramified; Bark - grayish white with numerous lenticels.

Leaves: Simple, sub-sessile, linear to lanceolate; phyllotaxy - alternate; petiole - short, 0.18 - 0.25 cm long, lamina - glabrous, 12.0 - 15.5 (L) × 1.0 - 1.3 (B) cm, acute, slightly leathery and glossy green on adaxial side, abaxial side dull green, tapering at both ends, margins slightly revolute, venation obscure; midrib - prominent and lateral nerves inconspicuous.

Inflorescence: Terminal, or axillary, leaf opposed, peduncled, cyme many flowered (8 - 10); bracteole - green, persistent, 0.2 - 0.4 cm long; peduncle - light green, 1.5 - 2.0 cm long.

Flower: Bracteate, pedicellate, regular, bisexual, actinomorphic, pentamerous, large, showy, non-fragrant, 8.7 - 9.1 cm long; pedicel - light green, 2.5 - 2.6 cm.
Plate 2.3.1 Flower anatomy

a) A portion of corolla split view showing corolline corona, two mature anthers connected by apical hood, broad anther base, downwardly directed hairs (YFP)
b) C.S of flower passing through stigmatic region showing five epipetalous anthers with 10 anther lobes, each lobe appressed on 10-lobed and incompletely fused stigma (YFP: 5 X)
c) appendiculate hairs on corolline corona (OFP: 40 X)
d) smooth hairs on either side of anther base (OFP: 40 X)
e) 2-cleft apical part
f) Unicellular glands at sterile apical dome (WFP: 40 X)
g) receptive stigmatic notch situated laterally, with germinating pollen grains and unicellular hairs (WFP: 40 X).

Calyx: Sepals 5, green, deeply lobed, glandular, acute, spreading, unequal, lanceolate, 0.7 - 0.9 cm long, 0.32 - 0.4 cm broad base, apex acute, persistent, light green, quincuncial.

Corolla: Petals 5, gamopetalous, bell shaped, lobes obovate, basal tube cylindrical, 1.5 - 1.6 cm long, proximal end slightly widened and pentagonal due to nectar disc, inner region of corolla tube is silky smooth, tube widening above the middle into a corolla throat (0.5 cm), corolla white, with an outer light green or purple shade starting from base to tip,
densely pubescent corona lobes near the throat opposing the stamens, give a star shaped look from the periphery, 2 types of unicellular hairs directed transversely and downwardly on coronal appendages and near connective base, upper expanded corolla lobes 4.2 - 4.9 cm long, sinistrally twisted.

Androecium: Stamens 5, completely inserted, epipetalous, 2-lobed; filaments - short; lobes - small, 2-loculed, oblong to cordate, mucronate; connective - broad below, narrow above, projects beyond the anther lobes and terminating into short membraneous appendages, united together and twisted in anticlockwise direction in buds above the pistil head, all 5 anthers cohering as a cone around the stigma, appressed but not adnate to it; dehiscence - longitudinal; pollen grains - numerous, uniform and in monads.

Gynoecium: Bicarpellary, partially syncarpous, distal ¼th portion of ovary free; nectar disc - around the ovary with 5 prominent lobes, each lobe again 2 - 3 divided; ovary - pale green, 4-chambered, single hemianatropous ovule inside each locule with axile placentation; style - smooth, narrow, 1.3 - 1.4 cm long, with a jointed appearance, basal region incompletely fused, fusion complete in the middle part, upper portion bifurcated just below the style head; stigma - large, broad and massive, apex dome shaped and 2-cleft, with numerous unicellular glands, margin 10-lobed, receptive surface marginal, with ridges, furrows and numerous small unicellular hairs. Flowers at noon were observed with germinating pollen grains carrying > 50 µm long pollen tubes.

Fruit: Fleshy follicle, triangular to sub-globose, bluntly 3 - 4 angled, glabrous, broadly turbinate, size 3.9 - 4.8 (L) × 3.5 - 4.3 (B) × 3.4 - 3.9 (H) cm, compressed laterally, light green, brownish black on ripening; pericarp - outer leathery and shining epicarp, middle fleshy and spongy mesocarp,
inner hard and stony endocarp 3.2 - 3.7 (L) × 1.53 - 1.78 (B) × 1.62 - 1.9 (H) cm size; usually 2, 3 or 4 seeded, rarely 1.

Seeds: Flattened, winged, radicle directed sideways, cotyledons thick, fleshy, oily and creamy white.

Fruiting: Seeds set throughout the year, but drop down during heavy downpour without maturing, mature fruits produced after the rainy season.

Propagation: By seeds; germination - epigeal.

![Fruit morphology](image)

**Plate 2.3.2 Fruit morphology**

a) Bunch of tender fruits of WFP, lowest one showing remnants of two styles, free at proximal end b) mature fruit with partly dehisced epicarp on ventral suture (WFP) c) endocarp with single seed in each mericarp (OFP) d) upper row: seeds without seed coats, lower row wings on the seed coat (OFP) e) emergence of radicle through opposite poles (YFP) f) germination of a 3-seeded fruit (YFP).

2.3.2 Anatomy

Internal features of young and mature stem, leaf lamina and petiole from three colour variants were studied for comparing the specimens.
2.3.2.1 Stem anatomy (Plate 2.3.3)

a) Young stem of YFP

Outline - circular; epidermis - single layered, cells radially elongated, cuticle heavily thickened; cortex - outer 3 - 4 layers of collenchyma, 62.5 - 75 µm thick, calcium oxalate (caox) prism crystals (8 - 18 × 4 - 8 µm) abundant, middle 6 - 8 layers of chlorenchyma, 137.5 - 165.5 µm thick, chloroplasts abundant, inner 4 - 6 layered round parenchyma, 100 - 125 µm thick, with starch grains, latex cells, prismatic crystals and crystal sands. Starch grains uniformly distributed, simple, hilum distinct, concentric type; sclerenchymatous fiber layer in inner cortex, discontinuous, groups (62.5 - 75 µm diameter) of varying number of cells (10 - 18), highly thickened, hard and strongly lignified, lumen reduced, oblong; vascular system - bicollateral; phloem - continuous layer, 35 - 50 µm thick; cambium - thickness 25 - 35 µm; xylem - tracheids prominent, 100 - 125 µm thick; pith - large, 1500 - 1600 µm diameter, rounded thin walled and thick walled parenchyma in small groups, with numerous laticifers and idioblasts, latex cells elongated with brown contents, evident in transverse and radial longitudinal sections.

Mature stem

Outline - roughly circular; periderm - lenticels occasional; phellem - heavily stained, 8 - 9 layers, 75 - 90 µm thick; phellogen - thickness 90 - 100 µm, 8 - 12 layers of tangentially elongated thin-walled cells, devoid of cytoplasm, prismatic crystals rare; phelloderm - thick walled cells, 3 - 4 layers, 25 - 30 µm; cortex - 400 µm thick, cells become oblong to elliptical; outer cortex with angular collenchyma, quadrangular to rhomboidal prisms of varying sizes (15 × 7.5 µm), concentrated in some
areas, fills about 70 - 80 % of the parenchyma cell lumen; middle cortex chlorenchymatous, crystal druses rare; inner cortex parenchymatous, with abundant crystal sands; sclerenchyma fibrous layer in the inner cortex, lumen cavity further reduced; vasculature - bicollateral; secondary phloem - with sieve cells and companion cells, 75 - 87.5 µm; cambium - layers 6 - 8, 50 - 62.5 µm; secondary wood - thickness 950 - 1250 µm, xylem vessels solitary (62.5 × 37.5 µm diameter, 210 - 275 µm long), short radial multiples of two, three, four and long radial multiples up to 8 - 9, occasional, more towards the inner wood, possess simple pits, end wall simple; tracheids with simple pits; fibers long, thread like, ends tapering, protoxylem (25 × 25 µm diameter) possess annular or spiral thickening, metaxylem with scalariform or reticulate side walls; medullary rays - uniseriate, 6 - 9 cells in each series (125 - 137.5 µm long); pith - prominent, groups of thin walled and thick walled rounded parenchyma, with latex cells and idioblasts.

b) Young stem of OFP

Epidermis - Single layered, thickly cuticularised; cortex - outer 2 - 3 layered angular collenchyma (75 - 87.5 µm), prism crystals common; middle 5 - 7 layered chlorenchyma (75 - 87.5 µm), druses frequent; inner 8 - 12 layered parenchyma (175 - 187.5 µm) with starch grains and occasional crystal sands, sclerenchymatous fibers in various groups (75 - 87.5 µm) of 18 - 22 cells with narrow lumen in inner cortex; vascular system - bicollateral; phloem - continuous, 35 - 50 µm thick; cambium layers 4 - 6, 25 - 37.5 µm thick; xylem - unbroken ring with many tracheids and few vessels, 110 - 125 µm; pith - large, parenchymatous, 1500 - 1600 µm diameter with laticifers and idioblasts.
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Mature stem

Outline - Roughly circular; periderm - tangentially elongated rectangular cells; outer phellem - thick walled, 5 - 7 layered, deeply stained, 50 - 62.5 μm; middle phellogen - thin walled, 10 - 14 layered, 75 - 87.5 μm; phelloderm - inner, 2 - 3 layered, thick walled, 25 - 30 μm thick; cortex - thickness 350 - 425 μm, outer 4 - 5 layers of collenchyma, single large prism of varying sizes and shapes, rectangular (18.75 × 3.75 μm), 5-angled, square type or 2 - 3 medium sized crystals in a single cell; middle 4 - 6 layers chlorenchyma with druses, inner 6 - 9 layers rounded parenchyma with latex cells, starch grains, prisms and crystal sands; sclerenchymatous fibers - discontinuous layer, groups (50 - 87.5 μm) of varying number (12 - 32) of isodiametric, thick walled cells, lumen narrow, intermingled with inner parenchyma; vasculature - bicollateral; secondary phloem - with sieve cells and companion cells, 112.5 - 125 μm, prism crystals in some phloem cells, regularly placed; vascular cambium - layers 8 - 10, thickness 112.5 - 125 μm; secondary xylem - 1250 - 1300 μm, in radial chains, vessels solitary (37.5 - 43.75 μm diameter, 87.5 - 112.5 μm long), in pairs, or in groups of 3, or radial multiples in some areas, whereas, chains of 6 - 8 multiples prominent in other areas, simple pits on side walls, aperture simple; tracheids with simple pits; fibers long, narrow, ends tapering; protoxylem with spiral and annular thickening, metaxylem with simple pits; medullary ray - uniseriate, elongated chains of 6 - 8 cells; pith - large, prominent, parenchymatous, latex cells numerous.

c) Young stem of WFP

Outline - circular; epidermis - single layered, thickly cuticularised; cortex - outer 3 - 4 layered collenchyma (75 - 87.5 μm), with abundant
rectangular caox prisms; middle 4 - 5 layered chlorenchyma (75 - 87.5 µm); inner 8 - 10 layered parenchyma (175 - 250 µm) with abundant druses, prisms and starch grains; vascular system - bicollateral; phloem - with sieve cells and companion cells, 37.5 - 50 µm; cambium - thickness 35 - 50 µm, 4 - 6 layers; xylem - continuous ring, 100 - 125 µm thick, radially elongated chains of tracheids, vessels few; pith - large, 1450 -1600 µm diameter, rounded parenchyma, laticifers and idioblasts frequent.

Plate 2.3.3 Stem anatomy
a) Calcium oxalate prism crystals in periderm and outer cortex (40 X) in YFP b) crystal sands in the inner cortex (60X) in YFP c) secondary wood showing single, double and radial multiples of vessels in YFP (10 X) d) OFP (5 X) e) WFP (5 X).

Mature stem

Outline - roughly circular; periderm - outer 4 - 6 layered (30 - 37.5 µm), thick walled phellem; middle 9 - 10 layered (112.5 - 125 µm) thin walled
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Biosystematics, Phytopharmacological and Tissue Culture Studies on *Thevetia peruviana*

phellogen; inner thick walled 3 - 4 layered phelloderm (25 - 37.5 µm); cortex - three zones (312.5 - 375 µm), usual round shape become lost as the secondary thickening progresses, and all cells in the cortical region appeared transversely oblong to elliptical; outer collenchyma with abundant prisms, middle chlorenchyma with few druses; inner parenchyma with crystal sands, starch grains, laticifers and groups of sclerenchymatous fibers in varying numbers; vasculature - bicollateral; secondary phloem - continuous, 100 - 125 µm; cambium - thickness 112.5 - 125 µm, 8 - 12 layered; secondary xylem - well defined regular radial rows (1250 - 1375 µm), single vessels more in outer wood, radial multiples of 6 - 8 in inner wood, radial pairs prominent in some areas, vessel length vary from short (75 µm) to long (400 µm), lumen almost uniform (37.5 x 37.5 µm), protoxylem with annular thickening, metaxylem possess scalariform side walls; tracheids with simple pits, fibers long, narrow; medullary ray - uniseriate, rarely multiseriate, narrow; pith - prominent, groups of thick and thin walled rounded parenchyma, latex cells abundant.

2.3.2.2 Leaf anatomy (Plate 2.3.4)

a) Leaf of YFP

Outline - adaxial surface straight, almost parallel with the lamina, smooth, hairs absent, abaxial midrib region with a prominent downward ridge, often conical; adaxial epidermis - single layered, 12.5 - 15 µm thick, regularly arranged rectangular cells with thick cuticle on the lamina; polygonal and axially elongated smaller cells with ridges on upper midrib region, leaf margin slightly revolute; midrib - thickness 688 - 700 µm, 2 - 3 layers of collenchymatous hypodermis above and below the abaxial and adaxial epidermis; mesophyll - bifacial; palisade tissue - single layered, 80 µm long with rich chloroplast, extended into the
midrib from both lamina leaving a small gap; spongy tissue - transversely elongated, 3 - 4 layered, less compact, air chambered, 187.5 - 200 µm thick, crystal druses medium distribution, usually solitary, latex cells present; vascular bundle - bicollateral, open arc shaped, arms slightly wider, xylem groups 16 - 24; phloem in small patches, external and internal; collateral vascular bundle for minor veins; ground tissue - round parenchyma, few druses and latex cells; abaxial epidermis - single layered, smaller cells than adaxial one; stomata - restricted to abaxial epidermis, paracytic in high frequency, anomocytic occasional.

Plate 2.3.4 Leaf anatomy

Adaxial surface view of a) YFP showing axially elongated epidermal cells (60 X) b) OFP showing axially elongated wavy epidermal cells (60 X) c) WFP epidermal cells with radially elongated undulate cells (60 X) d) abaxial view of OFP with paracytic stomata and caox druses in groups of 2 or 3 (40 X) e) WFP with paracytic and anomocytic stomata and druses (40 X) f) YFP leaf lamina with highly thickened cuticle and druses in mesophyll tissue (40 X) g) internal structure of OFP (10 X) h) midrib region of WFP (10 X).
b) Leaf of OFP

Outline - adaxial surface slightly raised on the midrib region along with lamina, surface smooth, without hairs, abaxial midrib ridge prominent and almost U-shaped; adaxial epidermis - single layered, regularly arranged rectangular cells on the lamina with highly thickened cuticle, slightly pointed small cells at the midrib region with thin cuticle, epidermis cells highly wavy on surface view; midrib - thickness 850 - 900 µm, 2 layers of collenchymatous hypodermis; leaf lamina 325 - 350 µm thick, slightly down curved margins; mesophyll - bifacial; palisade tissue - single layered, 70 - 75 µm thick, extended into the midrib from both lamina, chloroplast less than YFP; spongy tissue - transversely elongated, less compact, with 250 - 270 µm thick, crystal prisms single or in pairs, medium distribution; vascular bundle - bicollateral, open arc shaped, 12 -14 phloem patches above and below the xylem groups (14 - 16), 1 - 2 parenchyma cells separate each xylem group; ground tissue - round parenchyma, prismatic crystals towards peripheral region, druses towards inside, two rows of tangentially elongated cells below vascular tissues, abaxial collenchyma 2-layered; abaxial epidermis - single layered, smaller cells, thin cuticle, abundant paracytic stomata.

c) Leaf of WFP

Outline - adaxial surface slightly raised at the midrib region, lamina smooth, without hairs, abaxial midrib region projected, almost U-shaped; adaxial epidermis - single layered, regularly arranged, cuticle heavily thickened, highly undulate epidermal cells on surface view, slightly pointed cells at midrib region with thin cuticle; midrib - thickness
800 - 825 µm, 5 - 6 layers of collenchyma below the elevated region, leaf lamina 375 µm thick, margins revolute; mesophyll - bifacial; palisade tissue - single row, 90 µm, abundant chloroplast; spongy tissue - air chambered, 4 - 5 layered, 275 - 300 µm, druses frequent - in singles, pairs or groups of 3; vascular bundle - bicollateral, crescent shaped, arms extend beyond the level of 3 - 4 palisade cells on either side; ground tissue - round parenchyma, two rows of elongated cells below vascular tissue, 2 layers of abaxial peripheral collenchyma; abaxial epidermis - single layered with smaller cells and thin cuticle, paracytic stomata abundant; crystals and druses in lower ground tissue; laticifers - in minor veins and ground tissue.

2.3.2.3 Petiole anatomy (Plate 2.3.5)

a) Petiole of YFP

Outline - adaxial surface straight, abaxial convex, 1.13 × 1.08 mm thick, adaxial surface with 15 - 18 colleters of varying sizes in leaf axil; vascular bundle - bicollateral, C-shaped, arc shallow, more druses concentrated below the bundle and near the colleter tufts, latex present, prism crystals even in colleters; ground tissue - multilayered, rounded parenchyma; colleters - elongated parenchyma as epithelial cells, tapering with broad base and pointed apex, with chlorophyll, converged as a cone, with full of dust, persistent even after the senescence of leaves, seen as leathery purple coloured structure above the scar at each node.
Plate 2.3.5 Petiole anatomy

a-c) Nodal colleters of YFP, OFP and WFP (5 X) d) ground tissue with druses above and below the bicollateral crescent shaped vasculature in YFP (10 X) e) OFP petiole (10 X) f) WFP petiole showing colleter cells on the adaxial surface and bicollateral arc shaped vasculature in parenchymatous ground tissue (5 X).

b) Petiole of OFP

Outline - adaxial surface slightly raised, abaxial convex, 1.5 x 1.25 mm thick, margins rounded; outer epidermis with thin cuticle; vasculature - bicollateral, upright C-shaped, arms of arc more deeper than YFP, druses below the bundle; ground tissue - parenchymatous with 2 - 3 vein traces on either side of open bundle; colleters - converging as a cone, 15 - 20 numbers of varying sizes, small (125 µm long) and large (up to 1000 µm), arises directly from upper epidermal layer, rarely stalked, marginal ones broad and stout, intermediate ones long, narrow and tapering.
c) **Petiole of WFP**

Outline - adaxial surface slightly convex, 1.37 × 1.12 mm thick, semicircular to sub-globose, covered with single layered epidermis, thin cuticle on adaxial surface; vasculature - bicollateral, arc shaped; xylem in groups, up to 20, patches of phloem tissue on either side of xylem groups, curvature of arms deep; ground tissue - parenchymatous, caox crystals abundant, concentrated below the vasculature, arranged either in the form of rosettes (druses) or prism crystals of varying sizes, packed in single parenchyma cell, sands rare, laticifers and minor accessory bundles towards the wings; colleters - small to large, 15 - 24 colleters of varying length, middle ones elongated, narrow and tapering, marginal ones (1 - 2) blunt and stout with rounded ends, converging towards the centre.

2.3.3 Embryology

Embryological features of anther and ovary was evaluated and presented in separate headings.

2.3.3.1 Anther (Plate 2.3.6)

a) **Anther of YFP**

Bisporangiate, 4-loculed, locules longitudinally elongated; connective - massive, broad, parenchymatous, protruded into locule, so locules appeared ‘C’ shaped in cross section, vasculature in the center; epidermis - single layered, cells columnar; wall layers 3 - 4, stomium present, tapetum - uniseriate, glandular; microspore formation - in tetrads; pollen grains - numerous, immature anther contains two types of pollen grains, majority normal, regular grains, and few smaller.
b) **Anther of OFP**

Bisporangiate, young anther 4-loculed, when mature it becomes 2-chambered, locules longitudinally elongated; connectives - broad, parenchymatous, protruded into the locules, so appeared ‘C’ shaped in cross section, vasculature in the center; epidermis - single layered, narrow, thin walled and radially elongated; 3 - 4 wall layers, stomium present, endothecium fibrous, tapetum - uniseriate, glandular; microspores - in tetrads; two types, larger ones abundant, smaller ones less frequent, pollen grains - numerous.

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**Plate 2.3.6 Embryology of anther**

YFP showing a) 5 stamens of androecium joined together by apical hood b) inner anther locule with glandular tapetum (40 X) c) inner wall of anther locule showing fibrous columnar endothecial cells (40 X) d) T.S of mature anther (10 X) e) microspore mother cell at tetrad stage (60 X) f) dimorphic grains during development (10 X) g) equatorial view of pollen showing transversely elongated ectoaperture and surface ornamentation under LM (60 X) h) polar view showing three germ pores (60 X).
c) Anther of WFP

Anther - bisporangiate, 4-loculed, connective - parenchymatous, broad and protruded into locule, locules ‘C’ shaped, vasculature in the center; epidermis - single layered; wall layers - multilayered (2 - 3), stomium present, tapetum - uniseriate, glandular; microspores - numerous in tetrads; pollen grains - monads.

2.3.3.2 Ovary (Plate 2.3.7)

a) Ovary of YFP

Bicarpellary, 2-chambered initially, placenta enlarged and become 4-chambered during development, single ovule on each locule, funicle attached to the placenta on axial position; integument - unitegmic; chalaza broad, archesporial cells are deeply seated, ovules hemianatropous.

b) Ovary of OFP

Bicarpellary, 2 ovules in each carpel, placenta enlarged to become false septa, so 4-chambered, ovule attached to the placenta by means of funicle, the position of funicle is midway in the chamber, placentation axile, ovules hemianatropous, single per chamber; integument - unitegmic.

c) Ovary of WFP

Bicarpellary ovary with 2 ovules in each carpel, ovules - hemianatropous, placentation axile, the position of funicle is midway in the chamber; integument - unitegmic; embryo sac deep seated.
2.3.4 Palynology

Pollen morphological characterization based on features recognized in various magnifications of LM (10 to 100 X) and SEM (1100 to 1800 X) was studied on the characteristics of the aperture, exine stratification, grain size, shape, diameter, polar (P) and equatorial view (E).

2.3.4.1 Light Microscopy (LM)

In fully opened flowers, the pollen grains appeared as five sticky masses between the longitudinally dehisced anther lobes. LM studies revealed that grains were uniform in appearance, size and colour in all three morphovariant plants. Grains are non-sticky, well spread with
glycerin, polar view revealed 3-colpate nature and equatorial view showed transversely elongated aperture. Exine showed 10 - 12.5 µm thickness (60 X) with network stratification (100 X).

2.3.4.2 Scanning Electron Microscopy (SEM) (Plate2.3.8)

a) Pollen of YFP

Pollen grains 3-zonocolporate, colpus (aperture) transversely elongated, 22.66 µm long, margins straight and thickened, tapering with rounded ends; endoaperturate, ora quadrangular 6.50 × 5.02 µm, lolongate; exine ornamentation uniform throughout, foveolo-reticulate; 61.59 × 47.01 µm, P/E ratio – 131, subprolate.

b) Pollen of OFP

Pollen grains 3-zonocolporate, colpus transversely elongated (24.31 µm), ends of ectoaperture rounded, margins straight and thickened, tapering to blunt or rounded ends; ora (endoaperture) rectangular, 6.29 × 4.52 µm, lolongate; exine ornamentation uniform throughout, two types of sculptures - dimorphofoveolate (smaller and larger circular foveoles) and fossulate; 63.38 × 48.90 µm, P/E ratio - 129.6, subprolate.

c) Pollen of WFP

Pollen grains 3-zonocolporate, colpus 19.68 µm transversely elongated, ends broad and rectangular, margins wavy; ora quadrangular, 5.27 × 4.00 µm, lolongate; exine ornamentation not uniform, more in polar regions and less in equatorial regions, dimorphofoveolate; 53.93 × 46.39 µm, P/E ratio - 116.25 with subprolate shape.
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Plate 2.3.8 Palynology

SEM images of three morphovariants: a-c) polar view, equatorial view and foveolo-reticulate surface ornamentation of YFP d-f) polar view, equatorial view, dimorphofoveolate and fossulate surface ornamentation of OFP g-h) polar view, equatorial view and dimorphofoveolate surface ornamentation of WFP.

2.3.5 Phytochemistry

Presence of primary and secondary metabolites were detected in different plant parts like leaves, flowers, fruit rinds and seed kernels of three morphovariants. The extracts were prepared with different solvents using a soxhlet extractor. The results were compared between the same
organs of different plants, revealed that their intensity varied slightly in various fractions. A comparison of metabolite analysis of flower extracts is presented in Table 2.3.1, and those of leaf extracts in Table 3.3.8. A detailed phytochemical analysis is discussed in chapter 3.

### Table 2.3.1 Preliminary phytochemical screening of primary and secondary metabolites in flower extracts of three morphovariants

<table>
<thead>
<tr>
<th>Metabolite groups</th>
<th>Yellow Flower (YF)</th>
<th>Orange Flower (OF)</th>
<th>White Flower (WF)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PE</td>
<td>CH</td>
<td>EA</td>
</tr>
<tr>
<td>Primary metabolites</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbohydrates (Fehling’s test)</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Benedicts test</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Starch (Iodine test)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Proteins (Biuret test)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Secondary metabolites</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaloids (Marqui’s test)</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Dragendorf’s test</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Phenolic compounds (FeCl₃)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids (H₂SO₄ test)</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Tannins (FeCl₃ test)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Coumarins (NaOH test)</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Terpenoids (SK test)</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Steroids (LB test)</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins (Froth test)</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Cardiac Glycosides (KK test)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

PE - Petroleum Ether, CH - Chloroform, EA - Ethyl Acetate, MT- Methanol; SK - Salkowski test, LB - Liebermann Burchard, KK - Keller Kiliani; +++ appreciable, ++ average, + minimum, - absent.

Presence of carbohydrates in chloroform (CH) fractions of yellow (YF), orange (OF) and white (WF) flowers was confirmed by the appearance of orange red precipitate after fehling’s and benedict’s reactions. Fehling’s solution gave minimum (+) colour to YF and gave an average (++) colour to both OF and WF. The intensity was average (++).
in Methanol (MT) fractions of OF and WF than YF (+) with benedict’s solution. Iodine test gave negative results to all fractions of three colour variants, but proteins were detected in minimum (+) amount in the MT fractions after biuret test.

The intensity of colour produced by alkaloids after Marqui’s reaction was average (+++) in CH fractions of OF, but found minimum (+) in YF and WF samples. Likewise, phenolic compounds were present in average amounts in the MT fractions of OF and WF, but minimum (+) in YF. Terpenoids and cardiac glycosides in the ethyl acetate (EA) fraction of YF and WF also showed variation in intensity than OF. However, the saponin group was found absent (-) in the CH fraction of OF samples.

2.3.6 \textit{rbcL} gene sequencing

The \textit{rbcL} gene fragment isolated from the genomic DNA of tender leaves was used for the present analysis. Successful double stranded amplified DNA obtained after PCR analysis, showed that the length of the sequenced fragments of YFP, OFP and WFP were 525, 534 and 535 base pairs. The sequences were compared with \textit{Accession No: EU 916732.1} in the NCBI Genbank database using a BLAST search tool for pair wise comparison. The sample YFP showed 100 \% similarity (Fig. 2.3.1) to the records available in the database for \textit{T. peruviana}, whereas both OFP (Fig. 2.3.2) and WFP (Fig. 2.3.3) were 99 \% similar to the database records of the NCBI DNA databank, accessed on June 2012.
Consensus sequence data of YFP (525 bp)

```
CAATTGCTTTATATATCCCTGATACTCAGGAACTAAGATGATCTCTTTGGAATCT
TCCGAGTACTCTCTGACAACCAGAGGTTTCATCCACCCGAAGAGCAGGAGGGCTGTCGATGC
GAACTCTTCATCTGCACTTGACAGCTGCCAACTTGATGTAGCTGACCTACCGCTCTGAGC
TTAACAAAGGGCGGAGTCTACCAACATCGAAAACCCTGCTCGAGGAGGAGAATCATTATTG
CTTATGCTAGCTTAACCTTTTATCTTTGCTAAGGAAGGTTCTGTACTCAACTAAGTTTCT
TCATGTCAGGTGAATGTATGTTTCTATACCTGACATGGCTGTCGAGGAGATTTG
GCCAACTCCATAGCTTTATATATCTAAAACCTCTTCAGAGGCGCTCTCTAGTTAAG
AAGGAGATTAAATTTACACAATAATGCTGTCCTCCCTATTGTTGAGTACTAATACCTAA
TTGGGTTATCCGCTAAAAACACTCGTAGGGCAATTTAGATAATGCCTCTCCTGG
```

BLAST result of YFP

![BLAST result](image)

Result: based on the rbcL-PCR analysis, sample showed 100% similarity with *Thevetia peruviana*. (Accession No: EU916732.1).

Fig. 2.3.1 Consensus sequence data and BLAST analysis based on rbcL marker gene of YFP
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Consensus sequence data of OFP (535 bp)

```
GTTAGGCTACCAATTTGCACTTATATTACCTCGAATACGAAACTTAAGATACGTATGCCTCGTGC
ACATTCGCTAGTTCCCTACACCCGACTATGCTACGCAAGGAGATCACTTATACATTTACATAGG
TCGATTTGCTGTTCAAGCGTGGCTGCTGGAGATTGAGTAGTTTAATACGTATTTTGTTCTCTAT
TAAAACCTTAAAGGAGGCAATGCTAGTGTAAGGAGATGATATTAGAATGAGGTGAGGTGAGGTG
TGTCCCTTATGGGATGATGCTATATATTTTACATTTATTTATTTATTTATTTATTTATTTATTT
GCAATTTATGATGCTCTCGTG
```

BLAST result of OFP

Result: based on the rbcl-PCR analysis, sample showed 99% similarity with Thevetia peruviana. (Accession No: EU916732.1).

Fig. 2.3.2 Consensus sequence data and BLAST analysis based on rbcl marker gene of OFP
Consensus sequence data of WFP (534 bp)

```
GT7AAGGTAACAATTGACTTTATTACTCTCTGAGATTACGAAGACTAAAGATAGACTGAATCTTGGC
AGACATCCGAGATTACCTCCTCAACCCGAGATTCCCTACCCGAAAGAACGAGACGGCTGCTGTAAGTGC
GAAATCTCTACTGATAGACACTCGTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTG
AGGCGCATGCTACCATCCATAGCGACGCTTCGAGAGAAATGCAATTAGATTGCTATTGCTTTGGCTCCG
TTACCCCTTATAAGCCCTTTTGAAGAGGCTGTTACTAATCATGTTTCCATCCCTATGKGATAT
```

BLAST result of WFP

**Result:** Based on the `rbcL`-PCR analysis, sample showed 99% similarity with *Thevetia peruviana*. (Accession No: EU916732.1).

**Fig. 2.3.3** Consensus sequence data and BLAST analysis based on `rbcL` marker gene of WFP
2.4 Discussion

The Genus *Thevetia* was placed under the ‘dogbane family’ after its discovery (Linnaeus, 1753), as it possesses all major distinguishing features of Apocynaceae. The taxon can be easily identified due to the peculiar features like: presence of milky latex; narrow, lanceolate, alternate, densely crowded, glossy green leaves; large, showy, bright yellow, fragrant, bell shaped flowers with 5 sinistrally twisted petals; and fleshy, angular, depressed fruits developed in 10 - 15 ft small ornamental shrubs.

The present study focuses the biosystematics analyses of three plants with similar morphological features, for their resemblances and differences. Vegetatively, all three specimens are identical and possessed simple, alternate, dorsi-ventral lanceolate leaves (16.5 x 10.5 cm). The internodes are very short; inflorescence appears extra-axillary, born in the axils of sub-sessile leaves. Minor biometric variations observed among the specimens were possibly due to variations in environmental factors such as water availability, sunlight prevalence, soil texture and nutrient composition in their growing locality or due to some genetic reasons.

Morphology of yellow flowers has been described frequently (Subrahmanyan, 1995; Sambamurthy, 2005; Sharma, 2009; Gupta, 2012), but the descriptions of other two related specimens are found limited. The present study revealed that the floral anatomy of yellow flowers is identical to the orange and white flowers, and showed nearly 100 % resemblances in majority of characters. The taxa are bicarpellate, bisexual, hypogynous, actinomorphic and gamopetalous with epipetalous stamens, features similar to the other 21 genera of the family Apocynaceae studied by Sennblad *et al*. (1998). Apart from the regular descriptions of distinguishing features, a
thorough systematics of yellow flowers was provided by many taxonomists during their classification and cladistic analysis (Endress et al., 1996; 2007; 2014; Simoes et al., 2007).

Usually the flowers are blessed with fragrance, but *Thevetia* flowers vary differently in having this amazing peculiarity. The yellow flowers have a serene, sweet scent, but the orange flowers with a lesser intensity. Another variant, the white flowers grown in the same environmental conditions owed no fragrance at all, which could be considered as a significant taxonomic variation.

The most important character to be discussed is the structure and adnation of essential whorls rather than the non essential calyx and corolla. The main part of the proper corolla tube is post-genitally fused, which starts at an adnation point of two adjacent petals, some distance away from the base of the petals, and then proceeds basipetally. Using the broad anther lobe, the staminal unit adnate to the angular style head, and the prolonged connectives joined together post-genitally in an anticlockwise direction above the stigmatic head, up to the phase of flower opening. Just above each anther, 5 large coronary lobes protruding into the center with numerous coarse white marginal hairs, created 5 openings above the style head, through which pollinators could enter. Because of the latrose dehiscence and lobes of same anther were separated by broad connectives, pollen from adjacent anther lobes mix and appeared as 5 masses, with a foamy adhesive (Simoes et al., 2007).

Even though, the gynoecium of most taxa of Apocynaceae are usually referred as apocarpous (Sennblad et al., 1998), the two carpels
often congenitally fused for a short distance from the base and the upper part of the ovary remains apocarpous. The post-genital fusion of two parallel styles and the style head also remains incomplete. The style-head is the enlarged specialized product of the apical part of the carpel, with zones covered by secretory epithelium (Fallen, 1986); and the body of style-head with epidermis clearly differentiated into distinct regions specialized in the production of pollen transport adhesive or for receptive function (Simoes et al., 2007).

In all three flowers, both style ends are incompletely fused; and the uppermost parts of the stigmatic head remain free giving the appearance of a cleft at its distal end. This sterile apical part is conical (term ‘stigmata’ is used by Boiteau and Allorge, 1978), with its two lobes parallel and independent, not touching each other, and all the stigmata cells uniformly arranged on it.

The pistil head is 10-lobed and the receptive surface is directed obliquely downwards from margin, with a ring of unicellular elongated hairs distributed regularly in the upper part of clefts, in small tufts. Sennblad et al. (1998) called these specialized structures in the style head, as ‘apical hair wreaths’. The stigmatic area is located below the region of anther adnation and is sometimes situated in a ‘stigmatic hollow’, an annular invagination of the style head; the resulting collar, equipped with a lower wreath of hairs, functions as a ‘pollen scraper’ (Fallen, 1986). But, Schick (1982) demonstrated that the receptive region of the style-head is typically at the base, often beneath a membranous collar or wreath of longer hairs. The present study clearly demonstrated that the receptive
surface is located marginally, within another 2-cleft region of the style head (Fig. 2.3.1 g).

The infrastaminal appendages are post-genitally fused to the style head, according to Endress *et al.* (1996), which is identical in all studied flowers. Infrastaminal and suprataminal appendages are the two terms coined by Pichon (1948) to represent outgrowths of the lower corolla tube, below and above the staminal sector, that are homologous to the corolline corona (Nilsson *et al.*, 1993). At the base of the gynoecium, nectar lobes sometimes fused into an annulus encircling the ovary (Simoes *et al.*, 2007).

In the studied samples, the stigmatic complex and related characters are identical, except a slight variation noticed in the longitudinal fusion of two styles at its proximal and distal ends in OFP. The stigmatic head complex has prime significance because it is considered as one of the most important criteria in Apocynaceae-Asclepiadaceae demarcation (Endress and Bruyns, 2000).

Cross pollination is affected by insects with long proboscis or by humming birds. The hairs on the corolline corona (Fig. 2.3.1.c) and around the stigmatic complex (Fig. 2.3.1.d) are meant for effective entomophily. Ripened fruits carrying fleshy and spongy mesocarp are often dispersed by birds or bats. Nevertheless, all four ovules get fertilized to produce 4 - 1 seeds, with wings in the region of the micropyle covering it.

Matured fruits are brownish black and indehiscent. However, the fleshy mesocarp split apart minimally on its ventral suture without
exposing the stony sclerified endocarp. The fruit characters are also significant because, fruit wall or pericarp structure of some Apocynacean follicles (*Alstonia scholaris* R. Br., *Catharanthus pusillus* (Murr.) G. Don., *Catharanthus roseus* (L.) G. Don., *Holarrhena antidysenterica* (L.) Wall., *Ichnocarpus frutescens* L., *Strophanthus wallichii* A. DC., *Vallaris solanacea* (Roth.) O. Ktze., *Wrightia tinctoria* (Roxb.) R. Br., *etc.* have played much taxonomic and phylogenetic role in genera and species identification (Thomas and Dave, 1994). The fruit structure of all three taxa is similar with a negligible variation in the size of endocarp as well as the whole fruit.

The number of flowers developed in each inflorescence is almost similar in two specimens (YFP and OFP: 6 - 8), but a maximum of 8 - 10 blooms of different stages were observed in WFP, which is reflected in the fruit setting also (Fig. 2.3.2.a). This character is considered as another significant aspect in taxa demarcation.

Another major area analyzed for similarity studies among the three specimens is the internal characteristics. Anatomy of several taxa of Apocynaceae like *Forsteronia glabrescens* Mull. Arg., (Larrosa and Duarte, 2006), 3 *Himatanthus sps*: *H. sucuuba* (Spruce. ex Mull. Arg.) Woodson, *H. bracteatus* (A. DC.) and *H. stenophyllus* Plumel. (Ferreira *et al.*, 2009), *Mandevilla coccinea* (Hook.) et Arn. Woodson (Duarte and Larrosa, 2011), 2 *Aspidosperma sps*: *A. olivaceum* Mull. Arg. and *A. polyneuron* (Krentkowski and Duarte, 2012) has been worked out, and is used widely for comparing variations among and between species, genus and at tribe levels (Lens *et al.*, 2008; 2009).
Stem anatomy from different regions of three investigating plants showed characteristic Apocynacean features like peripheral phellogen, discontinuous ring of sclerenchymatous fiber sheath that encircled the bicollateral vasculature, caox crystals in various forms: prisms, druses and crystal sands, idioblasts with phenolic substances and branched non-articulated laticifers in the cortex and pith. All three samples possessed a similar type of architecture in cross section. Anatomy is of restricted value in distinguishing groups below the species level, because the differences between them are usually more quantitative rather than qualitative (Pandey, 2005).

Generally the bark of *Thevetia* is thin and delicate with grayish surface, longitudinal fine striae and elongated lenticels; and the young cortex becomes tangentially compressed as the stem ages. The entire periderm (< 200 µm) and cortical region (> 400 µm) of all three samples were more or less similar structurally and quantitatively. Abundant caox prisms of varying sizes were concentrated in the outer collenchyma region in all specimens. Another form of caox, the druses were present in the middle cortex in WFP, whereas both druses and crystal sands were common in YFP. Mostly, the chlorenchyma region has drusy forms of oxalate crystals, and sandy forms were seen in the inner cortical region. The sclerenchyma fibers located in the inner cortical region were loosely arranged in YFP and WFP, but the cells of the OFP were more or less isodiametric in shape and were bounded as a single unit. According to Datta and Datta (1977), these sclerenchyma fibers are of brachysclereid type. They are polyhedral and have heavily thickened, lignified walls with reduced lumen, in all samples.
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Vascular system has always been a key role in various levels of hierarchy during taxonomic disputes. The outer phloem zone of bicollateral vasculature is broader in OFP and the phloem parenchyma has rather the greatest amount of prisms compared to other two varieties.

There are several wood features that are uniform throughout Apocynaceae, such as simple vessel perforations, alternate vestured intervessel pits, and vessel-ray pits that are similar in shape and size (Lens et al., 2008). On the other hand, the combination of vessel grouping, vessel element length, axial parenchyma distribution, uniseriate ray frequency, multiseriate ray fusion, and laticifer occurrence indicated that the wood structure of Apocynaceae is phylogenetically relevant at the tribal level ‘Rauvolfoioideae’ (Lens et al., 2009).

Xylem wood of *T. peruviana* is semi-ring porous or diffuse porous with medium sized vessels (37.5 - 43.75 µm) usually solitary, radially paired, or in radial multiples of 3 - 9. In YFP, vessels are grouped in solitary or in pairs frequently, and radial multiples of 6 - 8 are frequent only in the early wood. On the other hand, groups of 6 - 9 vessels in radial rows are more common in OFP and WFP. Both tracheids and vessels possessed simple pits in their side walls. The end walls of vessels are simple, without any spiral thickenings. The protoxylem bore annular or spiral thickening; and scalariform thickenings were met in metaxylem elements. Longest vessels among the three samples were noted in WFP, and 2-seriate medullary rays were also found more in number than the others, the only character agreeable with Datta and Datta (1977) in differentiating the samples.
Interestingly, these investigators claimed that the three plants could be distinguished morphologically based on stem bark characters, and were considered as three separate varieties. The distinguishing characters observed by them were surface colour, nature of wrinkles, cork depth, abundance of latex cells, the extent of secondary phloem, phloem fiber length, ray-height and ray abundance.

Previous reports showed that *T. peruviana* possessed simple perforated vessels and banded marginal axial parenchyma, which is considered as a general character because many of the investigated genera (*Allamanda cathartica* L., *Cerbera floribunda* K. Schum., *Chilocarpus suaveolens* Blume., *Cyclocotyla congolensis* Stapf., *Lacmellea edulis* H.Karst, *Saba comorensis* (Bojer.) Pichon) possessed the same features, revealed in a detailed study of the wood anatomy 103 specimens of Rauvolfioideae belonging to 91 species of 50 genera (Lens et al., 2008). Anatomical peculiarities are of great usefulness for diagnostic purposes, and enable one to distinguish even smaller samples of species from adulterants, from the pharmacological point of view (Lohar, 2007).

Wood of several members of this family has of less economic value. *Thevetia* wood is reported to make tool handles in Ghana and Uganda (Schmelzer, 2008), which reflects its hardness and toughness to insects, pests, weather conditions, and handy nature due to limited secondary growth.

The vasculature from the stem extends to the leaves, leaving gaps in the cortex as leaf traces. In general, anatomical characters of the leaves of all three samples comprised of regular adaxial epidermis with thick cuticle, mixtures of abundant paracytic and rare anomocytic stomata on abaxial epidermis, conspicuous and slightly convex midrib with a
bicollateral vasculature, dorsi-ventrally oriented mesophyll, adaxial and abaxial collenchymatous hypodermis, laticifers, druses and prism crystals in spongy mesophyll and ground tissue.

Stomatal characters having taxonomic significance are morphology, ontogeny, number, arrangement of subsidiary cells and their relationship with other epidermal cells (Sharma, 2009). In general, the subsidiary cells recognized in the family are paracytic; and anomocytic stomata are also fairly common (Omino, 1996). The cuticular preparations revealed the hypostomatic nature and the occurrence of high frequency of paracytic stomata with scanty anomocytic ones, was supported by previous reports of Watson and Dallwitz (1992). On the other hand, leaves with wavy epidermal cells with a moderate frequency of anisocytic (Doshi et al., 2012) or anomocytic stomata (Pongpijid et al., 2011) was also reported in Thevetia. Various investigators (Pongpijid et al., 2011; Korkijthamkul et al., 2013) differentiated T. neriifolia with Nerium oleander by noticing oblong leaf shapes, wavy epidermal margins, anomocytic stomata and rosette aggregates of caox crystals.

The leaf epidermal cells showed variations in size, shape and wall architecture, in the studied samples. The cells are polygonal, axially elongated, with slightly wavy anticlinal walls in YFP, whereas the walls are strongly undulate in both OFP and WFP. The undulations are consistently more pronounced in shade leaves, and according to Watson (1942) the epidermal wall undulation is affected by light intensity which inhibits the gene for waviness. Similarly, the intensity of brightness due to chlorophyll pigment also varied with environments, although all samples contained single layered palisade tissue of 60 - 75 µm thickness. Besides,
variations were also noticed in the thickness of the leaf lamina, which has not proved to be reliable in distinguishing the species as it also depends on the environment, texture and the size of the leaves (Omino, 1996).

Omino (1996) further opined that cuticular striations, stomatal outline, size and shape of epidermal cells have more taxonomic value than leaf margin, stomatal density, lamina thickness and minor veins, after the meticulous effort on anatomical characterization of 77 species under 30 genera of Apocynaceae. Consequently, he confidently separated many species on the basis of their leaf anatomy; and many genera are confirmed to be closely related. The leaf architecture of several taxa of ‘dogbane family’ has been worked out by many investigators (Inamdar et al., 1975; Mohan and Inamdar 1982; El-Kashef et al., 2015).

Another distinguishable feature connecting the samples is the shape of bicollateral vasculature in the midrib region. The crescent arms are a little wider in YFP, but somewhat shallow in OFP and WFP in most conditions. Likewise, the outline of the midrib region also makes YFP different from OFP and WFP, in that the former has slightly pointed convex abaxial side and the latter two has more or less rounded to conical outline. More specifically, Omino, (1996) pointed out that midrib outline and thickness, shapes of the vascular bundle and phloem arrangement in the midrib and petiole has some diagnostic value in distinguishing taxa at the species level. So, these characters are given lesser significance in the present study.

If all leaves are morphologically similar, the principal differentiating structure is the organization of the vascular bundles in the petiole, which provided a taxonomic resolution in the four species of the genus Aspidopserma Mart. & Zucc. (A. carapanauba, A. desmanthum, A. excelsum
and A. spruceanum) (Reis et al., 2013). The petiole of Thevetia is sub-globose in outline, with a bicollateral crescent bundle. The arms of vasculature showed shallow to deep curvature. Similar variations in epidermal outline and orientation of arms of the vasculature were also noticed in the leaf midrib. In YFP, the adaxial surface is somewhat straight and mostly arms of arc are widened. But, the upper surface is more raised in WFP than OFP, and the xylem arms have slightly closer ends. Moreover, caox prisms and druses were located below the vasculature region in all specimens, but their concentration was more in WFP and OFP than YFP in different regions of the ground tissue. Crystal location has been given less taxonomic significance in this study, because variations in location were noticed during repeated assessment of plant samples (stem, leaf and petiole) of all three taxa.

One of the common features of Apocynaceae is the presence of colleters in leaf axils, located very close to stem axis. They are observed in all samples, and their number varied from 12 - 20 in each leaf axil, either with blunt or pointed ends. These are devoid of vasculature, and small rectangular caox crystals were also noticed in the parenchyma of colleters. The colleters located centrally in each tuft are unequal, elongated, narrow and tapering with pointed ends, and almost similar types were observed in YFP, OFP and WFP. Apart from this, WFP possess 1 - 2 short, stout colleters with blunt ends at both lateral margins of the tufts, whereas they are blunt and longer with acute ends in OFP. No such types were observed in YFP samples.

In Apocynaceae, colleters normally appear in variable numbers, as small to conspicuous and conic to deltoid appendages. Each node of
Odontadenia lutea (Vell.) Markgr. bear 68 - 80 colleters; and the crystals were observed even in the parenchyma of the axillary colleters (Martins, 2012). In addition to the secretory appendages in the leaf petiole (Fjell, 1983), numerous calycine colleters were also noticed in Thevetia by Endress et al. (1996) and Sennblad et al. (1998).

Colleters are finger-like, multicellular glands, commonly occurring in the axils of the leaves and sepals in the families of Gentianales (Wagenitz, 1992). In the Apocynoideae s.l., leaf axil colleters may occur independently, sometimes at interpetiolar or intrapetiolar positions (Sennblad et al., 1998). These secretory structures are also seen attached to other organs like stipules, bracts, bracteoles, calyx and corolla to protect the developing meristem by secreting a viscous fluid. Laticifers and vasculature are present in many colleters of Apocynaceae (Thomas, 1991). Internally, all colleters consist of a central core of parenchymatous cells surrounded by radially elongated epithelial cells, covered externally by a cuticle (Appezzato-Da-Gloria and Estelita, 2000). Many genera like Allamanda neriifolia L., Thevetia peruviana Juss., Vinca minor L. (Fjell, 1983), Mandevilla illustris (Vell.) Woodson, M. velutina (Mart. ex Stadem.) Woodson (Appezzato-Da-Gloria and Estelita, 2000) and Odontadenia lutea (Vell.) Markgr. (Martins, 2012) are characterized by colleters on the adaxial face of the leaves. Nodal colleters were found in all studied 31 species, spread over 15 genera of Apocynaceae, except the genus Aspidosperma, which may be interpetiolar, intrapetiolar or occur uniformly occurring along the node, and Simoes and Kinoshita (2002) considered that their number and disposition are of great taxonomic value.
Another chief character of Apocynaceae is the presence of non-articulated laticifers. These are of thin-walled, elongated, branched and multi-nucleated tuber-like structures with milky latex, normally scattered throughout the cortex and ground tissue. They are located on both sides of the vascular strands in Allamanda neriifolia L. and T. peruviana Juss., while they are unbranched and confined to the abaxial side of the veins in Vinca minor L. (Fjell, 1983). Probably the laticifers being a secretory structure, use latex to seal the wounds to provide a physical defense (Dussourd, 1990), and the lipid nature of the latex secretion may inhibit the proliferation of microorganisms and protect the plant against the attacks from herbivores (Fahn, 1979). Generally, laticifers are situated in primary cortex, pericycle, phloem and pith cells (Omino, 1996), and are distributed all over the plant body at all stages of development, with proteolytic enzymes, dehydrogenases, isozymes and carbohydrates (Rao and Malaviya, 1965). Laticifers are mostly narrower than the surrounding cells; they ramify throughout the mesophyll, commonly following the veins. It is very difficult to identify them in cross section, but their position can be easily located by the hand puncturing of the whole leaf and making incisions in different parts of stem and petiole.

Members of Apocynaceae are characterized by the presence of calcium oxalate crystals either as prism or druse assemblage, or as crystal sands. In all the three studied organs, i.e. stem, leaves and petiole, their presence was observed greatly. They are more prominent in juvenile stem and their number and position vary as the plant ages. Large rectangular or rhomboidal prisms (18 × 4.8 µm) are well distributed in the outer stem cortex; prisms and druses in the middle cortex, and crystal sands are
frequent in inner cortex and pith region. Likewise, petiole is characterized by abundant prisms, druses and occasional sands in ground tissue, but leaf mesophyll is characterized by druses in singles, or groups of 2-3. These crystals can be located in specific tissues such as epidermis, cortex, phloem, xylem and pith or they may be distributed all over the plant (Konyar et al., 2014), and even in the periderm with thin or sclerified walls (Metcalf and Chalk, 1950). Both drusy and prism forms were reported in the leaves of Nerium oleander L., Strophanthus gratus Franch. and Thevetia peruviana (Pers.) K. Schum. by Pongpijid et al. (2011), but in Allamanda neriifolia L. and T. peruviana Juss., they differ conspicuously from each other both in shape and location, according to Fjell (1983).

Most of the crystals found in plants are composed of caox, which vary in shape, either simple prism shaped crystals or numerous prisms clustered into a single spiky body called the druses (Fry, 2003). Despite the shape, size and number that showed variations among taxa, they are grouped into five based on their morphology as prisms, druses, styloids, raphides and crystal sands (Webb, 1999). In plants, various physical, chemical and biological parameters such as light, temperature and pH possibly affect their location, size and other properties (Franceschi and Horner, 1980; Kuo-Huang et al., 2007; Meric, 2009), even though they are under genetic control (Ilarslan et al., 2001). Various functions are attributed to these crystals such as ionic balance, as a mean of removing oxalates, storage for calcium, structural supports or as a protective device (Franceschi and Horner, 1980). Crystal formation is usually associated with membranes, chambers or inclusions found within the cell vacuoles and the vacuole membranes may act to mold its shape (Franceschi and Horner, 1980).
Prismatic crystals and druses in the stem and leaves of *Rauvolfia sellowii* Mull. Arg. (Baratto et al., 2010), *Aspidosperma olivaceum* Mull. Arg., *A. polyneuron* Mull. Arg. (Krentkowski and Duarte, 2012), *Nerium oleander* L. (Franco et al., 2012; Konyar et al., 2014) and *Pachypodium lamerei* Drake. (El-Kashef et al., 2015) were reported; and their location, size, shape and number within a taxon are considered as a very specific taxonomic character by Genua and Hillson (1985), Prychid and Rudall (1999) and Lersten and Horner (2000).

The present investigation established that specimens OFP and WFP were highly concentrated with caox than YFP. The large rectangular single prisms are concentrated towards the periphery of stem and petioles, while sands and drusy assemblages either in rosette form or in clustered forms located deep in the inner cortex or ground tissue. The location and concentration of caox, in any form, varies in the same organ of the same plant from time to time, so it will be of little value in diagnosing their location as one identifying feature among the three morphoforms.

Apart from the above discussed disciplines, embryological features of both anther and pistil are useful to tackle many disputed situations, but only a few contributions to the family are available, and even today related studies remains scanty. In the early 19th century, noteworthy contributions in this field were made by Fyre and Blodgett (1905), Anderson (1931), Meyer (1938) and Rau (1940) on various family members.

The major resemblances noticed during embryological studies are: tetrasporangiate anther with broad connective, single layered glandular tapetum, tetrahedral pollen arrangement, few disintegrating microspores
and appearance of mature grains in monads. Among the pistil characters, most important structures are: 4-loculed ovary with single ovule per locule and axile position of hemianatropous ovule with unitegmic integument. No differences were observed among the studied samples, both in the structure of mature anther and ovary. A related study was quoted by Devi and Narayana (1975) between the cultivars of Nerium indicum Mull. with few embryological differences.

In several genera of Apocynaceae viz Alstonia scholaris R. Br., Thevetia neriifolia Juss. (Meyer, 1938), Cerbera odorollam (Rau, 1940), Carissa spinatum Linn., Catharanthus roseus (Linn.) G. Don, Holarrhena antidysenterica Wall., Rauwolfia serpentine L. Benth ex. Kurz. (Devi, 1971; 1974) and Trachelospermum fragrance Hook. (Sud, 1984), uniseriate parietal tapetum has been reported. Orbicules, a general character of Apocynaceae, located in the inner layer of anther locules were small, irregular, angular and folded in Thevetia bicornuta Mull. Arg., a character revealed in the SEM analysis by Vinckier and Smets (2002). Studies reported by Cousin, (1979) in Vinca rosea L. emphasized that the tapetal cells are of secretary type and are bounded by an acetolysis-resistant ‘pellicule’ sprinkled with ubisch bodies. The observed degeneration of a smaller number of undersized microspores, were presumably due to the failure of secretory tapetum to provide full nourishment to all the developing spores in the sporangium (Sud, 1984). In most of Apocynacean members, the tapetum observed is glandular type (Sud, 1984).

In T. peruviana, according to Indian taxonomists (Subrahmanyan, 1995; Verma, 2011) the ovules were axile in position; but the development of funicle on parietal position was reported by Endress et al. (1996).
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Presently, the funicle is attached to the placenta on the midway of the extended false partition and at right angle to the placental axis, clearly visible in cross sections. This type of hemianatropous and unitegmic ovules were present in *Trachelospermum fragrans* Hook. f. (Sud, 1984), and according to him the chalazal megaspore develops into 8-nucleated embryo sac of polygonum type, a usual character shown by the family members.

In an extensive study of microsporogenesis of *Rauvolfia serpentina* (L.) Benth. ex Kurz., Ghimir *et al.* (2011) noticed tetrasporangiate anther, dicotyledonous type of anther wall formation, occurrence of both successive and simultaneous cytokinesis during the meiosis of microspore mother cells, uninucleated and highly vacuolated glandular tapetum, tetragonal and decussate pollen tetrad and three-celled mature pollen grains during the shedding hours.

Pollen grains, the micro male unit of angiosperms has superior and significant role in plant taxonomy, phylogeny and evolution. Among various morphological characteristics, aperture features like structure, number, position and distribution were given prime importance; whereas exine ornamentation, pollen shape and size were of secondary and tertiary significance (Tewari and Nair, 1979).

Pollen identification is the foundation of palynological studies. Pollen morphology, which has not previously played much role in tribal delimitation, was shown to be the most useful morphological character for delimiting Alyxieae from other tribes of Rauvolfioidae (Endress *et al.*, 2007). Likewise, Verhoeven and Venter (2001) provided useful information
of pollinium and its wall structure in distinguishing the three subfamilies of Apocynaceae, namely Periplocoideae, Secamonoideae and Asclepiadoideae.

All three samples possessed striking dissimilarities in primary and supplementary characters. Basically, grains are 3-zonocolporate with transversely placed colpus, lolongate endoaperture and subprolate shape. The remarkable difference observed in the primary character was in the structure of ectoaperture in WFP, which have rectangular ends with wavy colpus margins. The other two forms (YFP, OFP) bore ectoaperture with blunt to rounded tapering ends, which exhibit significantly a lesser amount of palynological diversity. Regarding surface ornamentation, YFP has foveolo-reticulate; OFP has dimorphofoveolate and fossulate, whereas WFP has only dimorphofoveolate exine, another major point of taxa delimitation.

The present results agreed with previous reports in some basic features, like aperture number, position and symmetry. However, all three samples have subprolate shape with different surface stratification after SEM analysis of fresh acetolysed samples. The grains were reported as oblate-spheroidal (Korkijthamkul et al., 2013) with micro-reticulate (Endress et al., 1996; Vinckier and Smets, 2002) or micro-rugulate ornamentation; and elliptical pores having large annulus (Devarkar, 2011), besides similar features of isopolar, 3-colporate, radially symmetrical conditions. Numerous randomly spaced fissures were observed inside the exine, which is without a mesocolpial depression (Endress et al., 1996). Even, pollen grains of T. nerifolia collected from Lagoon swamp sediments were identified by lolongate colpus and reticulate surface, only because of the conservative nature of hard and tough outer wall, the exine (Adekanmbi and Ogundipe, 2009).
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The size of pollen grains of a taxon may vary naturally, i.e. within flowers or between individual plants, and also affected by means of preservation, chemical treatments or the degree of pollen maturity (Kodela, 2006). In prolate grains, the polar axis is greater than the equatorial axis and according to the degree of eccentricity (Erdtman, 1966), the grains of *Thevetia* appeared subprolate. Similarly, polar images of both LM and SEM clearly illustrated that all grains have radiosymmetric apertures; as they are arranged equidistantly and meridionally around the equator, if colpi are present (Moore and Webb, 1978). The chemically resistant outer layer of exine primarily composed of sporopollenin gives the characteristic sculpturing pattern to the grains. Nature of apertures can also vary, including the degree of ‘openness’ of colpi, or whether pores exceed the colpi margins due to ‘tearing off the wall’ in some tricolporate grains (Kodela, 2006).

On the basis of diversity of endoaperture, aperture number and pollen grain size, four types of pollen grains were distinguished among the 42 species of *Alstonia*. LM and SEM studies had shown that colporate grains are 2-aperturate (*Alstonia angustiloba* Miq.) or 3-aperturate (*A. scholaris* (L.) R. Br.) with rounded endoaperture, while the other two types (*Alstonia angustifolia* Wall. ex G.Don, *A. neriifolia* D.Don.) possess H-endoapertures (Kuijt and Ham, 1997). Pollen with harmomegathically inactive apertures and more or less flexible mesocolpium centers seems to be the case in all four pollen types within *Alstonia* (Kuijt and Ham, 1997). When comparing this literature with the present study material, *T. peruviana* goes one step ahead, because the same species possess pollen with rounded endoaperture and H-endoaperture, which can be considered as a tendency towards a separate ranking in the taxonomic hierarchy.
The taxonomic assessment of an investigated species is possible considering the pollen parameters such as polar axis, average diameter of lumen and muri, length of mesocolpium, width of colpus, form of lumen, shape of colpi and P/E ratio, all are valuable diagnostic features in species delimitation (Keshavarzi et al., 2012). Likewise, variation in pollen colour, pollen kitty, the biochemical nature, function and distribution of the surface coatings of pollen grains in some groups could be extremely valuable in understanding taxonomy and evolution (Ferguson, 1985). The similarities of exine stratification in unrelated groups, and the differences appeared in closely related groups have been assumed as the adaptive characters associated with flower pollinators (Ferguson and Skvarla, 1982). However, the main morphological characters are, as a rule, remain constant within the individual genera as may be seen from 3-corporate nature of 13 sps. of Cabucala, 6 sps. of Carissa, 4 sps. of Catharanthus and 12 sps. of Landolphia of Apocynaceae (Erdtman, 1952).

The architecture of aperture characteristics as well as exine stratification will help to distinguish the grains among the three flower colours. It is clear that pollen characters alone are insufficient to reconstruct phylogenetic relationships within the genus, but considering the overall morphological cleanness that characterizes the genus, the variation of pollen is important for future taxonomic revisions at the species level (Welsh et al., 2010).

Pollen will play an increasingly significant role in various taxonomic levels, hence these pollen characters will benefit enormously in the future from a systematic point of view. Pollen structure and morphology provides a significant contribution to the systematics at the
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tribe, generic and specific levels, ascertain the need for a pollen database, in understanding structural and functional homology of grains.

Genera of the family Apocynaceae exhibit palynological extremes indicated by an array of shapes, aperture character, size and ornamentation. Studies conducted in different genera like *Allamanda cathartica* L., *Alstonia booveni* De willd., *Alstonia scholaris* (L.) R. Br., *Catharanthus roseus* (L.) G. Don., *Nerium oleander* L., *Parsonsia straminea* (R. Br.) F. Muell, *Plumeria alba* L., *Rauvolfia vomitoria* (Afzel), *Tabernaemontana divaricata* (L.) R. Br. and *Thevetia neriifolia* Juss. have showed that the grains are mostly 3 to 4-colporate, isopolar, radially symmetrical with micro-reticulate, psilate, granulate to shallowly regulate exine sculpturing (Campo et al., 1979; Cousin, 1979; Kodela, 2006; Adekanmbi and Ogundipe, 2009; Pritha et al., 2014).

Classification based on morphology combined with molecular information is getting popular from two decades back, and now most widely accepted elsewhere (APG, 2009; Endress et al., 2007; 2014). The level of genetic variability and relationships among the genera and species using genetic markers like randomly amplified polymorphic DNA (RAPD) was extensively used for understanding phylogenetic relationships.

After DNA amplification of the selected region by PCR analysis, the obtained *rcbL* DNA sequences were compared with the gene sequences of *Thevetia peruviana* in the NCBI database. The ‘YFP’ sample sequences showed cent percent similarity to the one (*T. peruviana*) available in the DNA databank, means both plants are identical, whereas ‘OFP’ and ‘WFP’ showed only 99 % similarity with databank sequences. One percent difference observed at the genetic level is presumed to be reflected in various
phenotypic levels. A number of variations observable between samples, especially palynological characters, were considered as genetical and not environmental.

The banding sequences obtained showed a high genetic similarity or a low genetic diversity among the three samples. The higher similarity at the infraspecific level has also shown that all the samples are monophyletic, so rbcL genome sequences can be used for the evaluation of genetic variation both among and within species (Mahmood et al., 2011). The systematic status of *T. peruviana* under the subfamily Rauvolfioideae based on morphological characters was confirmed by Mahmood et al. (2011) using RADP markers. To elucidate deeper relationships within Rauvolfioideae (Apocynaceae), a phylogenetic analysis of 66 taxa, combined with morphology was conducted using sequences from five DNA regions of the chloroplast genome including *rbcL* sequences (Simoes et al., 2004; 2007).

Many taxa of Apocynaceae at generic level (*Alstonia scholaris, Catharanthus roseus, Nerium oleander, Rhazya stricta* by Ibrahim et al., 2014) and species levels (117 samples of *Asclepias* s.l. by Fishbein et al., 2011; 81 species of *Hoya* by Livia et al., 2011) have been evaluated using chloroplast genome, since its DNA can serve as a main source of information for phylogenetic analysis (Small et al., 2005).

‘Albus’ and ‘aureus’ are the two specific epithets linked with white and golden yellow colours. Earlier taxonomists used these terms descriptively to identify different colour variants of the same species, which are morphologically similar in the majority of the characters. Specific epithet, the second part of the scientific name, usually a
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descriptive adjective indicates the colour of the plant or plant part, eg. *alba* for white, *flava* for yellow and *nigra* for black (Subrahmanyan, 1995).

The International Code of Botanical Nomenclature recognizes twelve main ranks in the hierarchy with kingdom at the top and species at the base, and three infraspecific categories below the rank of the species: *viz.* subspecies (*ssp.*), variety (*var.*) and form (*forma*, plural: *formae*). If a greater number of ranks of taxa are desired, the terms for these are made by adding the prefix ‘sub-’ to the terms denoting the principal or secondary ranks: species, subspecies, variety, sub-variety, form and sub-form (IAPT, 2011). Of these, subspecies, variety and form have been widely used in literature (Singh, 2010).

The category forma, abbreviated as ‘f.’ is a trivial variant of sporadic specimens that shows distinctive variation in one or two characters (Pandey and Misra, 2008; Mukherjee, 2014); and they may be produced genetically or environmentally (Warren and Wagner, 1984). From the present study, it can be ascertained that both OFP and WFP are genetically created, and their DNA showed 1% difference from the YFP. So, it can be inferred that the original ancestor is YFP, from which mutants for flower colouration were created for phenotypic differentiation; so more variants can be expected during the course of evolution.

The species levels are usually represented with binomial nomenclature. With the development of ‘Biosystematics’ studies, when more than one infraspecific taxon is present, a trinomial or tetranomial form of nomenclature is followed with all described variants ascribed to that binomial (Naik, 1984). Since most of the garden cultivars with various
colour blossoms are recognized only with species names, it will be apt to rank them systematically at various infraspecific levels, adding appropriate connecting terms indicating the rank with suitable trinomial epithets.

2.5 Conclusion

Flora and fauna of the universe are becoming increasingly complex. More and more information is gathered for the last few decades due to the advancement in technology, so it is relevant to re-evaluate the old status of each taxon, by incorporating a greater amount of characters to assess the changes that would have happened during the path of succession. The present studies highlight the need for a major restructuring of classification below infraspecific level, and make it to be a part in the future taxonomic revision.

Morphological data are still the foundation of most of our classification today, although molecular data (DNA) are becoming increasingly more important at all levels of the hierarchy (Stuessy, 2008). More than that, data from anatomy and palynology also appeared as a major criteria by providing additional information in delimiting taxa than phytochemistry, since minute variations in the chemical constituents may expect from plant to plant from the same geographical location. Diversity observed among the morphovariants are consolidated in Table 2.5.1. A recap of the major observations noticed are:

- Gross morphological variations noted in the overall size of leaves, flower and fruits are regarded as negligible ones due to environmental factors. Corolla shades, of course, are of prime importance in taxa differentiation.
• Anatomical features such as colleter characters, vessel grouping in xylem wood, length of vessels and abundance of calcium oxalate crystals are the major distinguishable features; whereas the architecture of leaf epidermal cells, midrib outline, the vasculature shape of the leaf and petiole, the location of oxalate prisms, druses and crystal sands in different cells and tissues and quantitative internal features are considered less significant.

• No variations were observed in the embryology of anther and ovary structure. The characters common to morphovariants are: the tetrahedral microspores, glandular tapetum, hemianatropous and unitegmic ovule with axile placentation and single ovule in each locule.

• The 3-zonocolporate, subprolate grains have lolongate endoaperture in all samples. However, colpus ends, colpus margins and varied foveolate-fossulate-reticulate ornamentations are highly significant in relation to taxonomic delimitations.

• Chief secondary metabolites screened in leaf, flower, rind and seed kernel extracts of YFP, OFP and WFP were alkaloids, flavonoids, terpenoids, steroids and cardiac glycosides. The qualitative variations observed were primarily due to natural environmental conditions.

• BLAST analysis of genetic material showed 99 % similarity of OFP and WFP, whereas YFP was 100 % similar to *Thevetia peruviana* rbcL sequences deposited in NCBI database, which indicates that the YFP was originated in Peru in South America, and the other two are paraphyletic garden mutants or variants formed spontaneously during the course of time.
Table 2.5.1 Diversity among three morphovariants

<table>
<thead>
<tr>
<th>Characters</th>
<th>YFP</th>
<th>OFP</th>
<th>WFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distribution</td>
<td>Common</td>
<td>Rare</td>
<td>Rare</td>
</tr>
<tr>
<td>Flower colour</td>
<td>Yellow</td>
<td>Peachy orange</td>
<td>White</td>
</tr>
<tr>
<td>Fragrance</td>
<td>Sweetly fragrant</td>
<td>Mild fragrance</td>
<td>Non-fragrant</td>
</tr>
<tr>
<td>Inflorescence</td>
<td>Usually 6 - 8 flowered</td>
<td>6 - 8 flowered</td>
<td>Mostly many flowered (8 - 10)</td>
</tr>
<tr>
<td>Calcium oxalate Concentration</td>
<td>Less abundant in stem cortex, pith and petiole ground tissue</td>
<td>More abundant in stem cortex, phloem, pith and petiole ground tissue</td>
<td>More abundant in stem cortex, pith and petiole ground tissue</td>
</tr>
<tr>
<td>Xylem Vessel Length</td>
<td>Medium</td>
<td>Medium</td>
<td>Shorter and longer</td>
</tr>
<tr>
<td>Vessel Grouping</td>
<td>Single, doubles, and radial multiples concentrated in some areas</td>
<td>Radial multiples of 3-7 abundant than singles and doubles</td>
<td>Radial multiples of 3-7 abundant than singles and doubles</td>
</tr>
<tr>
<td>Protoxylem Thickening</td>
<td>Annular, Scalariform or reticulate</td>
<td>Annular or scalariform</td>
<td>Annular or scalariform</td>
</tr>
<tr>
<td>Leaf Epidermis</td>
<td>Angular with slight wavy anticlinal walls</td>
<td>Cells with strongly undulate anticlinal walls</td>
<td>Cells with strongly undulate anticlinal walls</td>
</tr>
<tr>
<td>Nodal Colleters</td>
<td>Elongated and narrow</td>
<td>Marginal ones (1-2) stout, blunt with an acute apex</td>
<td>Marginal ones (1-2) stout, short with blunt apex</td>
</tr>
<tr>
<td>Colpus Margin</td>
<td>Margins broad and tapering</td>
<td>Margins broad and tapering</td>
<td>Margins wavy, straight and broad</td>
</tr>
<tr>
<td>Colpus Ends</td>
<td>Rounded</td>
<td>Blunt to rounded</td>
<td>Rectangular</td>
</tr>
<tr>
<td>Pollen Ornamentation</td>
<td>Uniform throughout, foveolo- reticulate</td>
<td>Uniform throughout, dimorphofoveolate and fossulate</td>
<td>Less in equatorial region, dimorphofoveolate</td>
</tr>
<tr>
<td>DNA similarity with <em>T. peruviana</em> in ncbi database</td>
<td>100%</td>
<td>99%</td>
<td>99%</td>
</tr>
</tbody>
</table>
Chapter 2

To conclude, it is established that YFP is the original taxon represented by the binomial *Thevetia peruviana*, and the other two variants are recommended with trinomial names. The taxon OFP has given the status ‘sub-variety’ as: *Thevetia peruviana* subvar. *aurantiaca*; and WFP must be upgraded to the varietal hierarchy with the nomenclature: *Thevetia peruviana* var. *alba*, based on the above observations.

A taxonomic key for taxon differentiation is given below:

Distribution common, nodal colleters narrow and elongated; pollen wall foveolo-reticulate; DNA shows 100% similarity……..1. *Thevetia peruviana*

Distribution rare, nodal colleters elongated and blunt; pollen wall dimorpho-foveolate; DNA shows 99% similarity………………………..2.

2a. Inflorescence few flowered; corolla orange with mild fragrance; marginal colleters stout and pointed; pollen ornamentation throughout the surface, fossulate type present, colpus margin broad, ends rounded and tapering ……………………………*Thevetia peruviana* subvar. *aurantiaca*

2b. Inflorescence many flowered; corolla white without fragrance; marginal colleters stout and blunt; pollen ornamentation less in equatorial surface, fossulate type absent, colpus margin wavy, ends rectangular and straight …………………………………………………..*Thevetia peruviana* var. *alba*