Comparative Leaf Blade Anatomy and Genetic Relationship by iPBS Marker of *Clerodendrum* L., *Rotheca* Raf. and *Volkameria* L. (Lamiaceae) in Thailand

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ กายวิภาคเปรียบเทียบแผ่นใบและความสัมพันธ์ทางพันธุกรรมด้วยเครื่องหมายไอพีบีเอสของพืชสกุล *Clerodendrum* L. *Rotheca* Raf. และ *Volkameria* L. (Lamiaceae) ในประเทศไทย

ผู้เขียน นางสาวฟีนยา พุ่มประเสริฐ

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บทคัดย่อ

การศึกษากายวิภาคเปรียบเทียบแผ่นใบและความสัมพันธ์ทางพันธุกรรมด้วยเครื่องหมายไอพีบีเอสของพืชสกุล *Clerodendrum* L. จำนวน 21 ชนิด และ 1 พันธุ์ *Rotheca* Raf. 2 ชนิด และ *Volkameria* L. 1 ชนิด ในประเทศไทย โดยสำรวจและเก็บรวบรวมตัวอย่างพืชจากประชากรในธรรมชาติของประเทศไทย ระหว่างเดือนมกราคม พ.ศ. 2558 ถึงเดือนมีนาคม พ.ศ. 2560 ศึกษาลักษณะทางกายวิภาคโดยใช้เทคนิคพาราฟิน การลอกผิวใบ และการศึกษาด้วยกล้องจุลทรรศน์อิเลกตรอนแบบส่องกราด ได้จัดทำรูปวิธานจำแนกสกุลและชนิด คำบรรยายลักษณะกายวิภาคแผ่นใบในแต่ละชนิด รวมทั้งภาพแสดงลักษณะกายวิภาคที่สำคัญ โดยพืชทั้งสามสกุลมีลักษณะกายวิภาคของแผ่นใบโดยทั่วไปดังนี้ พืชสกุล *Clerodendrum* และ *Rotheca* มีรูปร่างเซลล์แบบไม่สมมาตร (รูปร่างคล้ายจิ๊กซอว์) ผนังเซลล์ด้านตั้งฉากกับผิวหยักเป็นคลื่น มัดท่อลำเลียงแบบเคียงข้างเรียงตัวเป็นวงกลมที่ไม่ต่อเนื่อง ในขณะที่พืชสกุล *Volkameria* มีรูปร่างเซลล์แบบหลายเหลี่ยม ผนังเซลล์ด้านตั้งฉากกับผิวตรง และมีลักษณะของมัดท่อลำเลียงแบบเคียงข้างที่มีรูปร่างครึ่งวงกลมต่างจากอีกสองสกุลอย่างชัดเจน ลักษณะปากใบสามารถแบ่งพืชออกได้เป็นสองกลุ่มคือ กลุ่มที่มีปากใบแบบแอนอโมไซติก ได้แก่ สกุล *Clerodendrum* และ *Volkameria* และกลุ่มที่มีปากใบแบบไดอะไซติก ได้แก่ สกุล *Rotheca* นอกจากนี้ชนิดและรูปแบบการปรากฏของขนต่อมสามารถนำมาใช้ร่วมกับลักษณะอื่นๆในการจัดกลุ่มและจำแนกพืชในระดับสกุลและ *Clerodendrum* บางชนิดได้ สำหรับการศึกษาความสัมพันธ์ทางพันธุกรรมโดยเทคนิคไอพีบีเอส พบว่าไพรเมอร์จำนวน 10 ไพรเมอร์จากทั้งหมด 30 ไพรเมอร์สามารถสังเคราะห์และให้แถบดีเอ็นเอที่มีความเสถียรและชัดเจนจำนวนทั้งหมด 735 แถบ โดยเป็นแถบดีเอ็นเอที่โพลีมอร์ฟิกจำนวน 734 แถบ คิดเป็นร้อยละของโพลีมอร์ฟิกเฉลี่ยที่ 99.9 จากทุกไพรเมอร์ จากการวิเคราะห์ความสัมพันธ์ทางพันธุกรรมโดยใช้รูปแบบของสายสัมพันธ์แบบ neighbor joining (NJ) และสร้างกลุ่มความสัมพันธ์แบบกลุ่ม (principal coordinates analysis; PCoA) พบว่าสามารถแบ่งพืชออกเป็นกลุ่มหลักซึ่งสอดคล้องกับการศึกษาทางด้านอนุกรมวิธานที่ผ่านมาทั้งที่ใช้ลักษณะทางสันฐานวิทยาและข้อมูลวิวัฒนาการเชิงโมเลกุล ผลการศึกษาแสดงให้เห็นว่าการใช้ข้อมูลลักษณะทางกายวิภาคของพืชร่วมกับข้อมูลทางพันธุกรรมโดยเทคนิคไอพีบีเอสสามารถสนับสนุนการศึกษาทางด้านอนุกรมของพืชทั้งสามสกุลและในบางชนิดของประเทศไทยได้

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ABSTRACT

The comparative leaf blade anatomical study and genetic relationship by iPBS technique of 21 species and one variety of *Clerodendrum* L., two species of *Rotheca* Raf. and one species of *Volkameria* L. were conducted. Plant specimens were collected from wild population throughout the country during their flowering period in January 2015 to March 2017. Leaf anatomy was examined by paraffin technique, peeling method and scanning electron microscopic observation. Key to genera and speecies, the species descriptions and illustrations of leaf blade anatomical characteristic are given. The significant characteristics are pointed out and discussed. Briefly, *Clerodendrum* and *Rotheca* species have irregular epidermal cell with sinuous anticlinal wall and discontinuous cylindrical vascular bundle. Only *Volkameria* species has isodiametric cell with straight anticlinal wall and continuous arc-shaped vascular bundle. Stomatal types can be used to classify studied plants into two groups; anomocytic stomata occur in *Clerodendrum* and *Volkameria*, whereas diacytic stomata occur in *Rotheca*. Moreover, trichome types and presenced are significantly helpful to differentiate the three genera and some taxa in *Clerodendrum*. For the molecular study by iPBS, ten primers out of thirty successfully generated a total of 735 scorable bands in the 24 studied taxa and 734 bands are polymorphic (%99.9). The neighbour joining dendrogram (NJ) based on genetic similarity and principle coordinate analysis (PCoA) can separate plant into the groups which agree very well with the previous taxonomic studies using both morphological and molecular phyletic information. The results indicate that anatomical data and iPBS molecular marker provide taxonomic supportive evidence for the three genera and some species of *Clerodendrum* in Thailand.

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ABBREVIATION

BEs	Bundle sheath Extensions
CTAB	Cethyltrimethyl Ammonium Bromide
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide Triphosphate
FAA II	Formalin Acetic Alcohol II
iPBS	inter-Primer Binding Site
LTR	Long Terminal Repeat
$MgCl_2$	Magnesium Chloride
NJ	Neighbour Joining
PCoA	Principal Coordinates Analysis
PCR	Polymerase Chain Reaction
SEM	Scanning Electron Microscope
TBA	Tertiary Butyl Alcohol
TBE	Tris-Borate Ethylenediaminetetraacetic acid

CHAPTER 1

INTRODUCTION

Clerodendrum L. belongs to the family Lamiaceae and comprises around 150 species in tropical Old World distributing from China and Japan down to Australia. According to the molecular phylogenetic studies, *Clerodendrum* is polyphyletic and to circumscribe the genus as monophyletic, several species were separated from *Clerodendrum* and then placed into the resurrected genera *Rotheca* Raf. and *Volkameria* L. (Yuan *et al*., 2010). In Thailand, 20–35 species of *Clerodendrum* were recorded (Leeratiwong *et al*., 2011), one species of *Volkameria* (Leeratiwong & Chantaranothai, 2014) and three represented species of *Rotheca* (Leeratiwong & Chantaranothai, 2010; Leeratiwong *et al*., 2018). Many species of *Clerodendrum* and *Rotheca* are traditionally used by people of East Asia and South-East Asia as a medicinal plant (Perry, 1980) and cultivated as an ornamental plant in India, Bangladesh, Asia and Europe (Sambamurty, 2005).

Rotheca and *Volkameria* species share various common characters with *Clerodendrum* species. The morphological similarity sometimes can lead to generic or species misidentification, especially in complex species. Despite a few taxonomic revision of these three related genera in Thailand were conducted, the delimitation of the genera and complex species using only morphological characters is still uncertain as there are only a few distinct characters to define the genus boundary. The different morphological characters between *Clerodendrum*, *Rotheca* and *Volkameria* (Steane & Mabberley, 1998; Harley *et al*., 2004; Yuan *et al*., 2010; Leeratiwong *et al*., 2011) are presented in Table 1.

Since the taxonomic issues still remain even after many morphological and molecular phylogenetic studies, several researchers have tried to investigate further alternative techniques and evidence to solve the problem, a particularly anatomical characteristics in the aim of taxonomic implication. Cantino (1990) and Metcalfe and Chalk (1950) demonstrated that leaf anatomical characters, particularly trichomes structure and vascular system, support morphological evidence for separating taxa in

the Lamiaceae and related families. Thus, anatomical information has provided valuable taxonomic implication at the subfamily to species levels for several groups of plants (Stuessy,1990). The leaf anatomy of *Clerodendrum* has been studied by several researchers. However, the leaf anatomy of *Clerodendrum*, *Rotheca* and *Volkameria* in Thailand has not yet been investigated. The aims of this study are to introduce anatomical characters of leaf blade to support the generic and species identification among the present members and to evaluate the usefulness of these characters for systematic purposes.

Table 1 Morphological comparison of *Clerodendrum*, *Rotheca* and *Volkameria*.

Character	*Clerodendrum*	*Rotheca*	*Volkameria*
Flowering bud	Both symmetrical and asymmetrical, if asymmetrical, corolla usually expanding abruptly on upper side due to resupination	Markedly asym-metrical, corolla expanding abruptly on lower side only	Mostly asym-metrical, corolla hypocrateriform
Anterior corolla lobe	Only slightly (if at all) larger than the others	Frequently much larger than the others	Only slightly (if at all) larger than the others
Anthers	Versatile	Usually basifixed (occasionally versatile)	Versatile
Stigma lobes	Equal	Frequently unequal	Equal
Leaf blade	Frequently longer than 6 cm	Frequently longer than 6 cm	Usually shorter than 6 cm
Inflorescence	Commonly terminal	Commonly terminal	Mostly axillary
Fruiting calyx	Accrescent, larger than fruits and brightly coloured	Rarely accrescent, smaller than fruits, enclosing the fruit base and not brightly coloured	Rarely accrescent, smaller than fruits, enclosing the fruit base and not brightly coloured
Fruit	Often fleshy, with bright colour contrasting with calyx	Often fleshy, brightly coloured	Usually dryish, not brightly coloured

Even though the molecular phylogenetic relationship in *Clerodendrum* and related genera were studied by several authors, only a few species found in Thailand were included in those works. Thus, the further genetic investigation of *Clerodendrum* and its related genera in Thailand is still necessary in order to clarify the genus boundaries. However, the classical molecular approach employing direct sequencing require wide rage of time and budget. Alternatively, reliable and uncomplicated PCR-based techniques are utilised to provide helpful molecular markers for coping with plant taxonomic issues. The markers rely on PCR amplification of either anonymous or specific regions in the whole genome and the genetic analysis base on electrophoretic size-fractioning to reveal size variations. The PCR-base molecular markers allow larger volumes of sequence data to be obtained in shorter times and lower costs (Kalendar *et al*., 2010).

In the recent decade, several molecular markers were developed to achieve genetic information in plant. iPBS molecular marker is one of the newest technique and has been demonstrated as a simple, reproducible and inexpensive method which provides informative DNA fingerprint without the requirement for prior sequence information (Kalendar *et al*., 2010). Though iPBS was widely applied in phylogenetic studies and genetic diversity analysis, not many studies have been done and criticized on taxonomic purpose. The present study attempt to elucidate the genetic relationship among the species of *Clerodendrum* and related genera, in which there is scarce molecular data in Thailand, using inter Primer Binding Site (iPBS) to support phenotypic identification using morphological characters.

Objectives:

1. To compare and evaluate the sixnificant leaf blade anatomical characteristics of *Clerodendrum*, *Rotheca* and *Volkameria* (Lamiaceae) in Thailand.

2. To examine the genetic diversity of *Clerodendrum*, *Rotheca* and *Volkameria* (Lamiaceae) in Thailand using inter Primer Binding Site (iPBS).

CHAPTER 2

LITERATURE REVIEW

2.1 Taxonomic studies of *Clerodendrum*, *Rotheca* and and *Volkameria*

Clerodendrum was first described by Linnaeus (1753) and previously placed in the family Verbenaceae. According to morphological and molecular phylogenic evidence, the genus was then moved to the family Lamiaceae based on the family boundary recircumscription (Cantino *et al*., 1992; Harley *et al*., 2004; Leeratiwong *et al*., 2011).

Rotheca and *Volkameria* used to be sunk in the genus *Clerodendrum* as the members share similar morphological characteristics described as follow: shrub, small tree or woody climber, rarely perennial herb. Stem and twigs mostly quadrangular and pubescent. Leaves simple, decussate or rarely whorled, exstipulate. Inflorescence cymose, cyme or thyrsoid, terminal or axillary. Flowers bisexual, mostly zygomorphc, subtended by bracts or bracteoles. Calyx of 5 fused sepals, campanulate, cup-shape or tubular, 5-lobed or truncate, persistent. Corolla sympetalous, lobes 5 (except more than 5 lobes in *C. chinense*), unequal size. Stamens 4, didynamous, inserted within corolla tube, filaments usrally long-exserted, alternate. Ovary superior, bicarpellate, syncarpous, 4 locular, with one ovule in each locule; style terminal, long exserted, glabrous with shortly bifid. Fruits drupaceus, endocarp separating into 2–4 pyrenes, each with one exalbuminous seed (Leeratiwong, 2011). However, several species have been separated from *Clerodendrum* and then placed into the resurrected genera *Rotheca* and *Volkameria* based on molecular phylogenetic evidence.

According to Leeratiwong *et al*. (2011), there are four major groups of *Clerodendrum* complex species in Thailand based on their similar morphological characteristics; 1) *C. intermedium* Cham., *C. japonicum* (Thunb.) Sweet and *C. paniculatum* L. 2) *C. godefroyi* Kuntze and *C. lloydianum* Craib 3) *C. calamitosum* L. and *C. longisepalum* 4) *C. nutans* Wall. ex Jack and *C. umbratile* King & Gamble.

2.2 Previous anatomical studies

Metcalfe and chalk (1950) recorded and mentioned diagnostic leaf anatomical characters in Lamiaceae. The general characters were described. Leaves isobilateral, dorsiventral or centric. Hair vary variable, but affording valuable character for the identification of genera and species. The occurrence together of various kind of hairs and characteristic short-stalked glands with uni- or multicellular heads (1–16 or more cells) is characteristic of the whole family. Non-glandular hairs are also frequent, and may be uniseriate, tufted or branched. Epidermal cell in surface view have more or less sinuose anticlinal wall or polyhedral with straight anticlinal walls. Stomata occure on one or both surfaces, but more frequenly restrict to the lower side and are commonly of the caryophyllaceous (diacytic) type, although often intermixed with others that are ranunculaceous (anomocytic). Vascular bundle of the veins accompanied by sclerenchyma in some species, especially in those showing xerophytic features, in others frequently surrounded by parenchymatous sheath. Crytal, when present, excrete in the form of small needles, rods, or octahedral, often occurring in considerable numbers in one cell.

Inamdar (1968) observed nectaries and vascular cambium in leaf and petiole in anatomy of *Clerodendrum splendens* G. Don. He reported the foliar nectaries are patelliform and originate from a single epidermal papillate cell to secrete sugary fluids. The spheroidal crystalliferous sclereids occur in the mesophyll, midrib region of the leaf and the ground tissue of the petiole. The vascular bundles in the midrib region and the petiole are arranged in a ring. The phloem does not form a continuous arc. The smaller vascular bundles in a ring originate from vascular meristems with delayed differentiation. The vascular cambium with radial alignment of cells presents in between xylem and phloem in the leaves and the petiole.

Kaushat and Tripathi (1984) studied the foliar epidermis, stomatal patterns and floral trichomes in *Clerodendrum indicum* O. Ktze., *C. inerme* (L.) Gaertn. (= *Volkameria inermis* L.), *C. squamatum* Vahl (= *C. japonicum* (Thunb.) Sweet) and *C. splendens* G. Don. They reported the epidermal anticlinal wall are sinuous, curvy to straight. Stomata occur more frequent on the lower side than on the upper side. Anomocytic stomata are predominant while anisocytic type is rarely found .

Rao and Ramayya (1985) studied the taxonomic significance of laminar stomatal distribution patterns in *Clerodendrum* They observed stomata in *Clerodendrum aculeatum* (Linn.) Griseb. (= *V. aculeata* L.), *C. inerme* (L.) Gaertn. (= *V. inermis* L.), *C. neriifolium* Wall. (= *V. inermis* L.), *C. philippinium* Schauer (= *C. chinense* (Osbeck) Mabb.), *C. phlomidis* L.f., *C. splendens* G. Don, *C. thomsonae* Balf., *C. viscosum* Vent. (= *C. infortunatum* L.). The stomatal distribution does not show any correlation with venation pattern in the floral parts. From all studied species, 45% have amphistomatic leaves.

Herman (1998) studied the anatomy of leaf in *Clerodendrum triphyllum* (Harv.) H.Pearson (= *Rotheca hirsuta* (Hochst.) R.Fern.) and *C. louwalberfsii* P.P.J.Herman (= *R. louwalbertsii* (P.P.J.Herman) P.P.J.Herman & Retief.). All species have amphistomatic leaves, diacytic stomata mainly occur with rarely anomocytic, anisocytic, or a few paracytic stomata. In transverse section, *C. louwalberlsii* has dorsiventral leaves but in *C. triphyllum* homogeneous mesophyll present. Peltate hairs distribute on lower and upper epidermis in both species. Secretary head of the hairs concist of 8-celled, with a a base cell and unicellular stalk sunken under the epidermis.

Bangar *et al*. (2011) studied the foliar epidermal in *Cleroderum aculeatum* (= *V. aculeata* L.), *C. inerme* (L) Gaeertn (= *V. inermis* L.), *C. philippinum* Schuer (= *C. chinense* (Osbeck) Mabb.), *C. serratum* (L) Moon (= *Rotheca serrata* (L.) Steane & Mabb.), *C. splenderns* G. Don. The five species of *Clerodendrum* were investigated for epidermal structure. All species has sinuous epidermal cells. The striations on lower epidermis were observed in *C. inerme*. Almost all species have hypostomatic leaves except *C. philippinum* with amphistomatic leaves and the lesser number of stomata on adaxial surface. Four types of stomata were found: anisocytic, anomocytic, diacytic and tetracytic. In *C. serratum*, diacytic stomata are dominant. Glandular trichomes are scale-like and sessile.

2.3 Molecular studies

2.3.1 Molecular marker and genetic diversity

Plant taxonomists primarily focus on the classification of species. For several decades, molecular approaches accelerate a clearer knowledge of plant evolutionary, especially in complex species groups. The remarkable benefit of molecular techniques is that analysis can be conduted at flexible range of plant stages, such as early stages of development, living individual or even from dry voucher specimens. This situation allows taxonomists to integrate present molecular data with reference morphology of studied species (Kalendar *et al*., 2011). However, molecular study is a delicate technique requiring immense and accurate knowledge in every processes such as, a reliable DNA extraction protocol, leaf tissue sampling methods etc.

In plant taxonomy, chloroplast DNA is commonly targeted. The nuclear sequences used in molecular taxonomic studies mostly locate in highly repetitive DNA. For example, the internal transcribed spacer (ITS) in nuclear ribosomal RNA genes This region is a common and interesting site to be targeted as the nucleotides locate at a few loci and are repeated tandemly in plant genomes. Consequently, the ITS genes have been succesfully applied to resolve taxonomic problem in plant by analising restriction initially and sequencing gene directly.

The previous phylogenetic studies of *Clerodendrum* employed nuclear ITS sequences (Steane *et al*., 1999), restriction site and fast-evolving region of cpDNA (Steane *et al*., 1997; Yuan *et al*., 2010) distinctly showed that *Clerodendrum* were devided into three main clade related to the geographical distribution: an African clade, an Asian clade and a Pantropical Coastal clade. The genus *Volkameria* was revived by Yuan *et al*. (2010) who proposed to separate the Pantropical Coastal clade and *C. spinosum* from *Clerodendrum*.

Steane and Mabberley (1998) and Steane *et al*. (1999) analized the sequence of internal transcribed spacers of the nuclear ribosomal DNA and combined with cpDNA restriction site data. They separated *Clerodendrum* subgenus *Cyclonema* (Hochst.) Gürke and *Clerodendrum* section *Konocalyx* Verdcourt and placed into the resurrected genus *Rotheca*.

The classical molecular investigation in previous studies is effective and accurate but can also consume wide range of time and budget. Alternatively, reliable and uncomplicated PCR-based techniques are utilised to develop helpful molecular markers for coping with taxonomic problem. In the recent decade, PCR-base molecular markers allow genetic data to be obtained in smaller costs, greater amount, and faster times. These molecular techniques also rely on DNA amplification of anonymous or specific sites on plant genomic DNA and the analyses base on length-fraction electrophoresis. In such techniques, the importance is not a direct sequence target from a specific region, but rather allowing numerous loci used as markers to reveal size variations from the whole genome. The main objective is to provide an information of genome diversity as accurate as possible (Kalendar *et al*., 2011).

Transposable elements is a major component of plant genomes. These fraction can be replicate or move within a genome leading to the increase of genome size and effecting the regulation of gene expression. These events play a major role in plant evolutionary processes. The insertions of ancient retrovirus into plant genomes results in the existing of long terminal repeats retrotransposons (LTRs) in transposable element. These insertion sites occur repeatedly through the entire genome and are inherited genetically with no excision making the insertion polymorphisms useful as molecular markers. Several molecular markers based on retrotransposon insertion polymorphisms were developed such as, IRAP, REMAP, ISSR, SSAP, RBIP etc. However, main limitations of all these markers are the presence of proper LTRs and the its sequence data, obtained by genomic DNA cloning and sequencing, for element specific primers design.

Even though the PCR method using conserved primers have been developed for rapid retrotransposon isolation, a hundreds of genes are still necessary to be cloned and sequenced in order to obtain a few primer sequences and the general LTR sequences cloning method can not access autonomous regions. The iPBS (inter-primer binding site) method is a new technique which can target anonymous regions of the genome without the requirement for the LTR sequences information. Hence, iPBS is an alternative technique with several benefits for harvesting LTR sequences variations and examinating DNA fingerprint (Monden *et al*., 2014).

2.3.2 iPBS (inter-primer binding site)

iPBS is a technique to identify LTR sequences diversity and visualise its polymorphism at interspecific- and intraspecific level (Kalendar *et al*.,2010). The PBS region focused in the method locates next to the 5′ LTR (Fig 1.) and is conserved among different LTR retrotransposon families.

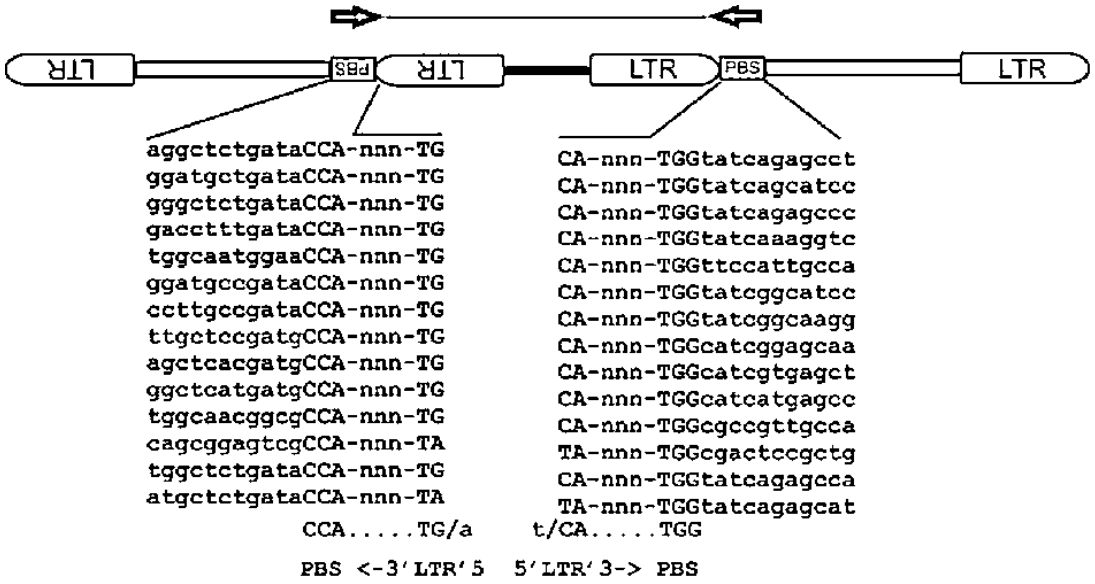

Figure 1 LTR retrotransposon and iPBS scheme (Kalendar *et al*., 2010)

The iPBS amplification technique, utilising the PBS conserved sequences to directly visualise polymorphism in transcription profiles and between individuals, establishs an effective method to search and clone LTR elements from the genome rapidly. After accessing LTR sequences of a selected retrotransposons, the most conserved region can be defined from the alignment and used for primers invertion in long distance PCR in order to clone the entire genome. Thus, the iPBS amplification is an effective method to detect complementary DNA clonal differences and polymorphism resulted by retrotransposon recombination or activities (Kalendar *et al*., 2011).

iPBS molecular marker has been generally applied in the studies of genetic diversity, genetic population, as well as, genetic relationship among several groups of plant. Although there are no report on using iPBS technique in *Clerodendrum*, *Rotheca*, *Volkameria* and other genera in the family Lamiaceae, several authors used such procedure to investigate genetic information in several groups of plant such as,

genetic analysis of the genus *Diospyros* (Raddová, 2012), genetic diversity and relationship among wild annual *Cicer* species (Andiden, 2013), molecular diversity analysis of grape varieties (Guo *et al*., 2014), genetic diversity and relationship among wild *Lens* species from Turkey (Baloch *et al*., 2015), molecular variability and phylogenetic relationships of guava (*Psidium guajava* L.) cultivars (Mehmood, 2015), genetic diversity in populations of the medicinal plant *Leonurus cardiaca* L. (Borna *et al*., 2016), genetic diversity of tea (*Camellia sinensis* (L.) O. Kuntze) grown in Vietnam (Phong *et al*., 2016), determination of the population structure of *Fig* genotypes from Algeria and Turkey (Belttar *et al*., 2017), genetic diversity in phytopathogenic Sclerotiniaceae (Özer *et al*., 2017), genetic information of 25 Fagaceae species in *Fagus, Castanea* and *Quercus* (Coutinho, 2018). All studies proposed that iPBS marker provide reliable genomic fingerprints which is effective in differentiation of plant variety and their related species.

CHAPTER 3

MATERIALS AND METHODS

3.1 Plant collection and plant materials

Field surveys and plant collection were conducted during January 2015 to Febuary 2017. Plant specimens were collected during the flowering period from natural populations throughout Thailand (Fig. 2) following the information of previous taxonomic studies. Leaf samples were collected in three different ways; 1) young shoot in CTAB for molecular study 2) fresh mature leaf in 70% alcohol for anatomical study and 3) dry leaves in voucher specimens for SEM observation, at least three replications from each plant individual or population were collected. The mature leaves (approximately leaves number 10–15 from the shoot) with no fungus and insect attack were selected. Voucher samples (Table 2) were kept in the Herbarium of Faculty of Science, Prince of Songkla University, Thailand (PSU). The photographs of studies species are given (Fig. 3)

Table 2 Samples of *Clerodendrum*, *Rotheca* and *Volkameria* used in present study.

Sample code	Taxa	Locality (Province)	Voucher specimens
C1	*Clerodendrum calamitosum* L.	Songkhla	FP064
C2	*C. chinense* (Osbeck) Mabb.	Songkhla	FP065
C3	*C. chinense* var.*simplex* (Moldenke) Chen	Chiang Rai	FP047
C4	*C. colebrookianum* Walp.	Phitsanulok	FP017
C5	*C. deflexum* Wall.	Yala	FP004
C6	*C. disperifolium* Blume	Nakhon Si Thammarat, Songkhla	FP036 FP003
C7	*C. garrettianum* Craib	Chiang Mai	FP020
C8	*C. godefroyi* Kuntze	Ubon Ratchathani	FP057
*	*C. haematolasium* Hall.f.	Narathiwat	FP002
C9	*C. indicum* (L.) Kuntze	Songkhla	FP058
C10	*C. infortunatum* L.	Chiang Mai, Songkhla, Nan	FP030 FP069 FP074

Table 2 Samples of *Clerodendrum*, *Rotheca* and *Volkameria* used in present study (continued).

Sample code	Taxa	Locality (Province)	Voucher specimens
C11	*C. intermedium* Cham.	Chiang Mai	FP027
C12	*C. japonicum* (Thunb.) Sweet	Ubon Ratchathani	FP024
C13	*C. lankawiense* King & Gamble	Satun, Phang Nga	FP043
C14	*C. lloydianum* Craib	Lampang	FP055
C15	*C. longisepalum* Dop	Maha Sarakham	FP010
C16	*C. nutans* Wall. ex Jack	Songkhla, Chanthaburi	FP039 FP080
C17	*C. paniculatum* L.	Surat Thani	FP001
C18	*C. schmidtii* C.B. Clarke	Saraburi	FP011
C19	*C.* sp.	Songkhla	FP014
C20	*C. umbratile* King & Gamble	Songkhla	FP015
C21	*C. villosum* Blume	Songkhla	FP060
R2	*Rotheca macrostachya* (Turcz.) Leerat. & Chantar.	Chiang Rai	FP050
R1	*R. serrata* (L.) Steane & Mabb	Chiang Mai, Songkhla, Loei	FP006 FP078 FP026
V1	*Volkameria inermis* L.	Surat Thani, Songkhla	FP033 FP072

Note: * **=** not included in molecular study (no code)

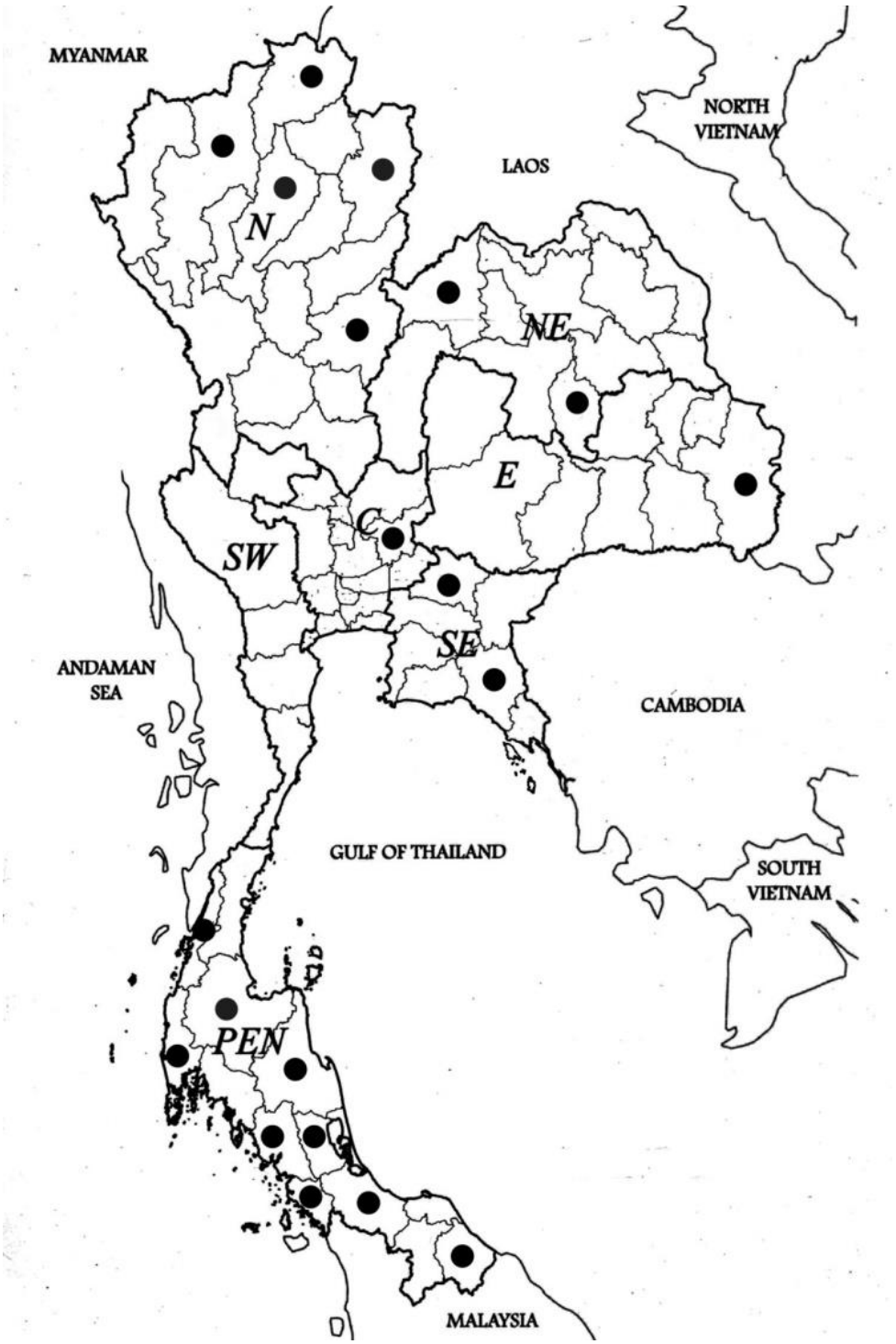

Figure 2 Map of Thailand showing the study sites: N = Northern, NE = North-eastern, E = Eastern, SW = South-western, C = Central, SE = South-eastern, and PEN = Peninsular.

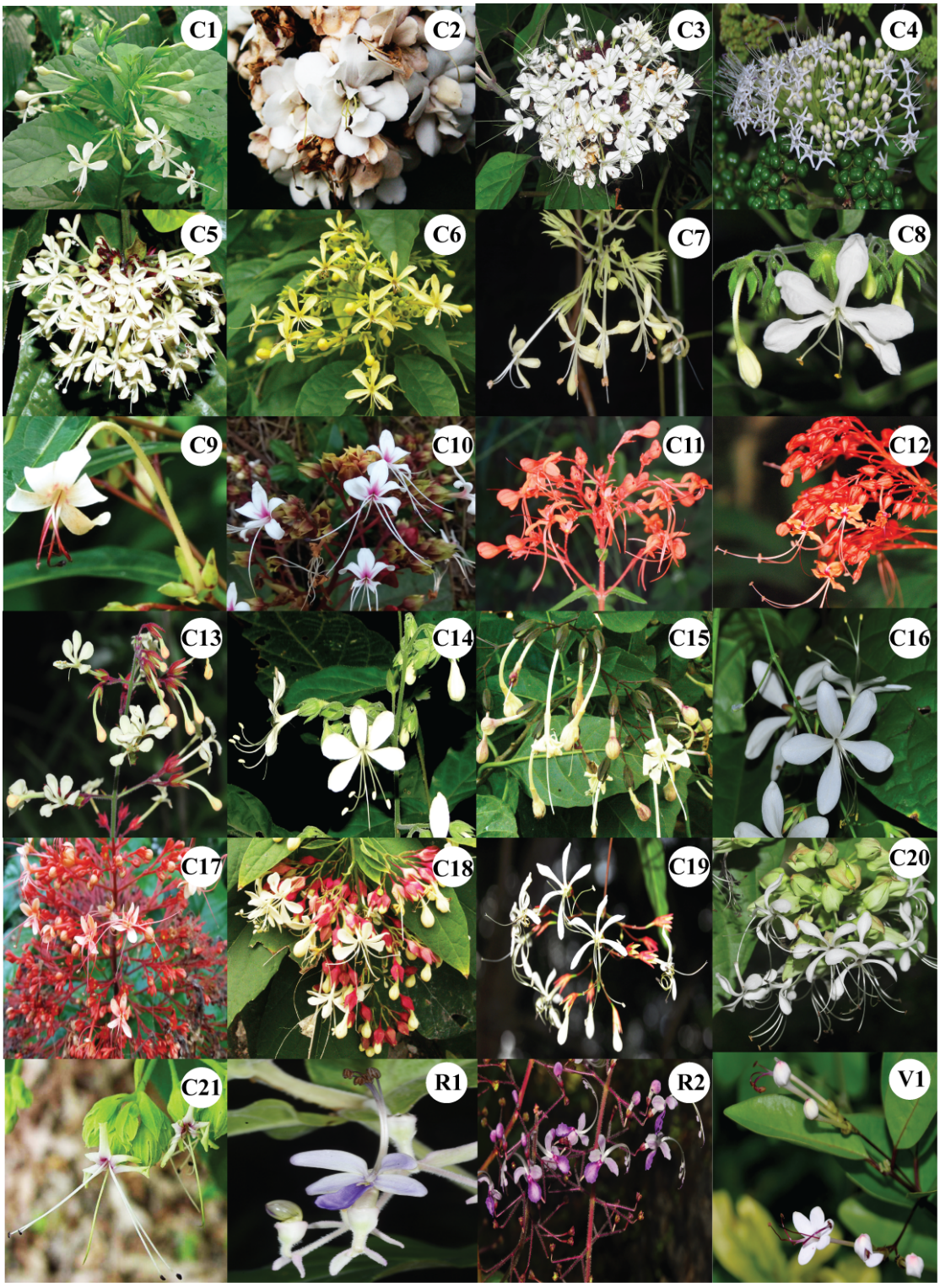

Figure 3 Morphology of flowers in each species; C1–C21, R1–R2 and V1 stand for individual species of studied taxa, see table 3 for details).

3.2 Anatomical study

3.2.1 Anatomical study of leaf blade

For anatomical studies, cross-sections of two regions of leaf blade were prepared; 1) midrib and 2) the region between midrib and leaf margin. The samples of some species were immediately observed after collecting from the field using transverse section of fresh leaves by MT-3 plant microtome, whereas most samples needed to be kept in 70% alcohol and observed later using paraffin technique dua to the long period of field survey.

For paraffin technique (appendix A), the samples were moved from the alcohol fixative to FAA II. The samples were then dehydrated with 12 sequences increasing concentration of TBA series before embed the samples into paraffin. Leaf blade cross-sections of 10 μm thickness were obtained by using sliding microtome. These sections were then deparaffinized and stained with Safranin and Fast green and mounted with Permount mounting media in order to prepare permanent slide. The anatomical characteristics of lamina such as, arrangement of vascular bundle, pattern and thickness of mesophyll cell etc. were examined under light microscope and photographed using OPTICAM HDMI-4083.13H. microscopic camera.

3.2.2 Anatomical study of leaf epidermal surface

For epidermal study, the thin membranous transparent layer was carefully pulled out from epidermis and cleaned using razor blade. Wet mounts were subsequently observed under light microscope without staining but some samples were stained with Safranin in order to obtain more distict and clearer photographs of some characteristics. Photographs of anatomical characters were captured by OPTICAM HDMI-4083.13H. microscopic camera. For SEM observation, dry leaves from voucher specimens were cut into small pieces and fixed on aluminum stubs with adhesive tape. The stubs were then sputter coated and observed under JEOL JSM-5800 LV scanning electron microscope.

3.2.3 Anatomical data analysis

The leaf blade anatomical characteristics as well as epidermal characteristics such as, stomata, epidermal cell, trichome types and distributions of trichomes were studied and compared among the species and genera to investigate diagnostic characteristics. The key to genera and species were constructed based on significant leaf blade and epidermal anatomical characteristics.

3.3 Molecular study by iPBS

3.3.1 DNA extraction

Genomic DNA from fresh young leave of studied species was extracted using a modified version of CTAB protocol (Doyle & Doyle, 1987; Vanijajiva *et al*., 2005) (Appendix B).

Quality of the DNA was evaluated by electrophoresis of 1% agarose gel with RedSafe™ nucleic acid staining solution and quantified using NanoDrop Microvolume spectrophotometer.

3.3.2 Optimization of PCR parameters

3.3.2.1 Primer optimization

Initially, thirty iPBS primers (Table 3) were screened in all plant samples. Primers which generated strong and clear visibly amplification band were selected for genetic diversity analysis.

3.3.2.2 Reagent concentration optimization

The total PCR mixture volume was 25 µl containing 10xPCR buffer (Promega), 0.4 mM each dNTP, 0.6 mM of 2398 iPBS primer along with the concentration gradient set of three following parameters in order to optimize the proper PCR condition.

I) $MgCl_2$: 2.0 mM, 3.0 mM, 4.0 mM and 5.0 mM

II) Genomic DNA: 30 ng/µl, 50 ng/µl, 70 ng/µl, and 90 ng/µl

III) *Taq* DNA polymerase: 0.5 U, 1.0 U, 1.5 U and 2.0 U

The proper concentration providing the strongest and clearest visibly amplification band were applied in iPBS technique.

Table 3 iPBS primers code and sequence.

Primer code	Nucleotide sequence 5’ to 3’
2076	GCTCCGATGCCA
2077	CTCACGATGCCA
2079	AGGTGGGCGCCA
2080	CAGACGGCGCCA
2081	GCAACGGCGCCA
2083	CCTCTAGCGCCA
2085	ATGCCGATACCA
2251	GAACAGGCGATGATACCA
2252	TCATGGCTCATGATACCA
2253	TCGAGGCTCTAGATACCA
2256	GACCTAGCTCTAATACCA
2272	GGCTCAGATGCCA
2273	GCTCATCATGCCA
2277	GGCGATGATACCA
2279	AATGAAAGCACCA
2295	AGAACGGCTCTGATACCA
2374	CCCAGCAAACCA
2378	GGTCCTCATCCA
2380	CAACCTGATCCA
2382	TGTTGGCTTCCA
2389	ACATCCTTCCCA
2391	ATCTGTCAGCCA
2392	TAGATGGTGCCA
2393	TACGGTACGCCA
2394	GAGCCTAGGCCA
2398	GAACCCTTGCCGATACCA
2400	CCCCTCCTTCTAGCGCCA
2401	AGTTAAGCTTTGATACCA
2402	CTCAAGCTCTTGATACCA
2415	CATCGTAGGTGGGCGCCA

3.3 iPBS technique

The iPBS-PCR amplification was carried out as the following cycle condition: initial denaturation at 95^{o}C for 3 minutes followed by denaturation at 94^{o}C for 30 seconds, annealing gradient temperature ranging from 45–50^{o}C for 30 seconds, elongation at 72^{o}C for 30 seconds, 40 cycles and final elongation at 72^{o}C for 5 minutes. PCR products were separated by electrophoresis of 2% agarose gel with RedSafe™ in 1xTBE buffer.

3.4 Data scoring and analysis

The electrophoretic patterns were visually analysed. DNA bands were scored as present (1) or absent (0) and were transformed into a binary metrix. The metrix obtained was then entered in PAST3 statistic solfware (Hammer *et.al*, 2001). Principal coordinates analysis (PCoA) and neighbour joining (NJ) dendograms were constructed based on genetic similarity (Dice Coefficient). The formular is given below.

$$\text{Dice (Sørensen) Coefficient} = S_D = \frac{2C}{N1+N}$$

Note: General parameters for binary data where determining the similarity between columns; C = number of positive matches between columns, A = number of negative matches between columns, T = total number of variables (rows), N1 = total number of presences in column 1, N2 = total number of presences in column 2

CHAPTER 4

RESULTS

The present study focused on 21 species and one variety of *Clerodendrum*, two species of *Rotheca* and one species of *Volkameria* in Thailand. Leaf blade anatomy was observed using mainly peeling method and paraffin technique (4.1). In addition, genetic relationship by iPBS marker was constructed using NJ and PCoA clustering method (4.2).

4.1 Anatomical study

In general, 25 species from three genera share common leaf blade anatomy as described below.

In surface view—*leaf indumentum* absent or consisting various types of hairs; capitate glandular trichomes, peltate glandular trichomes (sometimes at leaf base called scale-like glands (Fig. 4H)), unicellular trichome and multicellular uniseriate trichome (Fig. 4A–4D). Secretory gland composed of four to eight secretory cells or more than ten cells (Fig. 4E–4G). *Epidermal cell*: adaxial and abaxial epidermal cells are irregular (jigsaw puzzle like shape) or isodiametric, anticlinal wall various from straight to curved to sinuous, cuticle smooth, rarely striate. *Stomata*: anomocytic or diacytic.

In transverse section—*Epidermis*: uniseriate, adaxial cell usually wider than high or equal in width and height, rarely higher than wide. *Hypodermis* absent. *Mesophyll*: dorsiventral, adaxial palisade cells in one layers or 2–3 layers, 10–60 percents of mesophyll, spongy mesophyll in 3–8 layers. *Midrib outline*: adaxial varies from concave, convex, to flattened, abaxial convex to flattened, ground tissue consisted of 1–4 layers lacunar or lacunar collenchyma below the epidermis followed by parenchymatous tissue. *Crystal*: present or absent, occurring in ground tissue or closed to vascular bundle. *Fibres*: present or absent. *Vascular bundle*: collateral, cylindrical discontinuous ring or arc-shaped with incurved adaxial partition.

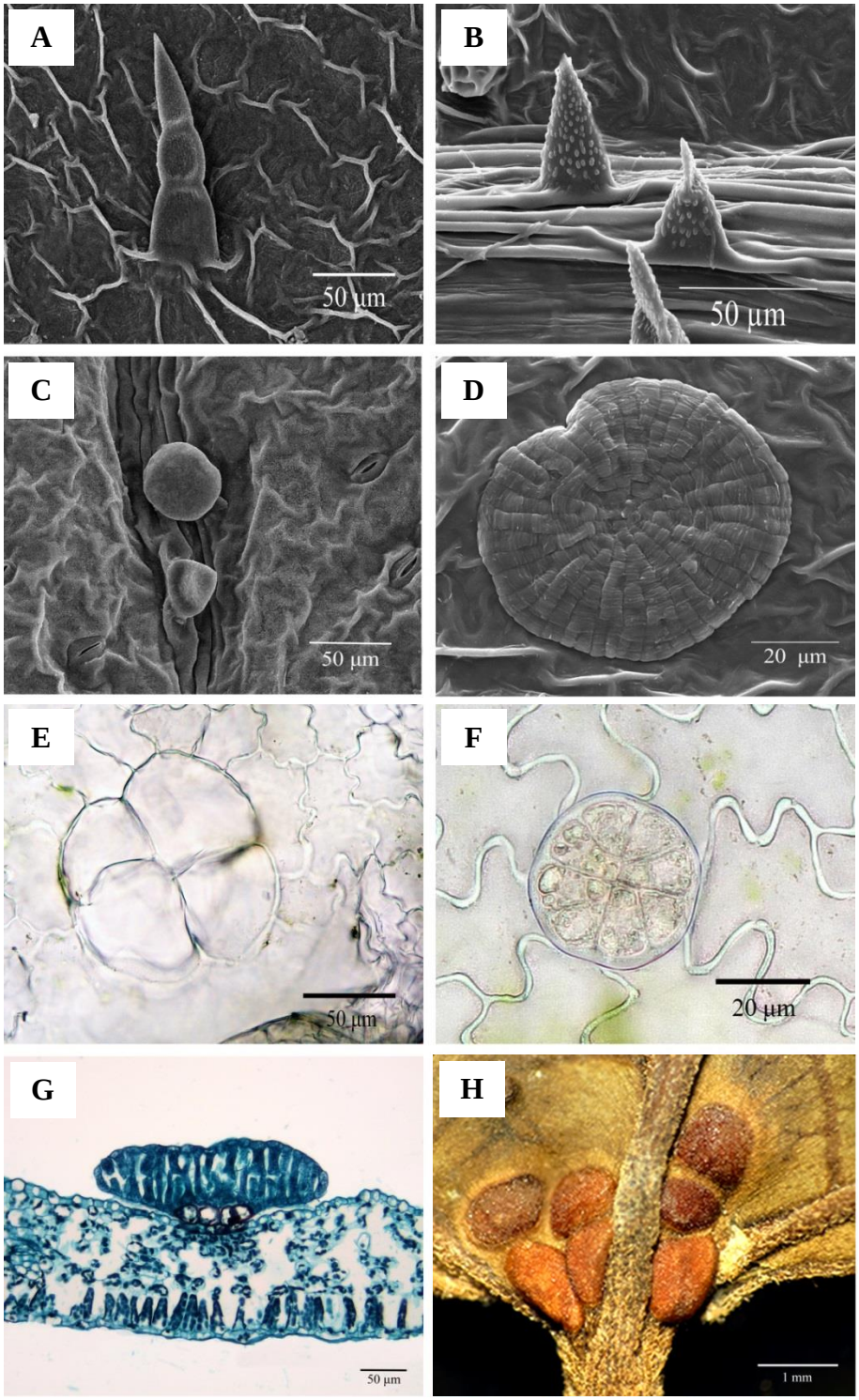

Figure 4 Trichomes (A–D) and secretory heads (E–H): A. multicellular uniseriate trichome; B. unicellular trichome co–occur with multicellular uniseriate trichome; C. capitate glandular trichome; D. peltate glandular trichome with multicellular head; E. secretory head with four cells; F. secretory head with eight cells; G. secretory head with more than ten cells; H. scale-like glands.

The key to genera and the generic describtions based on leaf blade anatomy are provided.

KEY TO GENERA

1 Multicellular uniseriate trichomes present; epidermal cell shape irregular with sinuous anticlinal wall; leaf amphistomatic, except in *C. longisepalum* with hypostomatic leaves; striation absent; vascular bundle cylindrical, arranged in discontinuous ring.…..2

2 Stomata diacytic…….…….…….…..……....…2. *Rotheca*

2 Stomata anomocytic……...…….…….…..……..1. *Clerodendrum*

1 Multicellular uniseriate trichomes absent; epidermal cell shape isodiametric with straight to curved anticlinal wall; leaf hypostomatic; striation on lower epidermis; vascular bundle arc-shaped with adaxial incurved partitions....……...3. *Volkameria*

1. *Clerodendrum* L.

Overall, the common leaf blade anatomy of 22 taxa of *Clerodendrum* was concluded as a generic describtion.

In surface view—*leaf indumentum* consisting various types of hairs; capitate, peltate, unicellular and multicellular uniseriate trichome. Secretory gland composed of four to eight secretory cells or more than ten cells. *Epidermal cell*: adaxial and abaxial epidermal cells are irregular (jigsaw puzzle like shape) with slightly (Fig. 5A) to strongly sinuous anticlinal wall (Fig. 5B), cuticle smooth. *Stomata*: anomocytic (Fig. 5C).

In transverse section—*Epidermis*: uniseriate, adaxial cell usually wider than high or equal in width and height, rarely higher than wide. *Hypodermis* absent. *Mesophyll*: dorsiventral, adaxial palisade cells in one layer, 10–60 percents of mesophyll, spongy mesophyll in 3–8 layers (Fig. 5E), bundle sheath extention present or absent (Fig. 5D). *Midrib outline*: adaxial varies from concave, convex, to flattened, abaxial convex to flattened, ground tissue consisted of 1–5 layers lacunar (Fig. 6C) or lamella collenchyma (Fig. 6D) below the epidermis followed by parenchymatous tissue. *Crystal*: absent or present, occurring in ground tissue (Fig. 6E) or closed to vascular bundle (Fig. 6F) if present. *Fibres*: absent or present (Fig. 6B). *Vascular*

bundle: collateral, cylindrical, xylem and phloem arranged in discontinuouse ring (Fig. 6A).

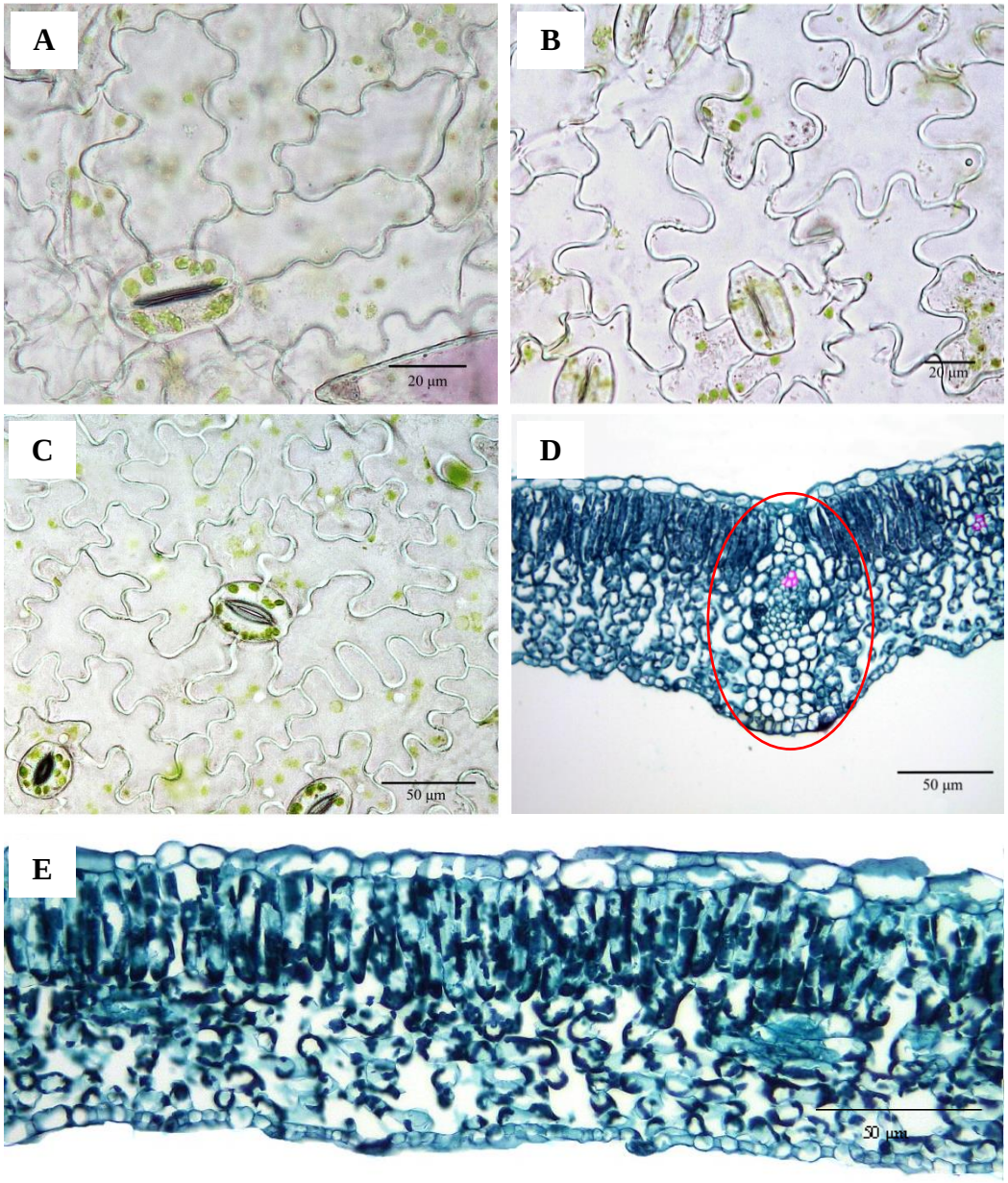

Figure 5 Anatomy of lamina in the genus *Clerodendrum*: A. slightly sinuous anticlinal wall; B strongly sinuous anticlinal wall; C. anomocytic stomata; D. the BEs (circle); E. lamina transverse section showing epidermis and mesophyll arrangement.

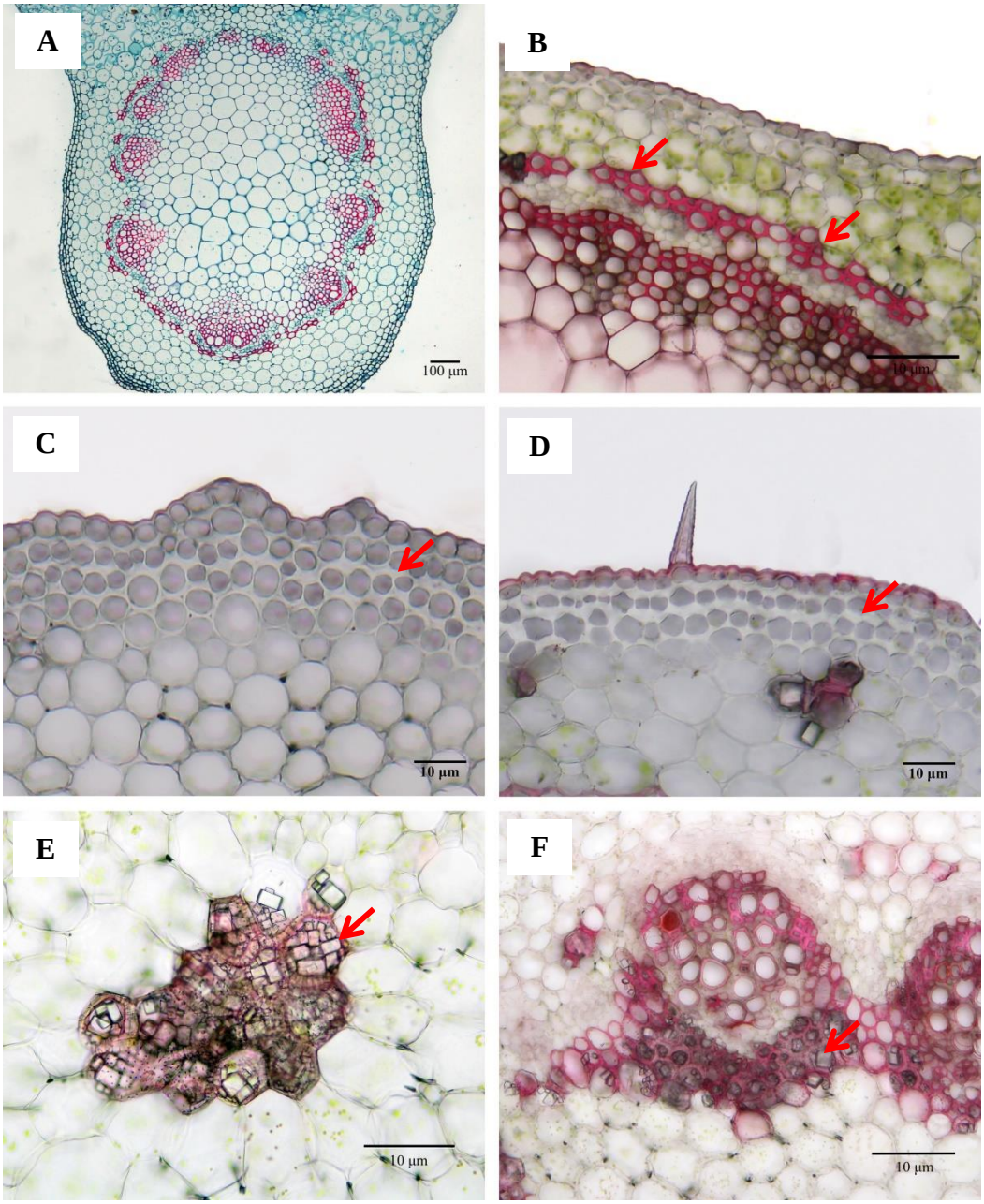

Figure 6 Anatomy of midrib in the genus *Clerodendrum*: A. cylindrical vasculabundle; B. sclerechymatous fibres; C. lacunar collenchyma; D. lamella collenchyma; E.prismatic crystals in parenchymatous tissue; F.prismatic crystals near vascular bundle.

Subsequently, the key to species and the species describtion based on leaf blade anatomical characters are provided.

KEY TO SPECIES

1 Leaves hypostomatic.....16. *C. longisepalum*

1 Leaves amphistomatic…….....…….……..2

2 Peltate glandular trichomes present…….....3

3 Leaf base with scale-like glands on abaxial surface.....4

4 Unicellular hairs present.....4. *C. colebrookianum*

4 Unicellular hairs absent.....2. *C. chinense* var. *chinense* and 3. *C. chinense* var. *simplex*

3 Leaf base without 2 clumps of scale-like glands on abaxial surface.....5

5 Unicellular hairs present.....15. *C. lloydianum*

5 Unicellular hairs absent.....…….….....…6

6 Collenchyma type lamella …….....……13. *C. japonicum*

6 Collenchyma type lacunar …….....….……...7

7 Crystal present.……...12. *C. intermedium* and 18. *C. paniculatum*

7 Crystals absent.…….....……..7. *C. garrettianum*

2 Peltate glandular trichomes absent…….....…..…….…8

8 Crystals present……..….....9

9 Curvature of abaxial anticlinal walls in epidermal cell strongly sinuous.....

..…….....11. *C. infortunatum* and 19. *C. schmidtii*

9 Curvature of abaxial anticlinal walls in epidermal cell slightly sinuous….....

.....22. *C. villosum*

8 Crystal absent…….....10

10 Collenchyma type lamella……...…..14. *C. lankawiensis*

10 Collenchyma lacunar…….....11

11 Anthocyanin present in abaxial epidermal cells….9. *C. haematolasium*

11 Anthocyanin absent in abaxial epidermal cells…….....12

12 Abaxial leaf surface glabrous except hairy on vein.....

.....10. *C. indicum*, 17. *C. nutans*, 20. *C.* sp. and 21. *C. umbratile*

12 Abaxial leaf surface hairy throughout the leaf blade.....

.....1. *C. calamitosum*, 5. *C. deflexum*, 6. *C. disperifolium* and 8. *C. godefroyii*

1. *Clerodendrum calamitosum* L.

- **Lamina surface view**

Indumentum: sparsely hairy**,** trichomes occur mostly on vein, consisting of capitate glandular trichomes and multicellular uniseriate trichomes. ***Epidermal cells***: irregular (jigsaw puzzle–like shape), anticlinal wall strongly sinuous, cuticle smooth. ***Stomata***: anomocytic, amphistomatic (Fig. 7A).

- **Lamina transverse–section**

Lamina: dorsiventral. The epidermis uniseriate. The shapes of adaxial and abaxial epidermal cells are irregular or round, usually wider than high, sometime equal in width and height, hypodermis absent. ***Mesophyll***: adaxial palisade cells present in single layer, 20–30 % of mesophyll, spongy mesophyll cells in 5–7 layers (Fig. 7C). ***Midrib***: adaxial convex, abaxial convex to flattened, ground tissue consisted of 1–2 layers lacunar collenchyma (Fig. 6C) below the epidermis followed by parenchymatous tissue, crystal absent. ***Vascular bundle***: collateral, cylindrical, xylem and phloem arranged in discontinuous ring (Fig. 7B).

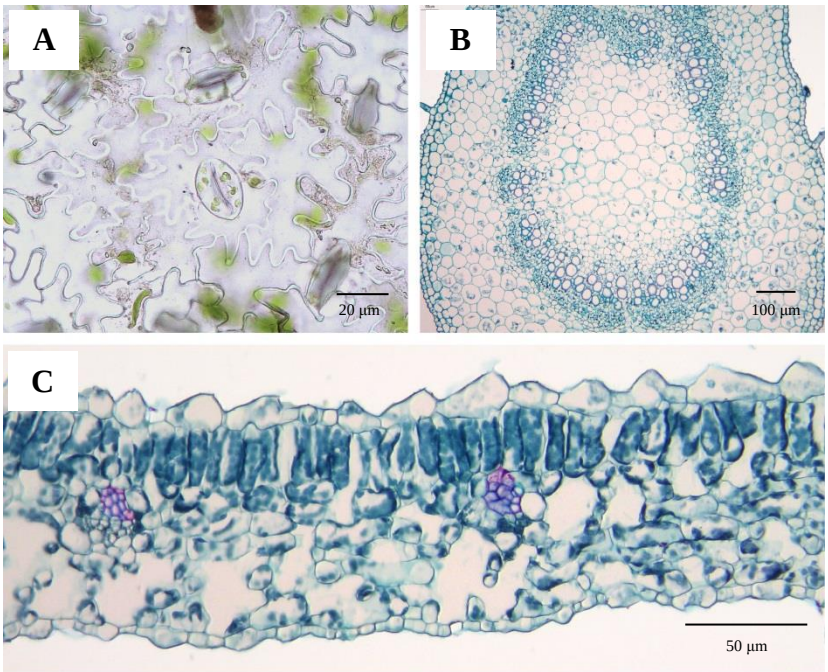

Figure 7 Leaf blade anatomy of *C. calamitosum*: A. anomocytic stomata and irregular epidermal cells with sinuous anticlinal wall; B. collateral discontinuous vascular bundle; C. lamina transverse section showing epidermis and mesophyll arrangement.

2. *Clerodendrum chinense* var. *chinense*(Osbeck) Mabb.

- **Lamina surface view**

Indumentum: moderately hairy**,** trichomes occur more densely on vein, consisting of both capitate and peltate glandular trichomes, multicellular uniseriate trichomes and clump of scale–like glands at leaf base of abaxial surface (Fig. 4H). ***Epidermal cells***: irregular (jigsaw puzzle–like shape), anticlinal wall strongly sinuous, cuticle smooth. ***Stomata***: anomocytic, amphistomatic (Fig. 8A).

- **Lamina transverse–section**

Lamina: dorsiventral. The epidermis uniseriate. The shapes of adaxial epidermal cells are irregular, usually wider than high, abaxial epidermal cells are smaller than adaxial cells, usually regular or round, equal in width and height, hypodermis absent. ***Mesophyll***: adaxial palisade cells present in one single layer, 40–50 % of mesophyll, spongy mesophyll cells in 6–7 layers (Fig. 8C), bundle sheath extension present (Fig. 5D). ***Midrib***: adaxial shape convex, abaxial shape convex to flattened, ground tissue consisted of 2–3 layers lacunar collenchyma below the epidermis followed by parenchymatous tissue, crystal absent. ***Vascular bundle***: collateral, cylindrical, xylem and phloem arranged in discontinuous ring (Fig. 8B).

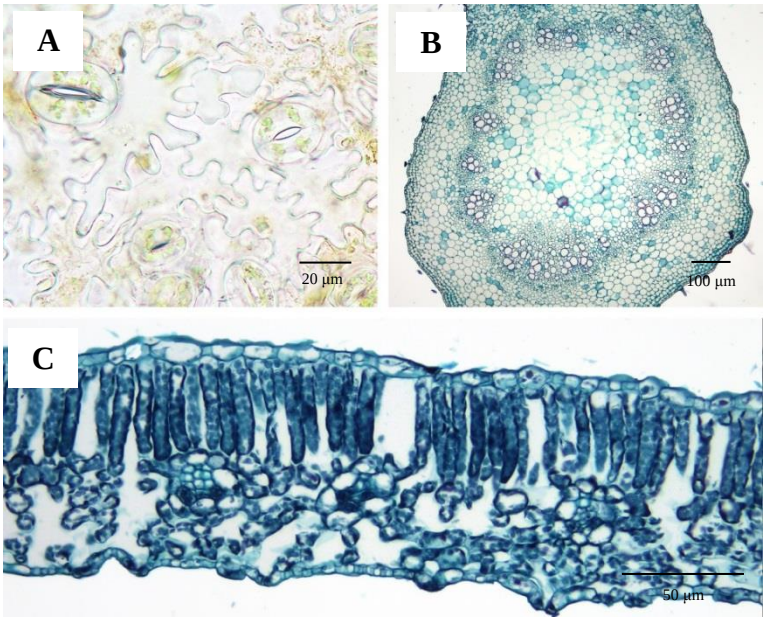

Figure 8 Leaf blade anatomy of *C. chinense* var. *chinense*: A. anomocytic stomata and irregular epidermal cells with sinuous anticlinal wall; B. collateral discontinuous vascular bundle; C. lamina transverse section showing epidermis and mesophyll arrangement.

3. *Clerodendrum chinense* var. *simplex* (Moldenke) Chen

- **Lamina surface view**

Indumentum: moderately hairy**,** trichomes occur more densely on vein, consisting of both capitate and peltate glandular trichomes, multicellular uniseriate trichomes and clump of scale–like glands at leaf base of abaxial surface. ***Epidermal cells***: irregular (jigsaw puzzle–like shape), anticlinal wall strongly sinuous, cuticle smooth. ***Stomata***: anomocytic, amphistomatic (Fig. 9A).

- **Lamina transverse–section**

Lamina: dorsiventral. The epidermis uniseriate. The shapes of adaxial epidermal cells are rectangular, usually wider than high, abaxial epidermal cells are smaller than adaxial cells, usually regular or round, equal in width and height, sometimes rectangular, hypodermis absent. ***Mesophyll***: adaxial palisade cells present in one single layer, 40–50 % of mesophyll, spongy mesophyll cells in 4–5 layers (Fig. 9C). ***Midrib***: adaxial convex, abaxial convex to flattened, ground tissue consisted of 3–5 layers lacunar collenchyma below the epidermis followed by parenchymatous tissue, crystal absent. ***Vascular bundle***: collateral, cylindrical, xylem and phloem arranged in discontinuous ring (Fig. 9B).

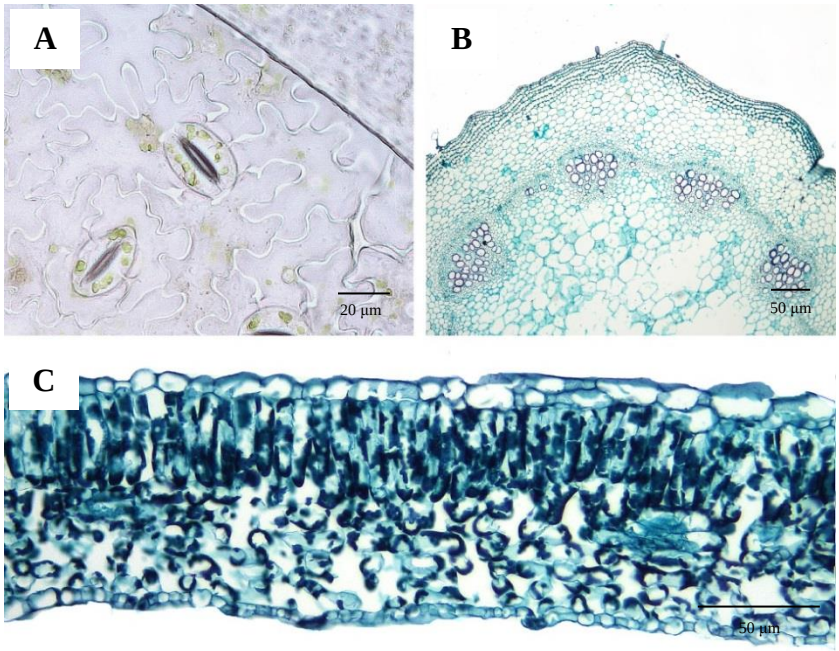

Figure 9 Leaf blade anatomy of *C. chinense* var. *simplex*: A. anomocytic stomata and irregular epidermal cells with sinuous anticlinal wall; B. collateral discontinuous vascular bundle; C. lamina transverse section showing epidermis and mesophyll arrangement.

4. *Clerodendrum colebrookianum* Walp.

- **Lamina surface view**

Indumentum: sparsely hairy**,** trichomes occur more densely on vein, consisting of both capitate and peltate glandular trichomes, multicellular uniseriate trichomes, unicellular trichome and scale-like glands at leaf base of abaxial surface. ***Epidermal cells***: irregular (jigsaw puzzle–like shape), anticlinal wall strongly sinuous, cuticle smooth. ***Stomata***: anomocytic, amphistomatic (Fig. 10A).

- **Lamina transverse–section**

Lamina: dorsiventral. The epidermis uniseriate. The shapes of adaxial epidermal cells are rectangular, usually wider than high, abaxial epidermal cells are regular or round, equal in width and height, sometimes rectangular, hypodermis absent. ***Mesophyll***: adaxial palisade cells present in one single layer, 20–25 % of mesophyll, spongy mesophyll cells in 7–8 layers (Fig. 10C), bundle sheath extension present. ***Midrib***: adaxial convex, abaxial convex to flattened, ground tissue consisted of 3–5 layers lacunar collenchyma below the epidermis followed by parenchymatous tissue, crystal absent, fibres absent or present (Fig. 6B). ***Vascular bundle***: collateral, cylindrical, xylem and phloem arranged in discontinuous ring (Fig. 10B).

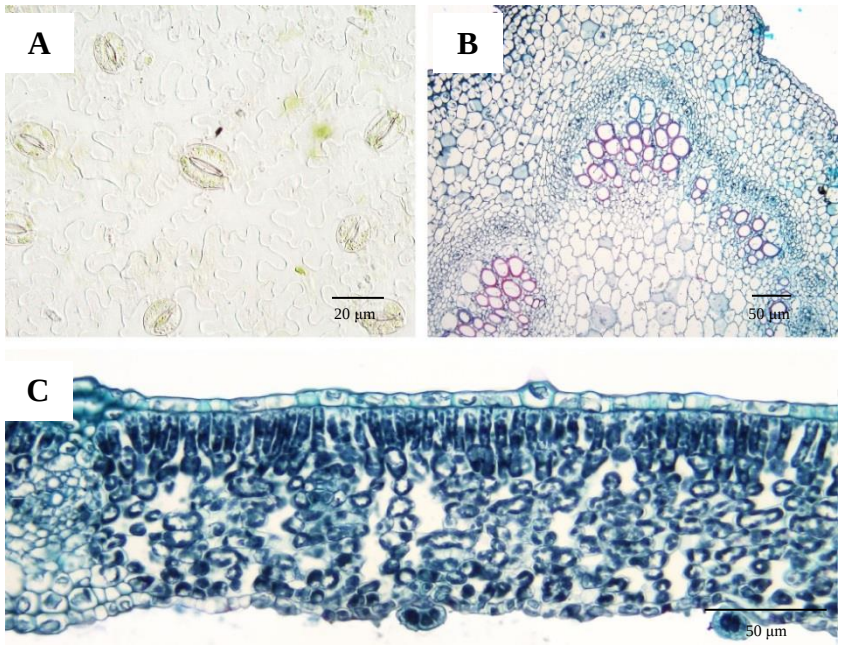

Figure 10 Leaf blade anatomy of *C. colebrookianum*: A. anomocytic stomata and irregular epidermal cells with sinuous anticlinal wall; B. collateral discontinuous vascular bundle; C. lamina transverse section showing epidermis and mesophyll arrangement.

5. *Clerodendrum deflexum* Wall.

- **Lamina surface view**

Indumentum: sparsely hairy**,** trichomes occur mostly on vein, consisting of capitate glandular trichomes and multicellular uniseriate trichomes. ***Epidermal cells***: irregular (jigsaw puzzle–like shape), anticlinal wall strongly sinuous, cuticle smooth. ***Stomata***: anomocytic, amphistomatic (Fig. 11A).

- **Lamina transverse–section**

Lamina: dorsiventral. The epidermis uniseriate. The shapes of adaxial epidermal cells are irregular, usually wider than high, abaxial epidermal cells are regular or round, equal in width and height, sometimes rectangular, hypodermis absent. ***Mesophyll***: adaxial palisade cells present in one single layer, 20–30 % of mesophyll, spongy mesophyll cells in 5–6 layers (Fig. 11C). ***Midrib***: adaxial convex, abaxial convex to flattened, ground tissue consisted of 2–3 layers lacunar collenchyma below the epidermis followed by parenchymatous tissue, crystal absent. ***Vascular bundle***: collateral, cylindrical, xylem and phloem arranged in discontinuous ring (Fig. 11B).

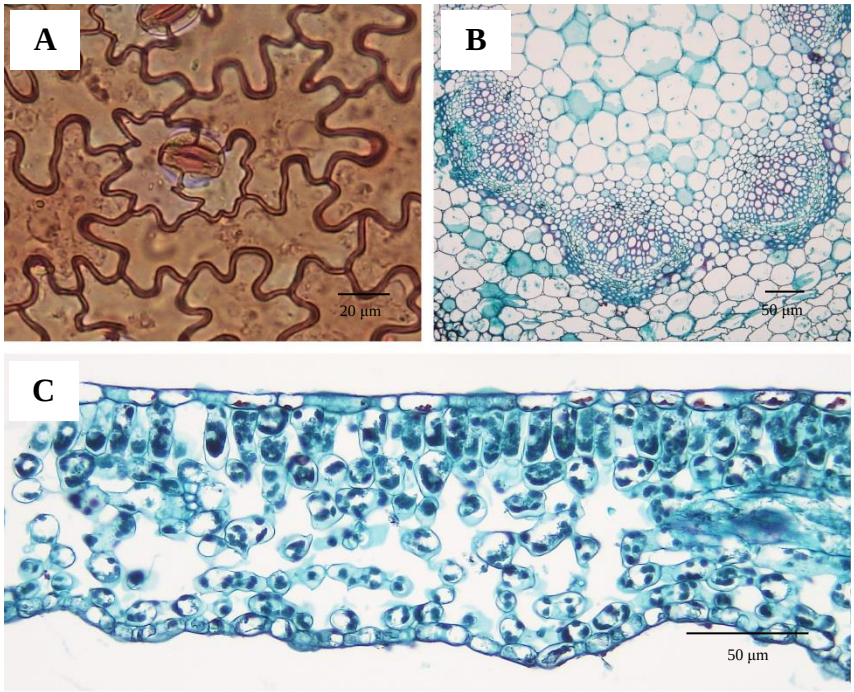

Figure 11 Leaf blade anatomy of *C. deflexum*: A. anomocytic stomata and irregular epidermal cells with sinuous anticlinal wall; B. collateral discontinuous vascular bundle; C. lamina transverse section showing epidermis and mesophyll arrangement.

6. *Clerodendrum disperifolium* Blume

- **Lamina surface view**

Indumentum: sparsely hairy**,** trichomes occur mostly on vein, consisting of capitate glandular trichomes and multicellular uniseriate trichomes. ***Epidermal cells***: irregular (jigsaw puzzle–like shape), anticlinal wall strongly sinuous, cuticle smooth. ***Stomata***: anomocytic, amphistomatic (Fig. 12A).

- **Lamina transverse–section**

Lamina: dorsiventral. The epidermis uniseriate. The shapes of adaxial epidermal cells are irregular, usually wider than high, abaxial epidermal cells are irregular, rectangular or round, hypodermis absent. ***Mesophyll***: adaxial palisade cells present in one single layer, 20–25 % of mesophyll, spongy mesophyll cells in 6–7 layers (Fig. 12C). ***Midrib***: adaxial convex, abaxial convex to flattened, ground tissue consisted of 1–2 layers lacunar collenchyma below the epidermis followed by parenchymatous tissue, crystal absent, fibres absent or present. ***Vascular bundle***: collateral, cylindrical, xylem and phloem arranged in discontinuous ring (Fig. 12B).

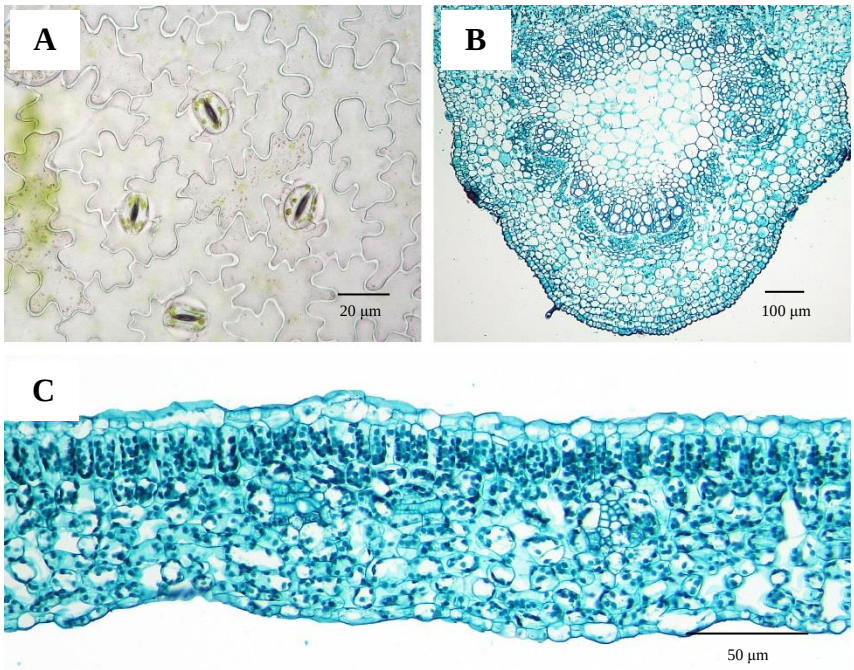

Figure 12 Leaf blade anatomy of *C. disperifolium*: A. anomocytic stomata and irregular epidermal cells with sinuous anticlinal wall; B. collateral discontinuous vascular bundle; C. lamina transverse section showing epidermis and mesophyll arrangement.

7. *Clerodendrum garrettianum* Craib

- **Lamina surface view**

Indumentum: glabrous**,** trichomes occur only on vein**,** consisting of both capitate and peltate glandular trichomes and multicellular uniseriate trichomes. ***Epidermal cells***: irregular (jigsaw puzzle–like shape), anticlinal wall strongly sinuous, cuticle smooth. ***Stomata***: anomocytic, amphistomatic (Fig. 13A).

- **Lamina transverse–section**

Lamina: dorsiventral or unifacial. The epidermis uniseriate. The shapes of adaxial epidermal cells are irregular, usually wider than high, abaxial epidermal cells are rectangular or round, hypodermis absent. ***Mesophyll***: adaxial palisade cells present in one single layer, 30 % of mesophyll, spongy mesophyll cells in 4–5 layers (Fig. 13C). ***Midrib***: adaxial convex, abaxial convex to flattened, ground tissue consisted of 2–3 layers lacunar collenchyma below the epidermis followed by parenchymatous tissue, crystal absent. ***Vascular bundle***: collateral, cylindrical, xylem and phloem arranged in discontinuous ring (Fig. 13B).

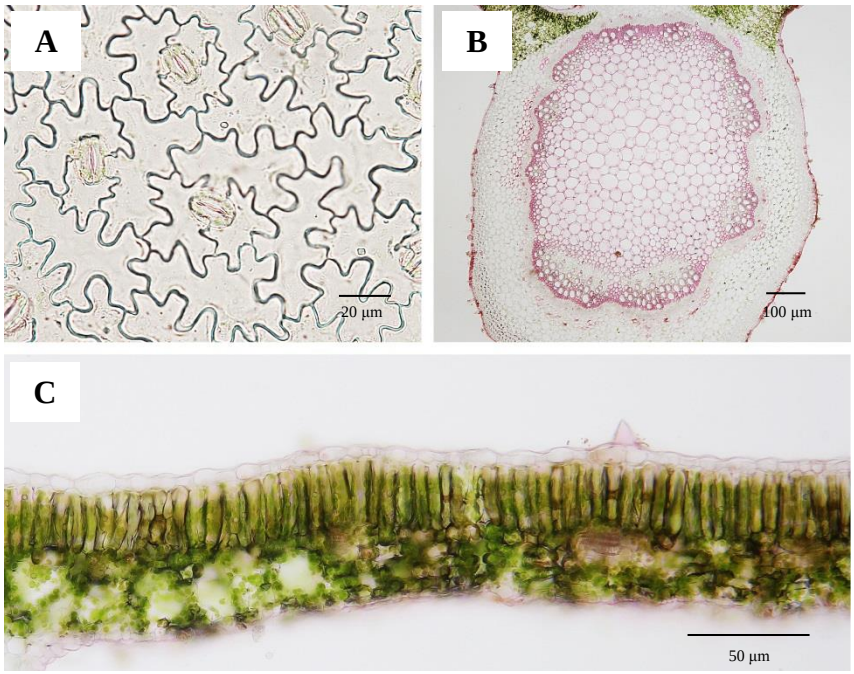

Figure 13 Leaf blade anatomy of *C. garrettianum*: A. anomocytic stomata and irregular epidermal cells with sinuous anticlinal wall; B. collateral discontinuous vascular bundle; C. lamina transverse section showing epidermis and mesophyll arrangement.

8. *Clerodendrum godefroyi* Kuntze

- **Lamina surface view**

Indumentum: sparsely hairy**,** trichomes occur mostly on vein, consisting of capitate glandular trichomes and multicellular uniseriate trichomes. ***Epidermal cells***: irregular (jigsaw puzzle–like shape), anticlinal wall strongly sinuous, cuticle smooth. ***Stomata***: anomocytic, amphistomatic (Fig. 14A).

- **Lamina transverse–section**

Lamina: dorsiventral. The epidermis uniseriate. The shapes of adaxial epidermal cells are irregular, usually wider than high, sometime higher than wide, abaxial epidermal cells irregular, rectangular or round, sometimes equal in width and height, hypodermis absent. ***Mesophyll***: adaxial palisade cells present in one single layer, 30–40 % of mesophyll, spongy mesophyll cells in 3–4 layers (Fig. 14C). ***Midrib***: adaxial convex, abaxial convex to flattened, ground tissue consisted of 1–2 layers lacunar collenchyma below the epidermis followed by parenchymatous tissue, crystal absent. ***Vascular bundle***: collateral, cylindrical, xylem and phloem arranged in discontinuous ring (Fig. 14B).

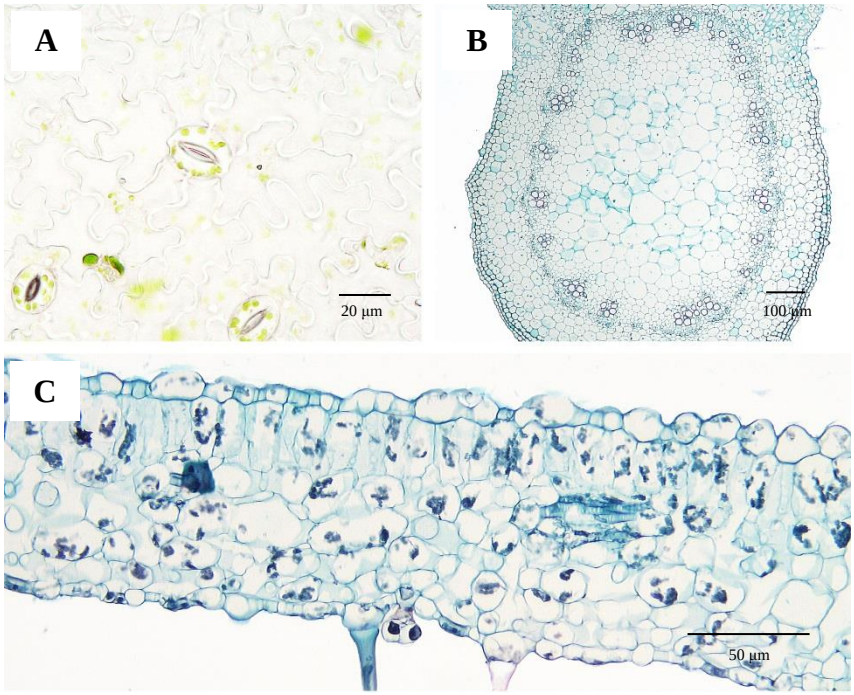

Figure 14 Leaf blade anatomy of *C. godefroyi*: A. anomocytic stomata and irregular epidermal cells with sinuous anticlinal wall; B. collateral discontinuous vascular bundle; C. lamina transverse section showing epidermis and mesophyll arrangement.

9. *Clerodendrum haematolasium* Hall.f.

- **Lamina surface view**

Indumentum: hairy**,** trichome distribute evenly throughout the leaf blade, consisting of capitate glandular trichomes and multicellular uniseriate trichomes. ***Epidermal cells***: irregular (jigsaw puzzle–like shape), anticlinal wall slightly sinuous, anthocyanin occurs in abaxial epidermis (Fig. 15B), cuticle smooth. ***Stomata***: anomocytic, amphistomatic (Fig. 15A).

- **Lamina transverse–section**

Lamina: dorsiventral. The epidermis uniseriate. The shapes of adaxial epidermal cells are regular, usually equal in width and height, abaxial epidermal cells regular, sometimes round, equal in width and height, hypodermis absent. ***Mesophyll***: adaxial palisade cells present in one single layer, 25–30 % of mesophyll, spongy mesophyll cells in 4–5 layers (Fig. 15D). ***Midrib***: adaxial convex to flattened, abaxial convex to flattened, ground tissue consisted of 1–3 layers lacunar collenchyma below the epidermis followed by parenchymatous tissue, crystal absent, fibres absent or present . ***Vascular bundle***: collateral, cylindrical, arranged in discontinuous ring (Fig. 15C).

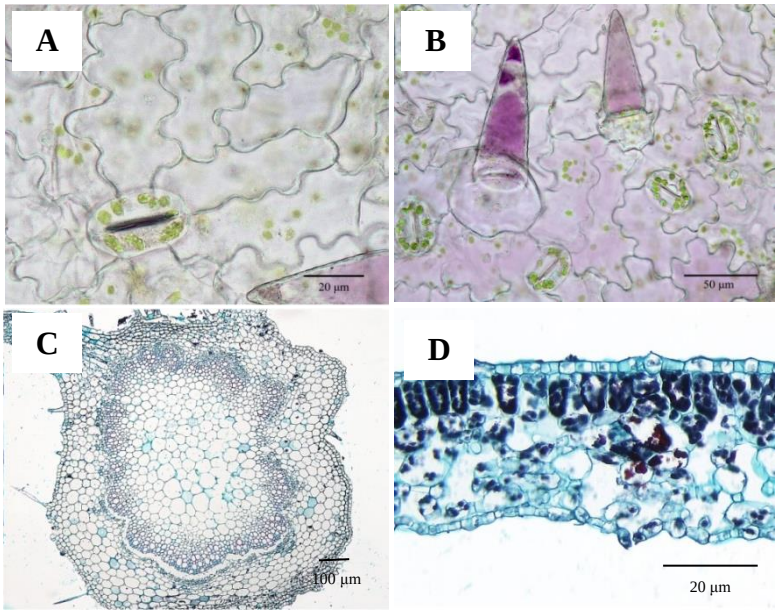

Figure 15 Leaf blade anatomy of *C. haematolasium*: A. anomocytic stomata and irregular epidermal cells with sinuous anticlinal wall; B. collateral discontinuous vascular bundle; C. lamina transverse section showing epidermis and mesophyll arrangement.

10. *Clerodendrum indicum* (L.) Kuntze

- **Lamina surface view**

Indumentum: glabrous**,** trichomes occur sparsely and only on vein, consisting of capitate glandular trichomes and multicellular uniseriate trichomes. ***Epidermal cells***: irregular (jigsaw puzzle–like shape), anticlinal wall strongly sinuous, cuticle smooth. ***Stomata***: anomocytic, amphistomatic (Fig. 16A).

- **Lamina transverse–section**

Lamina: dorsiventral. The epidermis uniseriate. The shapes of adaxial epidermal cells are regular, usually wider than high, abaxial epidermal cells regular, usually wider than high, sometimes equal in width and height, hypodermis absent. ***Mesophyll***: adaxial palisade cells present in one single layer, 25–30 % of mesophyll, spongy mesophyll cells in 5–6 layers (Fig. 16C). ***Midrib***: adaxial convex, abaxial convex to flattened, ground tissue consisted of 1–2 layers lacunar collenchyma below the epidermis followed by parenchymatous tissue, crystal absent. ***Vascular bundle***: collateral, cylindrical, xylem and phloem arranged in discontinuous ring (Fig. 16B).

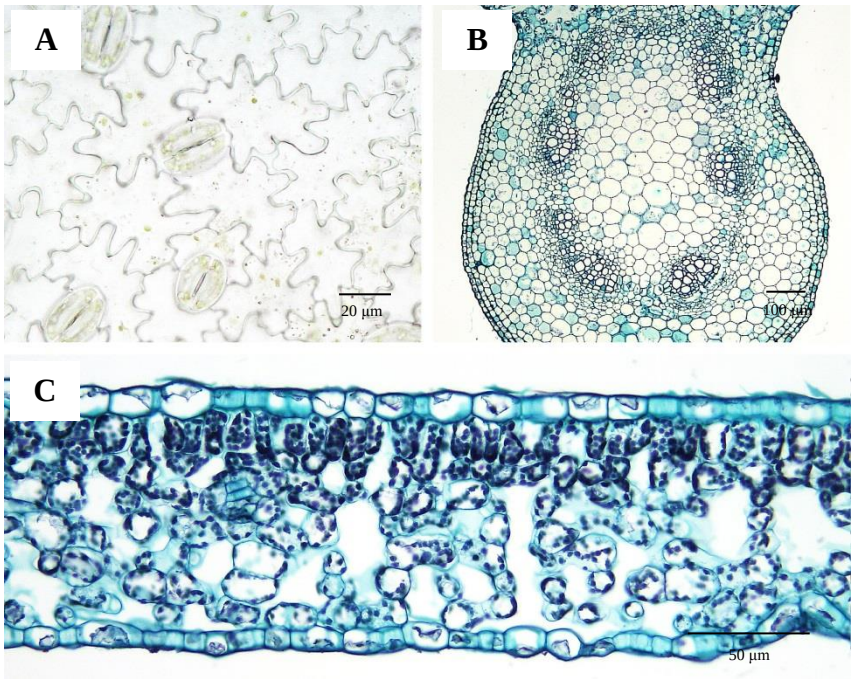

Figure 16 Leaf blade anatomy of *C. indicum*: A. anomocytic stomata and irregular epidermal cells with sinuous anticlinal wall; B. collateral discontinuous vascular bundle; C. lamina transverse section showing epidermis and mesophyll arrangement.

11. *Clerodendrum infortunatum* L.

- **Lamina surface view**

Indumentum: hairy**,** trichomes distribute evenly throughout the leaf blade, consisting of capitate glandular trichomes and multicellular uniseriate trichomes. ***Epidermal cells***: irregular (jigsaw puzzle–like shape), anticlinal wall strongly sinuous, cuticle smooth. ***Stomata***: anomocytic, amphistomatic (Fig. 17A).

- **Lamina transverse–section**

Lamina: dorsiventral. The epidermis uniseriate. The shapes of adaxial epidermal cells are regular, usually wider than high, abaxial epidermal cells irregular, rectangular or round, hypodermis absent. ***Mesophyll***: adaxial palisade cells present in one single layer, 40–50 % of mesophyll, spongy mesophyll cells in 4–6 layers (Fig. 17C). ***Midrib***: adaxial convex, abaxial convex to flattened, ground tissue consisted of 2–3 layers lacunar collenchyma below the epidermis followed by parenchymatous tissue, prismatic crystal present, randomly accumulated in parenchyma tissue (Fig. 27E), fibres present. ***Vascular bundle***: collateral, cylindrical, xylem and phloem arranged in discontinuous ring (Fig. 17B).

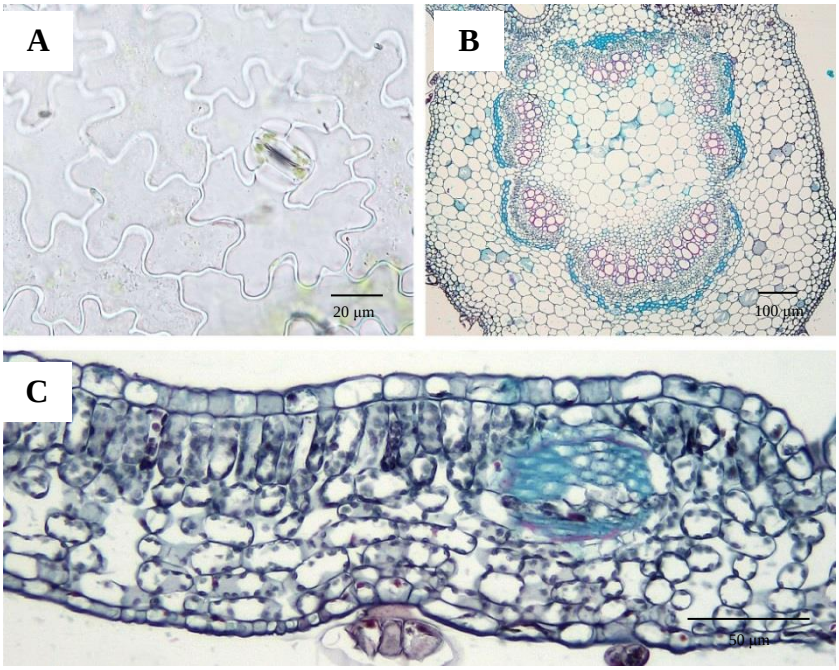

Figure 17 Leaf blade anatomy of *C. infortunatum*: A. anomocytic stomata and irregular epidermal cells with sinuous anticlinal wall; B. collateral discontinuous vascular bundle; C. lamina transverse section showing epidermis and mesophyll arrangement.

12. *Clerodendrum intermedium* Cham.

- **Lamina surface view**

Indumentum: moderately hairy**,** trichomes distribute evenly throughout the leaf blade, consisting of both capitate and peltate glandular trichomes and multicellular uniseriate trichomes. ***Epidermal cells***: irregular (jigsaw puzzle–like shape), anticlinal wall strongly sinuous, cuticle smooth. ***Stomata***: anomocytic, amphistomatic (Fig. 18A).

- **Lamina transverse–section**

Lamina: dorsiventral. The epidermis uniseriate. The shapes of adaxial epidermal cells are irregular, usually wider than high, abaxial epidermal cells smaller than adaxial cells, irregular, rectangular or round, hypodermis absent. ***Mesophyll***: adaxial palisade cells present in one single layer, 20–30 % of mesophyll, spongy mesophyll cells in 5–6 layers (Fig. 18C). ***Midrib***: adaxial convex, abaxial convex to flattened, ground tissue consisted of 2–4 layers lacunar collenchyma below the epidermis followed by parenchymatous tissue, prismatic crystal present, particularly accumulated near vascular bundle (Fig. 6F). ***Vascular bundle***: collateral, cylindrical, xylem and phloem arranged in discontinuous ring (Fig. 18B).

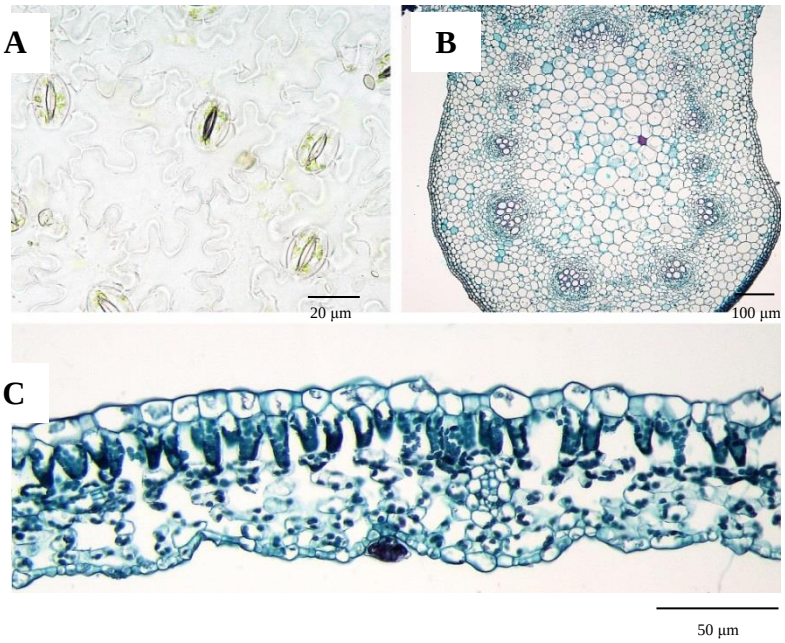

Figure 18 Leaf blade anatomy of *C. intermedium*: A. anomocytic stomata and irregular epidermal cells with sinuous anticlinal wall; B. collateral discontinuous vascular bundle; C. lamina transverse section showing epidermis and mesophyll arrangement.

13. *Clerodendrum japonicum* (Thunb.) Sweet

- **Lamina surface view**

Indumentum: sparsely hairy**,** trichomes distribute evenly throughout the leaf blade, consisting of both capitate and peltate glandular trichomes, multicellular uniseriate trichomes. ***Epidermal cells***: irregular (jigsaw puzzle–like shape), anticlinal wall strongly sinuous, cuticle smooth. ***Stomata***: anomocytic, amphistomatic (Fig. 19A).

- **Lamina transverse–section**

Lamina: dorsiventral. The epidermis uniseriate. The shapes of adaxial epidermal cells are irregular, usually wider than high, abaxial epidermal cells irregular, sometimes round, hypodermis absent. ***Mesophyll***: adaxial palisade cells present in one single layer, 30–40 % of mesophyll, spongy mesophyll cells in 5–6 layers (Fig. 19C). ***Midrib***: adaxial convex, abaxial convex to flattened, ground tissue consisted of 2–3 layers lamella collenchyma (Fig. 6D) below the epidermis followed by parenchymatous tissue, prismatic crystal present, randomly accumulated in parenchyma tissue. ***Vascular bundle***: collateral, cylindrical, xylem and phloem arranged in discontinuous ring (Fig. 19B).

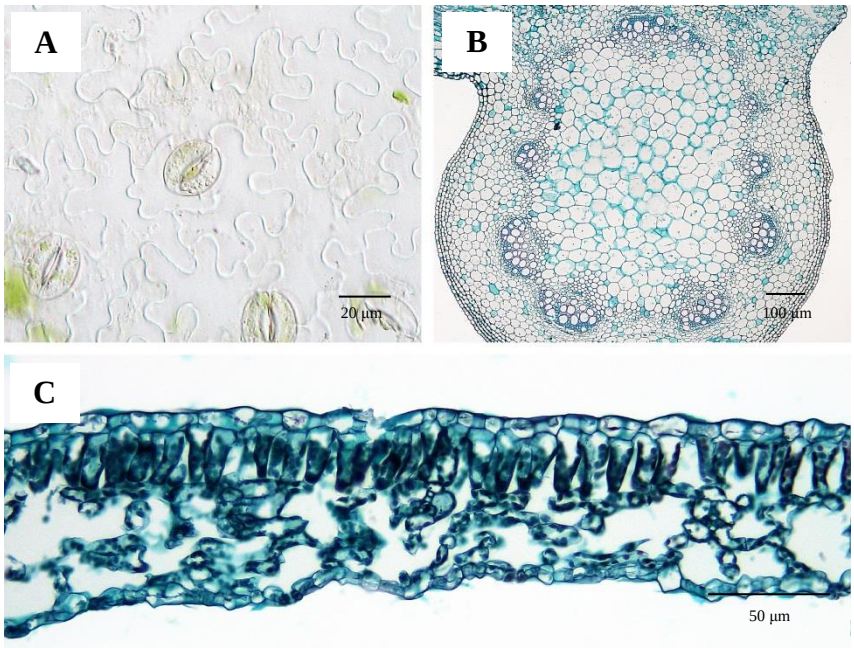

Figure 19 Leaf blade anatomy of *C. japonicum*: A. anomocytic stomata and irregular epidermal cells with sinuous anticlinal wall; B. collateral discontinuous vascular bundle; C. lamina transverse section showing epidermis and mesophyll arrangement.

14. *Clerodendrum lankawiense* King & Gamble

- **Lamina surface view**

Indumentum: hairy**,** trichomes distribute evenly throughout the leaf blade, consisting of capitate glandular trichomes and multicellular uniseriate trichomes. ***Epidermal cells***: irregular (jigsaw puzzle–like shape), anticlinal wall strongly sinuous, cuticle smooth. ***Stomata***: anomocytic, amphistomatic (Fig. 20A).

- **Lamina transverse–section**

Lamina: dorsiventral. The epidermis uniseriate. The shapes of adaxial epidermal cells are regular, usually wider than high, abaxial epidermal cells smaller than abaxial cells, irregular, sometimes round, hypodermis absent. ***Mesophyll***: adaxial palisade cells present in one single layer, 25–30 % of mesophyll, spongy mesophyll cells in 3–4 layers (Fig. 20C). ***Midrib***: adaxial convex, abaxial convex to flattened, ground tissue consisted of 2–3 layers lamella collenchyma below the epidermis followed by parenchymatous tissue, crystal absent, fibres present. ***Vascular bundle***: collateral, cylindrical, xylem and phloem arranged in discontinuous ring (Fig. 20B)**.**

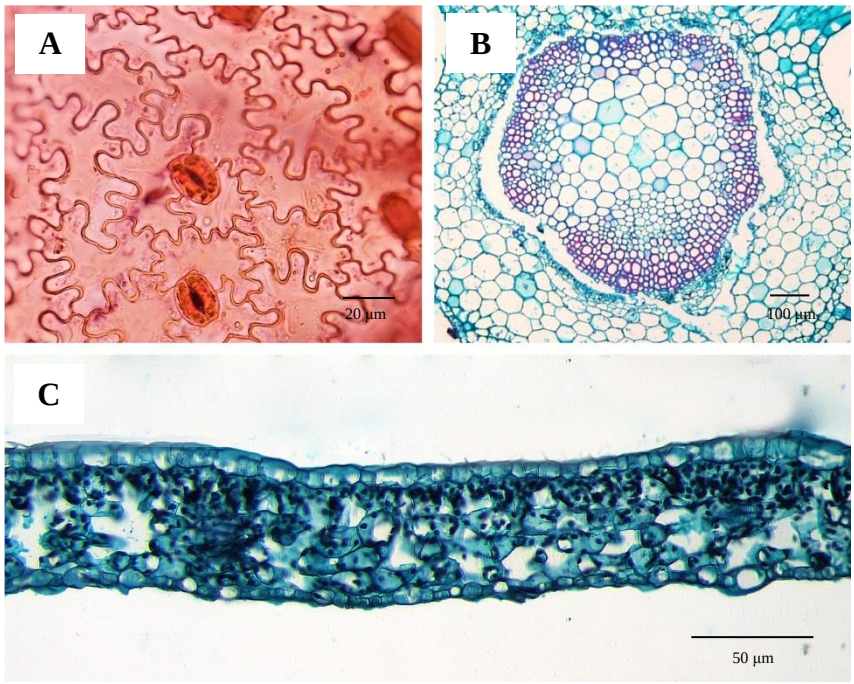

Figure 20 Leaf blade anatomy of *C. lankawiense*: A. anomocytic stomata and irregular epidermal cells with sinuous anticlinal wall; B. collateral discontinuous vascular bundle; C. lamina transverse section showing epidermis and mesophyll arrangement.

15. *Clerodendrum lloydianum* Craib

- **Lamina surface view**

Indumentum: moderately hairy**,** trichomes distribute more densly on vein, consisting of both capitate and peltate glandular trichomes, unicellular trichomes and multicellular uniseriate trichomes. ***Epidermal cells***: irregular (jigsaw puzzle–like shape), anticlinal wall strongly sinuous, cuticle smooth. ***Stomata***: anomocytic, amphistomatic (Fig. 21A).

- **Lamina transverse–section**

Lamina: dorsiventral. The epidermis uniseriate. The shapes of adaxial epidermal cells are regular, usually wider than high, abaxial epidermal cells smaller than abaxial cells, irregular, sometimes round, hypodermis absent. ***Mesophyll***: adaxial palisade cells present in one single layer, 30–40 % of mesophyll, spongy mesophyll cells in 3–4 layers (Fig. 21C). ***Midrib***: adaxial convex, abaxial convex to flattened, ground tissue consisted of 1–3 layers lacunar collenchyma below the epidermis followed by parenchymatous tissue, crystal absent, fibres present. ***Vascular bundle***: collateral, cylindrical, xylem and phloem arranged in discontinuous ring (Fig. 21B)**.**

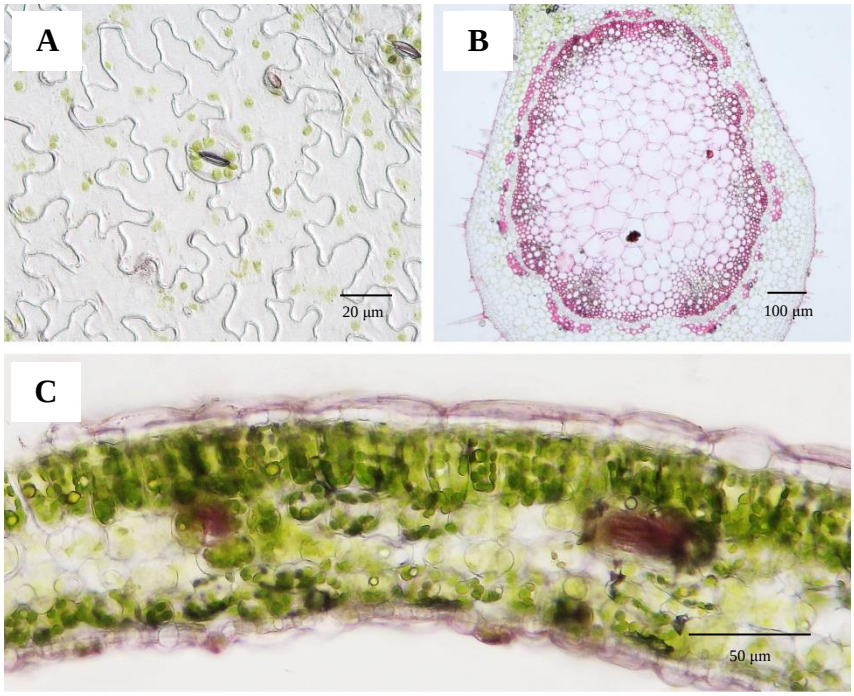

Figure 21 Leaf blade anatomy of *C. lloydianum*: A. anomocytic stomata and irregular epidermal cells with sinuous anticlinal wall; B. collateral discontinuous vascular bundle; C. lamina transverse section showing epidermis and mesophyll arrangement.

16. *Clerodendrum longisepalum* Dop

- **Lamina surface view**

Indumentum: glabrous**,** trichomes distribute only on vein, consisting of capitate glandular trichomes and multicellular uniseriate trichomes. ***Epidermal cells***: irregular (jigsaw puzzle–like shape), anticlinal wall strongly sinuous, cuticle smooth. ***Stomata***: anomocytic, hypostomatic (Fig. 22A).

- **Lamina transverse–section**

Lamina: dorsiventral. The epidermis uniseriate. The shapes of adaxial epidermal cells are irregular, rectangular, usually wider than high, sometimes round, abaxial epidermal cells irregular, hypodermis absent. ***Mesophyll***: adaxial palisade cells present in one single layer, 20–40 % of mesophyll, spongy mesophyll cells in 3–5 layers (Fig. 22C). ***Midrib***: adaxial convex, abaxial convex to flattened, ground tissue consisted of 1–2 layers lacunar collenchyma below the epidermis followed by parenchymatous tissue, crystal absent, fibres present. ***Vascular bundle***: collateral, cylindrical, xylem and phloem arranged in discontinuous ring (Fig. 22B)**.**

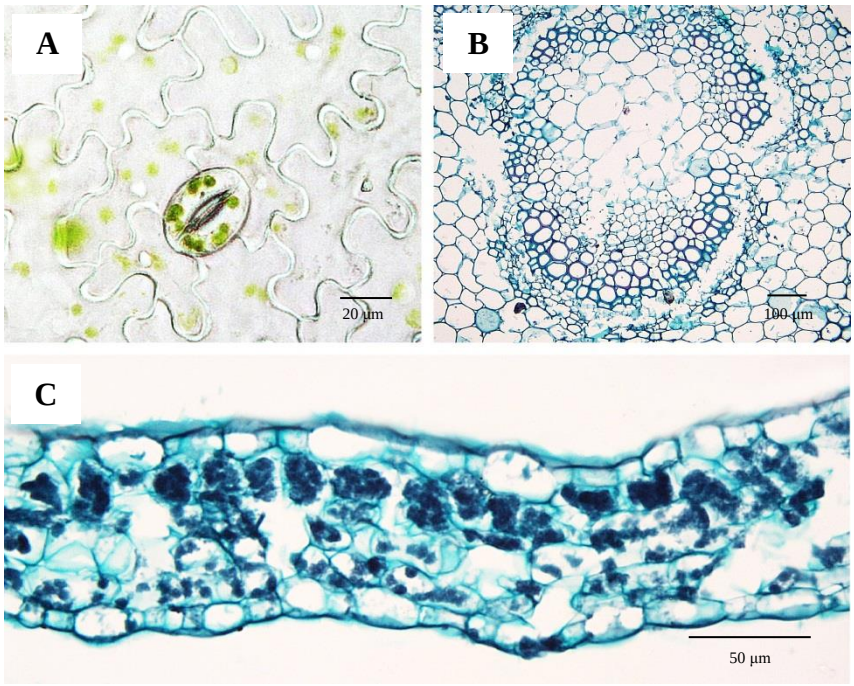

Figure 22 Leaf blade anatomy of *C. longisepalum*: A. anomocytic stomata and irregular epidermal cells with sinuous anticlinal wall; B. collateral discontinuous vascular bundle; C. lamina transverse section showing epidermis and mesophyll arrangement.

17. *Clerodendrum nutans* Wall. ex Jacke

- **Lamina surface view**

Indumentum: glabrous**,** trichomes distribute only on vein, consisting of capitate glandular trichomes and multicellular uniseriate trichomes. ***Epidermal cells***: irregular (jigsaw puzzle–like shape), anticlinal wall strongly sinuous, cuticle smooth. ***Stomata***: anomocytic, amphistomatic (Fig. 23A).

- **Lamina transverse–section**

Lamina: dorsiventral. The epidermis uniseriate. The shapes of adaxial epidermal cells are regular, usually wider than high, abaxial epidermal cells smaller than abaxial cells, irregular, sometimes round, hypodermis absent. ***Mesophyll***: adaxial palisade cells present in one single layer, 10–20 % of mesophyll, spongy mesophyll cells in 5–7 layers (Fig. 23C). ***Midrib***: adaxial convex, abaxial convex to flattened, ground tissue consisted of 2–3 layers lacunar collenchyma below the epidermis followed by parenchymatous tissue, crystal absent, fibres absent or present. ***Vascular bundle***: collateral, cylindrical, xylem and phloem arranged in discontinuous ring (Fig. 23B)**.**

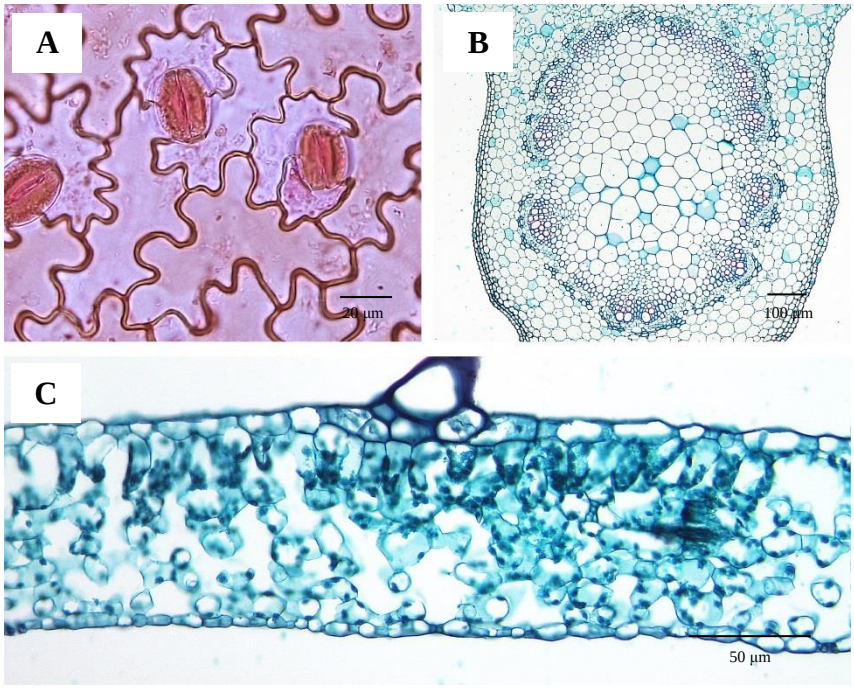

Figure 23 Leaf blade anatomy of *C. nutans*: A. anomocytic stomata and irregular epidermal cells with sinuous anticlinal wall; B. collateral discontinuous vascular bundle; C. lamina transverse section showing epidermis and mesophyll arrangement.

18. *Clerodendrum paniculatum* L.

- **Lamina surface view**

Indumentum: moderately hairy**,** trichomes distribute more densely on vein, consisting of both capitate and peltate glandular trichomes, multicellular uniseriate trichomes. ***Epidermal cells***: irregular (jigsaw puzzle–like shape), anticlinal wall strongly sinuous, cuticle smooth. ***Stomata***: anomocytic, amphistomatic (Fig. 24A).

- **Lamina transverse–section**

Lamina: dorsiventral. The epidermis uniseriate. The shapes of adaxial epidermal cells are irregular, usually wider than high, sometimes round, abaxial epidermal cells irregular, sometimes higher than wide or round, hypodermis absent. ***Mesophyll***: adaxial palisade cells present in one single layer, 25–30 % of mesophyll, spongy mesophyll cells in 5–6 layers, bundle sheath extension present (Fig. 24C). ***Midrib***: adaxial convex, abaxial convex to flattened, ground tissue consisted of 2–3 layers lacunar collenchyma below the epidermis followed by parenchymatous tissue, prismatic crystal present, randomly accumulated in parenchyma tissue. ***Vascular bundle***: collateral, cylindrical, xylem and phloem arranged in discontinuous ring (Fig. 24B)**.**

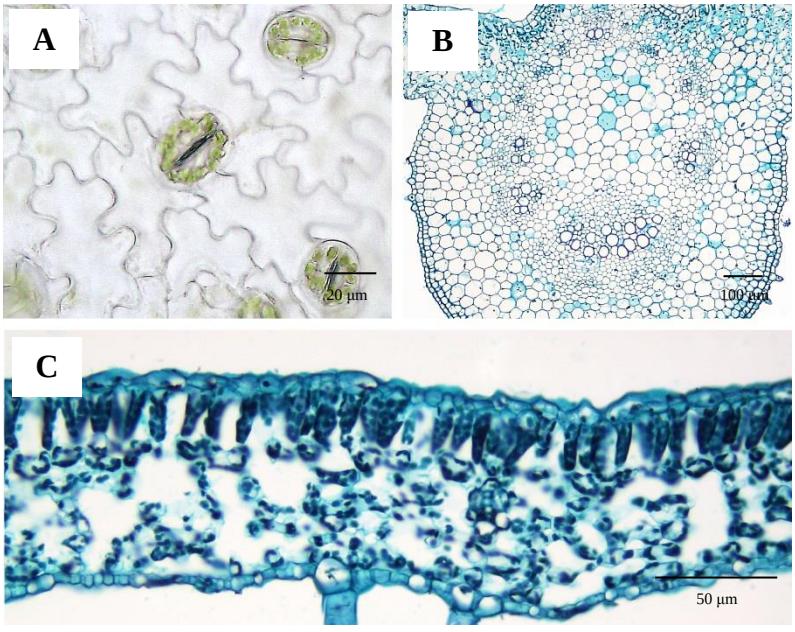

Figure 24 Leaf blade anatomy of *C. paniculatum*: A. anomocytic stomata and irregular epidermal cells with sinuous anticlinal wall; B. collateral discontinuous vascular bundle; C. lamina transverse section showing epidermis and mesophyll arrangement.

19. *Clerodendrum schmidtii* C.B. Clarke

- **Lamina surface view**

Indumentum: moderately hairy**,** trichomes distribute more densely on vein, consisting of capitate glandular trichomes and multicellular uniseriate trichomes. ***Epidermal cells***: irregular (jigsaw puzzle–like shape), anticlinal wall strongly sinuous, cuticle smooth. ***Stomata***: anomocytic, amphistomatic (Fig. 25A).

- **Lamina transverse–section**

Lamina: dorsiventral. The epidermis uniseriate. The shapes of adaxial epidermal cells are irregular, usually wider than high, abaxial epidermal cells irregular, usually wider than high, hypodermis absent. ***Mesophyll***: adaxial palisade cells present in one single layer, 20–30 % of mesophyll, spongy mesophyll cells in 5–6 layers (Fig. 25C). ***Midrib***: adaxial convex, abaxial convex to flattened, ground tissue consisted of 2–3 layers lacunar collenchyma below the epidermis followed by parenchymatous tissue, prismatic crystal present, randomly accumulated in parenchyma tissue, fibres present. ***Vascular bundle***: collateral, cylindrical, xylem and phloem arranged in discontinuous ring (Fig. 25B)**.**

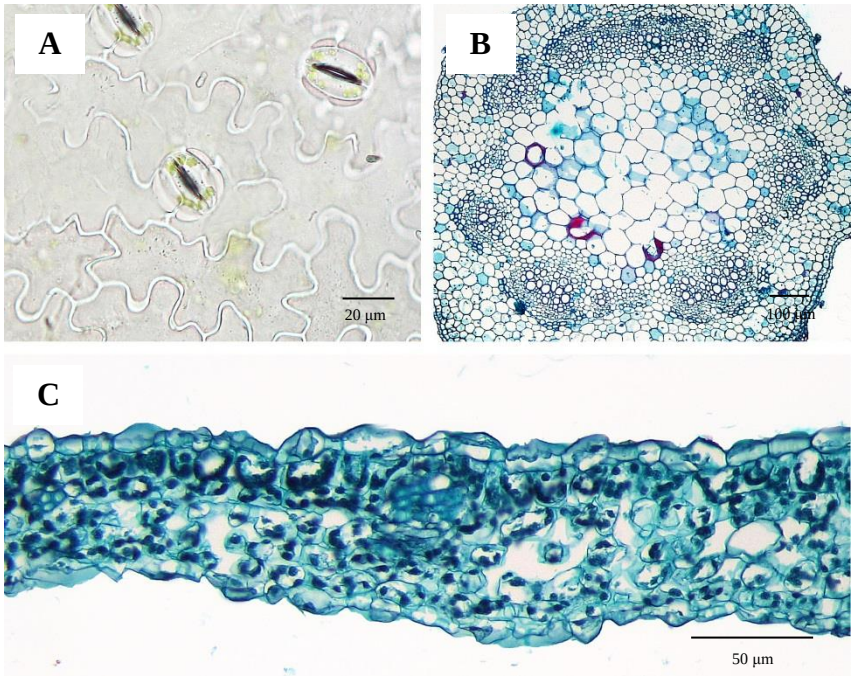

Figure 25 Leaf blade anatomy of *C. schmidtii*: A. anomocytic stomata and irregular epidermal cells with sinuous anticlinal wall; B. collateral discontinuous vascular bundle; C. lamina transverse section showing epidermis and mesophyll arrangement.

20. *Clerodendrum* sp.

- **Lamina surface view**

Indumentum: sparsely hairy**,** consisting of capitate glandular trichomes and multicellular uniseriate trichomes. ***Epidermal cells***: irregular (jigsaw puzzle–like shape), anticlinal wall strongly sinuous, cuticle smooth. ***Stomata***: anomocytic, amphistomatic (Fig. 26A).

- **Lamina transverse–section**

Lamina: dorsiventral. The epidermis uniseriate. The shapes of adaxial epidermal cells are irregular, usually wider than high, abaxial epidermal cells are rectangular or round, hypodermis absent. ***Mesophyll***: adaxial palisade cells present in one single layer, 20–30 % of mesophyll, spongy mesophyll cells in 3–4 layers (Fig. 26C). ***Midrib***: adaxial convex, abaxial convex to flattened, ground tissue consisted of 2–3 layers lacunar collenchyma below the epidermis followed by parenchymatous tissue, crystal absent. ***Vascular bundle***: collateral, cylindrical, xylem and phloem arranged in discontinuous ring (Fig. 26B).

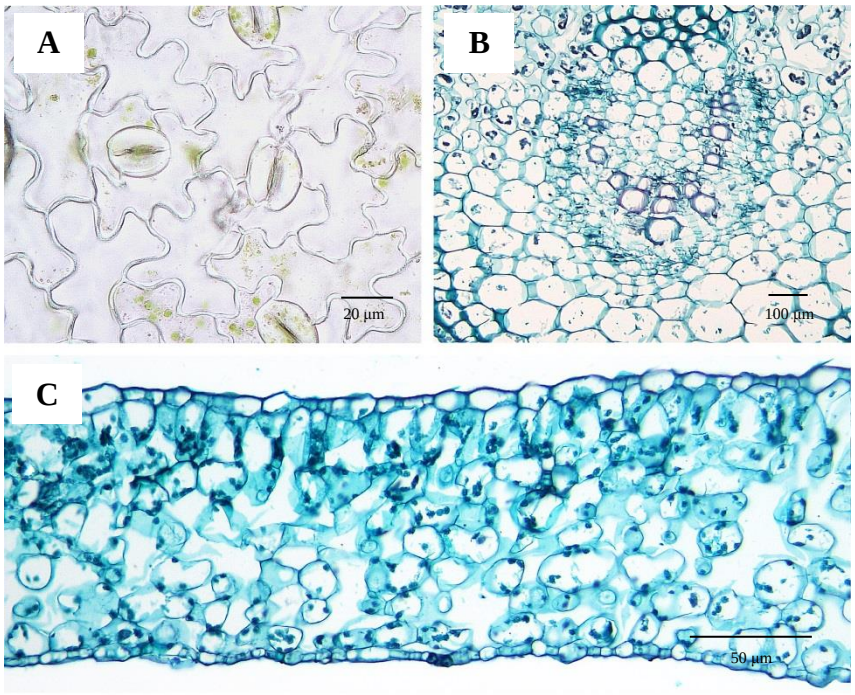

Figure 26 Leaf blade anatomy of *C.* sp.: A. anomocytic stomata and irregular epidermal cells with sinuous anticlinal wall; B. collateral discontinuous vascular bundle; C. lamina transverse section showing epidermis and mesophyll arrangement.

21. *Clerodendrum umbratile* King & Gamble

- **Lamina surface view**

Indumentum: glabrous**,** trichomes distribute only on vein, consisting of capitate glandular trichomes and multicellular uniseriate trichomes. ***Epidermal cells***: irregular (jigsaw puzzle–like shape), anticlinal wall strongly sinuous, cuticle smooth. ***Stomata***: anomocytic, amphistomatic (Fig. 27A).

- **Lamina transverse–section**

Lamina: dorsiventral. The epidermis uniseriate. The shapes of adaxial epidermal cells are regular, usually wider than high, abaxial epidermal cells regular, sometimes round, hypodermis absent. ***Mesophyll***: adaxial palisade cells present in one single layer, 10–20 % of mesophyll, spongy mesophyll cells in 6–7 layers (Fig. 27C). ***Midrib***: adaxial convex, abaxial convex to flattened, ground tissue consisted of 2–3 layers lacunar collenchyma below the epidermis followed by parenchymatous tissue, crystal absent, fibres present. ***Vascular bundle***: collateral, cylindrical, xylem and phloem arranged in discontinuous ring (Fig. 27B)**.**

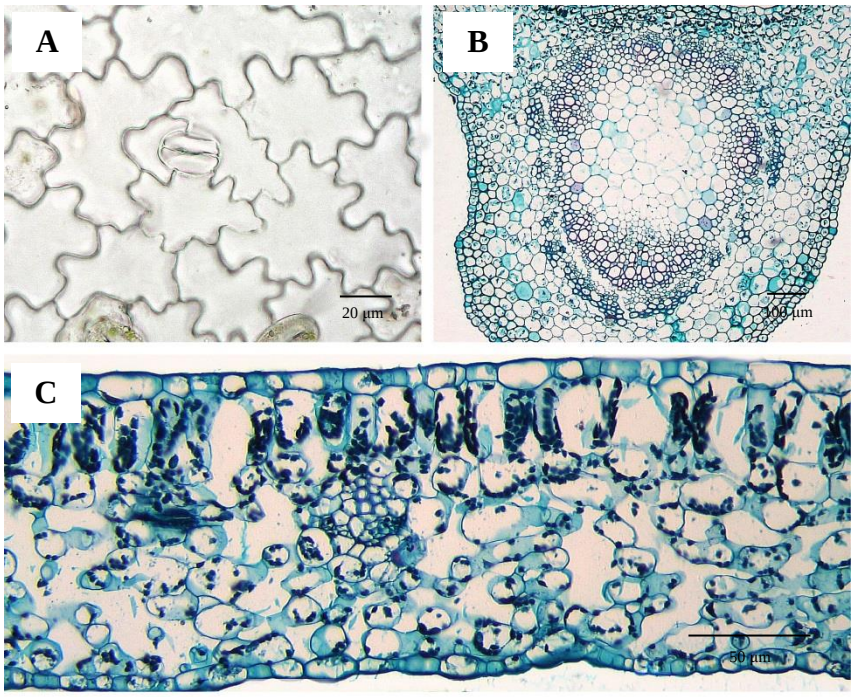

Figure 27 Leaf blade anatomy of *C. umbratile*: A. anomocytic stomata and irregular epidermal cells with sinuous anticlinal wall; B. collateral discontinuous vascular bundle; C. lamina transverse section showing epidermis and mesophyll arrangement.

22. *Clerodendrum villosum* Blume

- **Lamina surface view**

Indumentum: hairy**,** trichomes distribute evenly throughout the leaf blade, consisting of capitate glandular trichomes and multicellular uniseriate trichomes. ***Epidermal cells***: irregular (jigsaw puzzle–like shape), anticlinal wall slightly sinuous, cuticle smooth. ***Stomata***: anomocytic, amphistomatic (Fig. 28A).

- **Lamina transverse–section**

Lamina: dorsiventral. The epidermis uniseriate. The shapes of adaxial epidermal cells are iregular, usually wider than high, sometimes round, abaxial epidermal cells irregular, smaller than adaxial cells, mostly round, hypodermis absent. ***Mesophyll***: adaxial palisade cells present in one single layer, 50–60 % of mesophyll, spongy mesophyll cells in 3–5 layers (Fig. 28C). ***Midrib***: adaxial convex, abaxial convex to flattened, ground tissue consisted of 2–3 layers lacunar collenchyma below the epidermis followed by parenchymatous tissue, prismatic crystal present, randomly accumulated in parenchyma tissue, fibres present. ***Vascular bundle***: collateral, cylindrical, xylem and phloem arranged in discontinuous ring (Fig. 28B).

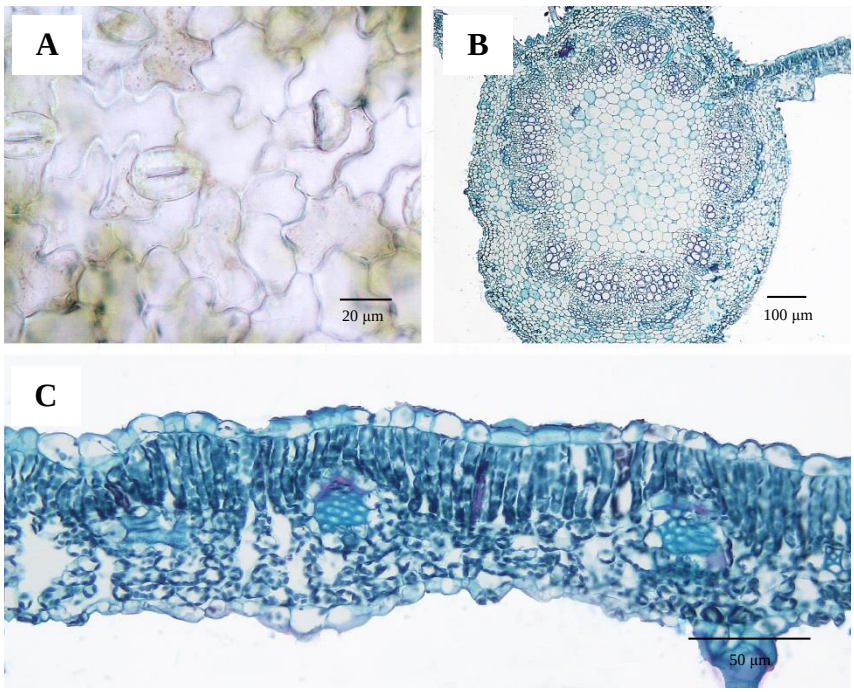

Figure 28 Leaf blade anatomy of *C. villosum*: A. anomocytic stomata and irregular epidermal cells with sinuous anticlinal wall; B. collateral discontinuous vascular bundle; C. lamina transverse section showing epidermis and mesophyll arrangement.

2. *Rotheca* Raf.

The common leaf blade anatomy of 2 taxa of *Rotheca* in present study were generally concluded.

In surface view—*leaf indumentum* consisting capitate glandular trichomes and multicellular uniseriate trichomes. Secretory gland composed of four to eight secretory cells. *Epidermal cell*: adaxial and abaxial epidermal cells are irregular (jigsaw puzzle like shape) with strongly to strongly sinuous anticlinal wall, cuticle smooth. *Stomata*: anomocytic.

In transverse section—*Epidermis*: uniseriate, adaxial cell irregular, usually wider than high, sometimes round, abaxial epidermis equal in size or smaller than adaxial cells. *Hypodermis* absent. *Mesophyll*: dorsiventral, adaxial palisade cells in one layer, 30–50 percents of mesophyll, spongy mesophyll in 3–6 layers. *Midrib outline*: adaxial varies from concave, convex, to flattened, abaxial convex to flattened, ground tissue consisted of 1–2 layers lacunar or lamella collenchyma below the epidermis followed by parenchymatous tissue. *Crystal*: absent. *Fibres* absent *Vascular bundle*: collateral, cylindrical, xylem and phloem arranged in discontinuouse ring.

Subsequently, the key to species and the species describtion based on leaf blade anatomical characters are provided.

KEY TO SPECIES

1 Trichomes distribute evenly throughout the leaf blade; color of subsidiary cells distinctly different from other cells in surface view by peeling method.....1. *R. macrostachya*

1 Trichomes distribute only on vein; color of subsidiary cells similar to other cells in surface view by peeling method.....2. *R. serrata*

1. *Rotheca macrostachya* (Turcz.) Leerat. & Chantar.

- **Lamina surface view**

Indumentum: moderately hairy**,** trichomes distribute evenly throughout the leaf blade, consisting of capitate glandular trichomes and multicellular uniseriate trichomes. ***Epidermal cells***: irregular (jigsaw puzzle–like shape), anticlinal wall strongly sinuous, cuticle smooth. ***Stomata***: diacytic, amphistomatic, subsidiary cells colourish, distinctly visible (Fig. 29A).

- **Lamina transverse–section**

Lamina: dorsiventral. The epidermis uniseriate. The shapes of adaxial epidermal cells are irregular, usually wider than high, abaxial epidermal cells irregular, smaller than adaxial cells, hypodermis absent. ***Mesophyll***: adaxial palisade cells present in one single layer, 40–50 % of mesophyll, spongy mesophyll cells in 3–4 layers (Fig. 29C). ***Midrib***: adaxial concave to flattened, abaxial convex to flattened, ground tissue consisted of 1–2 layers lacunar collenchyma below the epidermis followed by parenchymatous tissue, crystal absent. ***Vascular bundle***: collateral, cylindrical, xylem and phloem arranged in discontinuous ring (Fig. 29B).

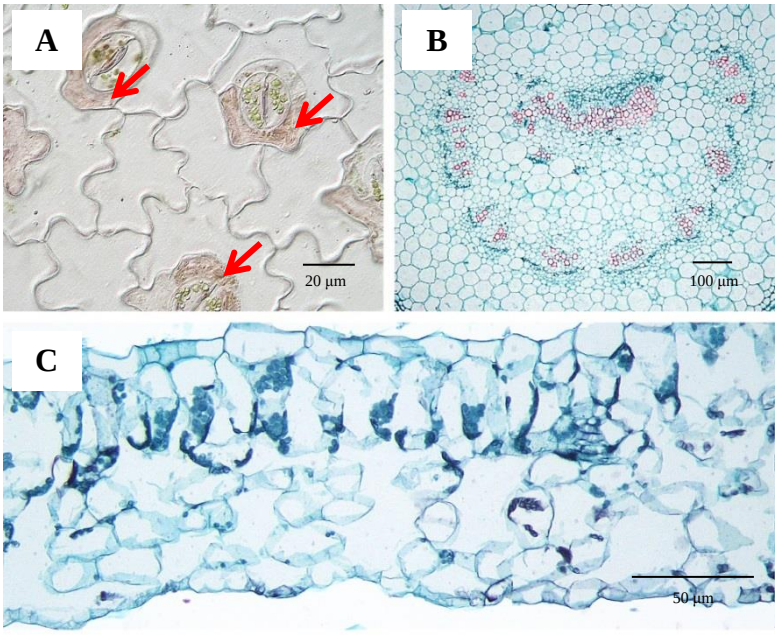

Figure 29 Leaf blade anatomy of *R. macrostachya*: A diacytic stomata, colourish subsidiary cells (arrows) and irregular epidermal cells with sinuous anticlinal wall; B collateral discontinuous vascular bundle; C lamina transverse section showing epidermis and mesophyll arrangement.

2. *Rotheca serrata* (L.) Steane & Mabb

- **Lamina surface view**

Indumentum: sparsely hairy, trichomes distribute only on vein, consisting of capitate glandular trichomes and multicellular uniseriate trichomes. ***Epidermal cells***: irregular (jigsaw puzzle–like shape), anticlinal wall strongly sinuous, cuticle smooth. ***Stomata***: diacytic, amphistomatic (Fig. 30A).

- **Lamina transverse–section**

Lamina: dorsiventral. The epidermis uniseriate. The shapes of adaxial epidermal cells are irregular, usually wider than high, sometimes round, abaxial epidermal cells irregular, mostly round, hypodermis absent. ***Mesophyll***: adaxial palisade cells present in one single layer, 30–40 % of mesophyll, spongy mesophyll cells in 4–6 layers (Fig. 30C). ***Midrib***: adaxial convex, abaxial convex to flattened, ground tissue consisted of 1–2 layers lacunar or lamella collenchyma below the epidermis followed by parenchymatous tissue, crystal absent, fibres absent or present. ***Vascular bundle***: collateral, cylindrical, xylem and phloem arranged in discontinuous ring (Fig. 30B)**.**

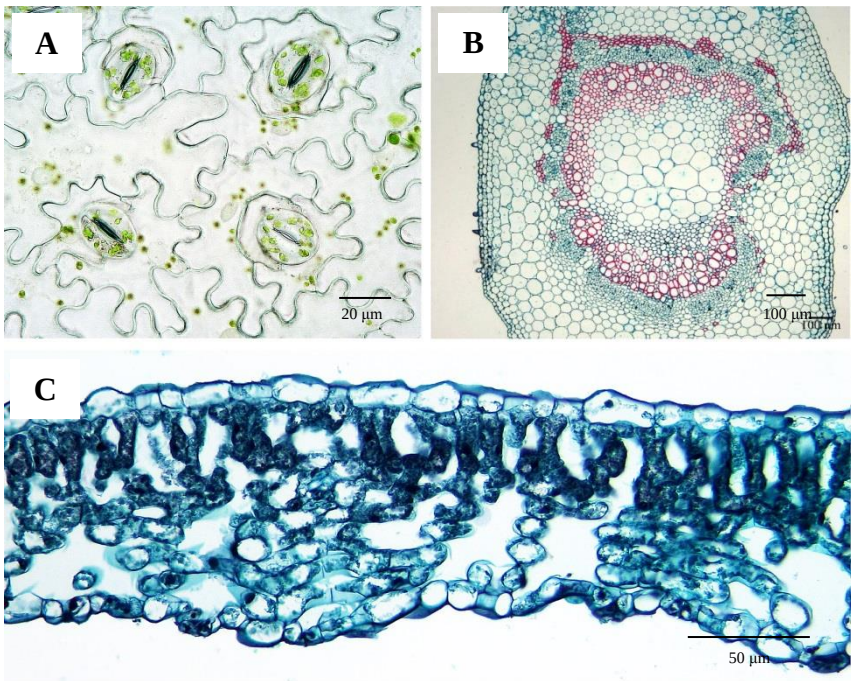

Figure 30 Leaf blade anatomy of *R. serrata*: A diacytic stomata and irregular epidermal cells with sinuous anticlinal wall; B collateral discontinuous vascular bundle; C lamina transverse section showing epidermis and mesophyll arrangement.

3. *Volkameria* L.

Since *Volkameria inermis* is the only species of the genus found in Thailand, the species describtion of leaf blade anatomical characteristics is presumably the representative of the genus in this study.

***Volkameria inermis* L.**

- **Lamina surface view**

Indumentum: glabrous**,** trichomes distribute only on vein, consisting of sunken capitate glandular trichomes (Fig. 31C) and very short unicellular trichomes (Fig. 31D). ***Epidermal cells***: isodiametric, anticlinal wall straight or slightly curve (Fig. 31A), cuticle striate (Fig. 31B). ***Stomata***: anomocytic, hypostomatic.

- **Lamina transverse–section**

Lamina: dorsiventral. The epidermis uniseriate. The shapes of adaxial epidermal cells are regular, equal in width and height, abaxial epidermal cells regular, sometimes round, hypodermis absent. ***Mesophyll***: adaxial palisade cells present in 2–3 layers, 50–60 % of mesophyll, spongy mesophyll cells in 6–7 layers (Fig. 31E). ***Midrib***: adaxial concave to flattened, abaxial convex to flattened, ground tissue consisted of 2–4 layers lacunar collenchyma below the epidermis followed by parenchymatous tissue, fibres present. ***Vascular bundle***: collateral, arc–shape with adaxial incurved partition (Fig. 31F).

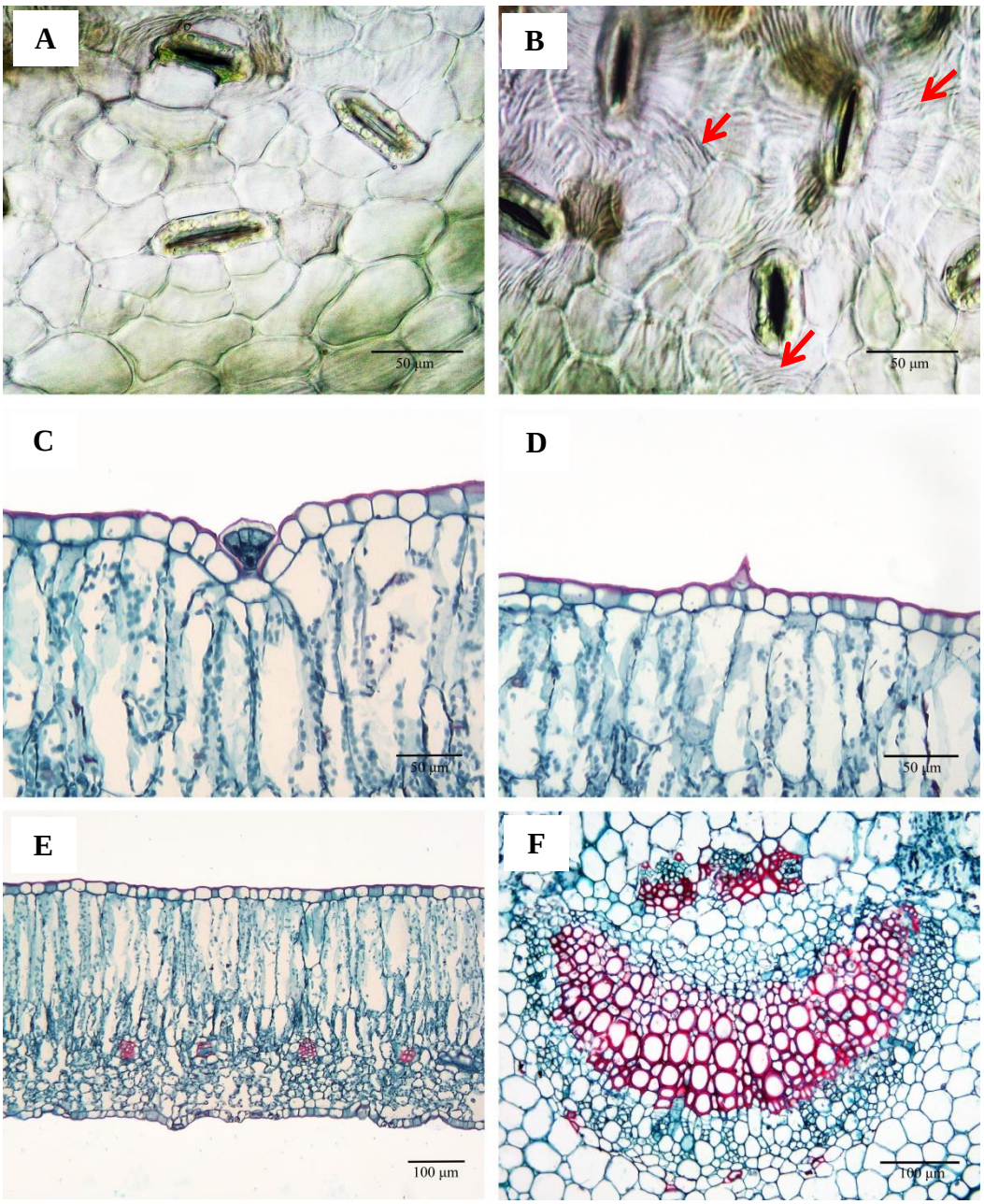

Figure 31 Leaf epidermal anatomy in *V. inermis*: A. anomocytic stomata with straight to curved anticlinal wall; B. striation on lower epidermis (arrows); C. sunken capitate glandular trichome; D. short small unicellular trichome; E. lamina in transverse section showing epidermis and mesophyll arrangement; F. arc-shaped vascular bundle with adaxial incurved partition.

4.2 Molecular study by iPBS

4.2.1 DNA isolation

DNA extracted from the leaf using the modified CTAB method give a good and sufficient quality DNA for PCR reaction. The quality of DNA tested by PCR confirm that the DNAs are suitable for PCR reaction (Figure 32). DNA isolated by minor modification method yields strong and reliable amplification products. The ratios of A260/A280 varie from 1.80 to 1.90. The parameters for the iPBS protocol from plant sample were also preliminarily optimized. The result shows that at 50 ng template DNA, 5 mM $MgCl_2$ and 0.5 U *Taq* polymerase concentration are suitable for further PCR analysis.

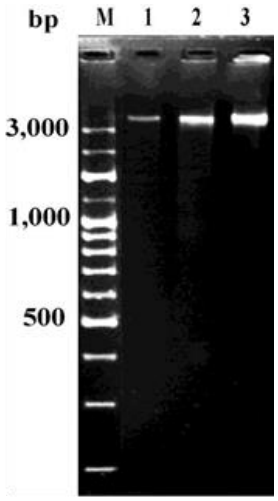

Figure 32 Pattern of DNA of *Clerodendrum* extracted from the leaf using a modified method (Vanijajiva, 2011); (M) Molecular ladder 100 bp (1) 25 mg, (2) 50 mg, (3) 100 mg of fresh leaves.

4.2.2 Genetic relationship of *Clerodendrum*, *Rotheca* and *Volkameria*

Initially, the DNA quality and quantity of *C. haematolasium* are probably low resulting in no amplification PCR product in all primers. Thus, the genetic data of the species could not be investigated and the number of studied taxa decrease to 24 taxa comparing to 25 taxa in anatomical study. Ten primers out of thirty successfully generated strong and clear visibly amplification band. The primers selected in this

study are 2076, 2079, 2095, 2224, 2232, 2237, 2271, 2272, 2273 and 2295. From 24 studied taxa, a total of 735 scorable bands were observed, which 734 band are polymorphic (%99.9). The fragment size ranged from 200 to 2500 bp. Almost all primer produced 100% of polymorphic bands (Table 4), while only primer 2232 produced one non-polymorphic band at 400 bp (Figure 37). The zymograms of iPBS amplification band are given (Figure. 33–42).

Table 4 Number of total and polymorphic bands bands generated by each primer and percent polymorphism.

Markers	Number of bands		Polymorphism (%)
	Total	Polymorphic	
2076	74	74	100
2079	85	85	100
2095	82	82	100
2224	72	72	100
2232	65	64	98.5
2237	69	69	100
2271	66	66	100
2272	71	71	100
2273	74	74	100
2295	77	77	100
Total	735	734	
Everage	73.5	73.4	99.9

The genetic similarity among 24 taxa examined using Dice similarity coefficients is shown in table 5. The highest genetic similarity is 0.560 observed between *C. japonicum* and *C. paniculatum*, whereas the lowest genetic similarity is 0.194 found between *V. inermis* and *R. serrata*.

Table 5 Pair–wise genetic similarity of 24 taxa according to Dice similarity index.

	C. nutans	*C. indicum*	*C.chinense* Var. *simplex*	*C. villosum*	*C. paniculatum*	*C. longisepalum*	*C. infortunatum*	*C. colebrookianum*	*C. disperifolium*	*C. umbratile*	*C. godefroyi*	*C. garrettianum*	*C. lankawiense*	*C. calamitosum*	*C. lloydianum*	*C. deflexum*	*C.* sp.	*C. schmidtii*	*C. japonicum*	*R. macrostachya*	*C. intermedium*	*C. chinense* var. *chinense*	*C. R. serrata*	*V. inermis*
C. nutans	1.000																							
C. indicum	0.311	1.000																						
C. chinense var. *simplex*	0.261	0.356	1.000																					
C. villosum	0.302	0.312	0.257	1.000																				
C. paniculatum	0.278	0.245	0.296	0.315	1.000																			
C. longisepalum	0.359	0.212	0.318	0.327	0.343	1.000																		
C. infortunatum	0.371	0.334	0.277	0.312	0.311	0.212	1.000																	
C. colebrookianum	0.367	0.316	0.301	0.331	0.315	0.215	0.393	1.000																
C. disperifolium	0.366	0.279	0.325	0.324	0.386	0.217	0.324	0.373	1.000															
C. umbratile	0.250	0.258	0.332	0.284	0.310	0.232	0.231	0.315	0.353	1.000														
C. godefroyi	0.398	0.342	0.267	0.331	0.331	0.327	0.229	0.359	0.377	0.407	1.000													
C. garrettianum	0.377	0.270	0.314	0.312	0.318	0.314	0.319	0.450	0.353	0.449	0.323	1.000												
C. lankawiense	0.322	0.359	0.339	0.338	0.311	0.329	0.342	0.332	0.303	0.363	0.362	0.364	1.000											
C. calamitosum	0.360	0.276	0.286	0.316	0.317	0.313	0.342	0.357	0.220	0.357	0.386	0.358	0.511	1.000										
C. lloydianum	0.395	0.315	0.233	0.262	0.328	0.365	0.373	0.270	0.326	0.373	0.399	0.331	0.334	0.313	1.000									
C. deflexum	0.331	0.233	0.254	0.273	0.280	0.316	0.283	0.358	0.312	0.341	0.315	0.346	0.362	0.382	0.264	1.000								
C. sp.	0.347	0.254	0.361	0.372	0.349	0.281	0.342	0.283	0.335	0.300	0.332	0.526	0.339	0.355	0.295	0.273	1.000							
C. schmidtii	0.379	0.320	0.273	0.328	0.395	0.373	0.443	0.357	0.356	0.377	0.239	0.304	0.360	0.365	0.238	0.295	0.324	1.000						
C. japonicum	0.319	0.236	0.249	0.279	0.560	0.346	0.370	0.318	0.332	0.339	0.355	0.282	0.353	0.320	0.293	0.329	0.302	0.389	1.000					
R. macrostachya	0.340	0.308	0.332	0.268	0.300	0.341	0.330	0.325	0.308	0.345	0.301	0.323	0.305	0.280	0.301	0.270	0.299	0.293	0.334	1.000				
C. intermedium	0.322	0.224	0.266	0.327	0.507	0.338	0.361	0.362	0.345	0.291	0.337	0.319	0.328	0.302	0.308	0.304	0.268	0.294	0.321	0.352	1.000			
C. chinense var. *chinense*	0.217	0.181	0.243	0.250	0.209	0.212	0.199	0.271	0.211	0.209	0.251	0.297	0.245	0.242	0.210	0.252	0.332	0.246	0.196	0.242	0.242	1.000		
R. serrata	0.256	0.198	0.217	0.232	0.226	0.203	0.220	0.229	0.258	0.288	0.252	0.298	0.247	0.223	0.234	0.294	0.266	0.290	0.279	0.272	0.221	0.458	1.000	
V. inermis	0.278	0.236	0.206	0.237	0.212	0.206	0.241	0.213	0.230	0.208	0.234	0.206	0.213	0.231	0.219	0.220	0.225	0.212	0.223	0.232	0.241	0.194	0.219	1.000

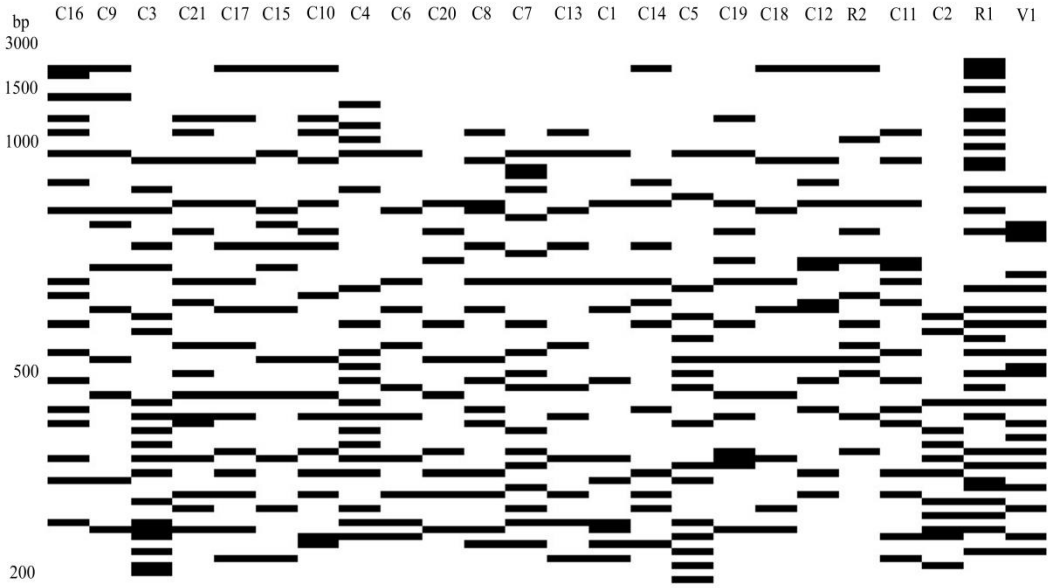

Figure 33 iPBS polymorphisms of 24 taxa revealed by primer 2076 (bp=molecular weight marker 1 kb ladder DNA; lanes C1–C21, R1–R2 and V1 stand for individual species of studied taxa, see material and method for details).

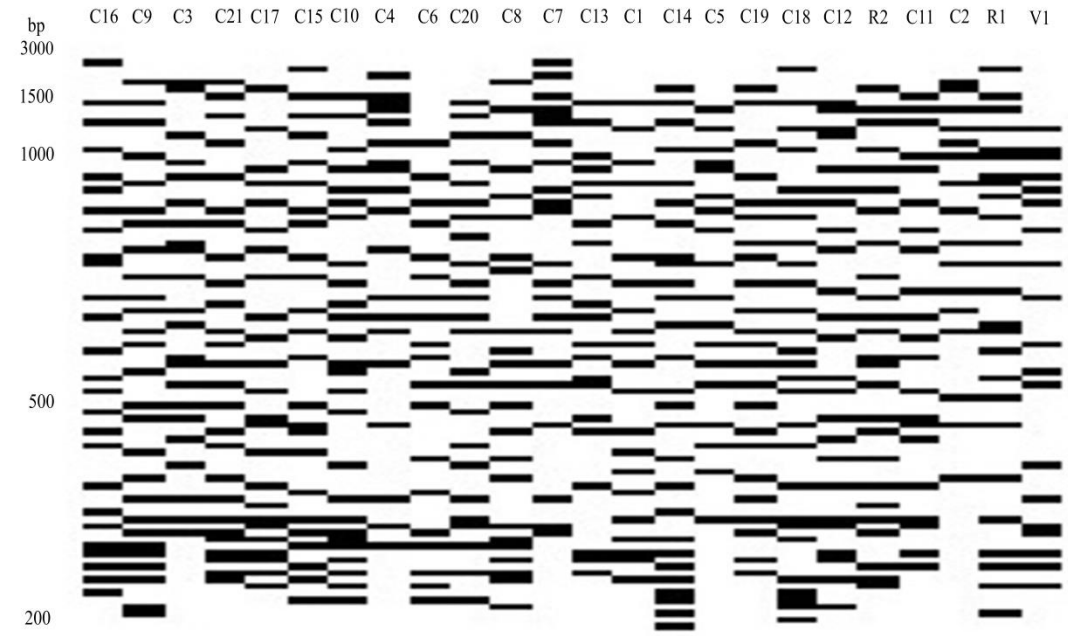

Figure 34 iPBS polymorphisms of 24 taxa revealed by primer 2079 (bp=molecular weight marker 1 kb ladder DNA; lanes C1–C21, R1–R2 and V1 stand for individual species of studied taxa, see material and method for details)**.**

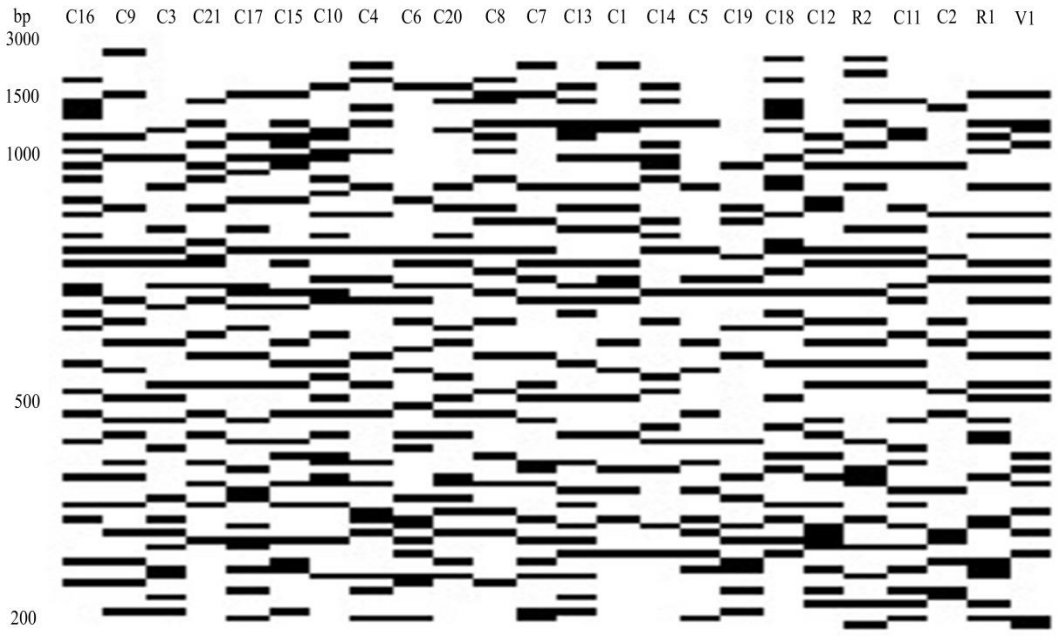

Figure 35 iPBS polymorphisms of 24 taxa revealed by primer 2095 (bp=molecular weight marker 1 kb ladder DNA; lanes C1–C21, R1–R2 and V1 stand for individual species of studied taxa, see material and method for details)**.**

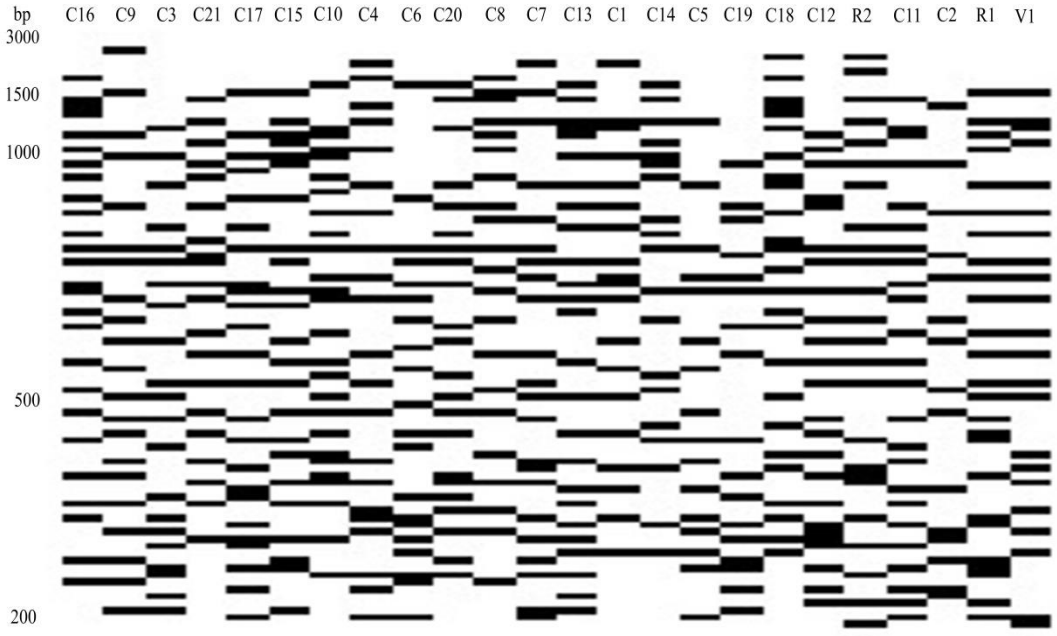

Figure 36 iPBS polymorphisms of 24 taxa revealed by primer 2224 (bp=molecular weight marker 1 kb ladder DNA; lanes C1–C21, R1–R2 and V1 stand for individual species of studied taxa, see material and method for details)**.**

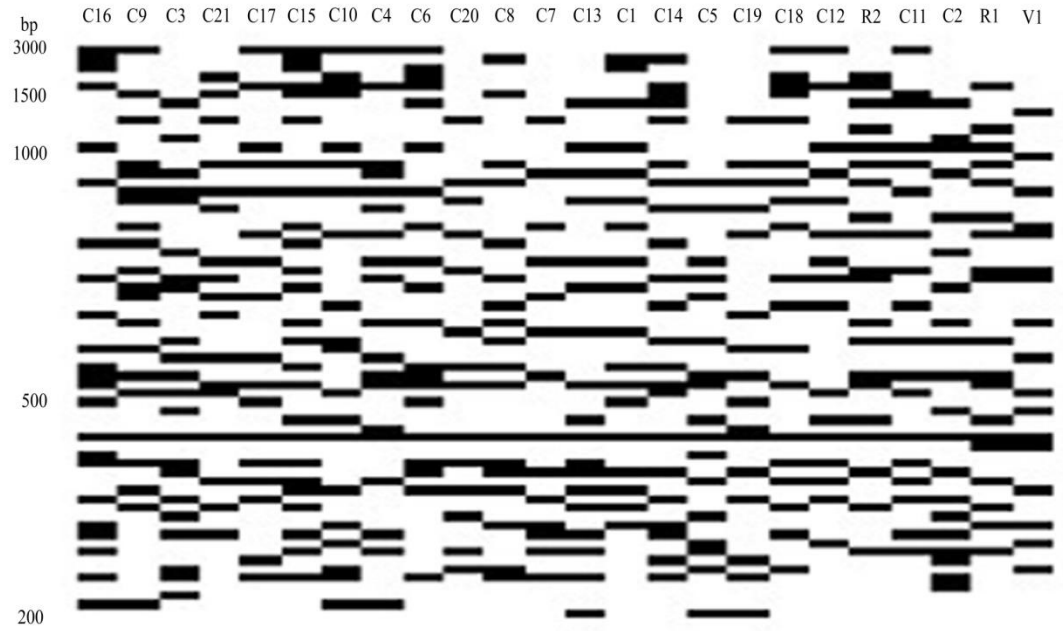

Figure 37 iPBS polymorphisms of 24 taxa revealed by primer 2232 (bp=molecular weight marker 1 kb ladder DNA; lanes C1–C21, R1–R2 and V1 stand for individual species of studied taxa, see material and method for details)**.**

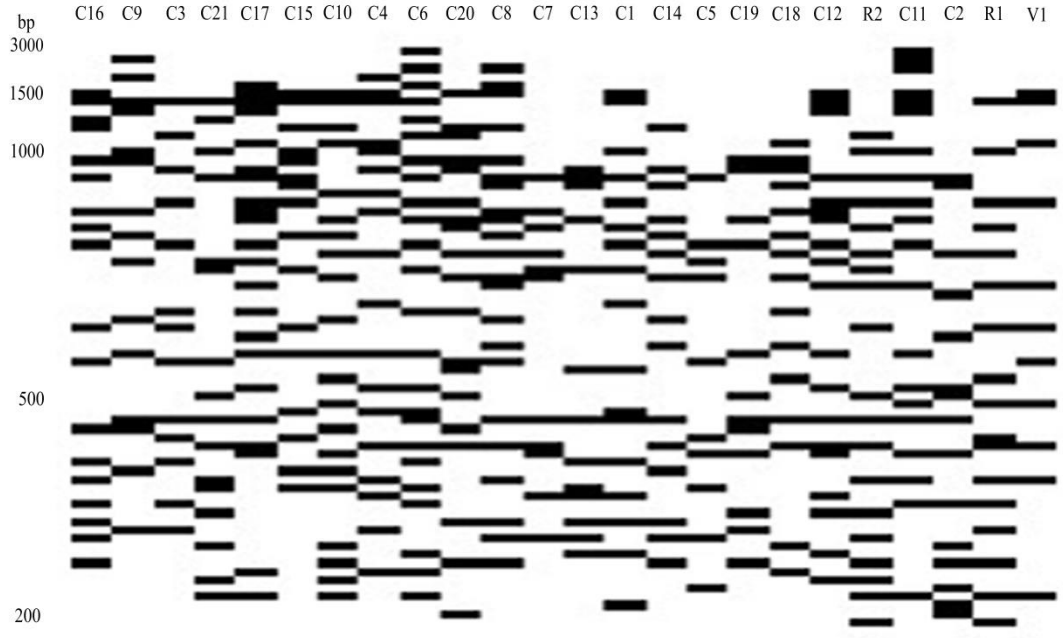

Figure 38 iPBS polymorphisms of 24 taxa revealed by primer 2237 (bp=molecular weight marker 1 kb ladder DNA; lanes C1–C21, R1–R2 and V1 stand for individual species of studied taxa, see material and method for details)**.**

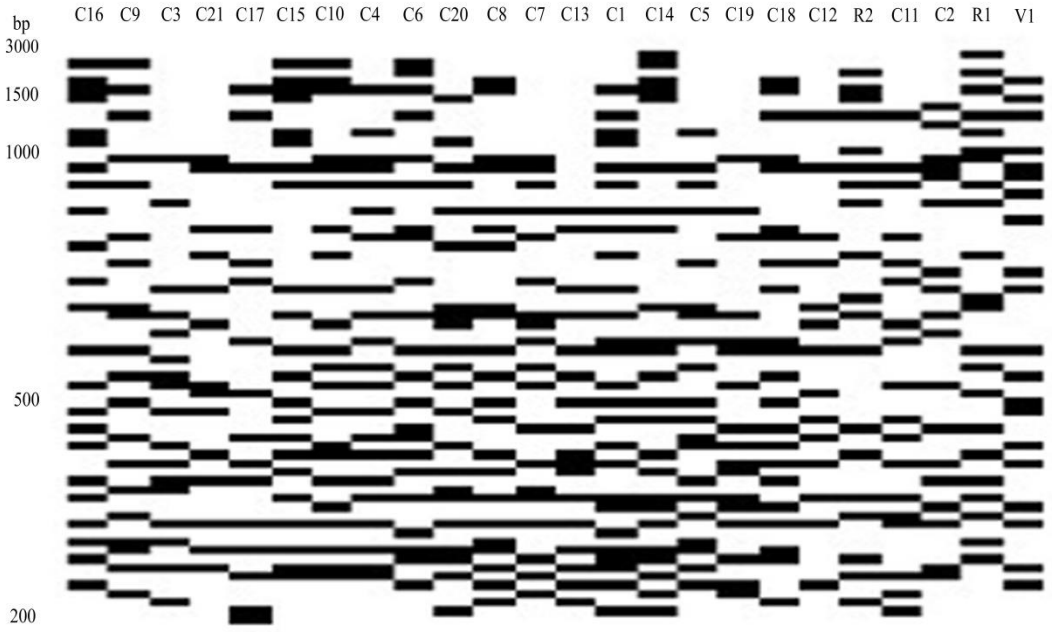

Figure 39 iPBS polymorphisms of 24 taxa revealed by primer 2271 (bp=molecular weight marker 1 kb ladder DNA; lanes C1–C21, R1–R2 and V1 stand for individual species of studied taxa, see material and method for details)**.**

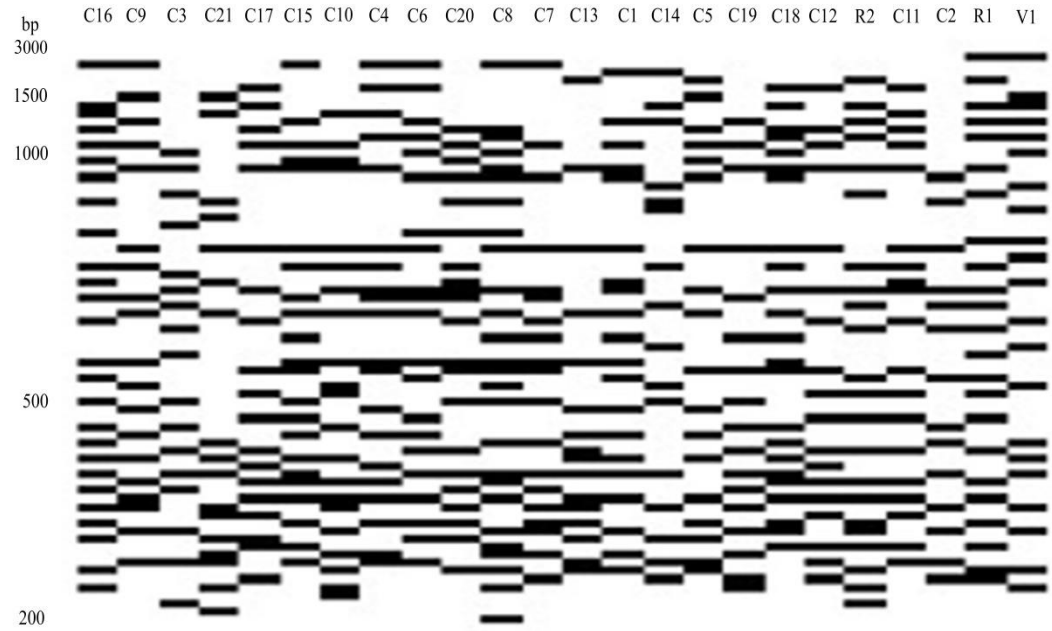

Figure 40 iPBS polymorphisms of 24 taxa revealed by primer 2272 (bp=molecular weight marker 1 kb ladder DNA; lanes C1–C21, R1–R2 and V1 stand for individual species of studied taxa, see material and method for details)**.**

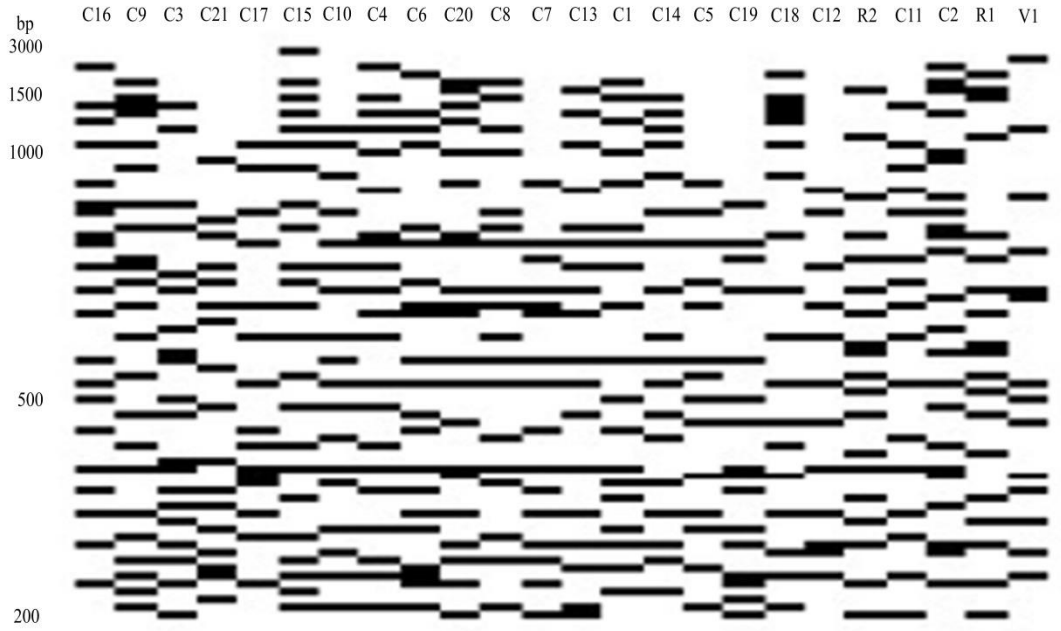

Figure 41 iPBS polymorphisms of 24 taxa revealed by primer 2273 (bp=molecular weight marker 1 kb ladder DNA; lanes C1–C21, R1–R2 and V1 stand for individual species of studied taxa, see material and method for details)**.**

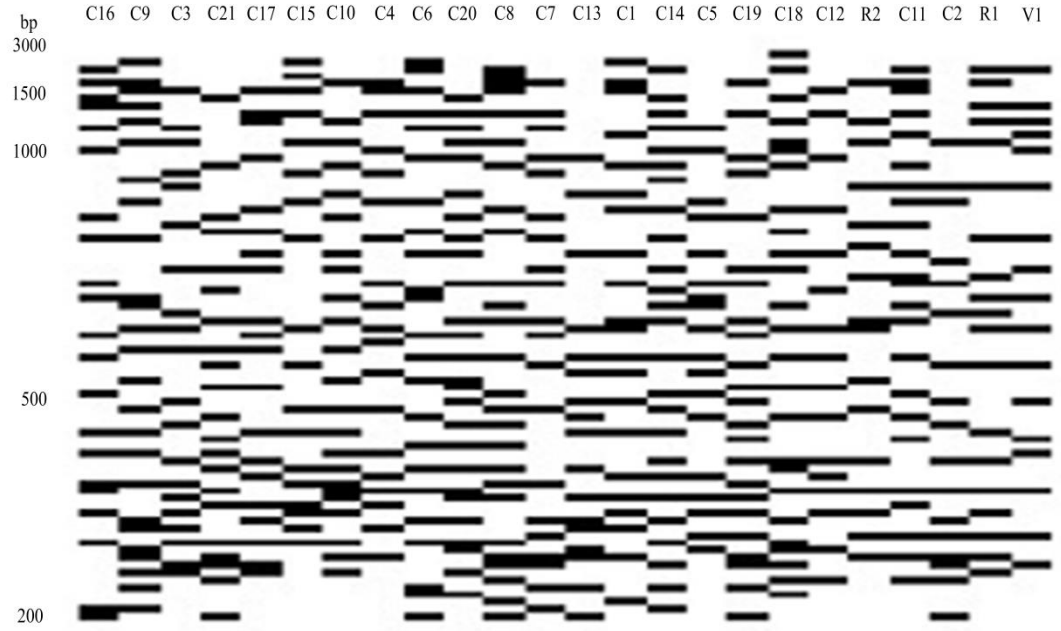

Figure 42 iPBS polymorphisms of 24 taxa revealed by primer 2295 (bp=molecular weight marker 1 kb ladder DNA; lanes C1–C21, R1–R2 and V1 stand for individual species of studied taxa, see material and method for details).

The neighbour joining (NJ) dendrogram and the principal coordinates analysis (PCoA) identically split the studied plants into two distinct groups. The first group comprises of 21 species of *Clerodendrum* (C1–C21), whereas the second group composes of *Volkameria inermis* (V1), *Rotheca serrata* (R1) and *R. macrostachya* (R2).

In NJ tree (Fig. 43), all *Clerodendrum* species is clearly separated from others outgroup species in the second group. Inaddition, the tree shows several devided subgroup in *Clerodendrum*. The first subgroup composes of *C. chinense* var. *chinense* (C2) and *C. chinense* var.*simplex* (C3). The second subgroup consists of *C. umbratile* (C20), *C. nutans* (C16), *C.* sp. (C19) and *C. garrettianum* (C7). The third subgroup comprises of *C. deflexum* (C5) and *C. colebrookianum* (C4). The fourth subgroup composes of *C. longisepalum* (C15), *C. indicum* (C9), *C. disperifolium* (C6), *C. calamitosum* (C1) and *C. lankawiense* (C13). The fifth subgroup consists of *C. paniculatum* (C17), *C. intermedium* (C11) and *C. japonicum* (C12). The last subgroup composes of *C. infortunatum* (C10), *C. villosum* (C21), *C. schmidtii* (C18), *C. lloydianum* (C14) and *C. godefroyi* (C8).

In PCoA plot (Fig. 44), *R. macrostachya* (R2), *R. serrata* (R1) and *V. inermis* (V1) are clearly splited from all remaining *Clerodendrum* species (C1–C21) similar to the relationship in NJ tree.

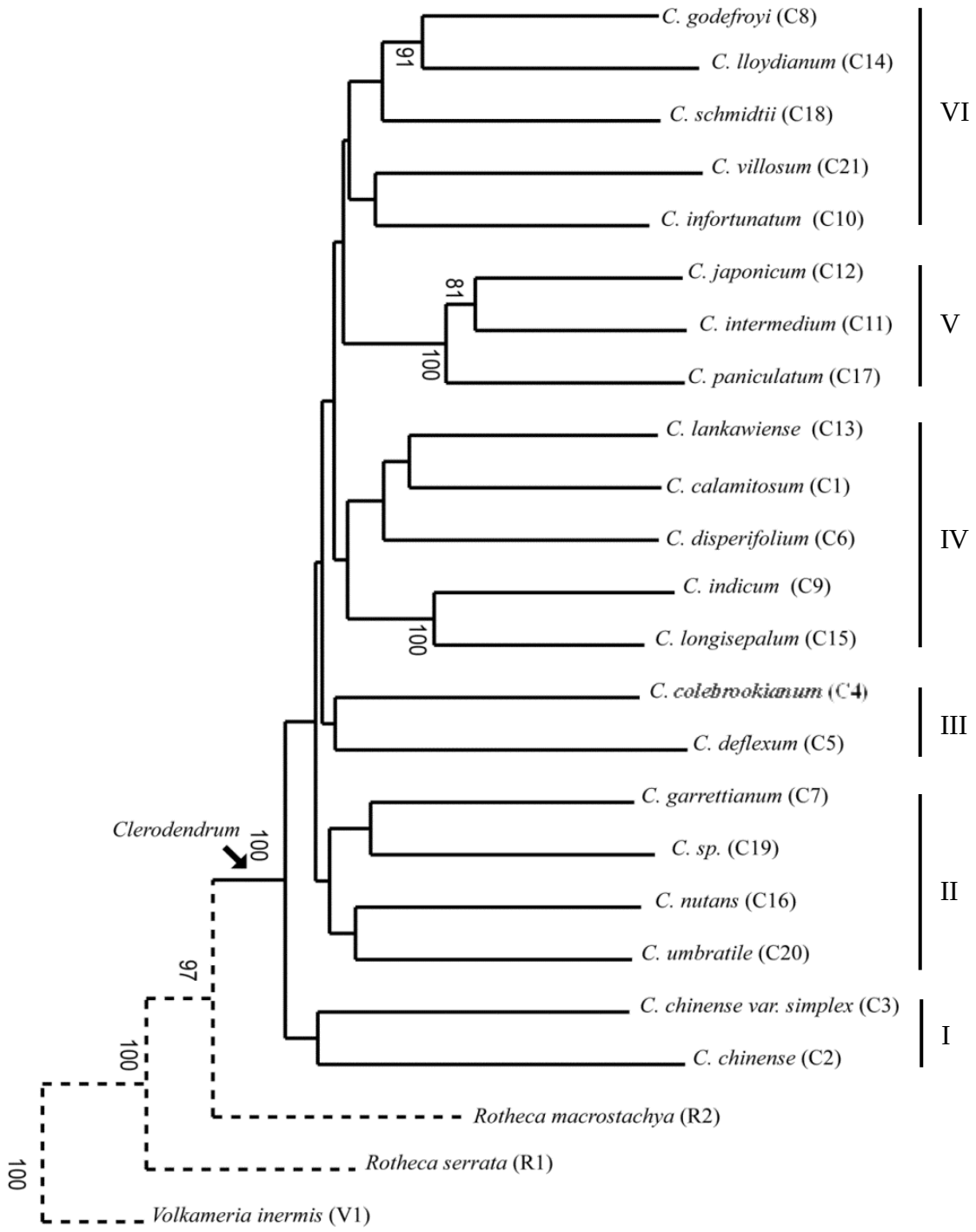

Figure 43 The neighbor joining dendrogram showing the genetic relationship among 24 taxa using iPBS molecular marker.

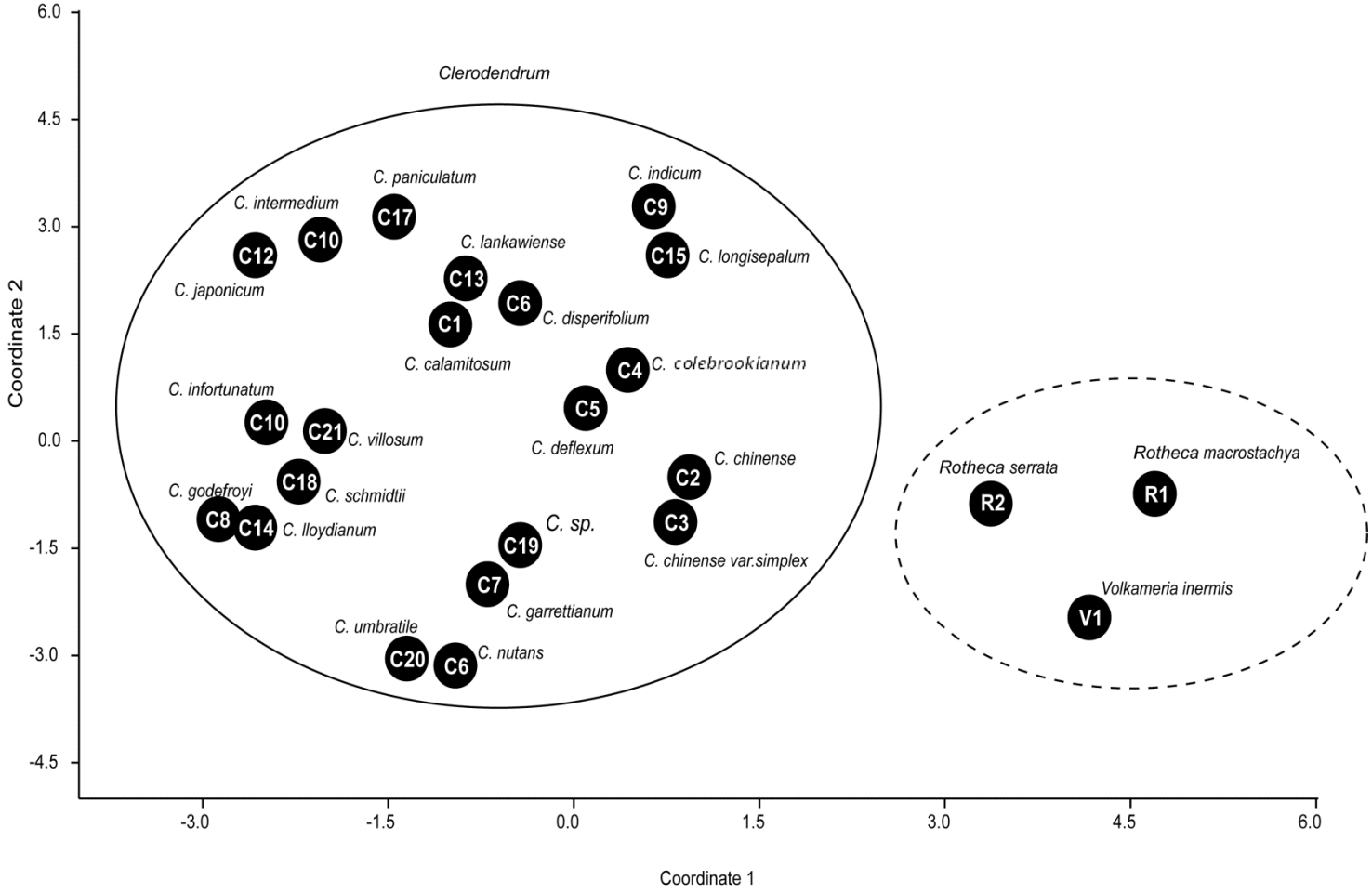

Figure 44 PCoA plot of genetic similarity based on iPBS molecular marker.

CHAPTER 5

DISCUSSIONS AND CONCLUSION

Significant leaf anatomical characters demonstrated in previous studies of Lamiaceae and *Clerodendrum* such as, trichome, stomatal types, epidermal cell, vascular bundle etc. were observed and compared (Table 7–8) in the present study. The differences of anatomical characters among the genera and among the species were discussed and pointed out (5.1). Furthermore, the genetic relationship by iPBS marker was interpreted and applied to differentiate the genera and species (5.2).

5.1 Anatomical study

Leaf blade anatomy were compared among the three genera and among the species. Each significant leaf blade anatomical characters mentioned before were discussed separately in order to easily clarify the boundaries among the three genera and apply in generic and species identification.

Trichomes

Metcalfe and Chalk (1979) and Moon *et al*. (2009) demonstrated taxonomic significance of trichome in family Lamiaceae. The present study also reveals the taxonomic implication of trichome related to these authors.

Briefly, four different types of trichomes were found and were defined into two categories based on the presence or absence of secretory gland: 1) glandular trichome and 2) non–glandular tirchome. Two types of glandular trichome were observed: capitate trichome and peltate (subsessile) trichome. Two types of non-glandular trichome were observed according to the number of cells in trichome: unicellular trichomes and multicellular uniseriate trichomes.

In *Clerodendrum*, all four types of trichome were found.

1) Unicellular trichomes occur only in *C. lloydianum* and *C. colebrookianum.*
2) Multicellular uniseriate trichomes occure in all *Clerodendrum* species.

3) Capitate glandular trichomes occur in all *Clerodendrum* species.

4) Peltate glandular trichomes occur only in some *Clerodendrum* species; *C.garrettianum*, *C. japonicum*, *C. lloydianum* and *C. paniculatum*. This type of trichome is sometimes called scale-like trichome as it enlarge and occur in a clump at leaf base of *C. chinense*, *C. chinense* var. *simplex* and *C. colebrookianum*. The trichomes function as extrafoliar nectaries to attract protective ants against pests and excrete superfluous fluids from the plant body (Chakravarty 1937, 1948; Maheshwari 1954; Inamdar, 1968).

In *Clerodendrum* complex groups, some complex species can be distinguished from others based on trichome structure. In the first group, *C. intermedium* is different from *C. japonicum* and *C. paniculatum* by the absence of peltate glandular trichomes on leaf blade. In the second group, *C. lloydianum* is different from *C. godefroyii* by the presence of peltate glandular trichomse and unicellular trichomes.

In *Rotheca*, only two types of trichome were found.

1) Multicellular uniseriate trichomes occur in both *R. macrostachya* and *R. serrata*.
2) Capitate glandular trichomes occur in both *R. macrostachya* and *R. serrata*.

Among the two species, the distribution of trichomes are different. In *R. macrostachy*, trichomes distribute evenly throughout the leaf blade whereas in *R. serrata*, trichomes distribute only on vein.

In *V. inermis*, two types of trichome were found.

1) Unicellular trichomes present in remarkably scarce number.
2) Capitate glandular trichomes present on both surface but noticeable sunken on abaxial surface.

The absence of multicellular trichomes is a diagnostic characteristic of *V. inermis* which can be used alongside other characteristics to diferentiate the genus and this species from others.

In conclusion, trichome types, structure and distribution are useful for generic and species differentiation in the present study.

Stomata

The variation of stomatal complex has been considered as plant characteristic which corresponds with ecological conditions and plays an important role in environmental adaptation (Haworth, 2011). As stomatal control is critical to a plant's adaptation to its environment, stomatal type of one species often varies through the different ecological gradients. However, our observation shows constant stomatal types in certain groups, despite they were collected from several populations under different ecological condition throughout Thailand.

In *Clerodendrum,* all studied taxa have anomocytic stomata and amphistomatic leaves as stoma present in both abaxial and adaxial leaf surface, except in *C. longisepalum* with hypostomatic leaves. The presence of amphistomatic leaves in *Clerodendrum* is confirmed by the reports of Rao and Ramayya (1985) and Herman (1998). The leaf type based on position of stomata is helpful to separate *C. longisepalum* from other *Clerodendrum* species, especially from *C. calamitosum* which are considered as its complex species.

In *Rotheca*, both *R. macrostachya* and *R. serrata* have diacytic stomata and amphistomatic leaves. The result is similar to Bangar *et al*. (2011), which reported that the diacytic stomatal type is dominant in *C. serratum* (=*R. serrata*). However, he also reported hypostomatic in *C. serrata* (=*R. serrata*) conflicted with the result from present study. However, *R. macrostachya* is different from *R. serrata* by having noticeable yellowish subsidiary cells.

In *V. inermis*, the stomata are anomocytic and leaves are hypostomatic. The result is similar to Bangar *et al*. (2011), which reported hypostomatic leaves in *C. inerme* (= *V. inermis*).

In conclusion, *Clerodendrum* and *Volkameria* have anomocytic stomata, while *Rotheca* possesses diacytic stomata. Hence, the diacytic stomatal type can be used as a diagnostic character of the genus *Rotheca*. In addition, the leaf type based on the position of stomata in *Volkameria* is different from all *Clerodendrum* (except in *C. longisepalum*) and *Rotheca* species. *Volkamaria inermis* has hypostomatic

leaves, whereas the remaining species show amphistomatic leaves. Thus, the stomatal types and position are useful for generic and species identification in *Clerodendrum* and related genera.

Epidermal cell and anticlinal wall

In *Clerodendrum*, all 22 taxa possess irregular (jigsaw puzzle-like) cell shape with slightly or strongly sinuous anticlinal wall. The sinuosity of anticlinal wall can be used to separate studied plants into two groups. The first group consisting of *C. haematolasium* and *C. villosum* has remarkably slightly sinuos anticlinal wall, while the second group consisting of all the remaining *Clerodendrum* species has strongly sinuous anticlinal wall. The result is congruent very well with the the study of Kaushat and Tripathi (1984) which reported sinuous anticlinal wall in *Clerodendrumm* species. In addition, *C. haematolasium* possesses the anthocyanin in adaxial epidermis which results in the purple colour of pubescence and lower leaf surface. According to the field observation, the anthocyanin occure since the early stage of young leaf bud to old leaves indicating that this character is stable and not vary dua to leaf age. Thus, the presence of anthocyanin in adaxial epidermis is a diagnostic characteristic of *C. haematolasium*.

In *Rotheca*, both *R. macrostachya* and *R. serrata* have similar irregular epidermal cell shape with strongly sinuous anticlinal wall.

In *V. inermis*, the epidermal cell shape is isodiametric with thick straight anticlinal wall. The result is congruent well with the study of Kaushat and Tripathi (1984) which reported that epidermal cells are curvy to straight in *C. inerme* (= *V. inermis*). Morover, *V. inermis* also presents the striation on lower epidermis which is similar to the report of Bangar *et al*. (2011).

In conclusion, the isodiametric cell shape with straight anticlinal wall and striation are the consistent diagnostic characteristics to identify *V. inermis* from other *Clerodendrum* and *Rotheca* species. Also, the sinuosity of anticlinal wall can be applied with other characteristics to separate *Clerodendrum* at species level.

For ecological implication, the shape of epidermal anticlinal wall is closely related to the climatic condition; Sinuous walls are characteristic of species growing in mesophytic habitat, whereas straight or curved walls are found in species inhabiting xerophytic areas (Gifford and Foster, 1989). Our study supports this hypothesis as *V. inermis* occurring in xerophytic costal area has thick straight epidermal anticlinal wall and isodiametric cell shape, while other *Clerodendrum* and *Rotheca* species distributing in more humid areas possess sinuous walls and irregular cell shape. However, further leaf anatomical study of mesophytic *Volkameria* is necessary in order to confirm generic trait and imply such character in taxonomic work.

Mesophyll

Both shade-tolerant and shade-intolerant species usually have one layer of palicade tissue when grow in the shade, but two to three layers when grow in full sun. Thus, the number of palisade layer could be various regardless of the light intensity in which they grow (Pallardy, 2008).

In *Clerodendrum,* all 22 taxa growing in both full sun and in shade develop only one layer of palisade mesophyll. Interestingly, the number of spongy mesophylls consequently change correlated to the thickness of palisade layer. For example, in *C. nutan*, palisade mesophyll covering 10–20% of leaf thickness results in the higher number of spongy mesophyll upto 5–7 layers. Meanwhile, in *C. villosum*, palisade mesophyll covering 50–60% of leaf thickness results in the decrease of spongy mesophyll to just 3–5 layers.

Among *Clerodendrum* taxa, bundle sheath extension (BEs) only occur in *C. chinense*, *C. colebrookianum* and *C. paniculatum* from some northern populations in Thailand, but absent in other northen populations and region. The presence of BEs in these three species which have distinct wide and big cordate leaves correlated with Wylie (1952). He reported that the BEs are of most frequent occurrence in the thin, broad, northern deciduous leaves and least common in the thick, evergreen leaves. However, the presence of BEs is mainly the ecological adaptive role to reduce the hydraulic resistance from the bundle sheath to the epidermis and thereby accelerate hydropassive stomatal movements. Low hydraulic resistance due to the presence of

bundle sheath extension would also increase the rate of stomatal opening (Kawai *et al*., 2017).

In *Rotheca*, *R. macrostachya* and *R. serrata* growing in both full sun and in shade also develop only one layer of palisade and the trend of mesophyll variation changes tend to be similar to *Clerodendrum*. However, the bundle sheath extension were not found in both species.

In *V. inermis*, growing in coastal habitat and exposing the strong sun probably result in development of thicker palisade mesophyll. The number of palisade cells increase to 2–3 layers. The bundle sheath extension were not found.

In conclusion, the result from both field and anatomical studies indicate that the number of palisade mesophyll layer and the presence of bundle sheath extension are highly correlated with ecological factors similar to the reported of Metcafe and chalk (1950) which demonstrated that the type of mesophyll is unreliable for diagnostic purposes owning to variations in response to environmental conditions. Thus, the anatomical characters of mesophyll are not applied in generic or species identification in this study.

Midrib and vascular bundle

In *Clerodendrum,* vascular bundle of all studied taxa are collateral cylindrical. Both xylem and phloem arrange in discontinuous ring. The result is different from Metcalfe and Chalk (1950). They reported the xylem appears in discrete group and the phloem arrange in continuous arc.

Among *Clerodendrum* taxa, midrib outline and structure of vascular bundle are not significantly different. However, prismatic crystal accumulation in midrib occure only in some *Clerodendrum* species. The crystals randomly accumulate in ground parenchyma tissue in *C. infortunatum*, *C. japonicum*, *C. paniculatum*, *C. schmidtii* and *C. villosum*, whereas in *C. intermedium*, prismatic crystals occure particularly and densly near vascular bundle. The crystal accumulating cells called crystalliferous sclereids are transformed parenchymatous tissue and can be found in ground tissue of midrib region and petiole (Roa, 1957; Inamdar, 1967).

Several studies reported that most plants, unlike animals, do not have well-developed excretory systems to dispose of excess calcium. Instead, higher plants appear to modulate differences between the natural abundance of environmental calcium and the very low levels required for cytosolic free calcium by controlling the distribution of calcium and its compartmentation within the cell (Clarkson, 1984; Kinzel, 1989; Leigh & Tomos, 1993; Webb, 1999). The direct relationship established between a certain crystal-forming plant species and it machinery are conserved and could be a useful tool for plant identification and chemotaxonomy (Monje & Baran, 2002). Thus, the presence of crystal in midrib is useful and can be applied in deferentiation of *Clerodendrum* species.

In addition, two types of collenchyma cells in groud tissue of midrib were observed. and used to separate *Clerodendrum*. The first type is lamella collenchyma found in *C. lankawiense* and *C. paniculatum*. The second type is lacunar collenchyma found in the rest taxa of *Clerodendrum*.

In *Rotheca*, both *R. macrostachya* and *R. serrata* have collateral cylindrical vascular bundle. Xylem and phloem arrange in discontinuous ring. Crystal were not found in both species. However, the types of collenchyma cells are different among the two species. In *R. macrostachya*, only lacunar collenchyma occurs whereas in *R. serrata*, both lacunar and lamella collenchyma occure.

In *V. inermis*, the species possesses the distinctive semicircular midrib outline and continuous arc-shape vascular bundle with adaxial incurved partition. Crystal were not found in midrib region and only lacunar collenchyma was observed.

In conclusion, *Clerodendrum* and *Rotheca* have cylindrical discontinuous vascular bundle different from *Volkameria* which has arc-shape vascular bundle with adaxial incurved partition. The present study then applied the characteristics in generic identification.

Table 6 Leaf anatomical characters in <u>*surface view*</u> of *Clerodendrum*, *Rotheca* and *Volkameria* in Thailand.

Taxon	Epidermal cell shape	Curvature of abaxial anticlinal walls	Types of Stomata	Types of trichome	Leaf type base on Stomata position
Clerodendrum calamitosum L.	Irregular	Strongly sinuous	Anomocytic	C, MU	Amphistomatic
C. chinense (Osbeck) Mabb.	Irregular	Strongly sinuous	Anomocytic	C, MU, P	Amphistomatic
C. chinense var. *simplex* (Moldenke) Chen	Irregular	Strongly sinuous	Anomocytic	C, MU, P	Amphistomatic
C. colebrookianum Walp.	Irregular	Strongly sinuous	Anomocytic	C, MU, UN, P	Amphistomatic
C. deflexum Wall.	Irregular	Strongly sinuous	Anomocytic	C, MU	Amphistomatic
C. disperifolium Blume	Irregular	Strongly sinuous	Anomocytic	C, MU	Amphistomatic
C. garrettianum Craib	Irregular	Strongly sinuous	Anomocytic	C, MU, P	Amphistomatic
C. godefroyi Kuntze	Irregular	Strongly sinuous	Anomocytic	C, MU	Amphistomatic
C. haematolasium Hall.f.	Irregular	Slightly sinuous	Anomocytic	C, MU	Amphistomatic
C. indicum (L.) Kuntze	Irregular	Strongly sinuous	Anomocytic	C, MU	Amphistomatic
C. infortunatum L.	Irregular	Strongly sinuous	Anomocytic	C, MU	Amphistomatic
C. intermedium Cham.	Irregular	Strongly sinuous	Anomocytic	C, MU	Amphistomatic
C. japonicum (Thunb.) Sweet	Irregular	Strongly sinuous	Anomocytic	C, MU, P	Amphistomatic
C. lankawiense King & Gamble	Irregular	Strongly sinuous	Anomocytic	C, MU	Amphistomatic
C. lloydianum Craib	Irregular	Strongly sinuous	Anomocytic	C, MU, UN, P	Amphistomatic
C. longisepalum Dop	Irregular	Strongly sinuous	Anomocytic	C, MU	Hypostomatic

Table 6 Leaf anatomical characters in *surface view* of *Clerodendrum*, *Rotheca* and *Volkameria* in Thailand (continued).

Taxon	Epidermal cell shape	Curvature of abaxial anticlinal walls	Types of Stomata	Types of trichome	Leaf type base on Stomata position
C. nutans Wall. ex Jack	Irregular	Strongly sinuous	Anomocytic	C, MU	Amphistomatic
C. paniculatum L.	Irregular	Strongly Sinuous	Anomocytic	C, MU, P	Amphistomatic
C. schmidtii C.B. Clarke	Irregular	Strongly Sinuous	Anomocytic	C, MU	Amphistomatic
C. sp.	Irregular	Strongly Sinuous	Anomocytic	C, MU	Amphistomatic
C. umbratile King & Gamble	Irregular	Strongly Sinuous	Anomocytic	C, MU	Amphistomatic
C. villosum Blume	Irregular	Slightly Sinuous	Anomocytic	C, MU	Amphistomatic
Rotheca incisa (Klotzsch) Steane & Mabb.	Irregular	Strongly Sinuous	Diacytic	C, MU	Amphistomatic
R. macrostachya (Turcz.) Leerat. & Chantar.	Irregular	Strongly Sinuous	Diacytic	C, MU	Amphistomatic
R. serrata (L.) Steane & Mabb	Irregular	Strongly Sinuous	Diacytic	C, MU	Amphistomatic
Volkameria inermis L.	Isodiametric	Straight to curved	Anomocytic	C, UN	Hypostomatic

Note: C = Capitate glandular trichome, MU = Multicellular uniseriate trichome, P = Peltate glandular trichome, UN = Unicellular trichome

Table 7 Leaf anatomical characters in *transverse–section* of *Clerodendrum*, *Rotheca* and *Volkameria* in Thailand.

Taxon	Leaf type	Palisade mesophyll		Spongy meso–phyll (layer)	Collenchyma		Fibre	Crys–tal	Vascular bundle	
		Number of layer	Percent cover(%)		Number of layer	type			Type	Shape
Clerodendrum calamitosum L.	Dorsiventral	1	20–30	5–7	1–2	Lacunar	–	–	collateral	cylindrical
C. chinense (Osbeck) Mabb.	Dorsiventral	1	40–50	6–7	2–3	Lacunar	–	–	collateral	cylindrical
C. chinense var.*simplex* (Moldenke) Chen	Dorsiventral	1	40–50	4–5	3–5	Lacunar	–	–	collateral	cylindrical
C. colebrookianum Walp.	Dorsiventral	1	20–25	7–8	3–5	Lacunar	✓	–	collateral	cylindrical
C. deflexum Wall.	Dorsiventral	1	20–30	5–6	2–3	Lacunar	–	–	collateral	cylindrical
C. disperifolium Blume	Dorsiventral	1	20–25	6–7	1–2	Lacunar	✓	–	collateral	cylindrical
C. garrettianum Craib	Dorsiventral	1	30	4–5	2–3	Lacunar	–	–	collateral	cylindrical
C. godefroyi Kuntze	Dorsiventral	1	30–40	3–4	1–2	Lacunar	–	–	collateral	cylindrical
C. haematolasium Hall.f.	Dorsiventral	1	25–30	4–5	1–3	Lacunar	✓	–	collateral	cylindrical
C. indicum (L.) Kuntze	Dorsiventral	1	25–30	5–6	1–2	Lacunar	-	–	collateral	cylindrical
C. infortunatum L.	Dorsiventral	1	40–50	4–6	2–3	Lacunar	✓	✓	collateral	cylindrical
C. intermedium Cham.	Dorsiventral	1	20–30	5–6	2–4	Lacunar	-	✓	collateral	cylindrical
C. japonicum (Thunb.) Sweet	Dorsiventral	1	30–40	5–6	2–3	Lamella	–	✓	collateral	cylindrical

Table 7 Leaf anatomical characters in *transverse–section* of *Clerodendrum*, *Rotheca* and *Volkameria* in Thailand (continued).

Taxon	Leaf type	Palisade mesophyll		Spongy meso–phyll (layer)	Collenchyma		Fibre	Crys–tal	Vascular bundle	
		Number of layer	Percent cover		Number of layer	type			Type	Shape
C. lankawiense King & Gamble	Dorsiventral	1	25–30	3–4	2–3	Lamella	✓	–	collateral	cylindrical
C. lloydianum Craib	Dorsiventral	1	30–40	3–4	1–3	Lacunar	✓	–	collateral	cylindrical
C. longisepalum Dop	Dorsiventral	1	20–40	3–5	1–2	Lacunar	✓	–	collateral	cylindrical
C. nutans Wall. ex Jack	Dorsiventral	1	10–20	5–7	2–3	Lacunar	✓	–	collateral	cylindrical
C. paniculatum L.	Dorsiventral	1	25–30	5–6	2–3	Lacunar	–	✓	collateral	cylindrical
C. schmidtii C.B. Clarke	Dorsiventral	1	20–30	5–6	2–3	Lacunar	–	✓	collateral	cylindrical
C. sp.	Dorsiventral	1	20–30	3–4	2–3	Lacunar	–	–	collateral	cylindrical
C. umbratile King & Gamble	Dorsiventral	1	10–20	6–7	2–3	Lacunar	✓	–	collateral	cylindrical
C. villosum Blume	Dorsiventral	1	50–60	3–5	2–3	Lacunar	✓	✓	collateral	cylindrical
R. macrostachya (Turcz.) Leerat. & Chantar.	Dorsiventral	1	40–50	3–4	1–2	Lacunar	–	–	collateral	cylindrical
R. serrata (L.) Steane & Mabb	Dorsiventral	1	30–40	4–6	1–2	Lamella or lacunar	✓	–	collateral	cylindrical
Volkameria inermis L.	Dorsiventral	2–3	50–60	6–7	2–4	Lacunar	✓	–	collateral	arc

Note: ✓ = present, – = absent

5.2 Molecular study of *Clerodendrum*, *Rotheca* and *Volkameria* by iPBS

5.2.1 DNA isolation

The extraction of high quality DNA from *Clerodendrum*, *Rotheca* and *Volkameria* is difficult because the presence of high polyphenolic compounds. The presence of polyphenols in leaf tissues can decrease DNA purity and amount by producing the covalent bond with the DNA making it ineffective in further implications (Vanijajiva 2011, Vanijajiva 2012). The alternative DNA extraction then were applied using modified CTAB protocol by adding phenol to the chloroform: isoamyl alcohol mixing solution. The phenolic compounds were simply removed from the DNA and better iPBS amplification bands were obtained. The quality of DNA was also measured to confirmed weather the DNAs are suitable for PCR reaction or not. Several parameters for the iPBS protocol from plant sample were also preliminarily optimized. The concentration of PCR mixture such as dNTPs, magnesium chloride, enzyme, primer and template DNA mainly had an effect on PCR reproducibility. According to the result of the obtimization, at 100 ng template DNA and MgCl2 5 mM concentration were proper for later PCR analysis.

5.2.2 Genetic relationship of *Clerodendrum*, *Rotheca* and *Volkameria*

The iPBS marker have been wildly applied for assessing genetic diffenreces among cultivars and wild species due to its reproducibility, inexpensive cost and no requirement for individual genetic sequence information. In fact, iPBS have high capacity to reveal polymorphism and offer great potential to determine intra and interspecific diversity (Andiden *et al*., 2013). In this study, iPBS marker were applied to confirm the genetic relationship among 24 taxa of *Clerodendrum*, *Rotheca* and *Volkameria*. The genetic similarity was examined using Dice similarity coefficients The hihgest genetic similarity is 0.526 observed between *C. japonicum* and *C. paniculatum*. The highest genetic similarity among *C. paniculatum* and *C. japonicum* is probably due to their close phylogentic relationship confirmed in the phylogenetic tree of Yuan *et al*. (2010) and the NJ dendogram by iPBS in this study. These two closely related species consequently share several similar morphological characteristics according to the taxonomic study by Leeratiwong (2011). Meanwhile,

the lowest genetic similarity is 0.194 found between *V. inermis* and *R. serrata* coresponsed with the phylogenetic study by Yaun *et al*. (2010) as these two species belong to clearly separated clades and geographical distribution history. Moreover, the species posse distinct different morphological characteristics (Leeratiwong, 2011)

The neighbor joining (NJ) and principal coordinate analysis (PCoA) identically split *R. macrostachya* (R2), *R. serrata* (R1) and *V. inermis* (V1) from all remaining *Clerodendrum* species (C1–C21). *R. macrostachya* (R2) is separated from other *Clerodendrum* species correlated with the taxonomic treatment of *C. macrostachyum* to *Rotheca macrostachya* as this species displays several diagnostic characteristics of *Rotheca* such as, asymmetrical flower buds, abruptly expanding only on the lower side of the corolla, the enlarge anterior corolla lobe compared to the others and the unequal stigma branches (Steane & Mabberley 1998; Harley *et al*. 2004 and Leeratiwong *et al*., 2018). Also, *R. serrata* (R1) is clearly distinct from those *Clerodendrum* species at 100% bootstarp correspondent with the previous molecular phylogentic studies by Steane *et al*. (1997, 1999) which separated *C. serratum* (*R. serrata*) and the rest member of subgenus *Cyclonema* (Hochst.) Gürke and section *Konocalyx* Verdcourt from others *Clerodendrum* and placed into the resurrected genus *Rotheca*. Moreover, *V. inermis* (V1) is also clearly splited from *Clerodendrum* at 100% bootstarp similar to the study of Yuan *et al*. (2010) which separated *C. inerm*e (*V. inermis*) from others *Clerodendrum* species and revised the genus *Volkameria*. Hence, the genetic relationship by iPBS technique highly support recent resurected genera such as *Rotheca* and *Volkameria*.

In *Clerodendrum*, the dendogram shows several devided subgroup which are highly congruent with the delimitation of species using morphological characteristics. For example, *C. nutans* (C16) and *C. umbratile* (C20) are grouped together. According to Leeratiwong (2011), these two species are closely relationship based on their similar morphological characteristics such as downward hanging panicle inflorescences, white flowers, bilaterally symmetrical flowers with 5 unequal ovate to lanceolate petals. Moreover, *Clerodendrum* sp., which douptfully identify as *C. nutan*, is separated from *C. nutans* in the dendrogram and suggested to be different species in this study. Another interesting clade composes of *C. intermedium* (C11) Cham., *C. japonicum* (Thunb.) Sweet (C12) and *C. paniculatum* L. (C17). These three taxa

considered as complex species are clearly grouped together related to morphological study as such species share several similar characteristic such as large subcordate leaves with 3–5 lobes, colorish terminal panicle inflorescences, red or orange flower, (Leeratiwong *et al*., 2011). The genetic relationship exposed by iPBS dendrogram supports morphological identification in current taxonomic system.

According to literature review, there is no previous molecular study using iPBS marker in *Clerodendrum*, *Rotheca*, *Volkameria* or any other genera in the family Lamiaceae. This study is the first report on iPBS genetic information of such genera and used almost 80% of all species found in Thailand (21 taxa out of 28 species and one variety). Even though, the number of species is sufficient for genetic study, further plant samples from neighbor countries are still necessary in order to strengthen and provide even more accurate genetic relationship among the genera and species. The results overall indicate that the technique exhibited high level of polymorphism percentage and low similarity among the taxa. More importantly, the dendrogram based on iPBS fingerprint can separate plant into the groups which agree very well with the previous taxonomic studies both using morphology and molecular phyletic information. Hence, this study support that iPBS technique is an effective and effordable tool to confirm the relationship of plant species in all generic, interspecific and intraspecific level and highly agree with several studies which have mentioned the usefulness and adventages of iPBS technique. For example, Coutinho *et al*. (2016) studied genetic characters of Fagaceae using iPBS technique and proposed that the iPBS primers discriminated the studied taxa and showed low genetic similarity. The individuals were clustered into the groups corroborating the genera and infrageneric groups in recent taxomomic system. The iPBS markers are suitable for DNA amplification, taxonomic and phylogeny delimitation of Fagaceae, and provide effective tools to identify natural hybrids that have similar morphology with the parents. Özer *et al*. (2017) studied genetic variation among phytopathogenic Sclerotiniaceae using iPBS. The results showed that the iPBS primers produced high polymorphic (98%) and clear band patterns specific to each specied. The primer amplified sufficient polymorphic bands for species differentiation. The polymorphism information content indicated better discriminating power of markers, the cluster analysis seperated all studied taxa into groups and subgroups in accordance with their

taxonomic and morphological studies. Principal coordinate analysis also highly supported this cluster pattern. Hence, the iPBS was an effective tool to evaluate genetic variation at several level for Sclerotiniaceae. Moreover, the markers provided simple delimitation of some species to another species which has similar morphological characteristics.

In conclusion, genetic relationship based on iPBS molecular marker and consistent anatomical characteristics provide taxonomic supportive evidence for the generic and species delimitation of *Clerodendrum*, *Rotheca* and *Volkameria* in Thailand.

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APPENDICES

APPENDIX A
PARAFFIN TECHNIQUE AND STAINING

1. FAA II solution

Formaldehyde	5% (v/v)
Glacial acetic acid	5% (v/v)
70 % alcohol	90% (v/v)

2. Dehydration series

No.	Total alcohol (%)	Composition (ml)				
		TBA	Ethanol		Water	Other
			95%	100%		
1	5	-	5	-	95	-
2	10	-	10	-	90	-
3	20	-	20	-	80	-
4	30	-	30	-	70	-
5	50	10	40	-	50	-
6	70	20	50	-	30	-
7	85	35	50	-	15	-
8	95	55	40	-	5	-
9	100	75	-	25	-	-
10	-	100	-	-	-	Eosin
11	-	100	-	-	-	-
12	-	100	-	-	-	Paraffin oil (50 ml)

3. Infiltration and embedding

3.1 Transfer fixed samples from dehydration solution No. 12 to hot melted paraplast 1(P1= solid paraplast melted in the oven) and leave in paraffin oven for at least 2 hours.

3.2 Pour P1 to paraffin waste bag (outside oven) and fill up samples with melted paraplast 2 (P2) and leave in the oven at 58^{o}C for 2 hours.

3.3 Replace with melted paraplast 3 (P3) and leave in the oven overnight.

3.4 Embed the samples into paraffin block using embedding center.

3.5 Let the samples cool completely and remove from the molds

3.6 Store in the refrigerator until use in microtome sectioning.

4. Deparaffinization protocol

After microtome sectioning, the samples need to be deparaffinized and rehydrated using the protocol below (the slides are placed in coplin jar fill with the following solution).

4.1 Xylene substitute I 3 minutes (Histochoice / Clear-rite^{TM} 3 / Xylene)

4.2 Xylene substitute II 3 minutes (Histochoice / Clear-rite^{TM} 3 / Xylene)

4.3 Absolute alcohol : Xylene substitute (1:1) 3 minutes

4.4 Absolute alcohol I 2 minutes

4.5 Absolute alcohol II 2 minutes

4.6 95% alcohol I 2 minutes

4.7 95% alcohol II 2 minutes

4.8 70% alcohol I 2 minutes

4.9 70% alcohol II 2 minutes

5. Safranin and fast green staining

Safranin staining

5.1 After deparaffinization, put the sample in 70% alcohol

5.2 Stain in Safranin for at least 18 hours (recommended 2–4 days)

5.3 Wash out excess with tap water for a few moments (2 changes)

Dehydration

5.4 Dip the slide in solution I (95% alcohol + 0.5 % picric acid) 10 second

5.5 Dip the slide in solution II (95% alcohol + 4 drops of ammonium hydroxide in 100 ml alcohol) 10 second

5.6 Dip the slide in solution III (absolute alcohol) 10 second

5.7 Dip the slide in solution IV (absolute alcohol*) 10 second

Note: solution I for safranin differentiation

solution II for stop action of picric acid

* After complete dehydration the slides may remain in absolute alcohol up to 10 minutes in case large number are being brought up and counterstained individually.

Counterstaining (with fast green)

5.8 Drop clove oil on samples and pour back into dropping bottle

5.9 Counterstain with fast green, leave for 10 second and pour counterstain back into dropping bottle

5.10 Rinse off excess stain with used clove oil fast green

5.11 Drop new clove oil and observe under light microscope

5.12 Wash the slide a few seconds in absolute alcohol : xylene substitute (1 : 1)

5.13 Put the slide in two changes of xylene substitute (5 minutes for each)

5.14 Mount the slide using Permout

APPENDIX B

CTAB PROTOCOL

CTAB protocol

1. Grind 200 mg of plant tissue to a fine paste in approximately 500 µl of CTAB with 10% β-mercaptoethanol.
2. Transfer plant extract in CTAB mixture to a microfuge tube.
3. Incubate the mixture at 65^{o}C for about 60 minutes.
4. After incubation, spin the mixture at 12,000 g for 5 minutes to spin down cell debris. Transfer the supernatant to clean microfuge tubes. Add 500 µl of Phenol : Chloroform : Isoamyl alcohol (25:24:1) and mix the solution by inversion. After mixing, spin the tubes at 13,000 rpm for 15 minutes.
5. Transfer the upper aqueous phase only (contains the DNA) to a clean microfuge tubes. To each tube add 2.5 µl of 20 mg/ml RNase A and leave at room temperature for 30 minutes.
6. Add 300 µl of Phenol : Chloroform : Isoamyl alcohol (25:24:1) and mix the solution by inversion. After mixing, spin the tubes at 13,000 rpm for 15 minutes (Repeat the processes until the upper aqueous phase is clear and has no color).
7. Transfer only the upper aqueous phase to a clean microfuge tube.
8. Add equal amount of Isopropanol to the solution (1:1) and invert the tubes slowly and gently several times to precipitate the DNA. Store at -20^{o}C for 1–2 hours, then spin the mixture at 13,000 rpm for 15 minutes to form a pellet.
9. Remove the supernatant and wash the DNA pellet by adding two changes of 1 ml ice cold 70% ethanol.
10. After the wash, add 1 ml of absolute ethanol and centrifuge at 13,000 rpm for 15 minutes. Remove all the supernatant and allow the DNA pellet to dry.
11. Resuspend the DNA in sterile DNase free water (The amount of water needed depends on how much DNA pellet is isolated)
12. After resuspending, the DNA can be store at -20^{o}C until the evaluation of the DNA quality and quantity before use in PCR process.

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