MACROSCOPIC, MICROSCOPIC AND MOLECULAR EVALUATIONS FOR THE IDENTIFICATION OF *ERYTHRINA* SPECIES DISTRIBUTED IN THAILAND

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การประเมินลักษณะทางมหทรรศน์ จุลทรรศน์ และอณูโมเลกุลของพืชสกุลอิริทรินาที่กระจายอยู่ในประเทศไทย

นายกิติพันธ์ ขาวนิ่ม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	MACROSCOPIC, MICROSCOPIC AND MOLECULAR EVALUATIONS FOR THE IDENTIFICATION OF *ERYTHRINA* SPECIES DISTRIBUTED IN THAILAND
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Field of Study	Public Health Sciences
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กิติพันธ์ ขาวนิ่ม : การประเมินลักษณะทางมหทรรศน์ จุลทรรศน์ และอณูโมเลกุลของพืชสกุลอิริทรินาที่กระจายอยู่ในประเทศไทย (MACROSCOPIC, MICROSCOPIC AND MOLECULAR EVALUATIONS FOR THE IDENTIFICATION OF *ERYTHRINA* SPECIES DISTRIBUTED IN THAILAND) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. ชนิดา พลานุเวช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ภก. ดร. นิจศิริ เรืองรังษี, 172 หน้า.

พืชสกุลอิริทรินา จัดอยู่ในวงศ์ FABACEAE พบจำนวน 6 ชนิดที่มีการกระจายอยู่ในประเทศไทย ได้แก่ *Erythrina fusca* Lour., *Erythrina stricta* Roxb., *Erythrina crista-galli* L., *Erythrina subumbrans* (Hassk) Merr., *Erythrina variegata* L., และ *Erythrina indica* Lam. เนื่องจากมีความคล้ายคลึงกันของลักษณะทางพฤกษศาสตร์และชื่อพื้นเมืองทำให้การจำแนกพืชชนิดนี้เกิดความไม่ชัดเจน ดังนั้นการจำแนกพืชชนิดนี้อย่างถูกต้องจึงเป็นเรื่องสำคัญ วัตถุประสงค์ในการศึกษาครั้งนี้เพื่อจำแนกความแตกต่างของพืชสกุลอิริทรินา จำนวน 6 ชนิด โดยใช้การศึกษาด้วยวิธีทางมหทรรศน์ จุลทรรศน์ (ลักษณะกายวิภาคของใบและค่าคงที่ของใบ) และการวิเคราะห์อณูโมเลกุล ลักษณะทางมหทรรศน์และลักษณะกายวิภาค ภาพตัดขวางของเส้นกลางใบแสดงไว้ในรูปแบบภาพวาดลายเส้น ผลการศึกษาพบว่าปากใบเป็นชนิด paracytic ซึ่งสอดคล้องกับลักษณะของพืชในวงศ์นี้ จำนวนปากใบ ดัชนีปากใบด้านล่างและอัตราส่วนแพลิเสด ทั้ง 6 ชนิดไม่แตกต่างกัน ในขณะที่ปากใบด้านบนพบเฉพาะใน *E. crista-galli*, *E. subumbrans* และ *E. variegata* (ช่วง 60-136, 12-44 และ 4-28 เซลล์ปากใบต่อตารางมิลลิเมตร ตามลำดับ) *E. crista-galli* มีจำนวนปากใบด้านบนมากที่สุดซึ่งสามารถใช้จำแนกความแตกต่างได้ ส่วน *E. subumbrans* และ *E. variegata* แยกความแตกต่างกันได้ด้วยจำนวนเซลล์ผิวใบด้านบน (ช่วง 1080-1820 และ 404-532 เซลล์ต่อตารางมิลลิเมตร ตามลำดับ และสามารถใช้จำนวนแอ่งเส้นใบเพื่อระบุ *E. subumbrans* ออกจากชนิดอื่นๆ ได้ (12.75-20.75 และ 3.50-11.00 เซลล์ต่อตารางมิลลิเมตร ตามลำดับ) นอกจากนี้ภาพตัดขวางของเส้นกลางใบทั้ง 6 ชนิดแสดงการจัดเรียงกลุ่มของเนื้อเยื่อที่แตกต่างกันอย่างชัดเจน การศึกษาครั้งนี้ไม่พบขนทั้งด้านบนและด้านล่างของใบในชนิดดังกล่าว การศึกษาลำดับนิวคลิโอไทด์ของยีน 5 บริเวณ ได้แก่ ITS, *mat*K, *psb*A_*trn*H, *rpo*C และ *ycf*1 พบความยาวของลำดับนิวคลิโอไทด์ของพืชสกุลอิริทรินาทั้ง 6 ชนิดมีความยาว 677, 794, 278, 375 และ 656 คู่เบสตามลำดับ เปรียบเทียบลำดับนิวคลิโอไทด์ของแต่ละยีนระหว่างพืชที่ศึกษา นำมาวิเคราะห์ความสัมพันธ์ทางพันธุกรรมและสร้างแผนภูมิความสัมพันธ์ทางพันธุกรรมพบว่าสามารถจำแนกพืชแต่ละชนิดออกจากกันได้เป็น 6 กลุ่ม โดย *E. stricta* กับ *E. subumbrans* มีความใกล้ชิดทางพันธุกรรมมากที่สุด ผลการศึกษาสรุปได้ว่าลักษณะทางมหทรรศน์ ทางจุลทรรศน์ของใบ และลักษณะทางอณูโมเลกุลสามารถใช้เป็นเครื่องมือในการจำแนกพืชสกุลอิริทรินาทั้ง 6 ชนิดที่กระจายอยู่ในประเทศไทยได้

สาขาวิชา วิทยาศาสตร์สาธารณสุข	ลายมือชื่อนิสิต ....
ปีการศึกษา 2560	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....
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KITIPAN KHAONIM: MACROSCOPIC, MICROSCOPIC AND MOLECULAR EVALUATIONS FOR THE IDENTIFICATION OF *ERYTHRINA* SPECIES DISTRIBUTED IN THAILAND. ADVISOR: ASST. PROF. CHANIDA PALANUVEJ, Ph.D., CO-ADVISOR: ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., 172 pp.

Plants in the genus *Erythrina* belong to the family FABACEAE. There are six species distributed in Thailand (*Erythrina fusca* Lour., *Erythrina stricta* Roxb., *Erythrina crista-galli* L., *Erythrina subumbrans* (Hassk) Merr., *Erythrina variegata* L., and *Erythrina indica* Lam.). Due to the similarity of the morphological characters and synonym in a vernacular name, the identification of these species is ambiguous. Therefore, an accurate investigation of their identities is essential. This research aimed to distinguish six *Erythrina* spp. through the macroscopic, microscopic, and molecular genetic analyses. The anatomical characteristics of each species (cross section of midrib) and the constant values of leaves including stomatal number, epidermal cell number, stomatal index, epidermal cell area, vein islet number and palisade ratio were investigated. The macroscopic characters and anatomical characteristics of the midrib of six investigated *Erythrina* species were illustrated. The stomatal type of all six species was paracytic type which was consistent with unique characteristics of plants in this family. In terms of microscopic leaf constant numbers, the stomatal number and stomatal indices in lower epidermis among these six species were overlapping, whereas the stomata in the upper epidermis were found only in *E. crista-galli*, *E. subumbrans* and *E. variegata* (60-136, 12-44 and 4-28 stoma/mm^2 respectively). *E. crista-galli* demonstrated the highest number of upper stomata which could be used as an indicator for the identification. In addition, *E. subumbrans* and *E. variegata* exhibited distinct upper epidermal cell number (1080-1820 and 404-532 cell/mm^2 respectively). This study revealed the overlapping of the palisade ratio among six *Erythrina* species. Nevertheless, vein islet number could be used to identify *E. subumbrans* from other species (12.75-20.75 and 3.50-11.00 cell/mm^2 respectively). Moreover, the cross sections of the midrib of six investigated *Erythrina* species revealed the distinguished arrangement of tissue especially the vascular bundle. None of the trichome was found in these species. Additionally, the nucleotide sequences of five regions; ITS, *mat*K, *psb*A_*trn*H, *rpo*C and *ycf*1 gene, were evaluated and compared among these species and 2 outgroups. The sequence lengths of gene among six *Erythrina* species were 650, 790, 375, 416 and 634 base pairs in length, respectively. The genetic relationship was demonstrated as phylogenetic tree constructed from each gene region. All studied of *Erythrina* were classified into 6 groups. *E. stricta* and *E. subumbrans* were close related these other species. In conclusion, leaf macroscopic and microscopic characteristics of six *Erythrina* species distributed in Thailand, both qualitative and quantitative, could be used as a tool for these plants authentication. Molecular genetic characteristics using ITS, *mat*K, *psb*A_*trn*H, *rpo*C and *ycf*1 gene sequences provided valuable information to evidently support the identification of six *Erythrina* species.

Field of Study: Public Health Sciences — Student's Signature ....

Academic Year: 2017 — Advisor's Signature ....

Co-Advisor's Signature ....

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LIST OF ABBREVIATIONS

°C	degree Celsius
A, T, C, G	nucleotide containing the base adenine (A), thymine (T), cytosine (C), and guanine (G)
Adh	alcohol dehydrogenase
bp	base pair
CBOL	a consortium for the barcode of life
cpDNA	chloroplast DNA
cm	centimeter
CTAB	cetyltrimethyl ammonium bromide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
E	epidermal cell
EA	epidermal cell area
EtBr	ethidium bromide
gap A	glyceraldehyde-3-phosphate dehydrogenase
g DNA	genomic DNA
ITS	internal transcribed spacer
Kb	kilobase
LSC	large single copy
M	molar
MEGA	molecular evolutionary genetics analysis
mg	milligram

LIST OF ABBREVIATIONS

$MgCl_2$	magnesium chloride
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
mm^2	square millimeter
MP	maximum parsimony
mRNA	messenger RNA
MSA	multiple sequence alignment
mtDNA	mitochondria DNA
μm^2	square micrometer
NCBI	national center biotechnology information
PCR	polymerase chain reaction
Pgi	phosphoglucose isomerase
Phy	phytochrome
rDNA	ribosomal DNA
rRNA	ribosomal RNA
RNA	ribonucleic acid
RNase A	ribonuclease A
rpm	round per minute
S	stomata number
SI	stomata index

SD	standard deviation
µl	microliter
µm	micrometer
µM	micromolar
Taq	*Taq* DNA polymerase
TBE	Tris Boric EDTA buffer
*Ycf*1	yeast cadmium factor

CHAPTER I

INTRODUCTION

Background and rationale

Up to now, the World Health Organization (WHO) estimates that 80 percent of the world's population still uses traditional remedies, including plants, as their primary health care tools. Medicinal plants have been consumed as food and used as medicinal remedies for a long time, and they are world-widely known for their medicinal significance over the past decades. Therefore, the identification of plant materials is the first priority for ensuring the standardization of herbal medicine.

The genus *Erythrina* belongs to the FABACEAE family. The origin of the name *Erythrina* comes from the Greek word "erythros" which means red, alluding to the bright red flowers of the trees in the genus [1]. The genus *Erythrina* consists of 110 species of trees, shrubs, and herbs. This genus is indigenous to the tropics and possibly originated from India and Malaysia [2]. There are six species of the genus *Erythrina* which have been recorded in Thailand [3]. Five of them are considered to be native to Thailand including *E. fusca* Lour. (= *E. glauca* and *E. ovalifolia*), *E. stricta* Roxb. (= *E. suberosa*), *E. subumbrans* (Hassk) Merr., *E. variegata* L., *E. indica* Lam., whereas the other one, *E. crista-galli* L., is exotic [3, 4].

Plants in *Erythrina* species have been used in the traditional system for the treatment of various ailments and found to have high medicinal values such as anti-inflammatory, antipyretic, neurosedative, anti-asthmatic, broken bones healing, antiepileptic, hypotensive, uterine stimulant, diuretic, antibacterial, antifungal, antiyeast and antimalarial activities [1, 5]. Because of the similarity in morphology and synonym in vernacular name of plants in this genus in Thailand, the identification of the *Erythrina* species is still a problem.

Macroscopic and microscopic examinations together with a genetic analysis have played a key role in medicinal plant identification. These are important parameters for plant authentication for the sake of standardization and quality assurance purposes, which are used for authenticity of plants. Macroscopic identities are based on the authentication of their gross morphological characters and organoleptic properties such as size, shape, colour, flowers or fruit that are visible with naked eyes. The microscopic examination is a conventional, rapid and inexpensive method to identify plant anatomical structures under a microscope based on midrib cross section of mature leaf, and to indicate histological characters of powdered crude drugs. The microscopic leaf constant numbers is one of the quantitative microscopic evaluations, which could be effectively used to distinguish some closely related species unclearly characterized by qualitative microscopic evaluations.

Recently, DNA technology is widely used because of the unique of genetic at genus and species levels. The identification using molecular markers has been widely applied in medicinal plant variation. Molecular based on polymerase chain reaction (PCR) has been extensively used because they are a powerful tool to evaluate genetic diversity and to provide a genetic relationship of plants [6]. Molecular markers are less affected by age, physiological and environmental conditions [7]. Moreover, a complementary with other analytical molecular methods provide important supporting evidence [8]. Plant genomes are more complex than other eukaryotic organisms due to the presence of multiple chromosome, nuclear genome, chloroplast genome and mitochondrial genome. A specific region of DNA sequence in plant genome has been used as a modern genomic tool for herbal plant identification. The sequence resulted from DNA markers such as ITS, *mat*K, *rpo*C, *psb*A_*trn*H and *ycf*1 are mostly used for plant identification. Chloroplast as well as mitochondrial and nuclear genes have been utilized for sequence variation study.

The quantitative microscopic evaluation of *E. fusca, E. stricta, E. crista-galli*, *E. subumbrans*, *E. variegata* and *E. indica* have never been established. Therefore, this research aimed to study the anatomical characteristics of each species using cross section of midrib and to investigate the constant values of leaves including stomatal number, stomatal index, epidermal cell number, epidermal cell area, vein islet number and palisade ratio, for the identification and molecular evaluation of five DNA markers (ITS, *mat*K, *rpo*C, *psb*A_*trn*H and *ycf*1), which is this phylogenetic relationship investigation among *Erythrina* species distributed in Thailand.

Research gap

1. The identification based on midrib cross section and the microscopic leaf constant numbers: stomatal number, stomatal index, palisade ratio, vein islet number and epidermal cell area of *E. fusca, E. stricta, E. crista-galli*, *E. subumbrans*, *E. variegata* and *E. indica* have never been established.

2. The molecular evaluation of *E. fusca, E. stricta, E. crista-galli*, *E. subumbrans*, *E. variegata* and *E. indica* using phylogenetic relationship regarding DNA sequencing data of the ITS, *mat*K, *rpo*C, *psb*A_*trn*H and *ycf*1 have never been investigated.

Objectives of the study

1. To establish leaf anatomical characteristics and microscopic leaf constant numbers of *E. fusca, E. stricta, E. crista-galli*, *E. subumbrans*, *E. variegata* and *E. indica*.

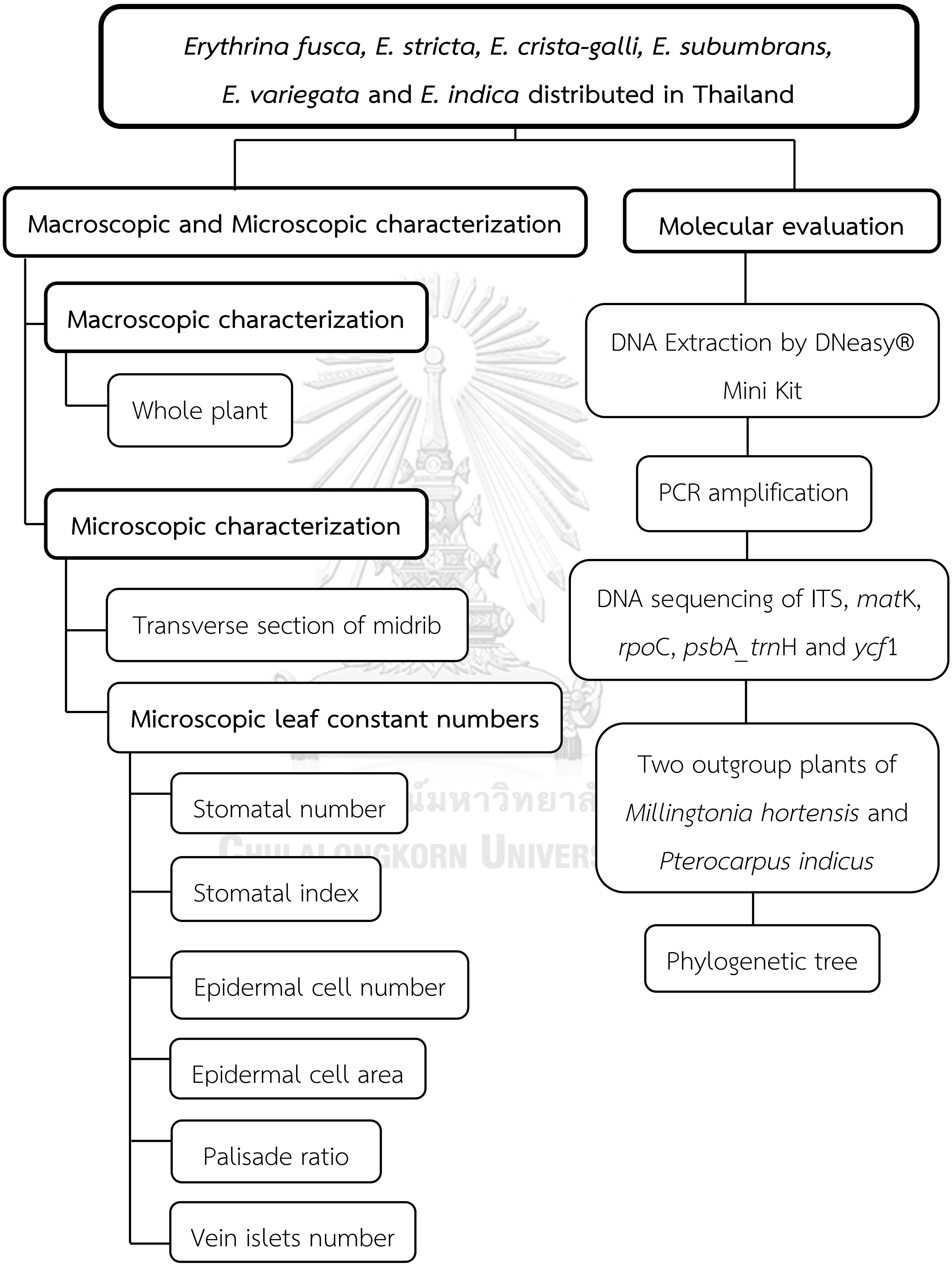
2. To distinguish *Erythrina* species using phylogenetic relationship regarding DNA sequencing data of the ITS, *mat*K, *rpo*C, *psb*A_*trn*H and *ycf*1.

Benefit of the study

1. This study provides the leaf characteristics among *E. fusca, E. stricta, E. crista-galli*, *E. subumbrans*, *E. variegata* and *E. indica*.

2. This study provides specific molecular markers for *E. fusca, E. stricta, E. crista-galli*, *E. subumbrans*, *E. variegata* and *E. indica*.

The conceptual framework



CHAPTER II

LITERATURE REVIEW

The taxonomic hierarchy of the genus *Erythrina* can be classified as follows;

Scientific classification [9]

Kingdom: Plantae

Subkingdom: Viridiplantae

Superdivision: Embryophyta

Division: Tracheophyta

Class: Magnoliopsida

Order: Fabales

Family: Fabaceae

Genus: *Erythrina*

The genus *Erythrina*

Erythrina is the genus of trees, shrubs and herbs. *Erythrina* species are distributed throughout the tropics and extended into warm temperate areas such as South Africa, the Himalayas, southern China, the Rio de la Plata region of Argentina and southern United States. Most species are trees or shrubs, but about ten species which occur in climates with pronounced dry and cool seasons are perennial herbs with large and woody rootstocks [10].

Morphology of the genus *Erythrina*

Because of their characteristic trifoliate leaves, *Erythrina* has been placed traditionally in the subtribe Erythrininae of the tribe Phaseoleae [11]. The trunk, young branches, petioles and petiolules are often armed with blunt, conical thorns or recurved prickles. Leaves are pinnately trifoliate, often clustered at the ends of branches; leaflets are broad-ovate, elliptic, often deltoid or rhomboid, entire, lateral leaflets often asymmetric, terminal leaflet largest, symmetric; stipels are fleshy, glandlike, turning black upon drying, usually one at base of lateral leaflets, paired stipels at base of terminal leaflet; stipels are small, ovate, or linear, caducous or persistent. *Erythrina* species exhibit great diversity in floral structure, inflorescence orientation, fruit morphology, seed coat coloration, and vestiture and epidermal ornamentation of foliage and calyces. Flowers appear before or with the first leaves, very showy, mostly red, some salmon, pink, orange or yellow, solitary, paired or fasicled in erect, terminal racemes leafy at the base or in axillary racemes [12].

The diversity of floral structure reflects adaptation to different pollination mechanisms. All *Erythrina* species have red or orange flowers with copious nectar and are adapted to pollination by nectivorous birds. All 42 paleotropical and some 15 of the neotropical species are pollinated by perching birds of the order Passeriformes; inflorescences of passerine-pollinated species are oriented in such a way that the birds can perch while feeding on the floral nectar. The corolla standard is usually broad, and the flowers are open with exposed reproductive parts. Pollen is deposited on the feeding bird's breast. The diversity of size, form and orientation of passerine-pollinated *Erythrina* flowers would appear to reflect variation in the size, morphology and behaviour of the pollinators [11]. The remaining 55 neotropical species are pollinated by hummingbirds. The corolla standard of hummingbird-pollinated *Erythrina* is narrow and conduplicately folded to form a pseudotube concealing the wing and keel petals as well as the reproductive parts. The flower resembles the tubular corollas of many gametopetalous hummingbird-pollinated plants, but the pseudotube is not sealed on the ventral side where the margins of the corolla standard meet. The inflorescence of the hummingbird-pollinated species is erect, and the flowers are oriented outward, providing no perch for the hummingbirds, which are the only nectivorous birds which hover while feeding [13].

Plant description of *Erythrina* species distributed in Thailand

Scientific name: *Erythrina fusca* Lour.

Synonyms: *Erythrina caffra*, *Erythrina glauca*, *Erythrina viarum*

English names: purple coraltree, gallito, bois immortelle, bucayo, and the more ambiguous "bucare" and "coral bean"

Thai Name: Thong long (ทองโหลง), Thong lang nam (ทองหลางน้ำ), Thong lang ban (ทองหลางบ้าน) [4]

Description: "*E. fusca* is a free-growing tree that can reach 20 m in height. The stems of young *E. fusca* have sharp thorns that become warts or very thick thorns in adult trees. Its leaves are trifoliate with green folioles on the front and whitish-green folioles on the back, coriaceous or semicoriaceous, ovate with a maximum width of 10 cm and a maximum length of 17 cm. Some authors cite that the tree is deciduous. In Colombia it is considered an evergreen species. In droughts a slight defoliation can be observed, but as soon as flowering begins new leaves appear [14]."

Location found in Thailand: All over the country, often planted as an ornamental

Distribution: Neotropics, Asia, Oceania, Madagascar, Mascarene and Africa

Figure 1 *E. fusca* Lour; (A) inflorescences, (B) leaves, (C) stem and (D) habitat

Scientific name: *Erythrina stricta* Roxb.

Synonyms: *Erythrina suberosa*, *Erythrina maxima*

English names: Indian coral tree, Corky coral tree

Thai Name: Thong duean ha (ทองเดือนห้า), Thong lang pa (ทองหลางป่า), Chao (เซ่า), Thong ki (ทองกี), Thong khae (ทองแค), Thong bok (ทองบก), Thong nam (ทองหนาม), Thong lueang (ทองเหลือง) [4]

Description: "*E. stricta* is a deciduous tree, to 10 m high, bark grey, corky, deeply cracked; branchlets tomentose, armed. Leaves trifoliate, alternate; stipules about 5 mm long, lateral, lanceolate; rachis 7.5-12.5 cm long, stout, puberulent, pulvinate; petiolule upto 10 mm; stipels gland like, leaflets 5.5-12 cm, rhomboid-ovate, base deltoid or truncate, apex acute or obtuse, margin entire or sinuate, glabrous above and wooly pubescent beneath, coriaceous; 3 ribbed from the base, lateral nerves 4-5 pairs, pinnate, prominent, intercostae reticulate, faint. Flowers bisexual, about 4 cm long, bright scarlet, in axillary and terminal racemes; bracts lanceolate, cauducous; calyx tube about 5 mm long, campanulate, splitting to become bilabiate, glabrous; corolla exserted; petals 5, standard oblong, 3.8 cm, sessile, the wings minute, keels about 1.8 cm long, connate; stamens 10, monadelphous, the vexillary filament free in the upper two thirds; filaments 6 and 8 mm; anthers uniform; ovary inferior, oblong, downy-pubescent, stipitate, 1 celled, ovules many; style to 1 cm, curved, subulate at apex, not bearded, stigma capitate. Fruit a pod, to 15 cm long, linear-falcate, torulose, follicular, with spongy packing between seeds; seeds 2-5, dark reddish-brown, subreniform [15]."

Location found in Thailand: Kanchanaburi, Loei, Chiang Mai, Nakhon Ratchasima, Prachin Buri, Saraburi

Distribution: China, India, Nepal, Bhutan, Myanmar, Cambodia, Laos, Thailand and Vietnam

Figure 2 *E. stricta* Roxb; (A) inflorescences, (B) leaves, (C) stem and (D) habitat

Scientific name: *Erythrina crista-galli* L.

Synonyms: *Erythrina speciosa*, *Corallodendron crista-galli*, *Erythrina fasciculata*

English names: Brazilian coral tree, cock's comb coral tree, cockspur coral tree, coral tree, crybaby tree, fireman's cap tree

Thai Name: **Thong lang hong kong (ทองหลางฮ่องกง)** [4]

Description: "Coral tree is a spiny, deciduous shrub or small tree that can grow 10 m tall. The older stems are brown or greyish in colour and have moderately rough bark. Younger stems are greenish in colour, shiny, and hairless (i.e. glabrous). The stems and leaf stalks (petioles) are sparsely covered with sharp thorns or prickles that are occasionally hooked (recurved). The alternately arranged leaves are borne on stalks (petioles) 10 cm long and are trifoliate. These leaflets are egg-shaped in outline (ovate) or oval (elliptic) in shape. They are hairless (glabrous), with entire margins and pointed tips (acute apices). The two side leaflets are borne on thin stalks that are 5-10 mm long, while the end leaflet has a stalk that is significantly longer (30-40 mm long). The flowers are scarlet red to dark red in colour and pea-shaped in appearance (5 cm long). They are borne in large, loose, elongated clusters (8-30 cm long). The largest and uppermost petal of each flower is bent upwards or backwards when the flowers are fully open. Flowers also have two inconspicuous side petals (laterals or wings), that are about 10 mm long, and a folded lower petal about 3.5 cm long. These flowers also have five sepals that are fused into a tube (calyx tube) about 10 mm long, and ten long stamens. The filaments of nine of these stamens are fused together into a tube, while the stamens are diadelphous. The ovary is very elongated in shape and is topped with a style and a small stigma. Flowering occurs mostly during spring and early summer. The fruit are large elongated pods (8-22 cm long) that turn from green to dark brown or blackish in colour as they mature. These pods are sometimes somewhat curved and gradually taper to a pointed tip (acute apex). They contain several seeds, with slight constrictions between each of the seeds, but are otherwise cylindrical in shape. The large and hard seeds (about 15 mm long) are slightly kidney-shaped (reniform), dark brown or blackish in colour, and often with a somewhat mottled appearance [16]."

Location found in Thailand: All over the country, especially Bangkok.

Distribution: South America (i.e. eastern Brazil, Bolivia, Peru, Paraguay, Uruguay and northern Argentina), Asia (Hong Kong, Thailand)

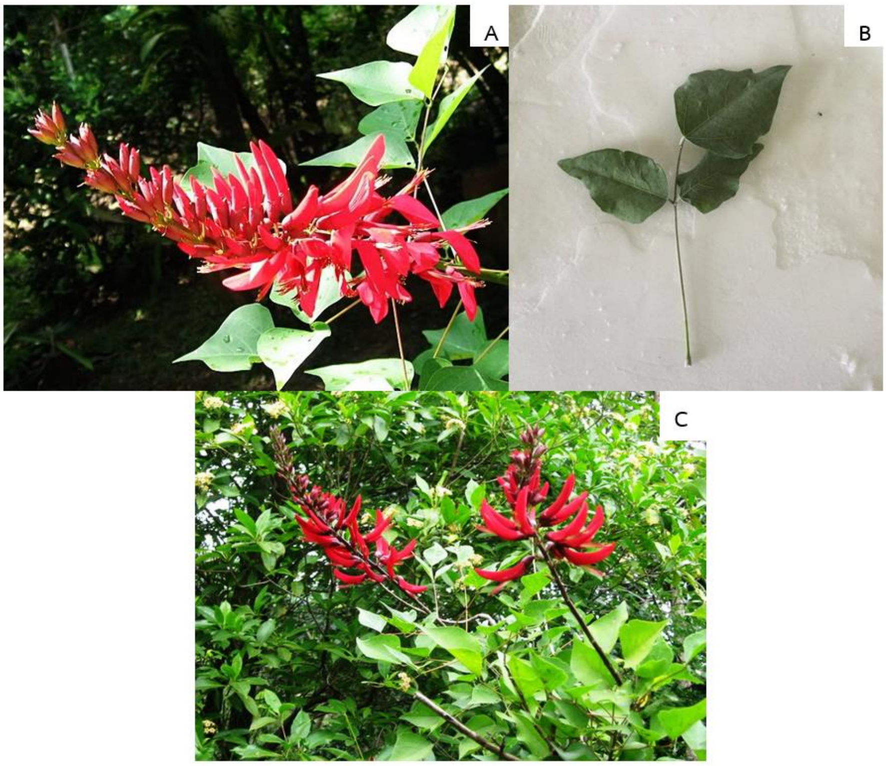


Figure 3 *E. crista-galli* L.; (A) Inflorescences, (B) leaves and (C) habitat

Scientific name: *Erythrina subumbrans* (Hassk.) Merr.

Synonyms: *Erythrina lithosperma*, *Erythrina holoserica*, *Erythrina sumatrana*

English names: December-tree, Dadap of Malaysia

Thai Name: Thong lang bai mon (ทองหลางใบมน), Thong mit khut (ทองมีดขูด), Thong lang (ทองหลาง) [4]

Description: "*E. subumbrans* is a deciduous, medium-sized tree which can reach 10-20 m tall. The crow spreads and the bark is whitish. The trunk and branches are armed with stout prickles while in cultivation. It is mostly unarmed. The leaves are arranged alternate and with three leaflets. The leaflets are ovate-triangular-rhomboid, with terminal one belong largest and measuring 8-16 cm. The base is rounded or cordate, acuminate at apex and hairless. The inflorescence is a racemes at the upper leaf axils. It is 5-23 cm long and brown-hairy. There are many flowers arranged in groups of 3. The peduncle is cylindrical, robust, measures 3-15 cm long and pubescent. The pedicel is 3 mm long, where in fruit it is up to 6 mm long. The sepal is bell-shaped. Measure 1-1.5 cm long, splits open halfway down, hairy and yellow green. The 5 petals are red where the upper part is broadly elliptical, shortly clawed, measure 3 cm, scarlet and with numerous white stripes at the base inside. The wings are as long as the keel or slightly longer. They are about 1.5 cm long, and pale red with a blackish at the upper margin. There are 10 stamens which are 3.5 cm long, monadelphous but with vexillary stamen slightly shorter than the other ones. The pistil is with a hairy ovary. The pod is flat, curved, measure 10-15 cm long and on a slender stalk 4.5 cm long. The lower part is seed and it 2-2.5 cm wide. While the upper part is thicker which is 1.5 cm wide and 1-5 seeded. It is septate between the seeds and dehiscent. The seed is ellipsoid, measuring 7.18 mm × 5-11 mm, smooth and dull black [17]."

Location found in Thailand: Chiang Mai, especially Bangkok

Distribution: Tropical Asia; Thailand, China, India, Sri Lanka, Myanmar, Laos, Vietnam, Malaysia, Indonesia, Philippines

Figure 4 *E. subumbrans* (Hassk.) Merr; (A) inflorescences, (B) leaves and (C) stem

Scientific name: *Erythrina variegata* L.

Synonyms: *Erythrina variegata var. orientalis*, *Corallodendron orientale*, *Chirocalyx candolleanus* Walp.*, Chirocalyx divaricatus* Walp.

English names: Indian coral tree, Tiger's claw, *Variegata* coral tree, *Variegata* tiger's claw

Thai Name: Thong lang lai (ทองหลางลาย), Thong lang dang (ทองหลางด่าง), Thong ban (ทองบ้าน), Thong phueak (ทองเผือก) [4]

Description: "*E. variegata* is a thorny deciduous tree growing up to 27 m in height. The dense, oblong to rounded crown is low-branching with many ascending branches. Inflorescence of many flowered fascicles occurs in terminal or axillary racemes up to 20 cm or more long. Calyx is top-shaped, deeply split along one side, 1–1.8 cm long, on a pedicel 2–5 mm long. Corolla is papilionaceous; standard is short-clawed, ovate to subelliptic, 3–4 cm long, red-orange with longitudinal white lines; wings are about half as long as the standard, greenish to pale red; keel is as long as the wings, greenish to pale red. Ovary is superior, stamens 10, diadelphous, with 9 fused together at the base, enclosed within the keel. Leaves are trifoliate, alternate, bright emerald-green; rachis is mostly 20 cm petiole and three leaflets, each leaflet up to 20 cm long and broad. Fruit a compressed, narrowly oblong pod 10–14 cm long, sterile in the basal portion, and not constricted between the 5–10 dark brown seeds. Seeds are kidney-shaped, dark purple to red, and 1–1.5 cm in length [2]."

Location found in Thailand: All over the country, often planted as an ornamental tree

Distribution: Distributed worldwide, especially in Africa, China, Japan, Taiwan, India, Thailand and Myanmar

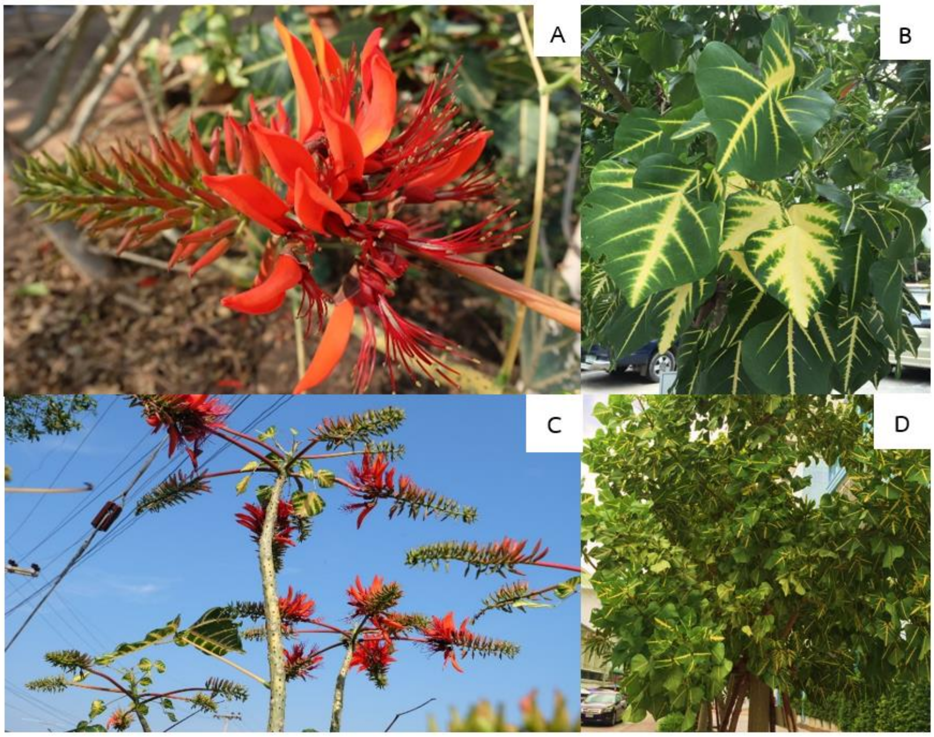


Figure 5 *E. variegata* L.; (A) inflorescences, (B) leaves, (C) stem and (D) habitat

Scientific name: *Erythrina indica* Lam.

Synonyms: *Erythrina variegata* orientalis, *Erythrina variegata* parcelli, *Erythrina variegata* picta

English names: Indian Coral Tree

Thai Name: Thong lang bai mon (ทองหลางใบมน) [18]

Description: "*E. indica* is a medium-sized, spiny, deciduous tree normally growing to 6-9 m (occasionally 28 m) tall and 60 cm. Young stems and branches are thickly armed with stout conical spines up to 8 mm long, which fall off after 2-4 years; rarely, a few spines persist and are retained with the corky bark. Bark smooth and green when young, exfoliating in papery flakes, becoming thick, corky and deeply fissured with age. Leaves trifoliate, alternate, Leaflets are green in colour [19], on long petioles 6-15 cm, rachis 5-30 cm long, prickly; leaflets smooth, shiny, broader than long, 5-15 cm, ovate to acuminate with an obtusely pointed end. Leaf petiole and rachis are spiny. Flowers in bright red to scarlet erect terminal racemes 15-20 cm long; stamens slightly protruding from the flower. Fruit a cylindrical torulose pod, green, turning black and wrinkly as they ripen, thin-walled and constricted around the seeds. There are 1-8 smooth, oblong, dark red to almost black seeds per pod [20]."

Location found in Thailand: All over the country, often planted as an ornamental tree

Distribution: Native to Asia such as Taiwan, southern China, Philippines, Indonesia, Malaysia, Thailand, India, as well as eastern Africa

Figure 6 *E. indica* Lam; (A) leaves

Ethnomedicinal uses of *Erythrina* species distributed in Thailand

Plants of the genus *Erythrina* have long been widely used as ethnomedicine in worldwide. This plants in *Eryhtrina* genus are utilized for a wide array of human diseases. The ethnomedicinal uses of selected *Erythrina* plants are summarized in Table 1.

Table 1 Ethnopharmacological uses of *Erythrina*

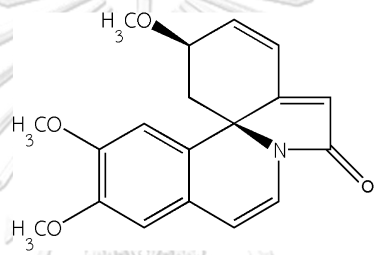
Species	Part utilized	Uses	Locality	Reference
E. fusca	Bark	Migraine	Peru	[21]
	Bark	Infection		
	Bark	wounds		
	Flowers	antifungal	Thailand	[22]
	Bark and Leaves	Anti-inflammatory		
	Leaves	Food (miang kham)		
	Seeds	Skin infections	Indonesia	[23]
	Seeds	Itching		
E. stricta	Bark	Epilepsy	India	[24]
	Bark	Leprosy		
	Stem bark	Edema	Thailand	[25]
E. crista-galli	Leaves	Anti-hemorrhoids	Argentina	[26, 27]
	Bark	Diarrhea		
	Bark	Respiratory tract		
	Bark	Infection		
	Stalk	Urinary tract		
	Stalk	infection		
		Antiseptic		
		Narcotic		

	Stalk and leaves	Antimicrobial Astringent in wound healing Throat infections	Brazil	[28]
	Leaves Leaves	Milk of Ascaris lumbricoides Analgesic	Thailand	[29]
E. subumbrans	Leaves	Menorrhagia	East Indian	[30]
	Leaves Leaves Leaves Leaves	Headache Broken bone healing Abscess Tuberculosis	Thailand	[25] [31] [32]
E. variegata	Bark	Antipyretic	Andamans Islands	[33]
	Bark Bark	Epilepsy Stomachache	India	[34]
	Bark	Swelling	New Guinea	[35]
	Bark Bark Bark	Amenorrhea Conception Dysmenorrhea	Rotuma	[36]
	Flowers Flowers Flowers	Antipyretic Sedative Antiasthmatic	Brazil	[37]
	Leaves	Induce menstruation Febrifuge	India	[38] [39]
	Bark	Anti-inflammatory	Thailand	[40]

E. indica	Roots and bark	Menstrual	India	[41]
	Bark	regulator		
	Bark and	Antipyretic		
	leaves	Anthelmintic		
	Bark	Astringent		[42]
	Bark	Expectorant		[43]
	Bark	Eye drops		[34]
	Bark	Antibilious		
	Leaves	Stomach upset		
	Leaves	Stimulation of		
	Leaves	milk Laxative		
	Leaves	Diuretic		
		Aphrodisiac		
	All plant part	Drugs solution	Thailand	[44]
	Stem bark	Sedative		
	Root	Apthous ulcer		
Erythrina	Leaves	Antipyretic	Solomon	[45]
species	Leaves	Analgesic	Islands	[46]

Chemical constituents of *Erythrina* species distributed in Thailand

The phytochemical data analysis allowed the verification of a predominance of alkaloids, flavonoids and pterocarpan in the *Erythrina* genus. *Erythrina* alkaloids are characteristic of this genus with over one hundred structural derivatives described to date [47]. Some important alkaloids, flavonoids and pterocarpan that are distributed within plants from the *Erythrina* genus are erytharbine, erythrartine, erysotramidine, erysotrine [1] erythratidinone, erythrabyssinII [48] and new pterocarpan [49], respectively shown in Figure 7. It is noteworthy that a characteristic feature of these alkaloids is the spiro structure in the rings bearing the nitrogen atom.

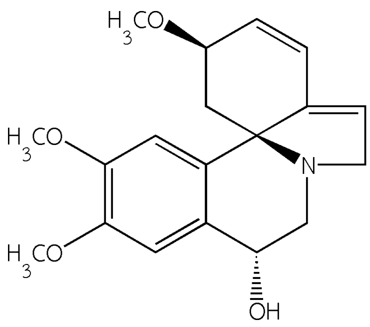


Chemical Name: Erytharbine

Molecular Formula: $C_{19}H_{19}NO_4$

IUPAC Name: (9b*S*,11*R*)-10,11-dihydro-7,8,11-trimethoxy-2*H*-Indolo[7a,1-a] isoquinolin-2-one

Molecular Weight: 325.36

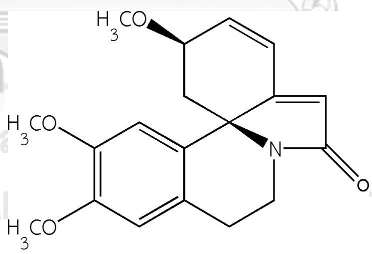


Chemical Name: Erythrartine

Molecular Formula: $C_{19}H_{23}NO_4$

IUPAC Name: (2*R*,9*R*,13b*S*)-2,6,8,9-tetrahydro-2,11,12-trimethoxy-1*H*-Indolo[7a,1-a] isoquinolin-9-ol

Molecular Weight: 329.39

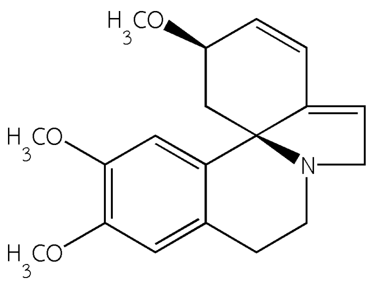


Chemical Name: Erysotramidine

Molecular Formula: $C_{19}H_{21}NO_4$

IUPAC Name: (3β)-1,2,6,7-tetradehydro-3,15,16-trimethoxy-,Erythrinan-8-one

Molecular Weight: 327.37

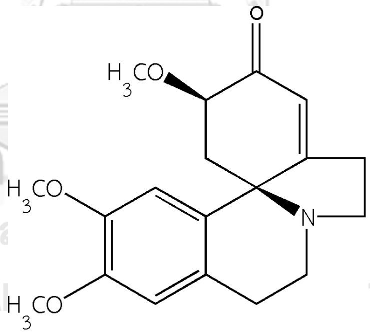


Chemical Name: Erysotrine

Molecular Formula: $C_{19}H_{23}NO_3$

IUPAC Name: (3ß)-1,2,6,7-tetradehydro-3,15,16-trimethoxy-Erythrinan

Molecular Weight: 313.39

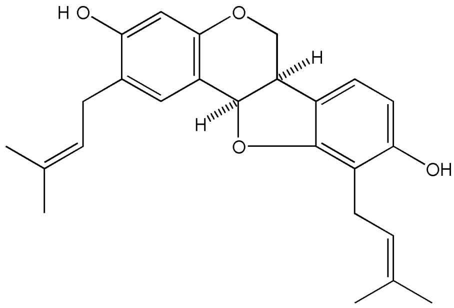


Chemical Name: Erythratidinone

Molecular Formula: $C_{19}H_{23}NO_4$

IUPAC Name: (3ß)-(9CI)1,6-didehydro-3,15,16-trimethoxy-Erythrinan-2-one

Molecular Weight: 329.39

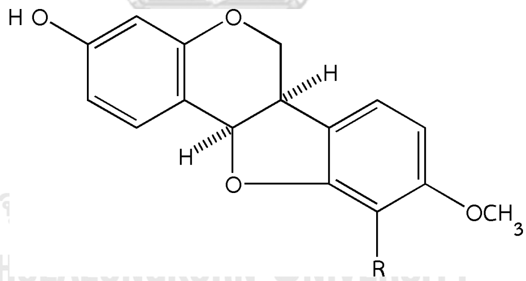


Chemical Name: Erythrabyssin II

Molecular Formula: $C_{25}H_{28}O_4$

IUPAC Name: 3,9-dihydroxy-2,10-diprenylpterocarpan

Molecular Weight: 392.495



Molecular Formula: $C_{21}H_{24}O_5$

IUPAC Name: 3-hydroxy-10-(3-hydroxy-3-methylbutyl)-9-methoxypterocarpan

Molecular Weight: 379.15

Figure 7 The structure of alkaloid, flavonoid and pterocarpan

Pharmacological investigations of *Erythrina* genus

Analysis of biological activity shows the wide variety of biological activity of plants from selected *Erythrina* genus are shown in Table 2.

Table 2 Biological activity of *Erythrina* genus

Species	Part of the plant	Uses	Location	Reference
E. fusca	Leaves	Hypotensive Uterine stimulant Diuretic	Thailand	[50]
	Seeds	Central Nervous System depressor	Indonesia	[23]
E. stricta	Stem	Spasmolytic Hypotermic Diuretic Anticonvulsant Analgesic Antiviral Anti-fungal Anti-yeast Anti-protozoan	India	[51]
	Leaves	Cytotoxic		[52]
	Leaves and seed oil	Anti-bacterial Anti-fungal	Thailand	[53] [54]
E. crista-galli	Bark	Anti-inflammatory Anti-bacterial Anti-fungal	Argentina	[26]
	Flowers	Anti-mutagenic	Unspecified	[55]

	Fresh fruit, leaves and stem	Anti-phagocytic	Greece	[56]
	Leaves	Anti-fungal Anti-bacterial	Egypt	[57]
	Leaves and stem	Cytotoxic Antiviral	Brazil	[28]
	Leaves and stem	Animal repellent	Germany	[58]
	Root and stem Bark	Anti-bacterial Anti-mycobacterial	Bolivia	[59] [60]
	Seeds	Trypsin inhibition	Uruguay	[61]
E. subumbrans	All plant part	Fetal anti-implantation Uterine stimulant Anti-tumoral Abortive effect	India	[62]
E. variegata	Bark	Anti-gastric ulcer	Japan	[63]
	Bark and leaves Seeds oil Stem	Inhibition of plant germination and growing Anti-bacterial Anti-fungal Juvenile hormone activity	India	[64] [65] [66] [67]
	Bark Stem bark	Phospholipase A2 Inhibitor	Samoa	[68] [69]

		Prostaglandin synthesis inhibitor Central Nervous System effects Spasmolytic		[70]
	Flowers	Anti-yeast Anti-bacteria	Thailand	[71]
	Fresh fruit	Anxiolytic	Brazil	[72]
	Flowers Leaves	Anti-inflammatory Skeletal muscle relaxing	Vietnam	[73] [74]
	Roots	Inhibitor of glutamate pyruvate transaminase	Taiwan	[75]
	Leaves Roots Stem bark	Antispasmodic Cytotoxic Anti-yeast Anti-bacterial Anti-mycobacterial Cytotoxic Antispasmodic	India	[52] [76] [52]
	Leaves	Anti-tumoral	Philippines	[77]
E. indica	Leaves Leaves	Anti-fungal Anti-bacterial	Egypt	[57]
		Central Nervous System	Sri Lanka	[78]

		Depressor		
	Unspecified	Stimulant and inhibitor of lymphocyte blastogenesis	India	[79]
	Root bark	Anti-mycobacterial	Nigeria	[80]
	Stem bark	Anti-bacterial Cytotoxic		[81]

Plant identification

The first step to categorize the herbal plant materials is the determination according to their macroscopic and microscopic characteristics for establishing the identity and the degree of purity of herbal plant materials. Visual by eyes based on the appearance of morphological characteristic provides the simplest and quickest inspection. However, macroscopic examination is sometime inadequate. It is often necessary to combine with other methods such as microscopic, chemical constituents or molecular analyses as shown in Figure 8.

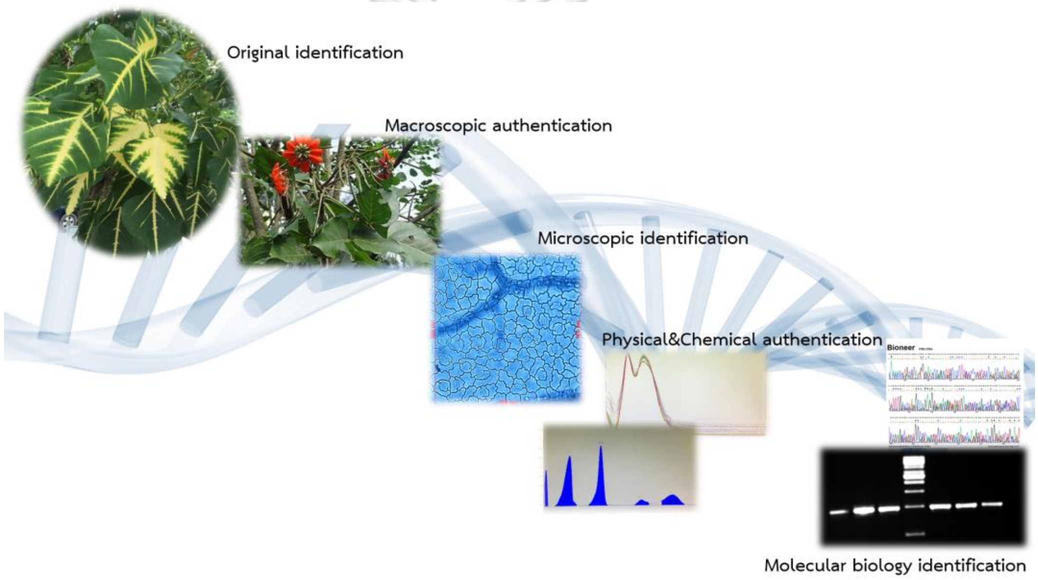


Figure 8 The medicinal plants authentication methods

Macroscopic and microscopic examinations

An examination to determine these characteristics is the first step towards establishing the identity and the degree of purity of such materials and should be carried out before undertaking any further tests. Wherever possible, authentic specimens of the material in question and samples of pharmacopoeia quality should be available to serve as a reference [82].

Macroscopic identity of medicinal plant materials is based on shape, size, colour, surface, for example as shown in Figure 9 characteristics, texture, fracture characteristics and appearance of the cut surface.

However, since these characteristics are judged subjectively and substitutes or adulterants may closely resemble the genuine material, it is often necessary to substantiate the findings by microscopic analysis [82]. Furthermore, microscopic inspection of medicinal plant materials is indispensable for the identification of broken or powdered materials. Both macroscopic and microscopic evaluations are acceptable to be the first step for identification of plants.

Figure 9 Leaf pattern of *Erythrina* species

Photomicroscope

Microscope evaluation is commonly conducted using a digital camera attached above the microscope. The photograph is recorded with an attached digital camera and examined under the photomicroscope using appropriated objective lens (5X, 10X, 20X and 40X magnifications) and eyepiece lens of a 10X magnification. The images are recorded using AxioVision Release 4.8.2 program. The photomicrography is uniquely qualified to be used for routine and advanced microscopic investigation of medicinal plant materials [83].

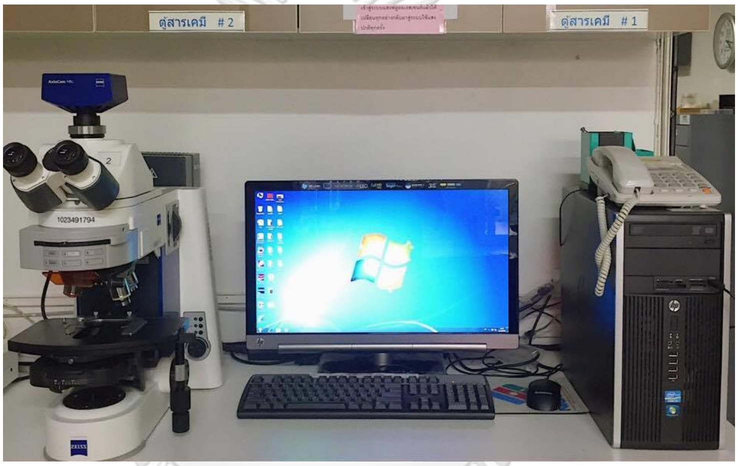


Figure 10 The photomicroscope (Zeiss Axioskop, Germany) with an attached digital camera (Cannon Power shot A640)

Reagents for microscopic examination

The presence of various contents within the cell such as starch grain, plastid and oil etc., may result in non-translucent section and obscure certain characteristics. There are some reagents that can dissolve these contents and have been used to make an infiltrating effect. Some of the most frequently used reagents are sodium hypochlorite and chloral hydrate as described below [84].

Sodium hypochlorite solution

Sodium hypochlorite is used for bleaching deeply colored sections for removing chlorophyll from the leaves [85]. The sections are immersed in sodium hypochlorite solution for a few minutes until sufficiently bleached, then washed with water and mounted with glycerol on the glass slide.

Chloral hydrate solution

Chloral hydrate is used as an aqueous solution, often added to glycerol to prevent crystallization of the reagent when used as a temporary mounting reagent for examination a variety of plant structures [86]. Chloral hydrate solution is gently heat. Chloral hydrate dissolves starch grains, plastids and volatile oils and expands collapsed and delicate tissue without causing any undue swelling of cell walls or distortion of the tissues.

Leaf microscopic characteristics

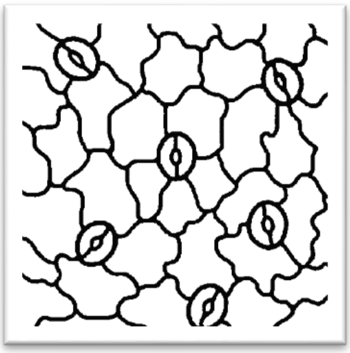
Transverses section of midrib

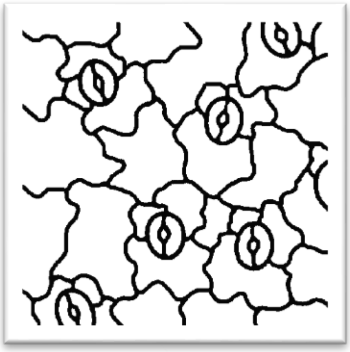
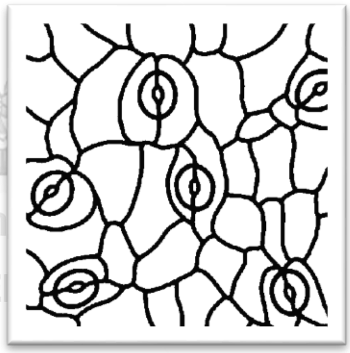
Regarding the qualitative microscopic evaluation, the transverse section of midribs, veins vascular tissues and particular surface cytomorphological characters (i.e., trichomes, palisade cells, stomata, etc) can be used to distinguish the identity for plant authentication based on each cell type, form, size and its distribution within midrib cross section. Moreover, midrib anatomical character enables to detect the contamination or adulteration in plant materials [87].

Types of stomata

In the mature leaves, five stomatal classification are consider the different types which are distinguished by their forms and arrangement in the surrounding cells, [82] as follows in Table 3.

Table 3 Type of stomata in plants are often available for mature leaves that are distinguished by their forms and arrangement in the surrounding cells [88]

Types of stomata	The arrangement of the surrounding cells	Surface view of epidermis
Anomocytic or ranunculaceous (irregular-celled) type	The stoma is surrounded by a varying number of cells, generally not different from those of the epidermis.	

Anisocytic or cruciferous (unequal-celled) type	The stoma is usually surrounded by three or four subsidiary cells, one of which is markedly smaller than the others.	
Diacytic or caryophyllaceous (cross-celled) type	The stoma is accompanied by two subsidiary cells, the common wall of which is at right angles to the stoma.	
Paracytic or rubiaceous (parallel-celled) type	The stoma has two subsidiary cells, of which the long axes are parallel to the axis of the stoma.	

Microscopic leaf constant numbers

Microscopic leaf constant numbers used to identify between some closely related species. It has great value for a quality of the medicinal plants based on their specific characters. Microscopic leaf constant numbers can be measured by as the stomatal number, stomatal index, cicatrix number, cicatrix index, trichome number, vein-islet number, vein termination number and palisade ratio [89].

The pieces of plant are mounted onto a slide in water for observing cells, tissue structures and microscopic leaf constant numbers under microscope with 10X, 20X and 40X objective lens magnifications.

(I) Stomatal number and stomatal index

The average stomatal number is the number of stomata per square millimeter of epidermis, midway between midrib of the leaf and its margin, while the stomatal index is a percentage ratio of stomata number (S) to the epidermal cell numbers (E) in the same unit area of leaf. The stomatal index (SI) is calculated by using formula: SI = (S / E + S) x 100. In recording results the range as well as the average value should be recorded for each surface of the leaf and the ratio of values for the two surfaces [90]. However, previous study reported that the stomatal density on the upper surface of *E. velutina* in Brazil was (264.60±16.83) whereas on the lower surface was (46.60 ± 8.82) [91].

(II) Upper epidermal cell area

The epidermal cell number per square millimeter of upper epidermis, midway between midrib of the leaf and its margin is counted. The epidermal cell area is calculated by using formula: EA = (1 / E + S) × 10^6 μm^2. Epidermal cell area = 1 mm^2/ E the surface of epidermal cells per square of leaf, where, E = number of epidermal cells per square millimeter (mm^2) in a given area of leaf, S = the number of stomata in a given area of leaf. The number of epidermal cell on upper surface of leaf in 1 square millimeter (mm^2) in each field is counted. The numbers of epidermal cells are counted as the epidermal cell number, and epidermal cell area using the above formula is calculated [88].

(III) Palisade ratio

Palisade cells contain most of the chloroplasts which are subjected to the photosynthesis. This is a type of photosynthetic cells in the mesophyll of leaves, mostly just beneath the upper epidermal surface layer. The cell are elongated and more cylindrical and arranged in one or more rather regular, relatively compact layer near the ventral, or upper side of the leaf with the long axis of the cells perpendicular to the leaf surface. Palisade ratio is the average number of palisade cells beneath one epidermal cell of a leaf by counting the palisade cell beneath four continuous epidermal cells [92].

Figure 11 Four upper contiguous epidermal cells with underlying palisade cells in surface view

(IV) Vein islet number

A vein-islet is the small area of green tissue surrounded by the veinlets. The vein-islet number is the average number of vein-islet per square millimeter of a leaf surface midway between midrib of the leaf and its margin [88].

Molecular identification

The molecular method or DNA-based techniques have been wildly used for herbal medicine technology and authentication of medicinal plant species. These methods useful in case of medicinal plants are frequently substituted or adulterated with other species or their morphological or phytochemically indistinguishable because of their variable sources and chemical complexity. These techniques have been found to be useful and accurate for determination of genetic variation in plants. DNA methods are suitable for identifying medicinal materials because genetic composition is unique for each individual irrespective of the physical forms of samples and are less affected by age, physiological conditions, environmental factors, harvest, storage and processing [93].

DNA (deoxyribonucleic acid) encode the genetic information of all known living organisms including viruses. Eukaryotic organisms keep most DNA inside nucleus and some DNA in organelles such as chloroplast genome, mitochondria genome, while prokaryotes keep their DNA only in the cytoplasm [94].

Plant genomes are all the genetic materials in plant cell consist of nuclear genome and organelle genome. The nuclear genome consists of inherited information: it is crowed with nongenomic DNA. The organelle genome can be divided into two parts; the mitochondrial genome which lacks inherited information, and the chloroplast genome which is crowed with gene [95].

Currently, sequence comparison analysis with universal primers for organelle DNA has been widely used in species identification, genetic diversity and phylogenetic studies in many different plant species.

DNA extraction

There are many alternative protocols for DNA extraction and the selected protocol affects the quality and quantity of DNA.

CTAB method

DNA isolation by CTAB method is one of the most popular protocols. Many different methods and technologies are available for the isolation of genomic DNA. In general, all methods involve disruption and lysis of the start material followed by the removal of proteins and other contaminants and finally recovery of DNA. This method is modified from Doyle [96, 97]. Fresh young leaves are rapidly frozen in liquid nitrogen and grounded into powder then lysed with the ionic detergent CTAB (cetyltrimetylammonium bromide), which form an insoluble complex with nucleic acid in a low-salt environment. Under these conditions, polysaccharides, phenolic compounds and other contaminants remain in the supernatant and can be washed away. Removal of proteins is typically achieved by organic solvent extraction. The DNA complex is solubilized by raising the salt concentration and precipitated with ethanol or isopropanol.

DNA extraction kit

One alternative to the DNA extraction kit method has been developed by Qiagen. Beside DNA isolation by CTAB method, the commercial instant DNA extraction kit is considered to be a widely isolation method. The technology makes use of spin columns, which contain a silica-gel-based membrane that binds the DNA. The DNA while bound to the membrane can be washed and cleaned from contaminants and then eluted from the column (membrane) using water. This method is relatively simple, saves time, does not contain harmful chemicals such as phenol or chloroform, involves minimal handling, higher percent yields and the high quality of DNA, but this method is expensive.

Determination of DNA quantity and purity

DNA quantity and purity is also checked by spectrophotometer analysis and agarose gel electrophoresis from the absorbance data of the sample DNA at 260 nm and 280 nm. The purity of DNA sample is calculated from OD260/OD280, and its ratio ranged from 1.8-2.0.

Agarose gel electrophoresis is a method to separate DNA or RNA molecules by size. This is achieved by moving negatively charge nucleic acid molecules and agarose matrix with an electric field electrophoresis. Shorter molecules move faster and migrate faster than longer ones.

The obtained genomic DNA is then used as a DNA template for amplification. There are several regions in the DNA from various origins that are used for studying the divergence or identity of plants, such as nuclear genome, chloroplast genome, and mitochondrial genome.

Nuclear genome

Nuclear genome is simply a DNA on the chromosome which has high capacity in the nucleus. It contains genetic information given directly from parents; for that reason, it is very useful, especially in forensic investigation. In terms of herbal drug, it is widely used in DNA fingerprinting. The commonly used regions of the nuclear genome is ITS [98].

Ribosomal DNA (rDNA)

Ribosomal DNA codes for ribosomal RNA (rRNA). The ribosome plays a role in protein synthesis or polypeptide chain production. It consists of a tandem repeat of a unit segment including non-transcribed spacer (NTS), external transcribed spacer (ETS), 18S, ITS1, 5.8S, ITS2, and 28S tract (Figure 12). rDNA tandem arrays are concerted evolution because it has low rate of polymorphism. Therefore, the comparison of the rDNA segments such as ITS region of the related species and phylogenetic analysis are applicable [99].

Internal transcribed spacers (ITS)

Internal transcribed spacers (ITS) contain two regions: 18S-5.8S rDNA coding regions (ITS1) and 5.8S-28S rDNA coding regions (ITS2) [100]. The sequences of ITS regions may vary because they are fast evolving. As a consequence, universal PCR primers are designed to make the amplification of ITS region easier by having highly conserved regions flanking the ITS and its relatively small size (600-700 base pairs), resulting in the high copy of rDNA repeats. The ITS, therefore, becomes the most widely used technique for evolutionary phylogenetic examination, as well as molecular systematics at the species level [101].

Apart from that, *phy* gene (phytochrome), *gap*A gene (glyceraldehydes-3-phosphate dehydrogenase), *adh* gene (alcohol dehydrogenase) and *pgi* gene (phosphoglucose isomerase) are other regions in the nuclear genome that can be applied in plant evolution analysis; however, they are not widely used in DNA fingerprinting of medicinal plants.

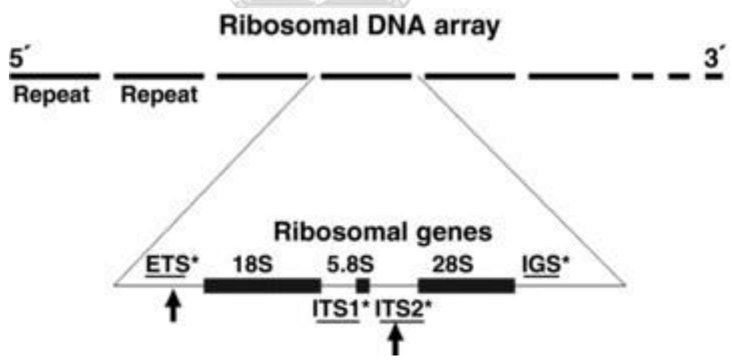


Figure 12 Structure of internal transcribed spacers (ITS) region of the nuclear ribosomal DNA

Chloroplast genome

The chloroplast DNA (cpDNA) are large in size (around 140 kb in higher plant) and code for rRNA and transfer RNA (tRNA) necessary for producing protein. The chloroplast genome is usually used to deduce plant phylogenies at various taxonomic levels. This allows the direct sequencing of polymerase chain reaction (PCR) products to be popularly used for plant systematics and evolution [102]. The cpDNA is uniparental inheritance, allowing the exact copies to be repeatedly produced. Examples of cpDNA are *mat*K, *rpo*C, *psb*A_*trn*H, and *ycf*1.

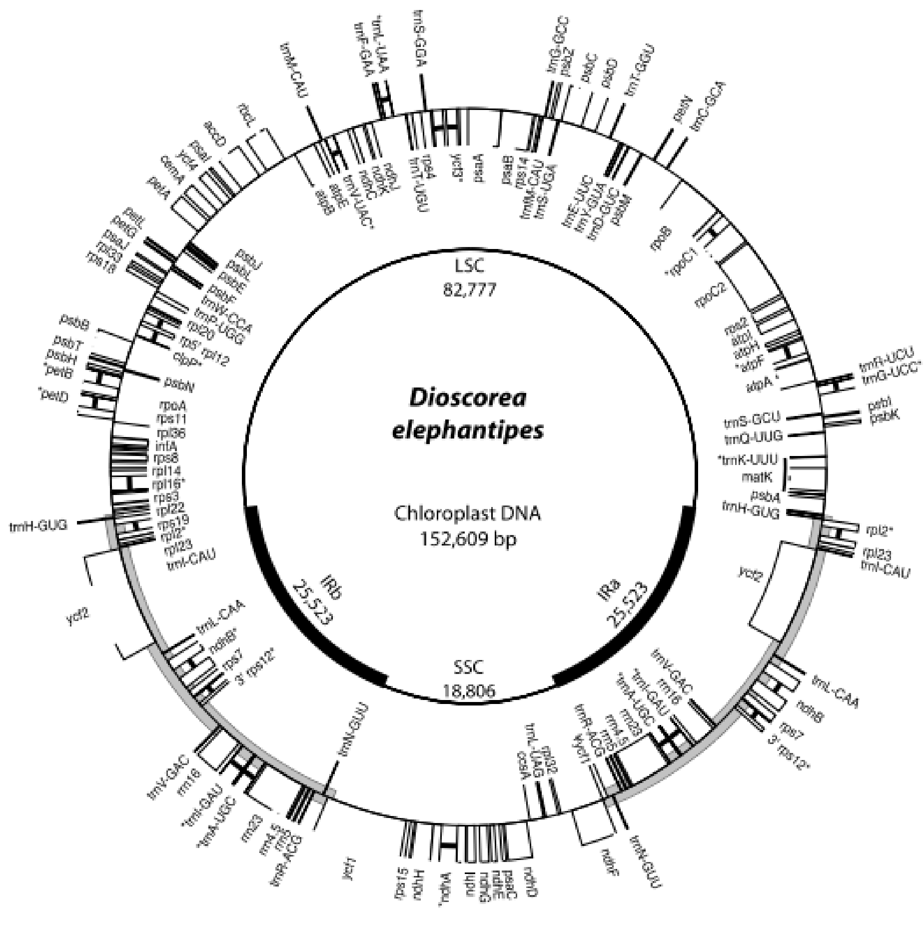


Figure 13 Gene map of *Dioscorea elephantipes* chloroplast genome illustrate location of many of the chloroplast regions [103].

The *mat*K gene

The *maturase* K (*mat*K) gene is within the intron of the chloroplast *trn*K gene (Figure 13) with approximately 1500 base pairs in size (Figure 14). The *mat*K gene encodes an enzyme *maturase*, folding the intron RNA into the catalytically-active structure. The 3’ end of the *mat*K is composed of a conserved region of about 100 amino. Because of its two flanking coding *trn*K genes, the *mat*K gene is interestingly found to have practical size, high substitution rate, evenly distributed codon position variation, low transition and transversion ratio, and the simplicity of amplification. Thereby, it can generate fast evolution only using the universal primer [104].

Figure 14 Structure of *mat*K gene which flanking between *trn*K gene regions.

The *rpo*C gene

The ß' subunit of RNA polymerase in *E. coli* K12, which encode the ß ' subunits of RNA polymerase in bacteria. In most plastid genomes rpoC is split into *rpo*C1 and *rpo*C2, which code for ß' and ß'' subunits [105]. *rpo*C gene is approximately ~840 base pairs [106] in size. The chloroplast *rpo*A, *rpo*B, *rpo*C1, and *rpo*C2 genes are all transcribed. The spinach chloroplast *rpo*A gene is expressed as determined by Northern hybridization and in vitro translation of chloroplast RNA. Transcripts of the tobacco genes were also reported [107]. The genes that encode the ß' and ß'' subunits of RNA polymerase (*rpo*B and *rpo*C) are found in a large transcription unit which also contains the genes for the ribosomal proteins L10 and L7/12 (*rpl* and *rpsl*). This unit is preceded by another transcription unit containing genes for ribosomal proteins L11 and L1 (*rpl*K and *rpl*A) [108].

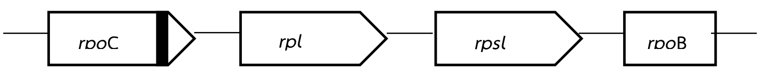


Figure 15 Structure of *rpo*C gene

The *psb*A_*trn*H intergenic spacer region

The chloroplast *trn*H gene has been sequenced in different plant species, and was found to be well conserved during cpDNA evolution. This gene is usually found located near the LSC/IRA junction in higher plant chloroplast genome, (Figure 13 and Figure 16) such as in common bean, soybean, spinach and tobacco. It is, however, located within the inverted repeats of the rice cpDNA and at the center of the LSC of the liverwort cpDNA. In pea and broad bean, the *trn*H gene is found downstream of the *psb*A gene. The length of the intergenic spacer between the *psb*A gene and the *trn*H gene varies from one plant to the other [109].

The *psb*A gene, along with three other chloroplast (cp) genes, namely *psb*B, *psb*C and *psb*D, encodes the core proteins complex in the chloroplasts. *psb*A_*trn*H intergenic spacer, tested on 99 species in 80 genera from 53 plant families, was exhibited high divergence levels and easy amplified [109, 110]. This spacer can also be used to test Ephedra in dietary supplement that sold in commercial markets [111].

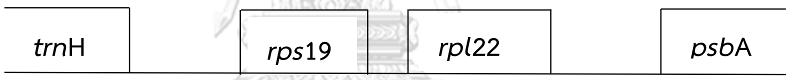


Figure 16 Structure of *psb*A_*trn*H gene

The *ycf*1 region

The chloroplast *ycf*1 gene has been sequenced in different plant species. The chloroplast genomes of higher plants contain two giant open reading frames designated *ycf*1 and *ycf*2. Although the function of *Ycf*1 is unknown, it is known to be an essential gene [112]. The yeast cadmium factor (*YCF*1) gene from *Saccharomyces cerevisiae* encodes a 1,515 aminos [113]. *Ycf*1 gene is approximately 6000 base pairs in size [114].

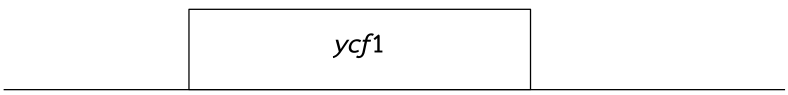


Figure 17 Structure of *Ycf*1b gene

Mitochondrial genome

Mitochondrial genome (mtDNA) is the DNA in mitochondria that synthesizes adenosine triphosphate (ATP). mtDNA is large and has variation in size. The substitution rate of the nucleotide in plant mtDNA is lower than that of animal, nuclear genome and cpDNA by 40-100, 12 and 3-4 times, respectively. Hence, the mtDNA is not commonly used in authentication of herbal drugs [115].

Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) or *in vitro* enzymatic gene amplification is the technique that increases DNA fragments. It is quick and easy method to characterize, analyze, and generate unlimited copies of any DNA or RNA pieces. The components of the PCR reaction consist of DNA template, thermostable DNA polymerase, deoxyribonucleotide triphosphates (dNTPs), oligonucleotide primers, suitable buffers and magnesium or manganese ions (Mg^2+) [116].

To obtain the copying method start, DNA template and two primers are required. The primers are short chain of four different chemical constituents to build the strand of genetic materials. These consist of the 3' ends of each of the sense and anti-sense strand of DNA target.

The efficacy of PCR can be stimulated from three causes. There are specificity, efficiency or yield and fidelity. There are three steps for cycle. First of all, the first step is denaturation. In this step, the target genetic material must be denatured. The strands of its helix should be splitted by heating around 90-96° C. Secondly, it is called annealing step; the primers bind to their target sequences on the template DNA. This step, the temperature is decreased to about 35 to 65° C (depend on primer sequence and technique). Lastly, it is elongation step; DNA is synthesized by polymerase. The temperature is chosen at which the activity of the thermostable polymerase is optimal (usually 65 to 72° C). To gain more DNA, repeating the three steps cycle 25 to 50 times resulting in the exponential amplification.

Although, there are some problems that can present with PCR technique such as the plant sample contamination. It might be contaminated with the external genetic material which can make many copies of unrelated DNA. For the reason, it may present an error result, but there are also have many applications of PCR such as cloning, genetic engineering, etc.

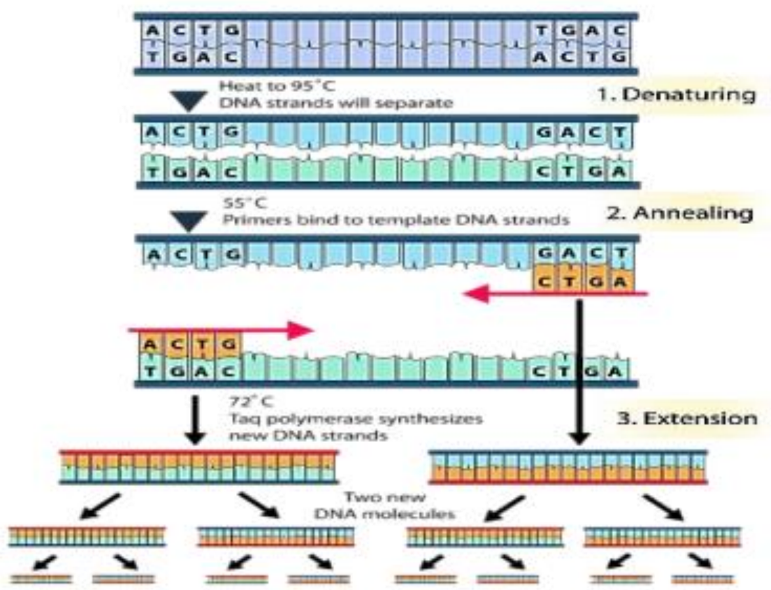


Figure 18 The polymerase chain reaction [117]

DNA sequencing

DNA sequencing is a technique used to determine the order of nucleotides in DNA. Polymorphism at the DNA level can be examined in various ways, but the direct method is to identify the nucleotide sequences of a defined region. Currently, DNA sequencing is a routine technique in molecular biological laboratory. DNA sequences can be applied in many fields such as diagnostic, biotechnology, forensic biology, botanical and biological systematics. For decades, the knowledge of DNA sequences have triggered many biological research, leading to invaluable discoveries [118]. Two major methods are described below;

Chemical method

Chemical method (also known as Maxam-Gilbert sequencing) requires radioactive labeling at one 5' end of the DNA by a kinase reaction using gamma-32P ATP and purification of the DNA fragment. Chemical treatment generates breaks at a small proportion of one or two of the four nucleotide bases in each of four reactions (G, A+G, C, and C+T). For example, the purines (A+G) are depurinated using formic acid, the guanines (and to some extent the adenines) are methylated by dimethyl sulfate, and the pyrimidine) C+T) are methylated using hydrazine. Sodium chloride or salt is added to the hydrazine reaction to restrain the methylation of thymine for the C-only reaction. The modified DNAs are then cleaved by hot piperidine at the position of the modified base. The concentration of the modifying chemicals is controlled to introduce on average one modification per DNA molecule. Thus, a series of labeled fragments is generated from the radiolabeled end to the first "cut" site in each molecule. The fragments in the four reactions are electrophoresed side by side in denaturing acrylamide gels for size separation. To detect the fragment, the gel is exposed to X-ray film for autoradiography, resulting in dark bands each corresponding to a radiolabeled DNA fragment, from which the sequence may be inferred [102]. Another non-radioactive labeling strategy which is stable during the chemical reactions uses a biotin marker molecule chemically or enzymatically attached to an oligonucleotide primer or an end-filling reaction of restriction enzymes sites [119]. After fragment separation by direct northern blot electrophoresis, the membrane-bound sequence pattern can be visualized by a streptavidin-bridged enzyme colour reaction [120].

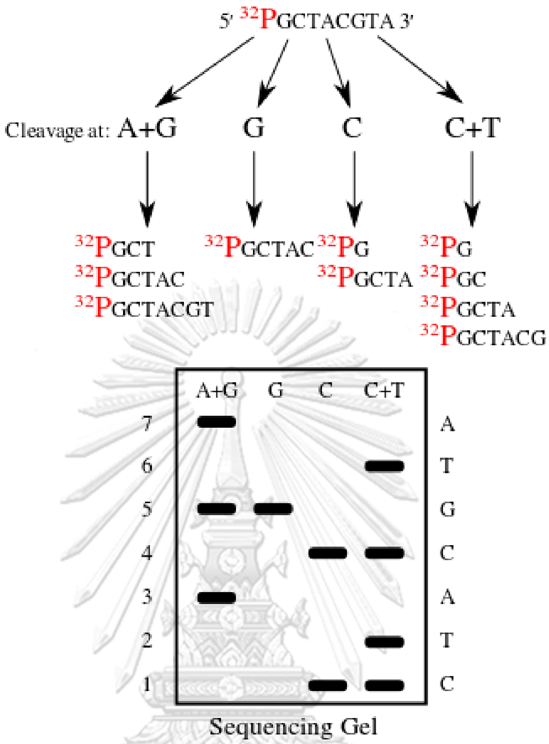


Figure 19 Chemical method Maxam-Gilbert sequencing [121]

Chain termination method (Sanger's method)

In this method, dideoxynucleotide triphosphates (ddNTPs) is applied as DNA chain terminators. Compared to the chemical method, this technique has a competitive advantage due to less hazardous materials and radioactivity. The method requires a single-stranded DNA template, a DNA primer, a DNA polymerase, normal deoxynucleotidetriphosphates (dNTPs; dATP, dGTP, dCTP and dTTP), and modified nucleotides (dideoxyNTPs; (ddATP, ddGTP, ddCTP, or ddTTP), lacking a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides, thus terminating DNA strand extension and resulting in DNA fragments of varying length. For the detection to be at ease, these ddNTPs are labelled fluorescently. The DNA sample is divided into four separate sequencing reactions, containing all four of the standard deoxynucleotides and the DNA polymerase. Only one of the four dideoxynucleotides is added to each reaction. The newly synthesized and labelled DNA fragments are heated, denatured and separated by size on a denaturing polyacrylamide gel electrophoresis with each of the four reactions run in individual lanes (lanes A, T, G, C); the DNA bands are then visualized by autoradiography or UV light, and the DNA sequence can be directly read off the X-ray film or gel image. In the image on the right, X-ray film is exposed to the gel, and the dark bands correspond to DNA fragments of different lengths. A dark band in a lane indicates a DNA fragment that is the result of chain termination after incorporation of a dideoxynucleotide (ddATP, ddGTP, ddCTP, or ddTTP). The relative positions of the different bands among the four lanes are then used to read (from bottom to top) the DNA sequence.

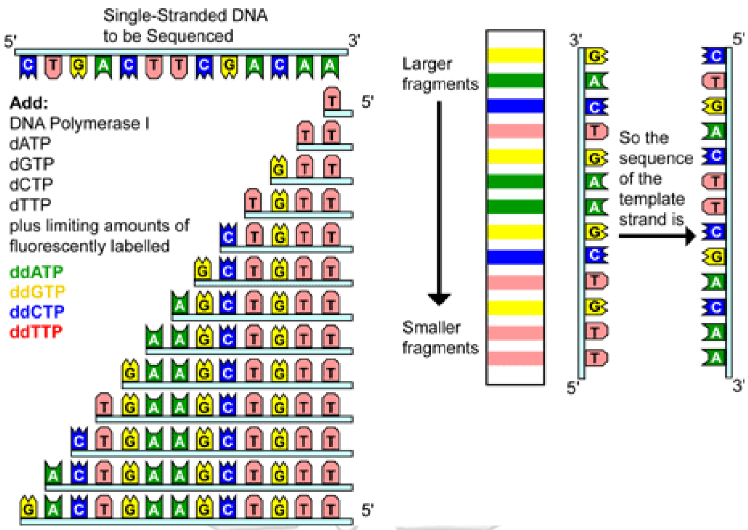


Figure 20 Chain termination method [122]

MALDI-TOF mass spectrometry method

MALDI-TOF mass spectrometry is also known as matrix-assisted laser desorption ionization time of flight .MALDI-TOF MS is to ionize a sample before the analysis by mixing the sample with matrices containing an acid or alkaline component, such as α-cyano-4-hydroxycinnamic acid (CHCA) and 2, 5 dihydroxybenzoic acid (DHB). Mass spectrometry is an experimental technique used to identify the components of a heterogeneous collection of biomolecules. By sensitive discrimination of their molecular mass, the sample to be analyzed is placed in a UV-absorbing matrix pad and exposed to a short laser pulse. The ionized molecules are accelerated off the matrix pad (desorption) and moved in an electric field towards a detector. The "time of flight" required to reach the detector depends on the mass/charge *(m/ z)* ratio of the individual molecules.

To use MALDI-TOF for DNA sequencing, for instance, the fragment ACGTACGATACGACT is considered to be sequenced. A PCR-derived DNA product is transcribed to RNA *in vitro* [ACGUACGAUACGACU] in four separate reactions, each with three rNTP bases and one specific dNTP. In the example, use of dC prevents cleavage of C positions by RNAse, which cleaves only after r*U* and produces three fragments of 4, 5, or 6 nucleotides. Each fragment has a characteristic *m/z* ratio, as indicated by a peak in the MALDI-TOF spectrum. Analogous reactions occur for each of the other three letters. The MALDI-TOF mass signal pattern obtained for any experimental DNA sequence is compared with the expected *m/z* spectrum for the reference sequence under consideration, which includes the products of all four cleavage reactions. Any SNP differences between the experimental and reference DNA sequences produced predictable shifts in the spectrum, and their exact nature can be deduced. In greater detail, four transcription reactions are done with two forward and two reverse primers. In each pair, either a dC or a dT is used along with the other three rNTPs. Since RNAse cleaves only after rC and rU, incorporation of dC protects those bases, and cleavage occurs only after rU. Use of dT allows cleavage only after rC. The same process on the complementary strand in the reverse reactions produces two fragment sets cleaved after rC and rU on the reverse strand, which corresponds to cleavage before rA and rG bases on the forward strand. The four reactions taken together include a collection of fragments terminated adjacent to every base in the sequence, as in the example [123].

Advantages of MALDI method are very rapid, high sensitivity, fast and low dosage. The substance that is examined does not need to be very pure and this technique can analyze many substances at the same time. However, disadvantages are its expensiveness and difficulty of quantitative analysis [124].

In addition, the MALDI-TOF technique can be applied to proteins as well. Protein study using the powerful mass spectrometry technique consists of several stages. Apart from good protein preparation, protein separation is one of the most important factors. There are two main methods used in the research: gel based and gel free [125].

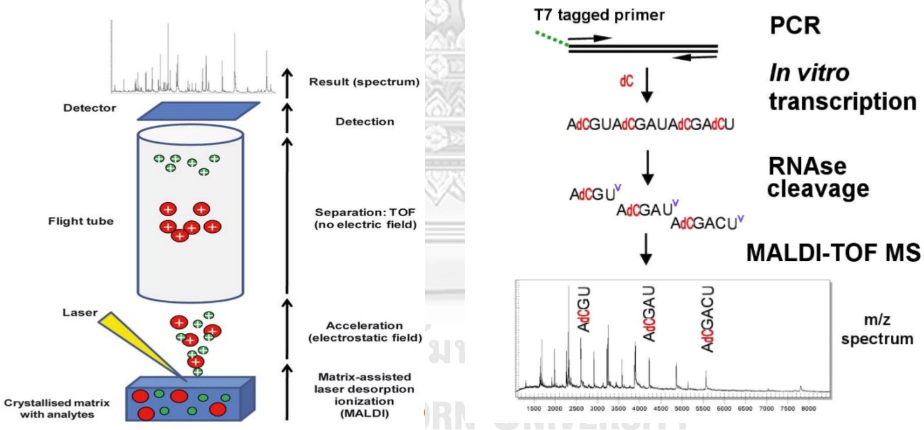


Figure 21 (A) Principle of MALDI-TOF and (B) DNA analysis by MALDI-TOF mass spectrometry [123]

Phylogenetic tree

Phylogenetic tree, also called Dendrogram, is a diagram showing the evolutionary interrelations of a group of organisms derived from a common ancestral form. The ancestor is in the tree “trunk”; organisms that have arisen from it are placed at the ends of tree “branches.” The distance of one group from the other groups indicates the degree of relationship; *i.e.,* closely related groups are located on branches close to one another. Phylogenetic trees, although speculative, provide a convenient method for studying phylogenetic relationships [126].

In a phylogenetic tree, the species or groups of interest are found at the tips of lines referred to as the tree's branches. For example, the phylogenetic tree below represents relationships between seven species, A, B, C, D, E, F, and G which are positioned at the ends of the branches [127].

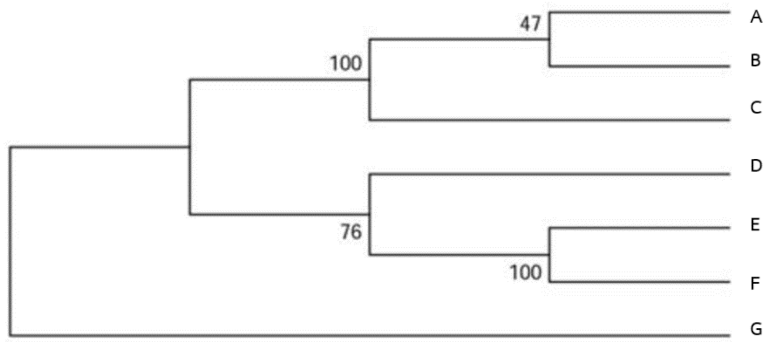


Figure 22 Modified from taxonomy and phylogeny [128]

Maximum parsimony method

Maximum parsimony predicts the evolutionary tree or trees that minimize the number of steps required to generate the observed variation in the sequences from common ancestral sequences. For this reason, the method is also sometimes referred to as the minimum evolution method. A multiple sequence alignment is required to predict which sequence positions are likely to correspond. These positions appeared in vertical columns in the MSA. For each aligned position, phylogenetic trees that require the smallest number of evolutionary changes to produce the observed sequence changes from ancestral sequences are identified. This analysis is continued for every position in the sequence alignment. Finally, those trees that produce the smallest number of changes overall for all sequence positions are identified. This method is best suited for sequences that are quite similar and is limited to small numbers of sequences [129].

Bootstrap analysis

Bootstrap analysis is a world-widely accepted tool, with computer based program, used to measure the accuracy of the species after being separated into clades in the phylogenetic tree. The value of bootstrap represents the % confidence of being repeatedly grouped into the same clade. The higher the bootstrap value, the greater the accuracy of the clade species in the tree. Apart from measuring the confidence level of the tree, the bootstrap value can be used to reasonably prove the errors of the estimated tree [130, 131].

CHAPTER III

MATERIALS AND METHODS

Chemicals and reagents

Agarose	Vivantis Inc., U.S.A
Boric acid	Merck, Daemstadt, Germany
Bromophenol blue loading dye	Invitrogen, U.S.A
Chloral hydrate	Ajax Finechem Pty. Ltd., New Zealand
DNA marker	Thermo Fisher Scientific Inc., U.S.A
DNeasy® plant mini kit	QIAGEN, U.S.A
dNTPs	Eurofins, Thailand
Ethylenediaminetetraacetic acid	Ajax Finechem Pty. Ltd., New Zealand
GeneRuler 100 bp, 1 Kb DNA ladder	Thermo Fisher Scientific Inc., U.S.A
Haiter™ solution (containing 6% sodium hypochlorite)	Kao Corp., Japan
Magnesium chloride	Eurofins, Thailand
PCR buffer	Eurofins, Thailand
Primers	Eurofins, Thailand
SYBR safe DNA gel stain	Invitrogen, U.S.A
Taq DNA polymerase	Eurofins, Thailand
Tris (hydroxymethly) aminomethane	Fluka, Biochemika, Germany

Instruments and equipments

-20°C Freezer	Sharp, Japan
AxioVision40 software (V 4.6.3.0)	Zeiss Inc., Germany
Centrifugation machine	Sigma, Germany
Dark Reader (DR22A Transilluminator)	Clare Chemical Research, Inc., U.S.A
Digital camera (Canon PowerShot A640)	Canon Inc., Japan
Gel electrophoresis apparatus and power supply	
Image Quant LAS4010	GE Healthcare Bio-Sciences AB., Sweden
Microscope (Axio image A2)	Zeiss Inc., Germany
PCR system C1000™ Thermocycler	Bio-Rad Laboratories, Inc., U.S.A
Ultrapure water	NW20VF, Heal Force, Chaina

Plant materials

Fresh mature leaves and fresh young leaves of *E. fusca, E. stricta, E. crista-galli, E. subumbrans*, *E. variegata* and *E. indica*, as well as fresh young leaves of two outgroups, *Pterocarpus indicus* (Family FABACEAE) and *Millingtonia hortensis* (Family BIGNONIACEAE), were collected from 3 different locations in Thailand. Plant specimens were authenticated by Associate Professor Nijsiri Ruangrungsi, Ph.D., The voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand. Their locations and collecting data of *Erythrina* species are shown in Table 4.

Table 4 List of six distributed *Erythrina* species and their different collecting localities

Sample no.	Species	Places of collection (Thailand)	Collecting date (Month, Year)	Voucher ID
1.	*E. fusca*	Chaiyaphum*	September, 2015	KEFCU.02092015
2.		Nakhon pathom	February, 2016	KEFCU.04022016
3.		Rayong	February, 2016	KEFCU.15022016
4.	*E. stricta*	Nakhon Ratchasima*	April, 2016	KESTCU.06042016
5.		Prachinburi	April, 2016	KESTCU.06042016
6.		Saraburi	April, 2016	KESTCU.06042016
7.	*E. crista-galli*	Bangkok*	November,2015	KECCU.25112015
8.		Nakhon pathom1	December, 2015	KECCU.25112015
9.		Nakhon pathom2	December, 2015	KECCU.25112015
10.	*E. subumbrans*	Chiang mai1*	February, 2016	KESUCU.19022016
11.		Chiang mai2	February, 2016	KESUCU.19022016
12.		Chiang rai	February, 2016	KESUCU.19022016

13.		Chiang rai*	November,2015	KECCU.06112015
14.	*E. indica*	Chaiyaphum		KECCU.04022016
15.		Nakhon	February, 2016	KECCU.09022016
		Ratchasima	February, 2016	
16.		Bangkok*	September, 2015	KEVCU.28092015
17.	*E. variegata*	Pathumthani	October, 2015	KEVCU.06102015
18.		Prachinburi	August, 2015	KEVCU.15092015

*** Selected fresh young leaves for DNA sequencing**

Part I Morphological characteristics

Macroscopic analysis of the leaves of *Erythrina* species

For macroscopic identity of characteristics and appearance of herbal drugs, visual characters of six *Erythrina* species were observed, and the whole plant was illustrated by hand drawing for its shape, size, and botanical morphology.

Microscopic analysis of the leaves of *Erythrina* species

Transverse section of the midrib

The fresh mature leaves from six *Erythrina* species were cleaned. Cross section was prepared by cutting the leaves in parallel including the midrib and lamina into pieces as thin as possible and transferred these tissue sections by a brush moistened with water. Selected satisfactory sections were prepared and mounted onto a slide in water for microscopic examination under photomicroscope observation with objective lens of 10X, 20X and 40X magnifications and eyepiece lens of 10X magnification by digital camera, scaled for labeling size of each character. Cross sections of midrib were drawn in the proportion size related to the original in drawing paper.

Determination of microscopic leaf constant numbers

Microscopic leaf constant numbers including stomatal number, stomatal index, epidermal cell number, epidermal cell area, vein islet number and palisade ratio were examined according to Mukherjee PK [83].

Fresh mature leaves were cleaned, cut into small pieces (1 cm x 1 cm), midway between midrib of the leaf and its margin, and the cut leaves were immersed in sodium hypochlorite solution for 24 hours, prepared by Haiter™ (containing 6% sodium hypochlorite) dilution with water 1: 1, to remove chlorophyll. The chlorophyll-less leaves were gently warmed with chloral hydrate solution until transparent [83]. After being washed with water 2-3 times, leaf samples were mounted in H_2O and the leaves were observed under a light microscope attached to a digital camera. Thirty fields of each species from 3 different locations were examined using AxioVision program.

Stomatal number and stomatal index

Both sides of leaf sample were observed under a microscope with a 20X objective lens magnification. The stomatal number is the number of stomata per square millimeter of epidermis. The stomatal index is a percentage ratio of stomatal number (S) to the epidermal cell numbers (E) in the same unit area of leaf. The stomatal index was calculated using formula:

$$SI = (S / E + S) \times 100$$

Upper epidermal cell area

The upper side of leaf sample was observed under a microscope with a 20X objective lens magnification. The epidermal cell area was calculated using formula:

$$EA = (1 / E + S) \times 10^6 \ \mu m^2$$

Where, E = number of epidermal cells per square millimeter (mm^2) of leaf.

S = the number of stomata in a given area of leaf

Palisade ratio

Group of four epidermal cells was traced under a microscope with a 40x objective lens magnification and 10x eyepiece lens. The palisade cells lying under the four epidermal cells were counted. The number of palisade cells obtained in each group divided by 4 gives the palisade ratio.

Vein islet number

The lower side of leaf sample was observed under a microscope with a 20X objective lens magnification. The number of vein islet were counted in 2 square millimeters of the leaf surface.

Data analysis

All microscopic leaf constant numbers were determined in thirty fields of each species from 3 different locations (ninety fields per each species) and the results were expressed as mean ± SD.

Part II Molecular identification

Extraction of genomic DNA

Preparation of DNeasy® Mini Kit

Genomic DNA was individually extracted from the fresh young leaves sample using DNeasy® plant Mini Kit. There are twelve steps involved in DNA extraction. Firstly, sample was disrupted using mortar and pestle. Secondly, 400 µl buffer AP1 and 4µl RNaseA were added and vortex further to remove clumps. The mixture was incubated to 65°C for 10 minutes. The tube was inverted for 2-3 times during incubation. Thirdly, 130 µl buffer P3 was added, mixed and incubated for 5 minutes on ice. Next, the sample the lysate is centrifuged for 5 minutes at 20,000 × g (14,000 rpm). After centrifugation, the lysate is transferred to the QIAshredder spin column placed in 2 ml collection tube. The sample is centrifuged for 2 minutes at 20,000 × g before transferred the flow-through into a new tube without disturbing the cell pellet. Buffer AW1 1.5 volumes were added to the sample and mixed by pipetting. Then, 650 µl of the mixture was transferred into a DNeasy Mini spin column placed in a 2 ml collection tube. The tube was centrifuged for 1 minute at 8000 × g (≥8000 rpm), and discarded the flow-through. This steps were repeated with the remaining sample. Moreover, the spin column was placed into a new 2 ml collection tube. 500 µl Buffer AW2 was added into and centrifuged at 8000 × g for 1 minutes. The flow-through was discarded. Next, another 500 µl buffer AW2 was added and centrifuged at 20,000 × g for 2 minutes. After that, the spin column was transferred to new microcentrifuge tube at 1.5 ml or 2 ml tube. Then, 100 µl Buffer AE is added into the tube for the elution. To incubate and for 5 minutes at the room temperature (15-25°C). The tube is centrifuged at 8000 × g for 1 minute. The last step is elution that 100 µl Buffer AE was added. The tube was incubated at the room temperature (15-25°C) for 5 minutes, and centrifuged at 8000 × g for 1 minute.

Determination of genomic DNA quantity

Five microliters genomic DNA were then analyzed by 1% agarose gel electrophoresis and compared with 100 bp and 1 Kb marker. Stained by SYBR safe and visualized under UV transilluminator.

PCR amplification

The universal primers of ITS [132, 133], *mat*K [134], *rpo*C [132, 133], *psb*A_*trn*H [135, 136], *ycf*1 [137] regions chosen for PCR amplification are listed in Table 5.

Table 5 Detail of the universal primers used in this PCR

Primer	Direction	Sequencing (5'-3')	Length (bp)	Tm (°C)
ITS5	Forward	GGAAGTAAAAGTCGTAACAAGG	22	55
ITS4	Reverse	TCCTCCGCTTATTGAGC	20	56
*mat*K 3f	Forward	CGTACAGTACTTTTGTGTTTACGAG	25	52
*mat*K 1r	Reverse	ACCCAGTCCATCTGGAAATCTTGGTTC	27	52
*rpo*C 2f	Forward	GGCAAAGAGGGAAGATTTCG	20	57
*rpo*C 4r	Reverse	CCATAAGCATATCTTGAGTTGG	22	57
*psb*A3' f	Forward	GTTATGCATGAACGTAATGCTC	24	55
*trn*Hf_05	Reverse	CGCGCATGGTGGATTCACAATCC	23	55
*ycf*1b	Forward	TCTCGACGAAAATCAGATTGTTGTGAAT	28	60.7
*ycf*1b	Reverse	ATACATGTCAAAGTGATGGAAAA	23	53.5

Genomic DNA was extracted from the fresh young leaf tissues following a DNeasy® Kit method. The obtained DNA was amplified using five primers Table 6, with various PCR conditions for each primer as listed in Table 7.

Table 6 Detail of the PCR reaction used in this PCR amplification

	Primer				
PCR reaction	ITS	*mat*K	*rpo*C	*psb*A_*trn*H	*ycf*1
PCR buffer	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl
$MgCl_2$	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl
$dNTP_s$	0.5 µl	0.5 µl	0.5 µl	0.5 µl	0.5 µl
Forward primer	0.8 µl	0.8 µl	0.8 µl	0.8 µl	0.8 µl
Reverse primer	0.8 µl	0.8 µl	0.8 µl	0.8 µl	0.8 µl
Taq polymerase	0.2 µl	0.2 µl	0.2 µl	0.2 µl	0.2 µl
H_2O	16.7 µl	16.7 µl	16.7 µl	16.7 µl	16.7 µl
DNA template	1 µl	1 µl	1 µl	1 µl	1 µl
Total	25 µl				

Table 7 Detail of the PCR reaction for each primer used in this PCR amplification

	Primer				
PCR reaction	ITS	*mat*K	*rpo*C	*psb*A_*trn*H	*ycf*1
Pre denaturing	95°C/300S	95°C/300S	95°C/180S	95°C/240S	94°C/240S
Denaturing	95°C/60S	95°C/60S	95°C/30S	95°C/30S	94°C/30S
Annealing	50°C/60S	53°C/40S	50°C/45S	55°C/30S	52°C/40S
Extension	72°C/60S	72°C/60S	72°C/60S	72°C/60S	72°C/60S
Final extension	72°C/300S	72°C/300S	72°C/600S	72°C/300S	72°C/600S
Hold	4°C/forever	4°C/forever	4°C/forever	4°C/forever	4°C/forever

The success of each PCR reaction was verified by electrophoresis of 5 µl of the reaction products on 1% agarose gels in 1X TBE buffer and stained with SYBR safe DNA stain. Fragment patterns were analyzed under UV transilluminator and photographed, and size was also estimated using GeneRuler 1 Kb DNA ladder.

1 % identity agarose gel electrophoresis

1 % agarose was prepared by adding 1 g of agarose to 100 ml of 1X TBE buffer and solubilized by heating in microwave. The medium warm gel solution was poured into a plastic tray. After the gel become solid, removed the comb and put the tray into a gel electrophoresis apparatus fulfilled with 1x TBE buffer in chamber. Five µl of each amplified PCR products were analyzed in 1% agarose gel electrophoresis compared with 1 Kb molecular weight marker. Electrophoresis was performed at constant voltage of 100 volts until the faster migration dye (bromophenol blue). Agarose gels in 1XTBE buffer and stained with SYBR safe DNA stain. The agarose gel was visualized under UV transilluminator and photographed.

DNA sequencing analysis

The PCR products of the ITS, *mat*K, *rpo*C, *psb*A_*trn*H and *ycf*1 region sequences from both sense and antisense stand were analyzed using chain termination method with ABI378 detector (U2Bio, Thailand), following with Multiple sequence alignment by Florence Corpet software sequence alignment version 5.4.1 for Windows.

Data analysis

Several methods have been used for the analysis of data and species resolution. Then the sequence was analyzed with the NCBI database. Based on the complete alignment with Multiple sequence alignment software and Phylogenetic tree constructed. Maximum parsimony (MP) method with the MEGA7 program analysis was performed using a branch and bound searching method with a number of bootstrap 1000 replications.

CHAPTER IV

RESULTS

This study was performed on 2 parts including macroscopic, microscopic characterizations and molecular evaluation of six *Erythrina* species distributed in Thailand. The results were described as follows:

Part I Macroscopic and microscopic characterizations

Macroscopic characteristics

Total six *Erythrina* species as *E. fusca*, *E. stricta*, *E. crista-galli*, *E. subumbrans*, *E. variegata* and *E. indica* distributed in Thailand were collected from Bangkok, Pathum Thani, Prachin Buri, Nakhon Pathom, Rayong, Chaiyaphum, Saraburi, Nakhon Ratchasima, Chiang Rai and Chiang Mai provinces, during September 2015 to April 2016. The macroscopic characteristics of *Erythrina* species were shown in Table 8 and hand drawing representing botanical characters were shown in Figure 23-28.

Table 8 Macroscopic characters of *Erythrina* species

Plants	Leaves	Stem	Fruit
E. fusca	Leaves are ovate, acute or acuminate apex. Margins are entire and rounded at the base.	Stems have sharp thick thorns in adult trees. Stems and branch are green to brown in colour.	A pod is 10 cm long, linear-falcate, torulose, follicular, with spongy packing between seeds.
E. stricta	Leaves are trifoliate, alternate; stipules about 5 mm long, rhomboid-ovate,	Young stems have sharp thorns that become warts or	A pod is linear-falcate, torulose, follicular, with

	base deltoid or truncate, apex acute or obtuse, margin entire or sinuate	very thick thorns in adult trees.	spongy packing between seeds.
E. crista-galli	The alternately arranged leaves are petioles and are made up of trifoliate. The shape is ovate or elliptic.	Adult stems are brown or greyish in colour and have moderately rough bark. Younger stems are greenish in color.	A pod is linear-falcate, torulose, follicular, with spongy packing between seeds.
E. subumbrans	Leaves are arranged alternate and trifoliate. The leaflets are ovate-triangular-rhomboid, with terminal one belong largest. The base is rounded or cordate, acuminate at apex.	Young stems have sharp thorns that become very thick thorns in adult trees. Stems and branch are brown or greyish in colour.	A pod is flat, curved, linear-falcate, torulose, follicular, with spongy packing between seeds.
E. variegata	Leaves are trifoliate, alternate, cordate, caudate apex. Margins are entire. Cordate at the base are bright emerald-green.	Stems of adult trees become low thick thorns. Stems and branch are brown or greyish in colour.	A compressed narrowly oblong pod is 10–14 cm long, sterile in the basal portion, and not constricted

			between the 5–10 dark brown seeds.
E. indica	Leaves are cordate, caudate apex. Margins are entire and cordate at base. The leaflets are green in colour.	Stems have sharp thick thorns in adult trees. Stems and branch are green to brown colour.	A pod is linear-falcate, torulose, follicular, with spongy packing between seeds.

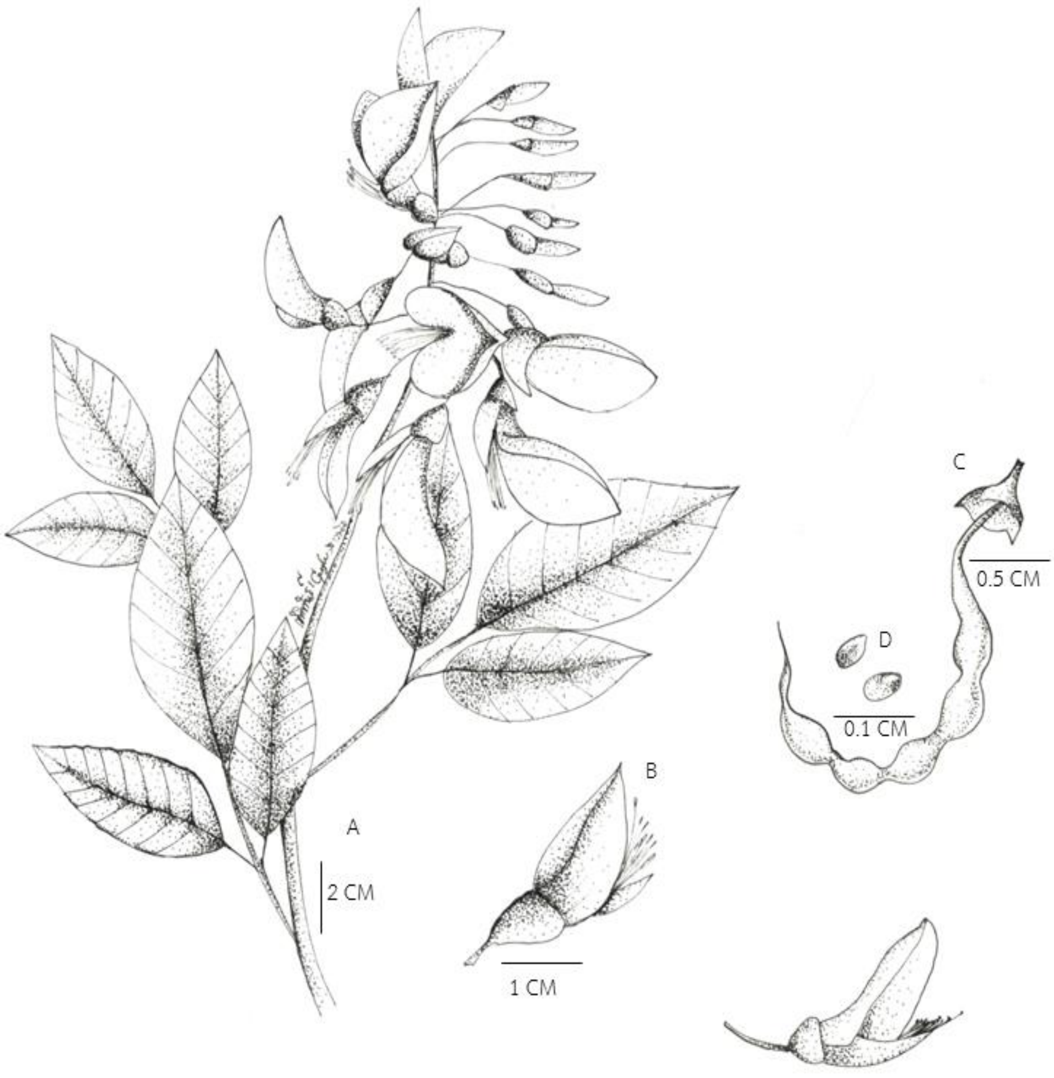


Figure 23 *Erythrina fusca* with flower (A), petal (B), pod (C) and seed (D)

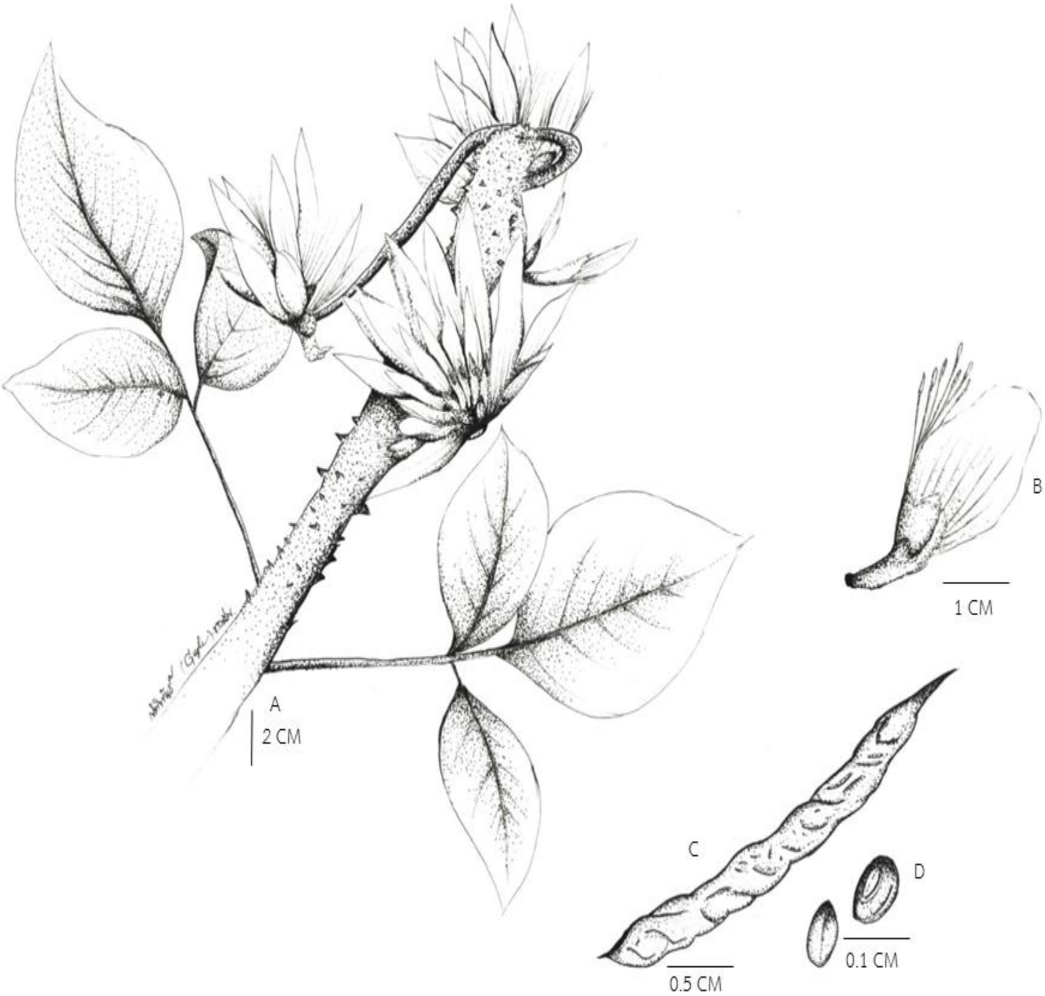


Figure 24 *Erythrina stricta* with flower (A), petal (B), pod (C) and seed (D)

Figure 25 *Erythrina crista-galli* with flower (A), petal (B), pod (C) and seed (D)

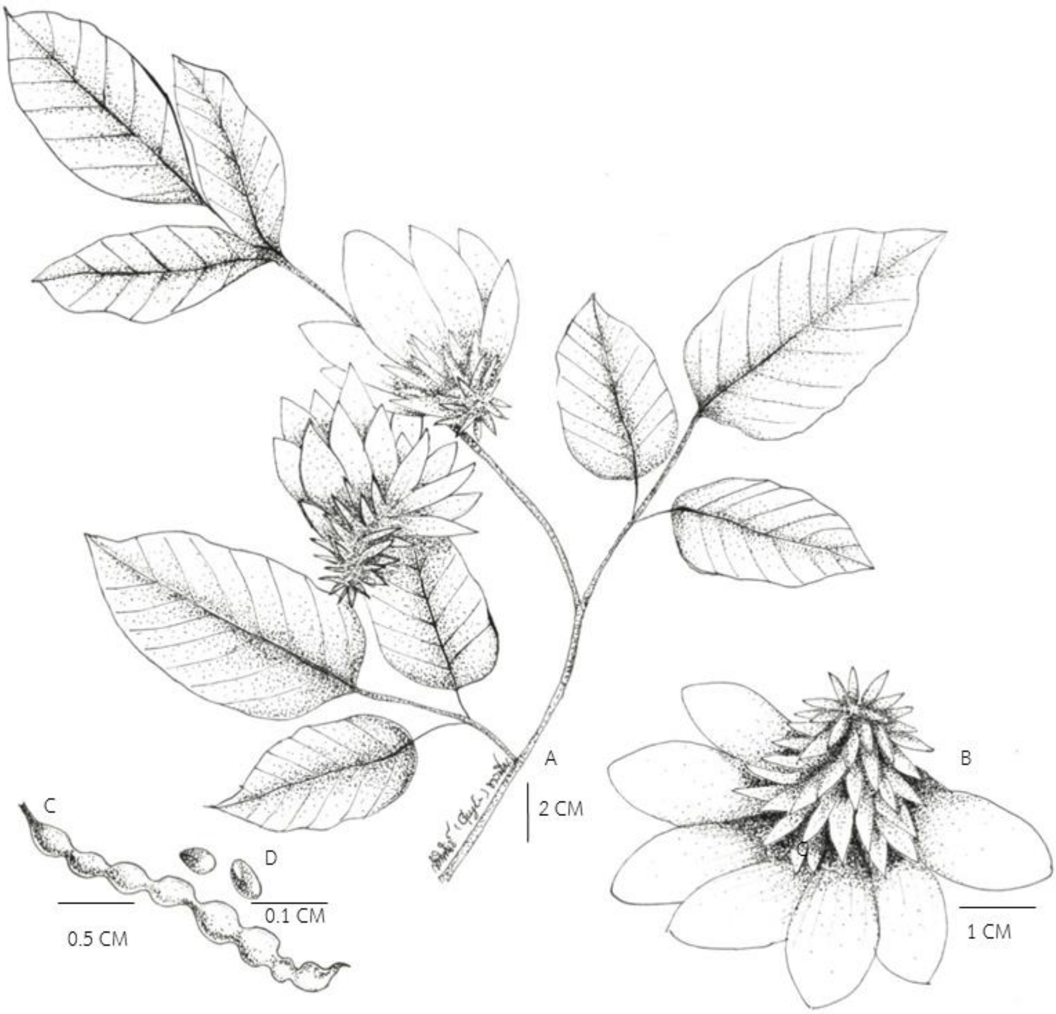


Figure 26 *Erythrina subumbrans* with flower (A), petal (B), pod (C) and seed (D)

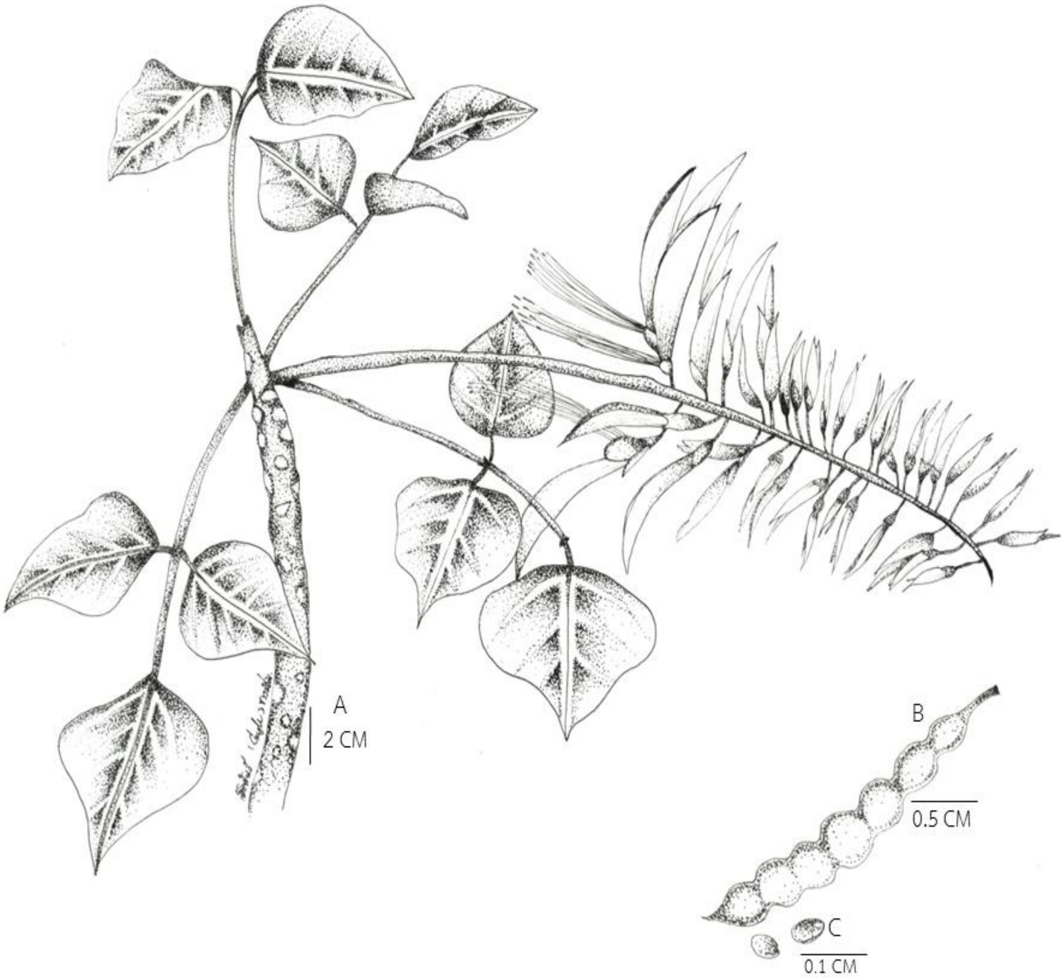


Figure 27 *Erythrina variegata* with flower (A), pod (B) and seed (C)

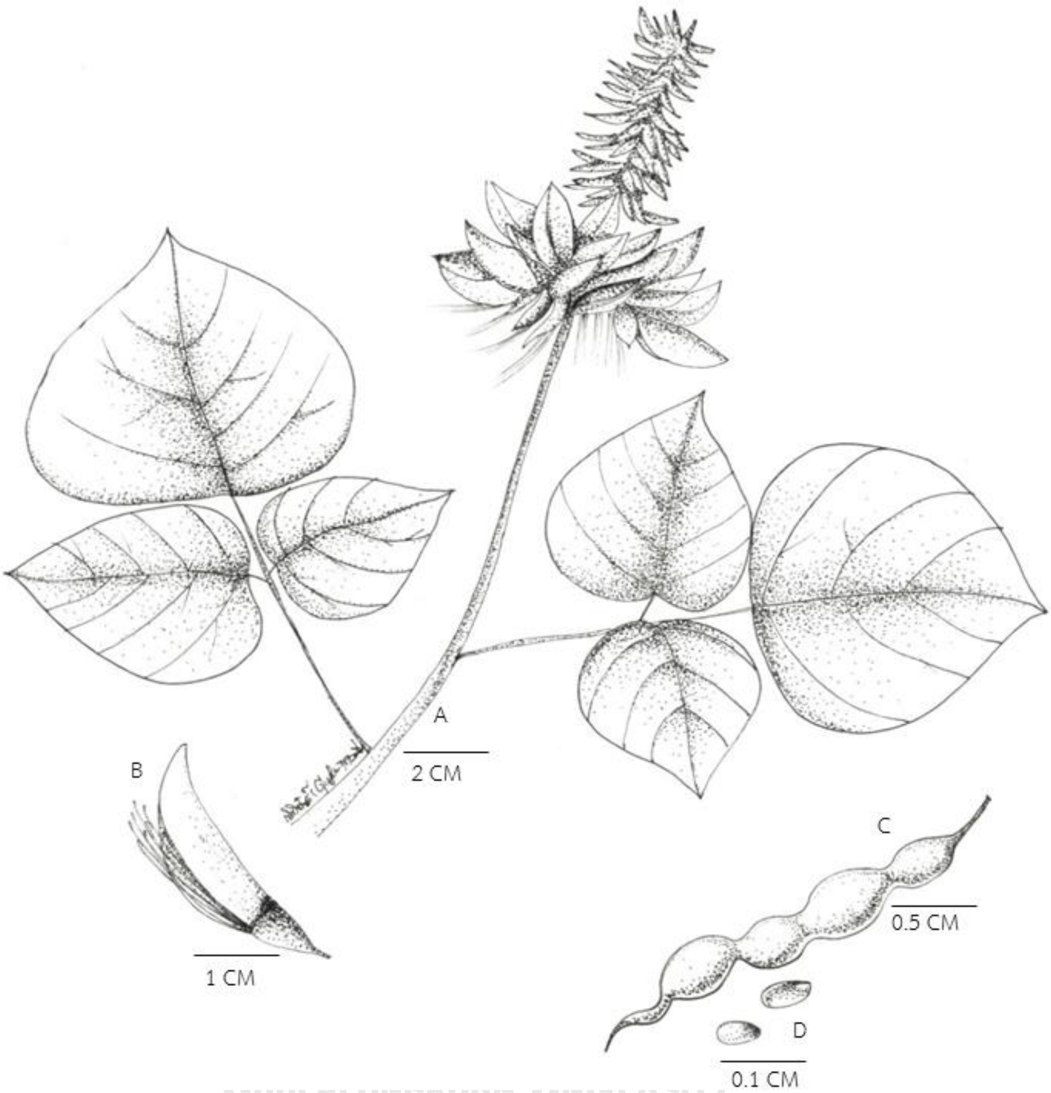


Figure 28 *Erythrina indica* with flower (A), petal (B), pod (C) and seed (D)

Microscopic characteristics

Transverse section of the midrib

The anatomical characteristics of the midrib of six investigated *Erythrina* species were illustrated (Figure 29-34). The results revealed the distinguished arrangement of tissue especially the vascular bundle (xylem and phloem tissues). None of the trichome was found in these species.

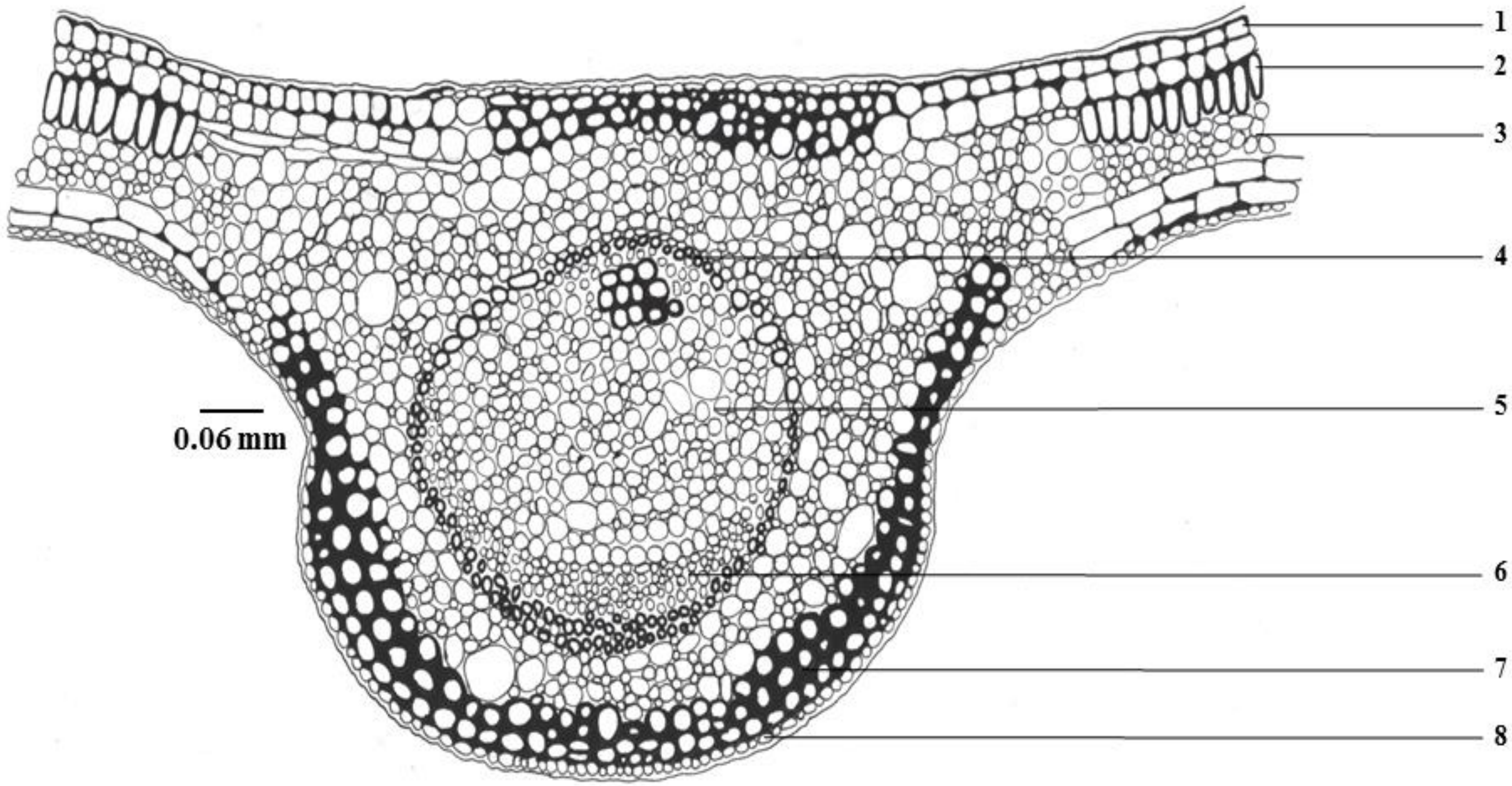


Figure 29 Midrib cross section of *E. fusca* leaf

1. Upper epidermis, 2. Palisade cell, 3. Spongy cell, 4. Group of fiber, 5. Phloem tissue, 6. Xylem tissue, 7. Collenchyma and 8. Lower epidermis

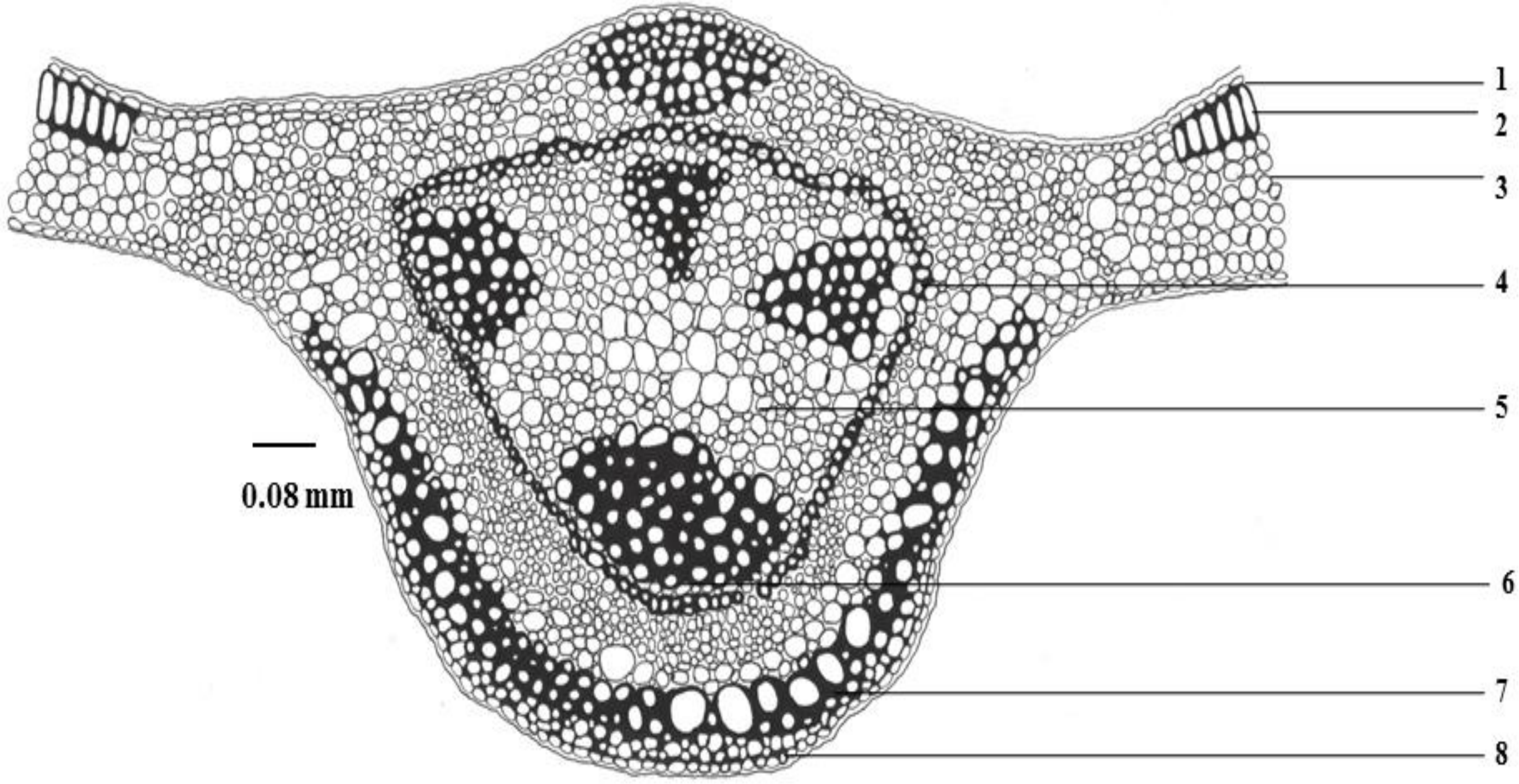


Figure 30 Midrib cross section of *E. stricta* leaf

1. Upper epidermis, 2. Palisade cell, 3. Spongy cell, 4. Group of fiber, 5. Phloem tissue, 6. Xylem tissue, 7. Collenchyma and 8. Lower epidermis

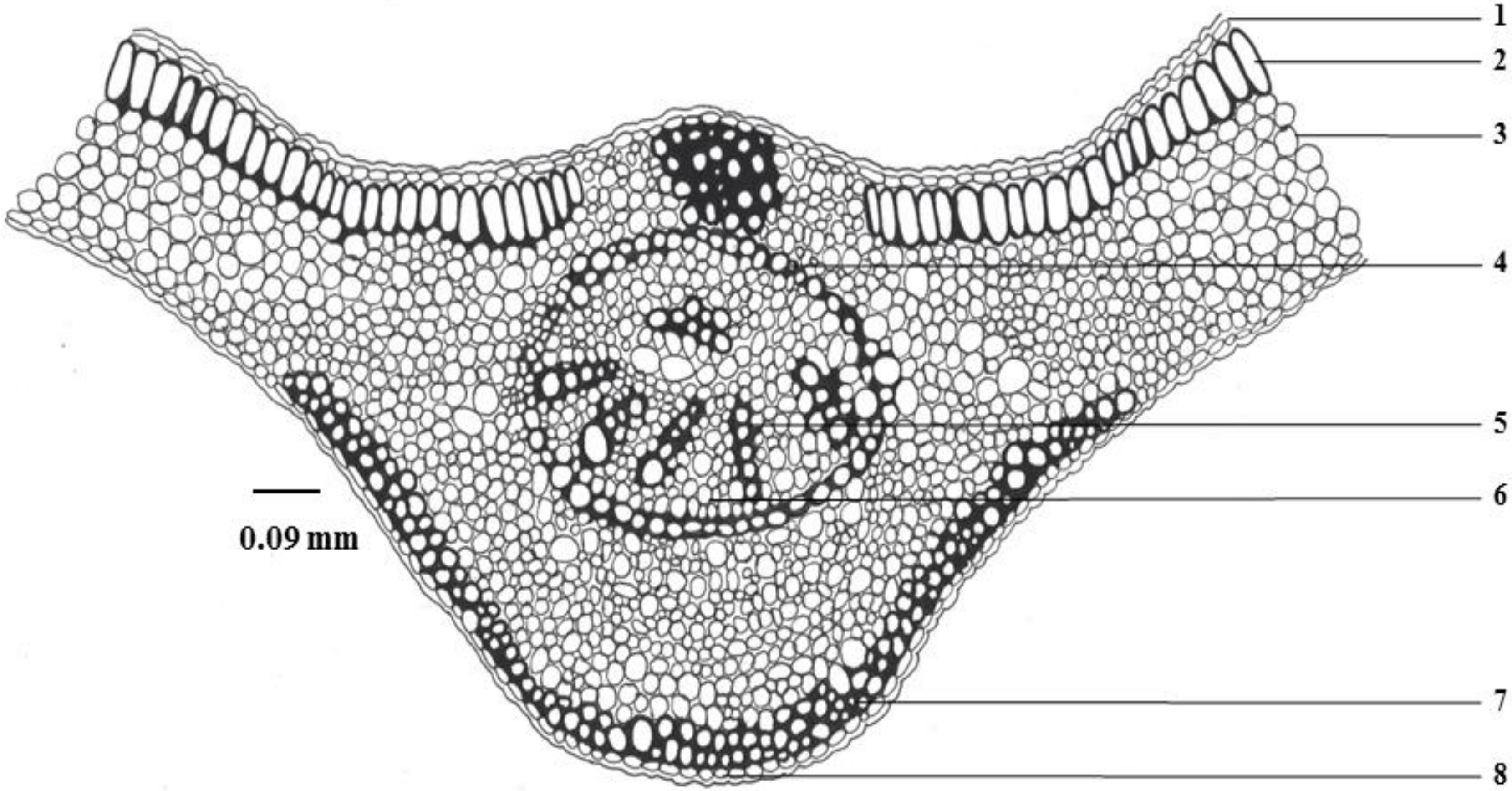


Figure 31 Midrib cross section of *E. crista-galli* leaf

1. Upper epidermis, 2. Palisade cell, 3. Spongy cell, 4. Group of fiber, 5. Phloem tissue, 6. Xylem tissue, 7. Collenchyma and 8. Lower epidermis

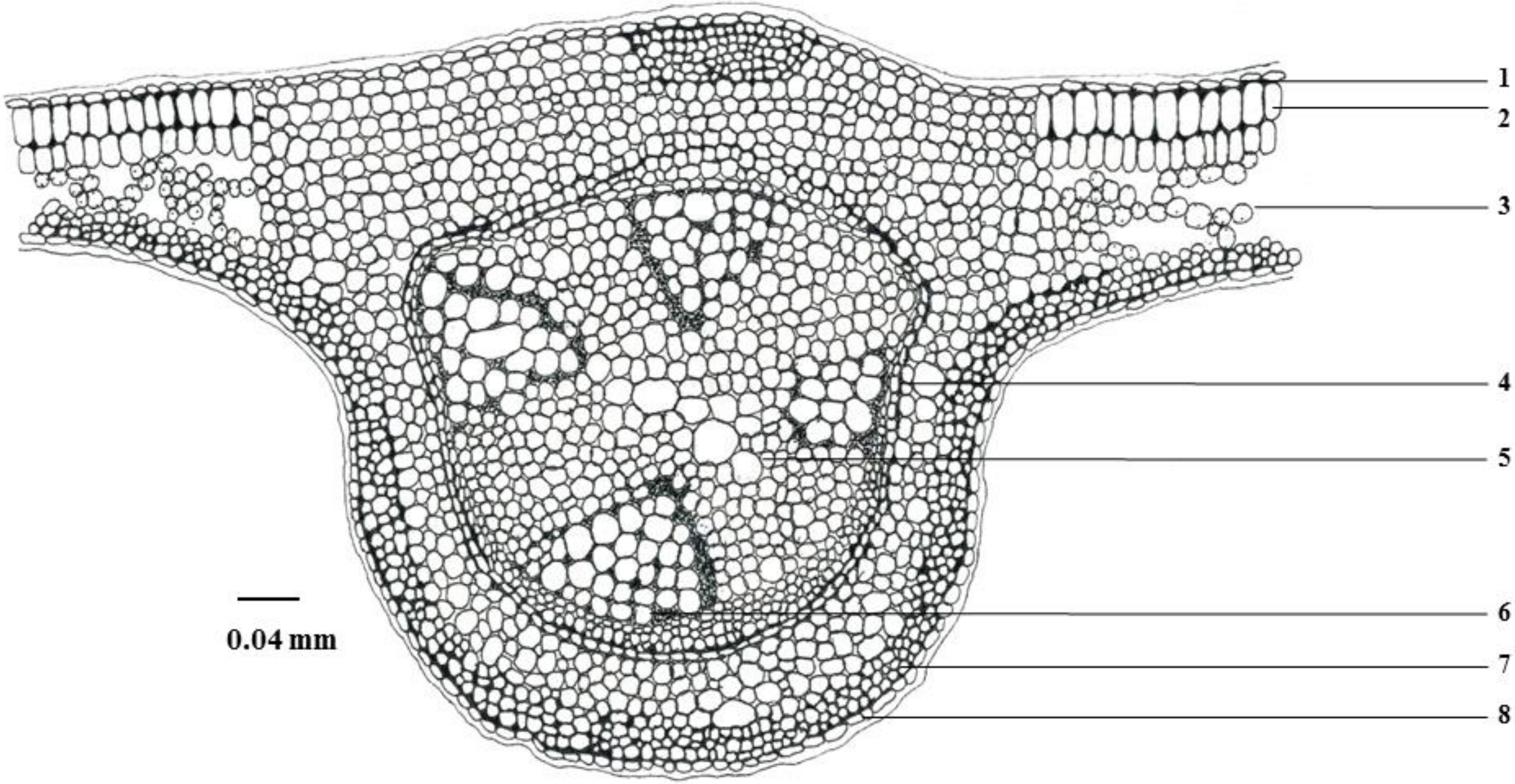


Figure 32 Midrib cross section of *E. subumbrans* leaf

1. Upper epidermis, 2. Palisade cell, 3. Spongy cell, 4. Group of fiber, 5. Phloem tissue, 6. Xylem tissue, 7. Collenchyma and 8. Lower epidermis

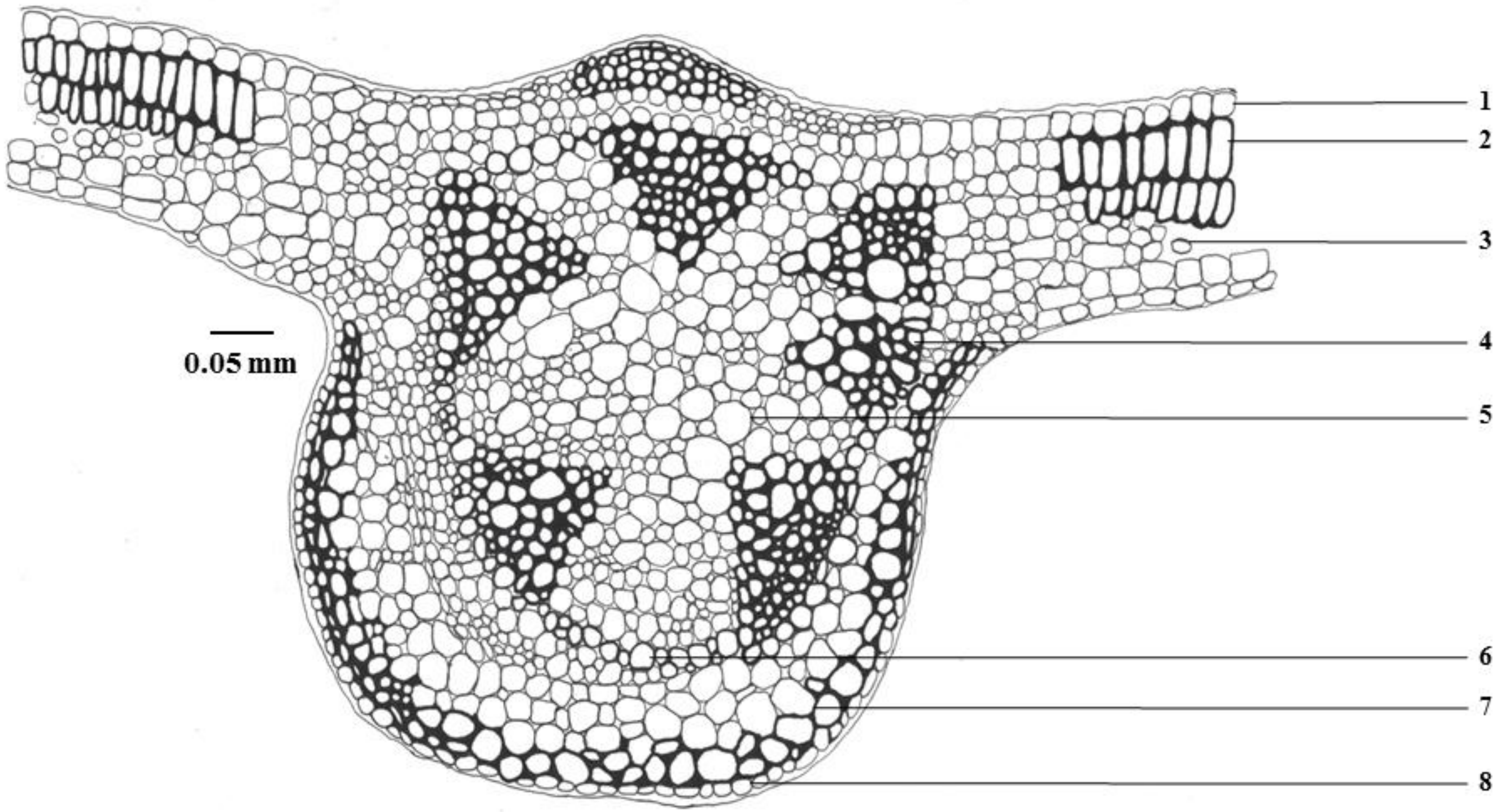


Figure 33 Midrib cross section of *E. variegata* leaf

1. Upper epidermis, 2. Palisade cell, 3. Spongy cell, 4. Group of fiber, 5. Phloem tissue, 6. Xylem tissue, 7. Collenchyma and 8. Lower epidermis

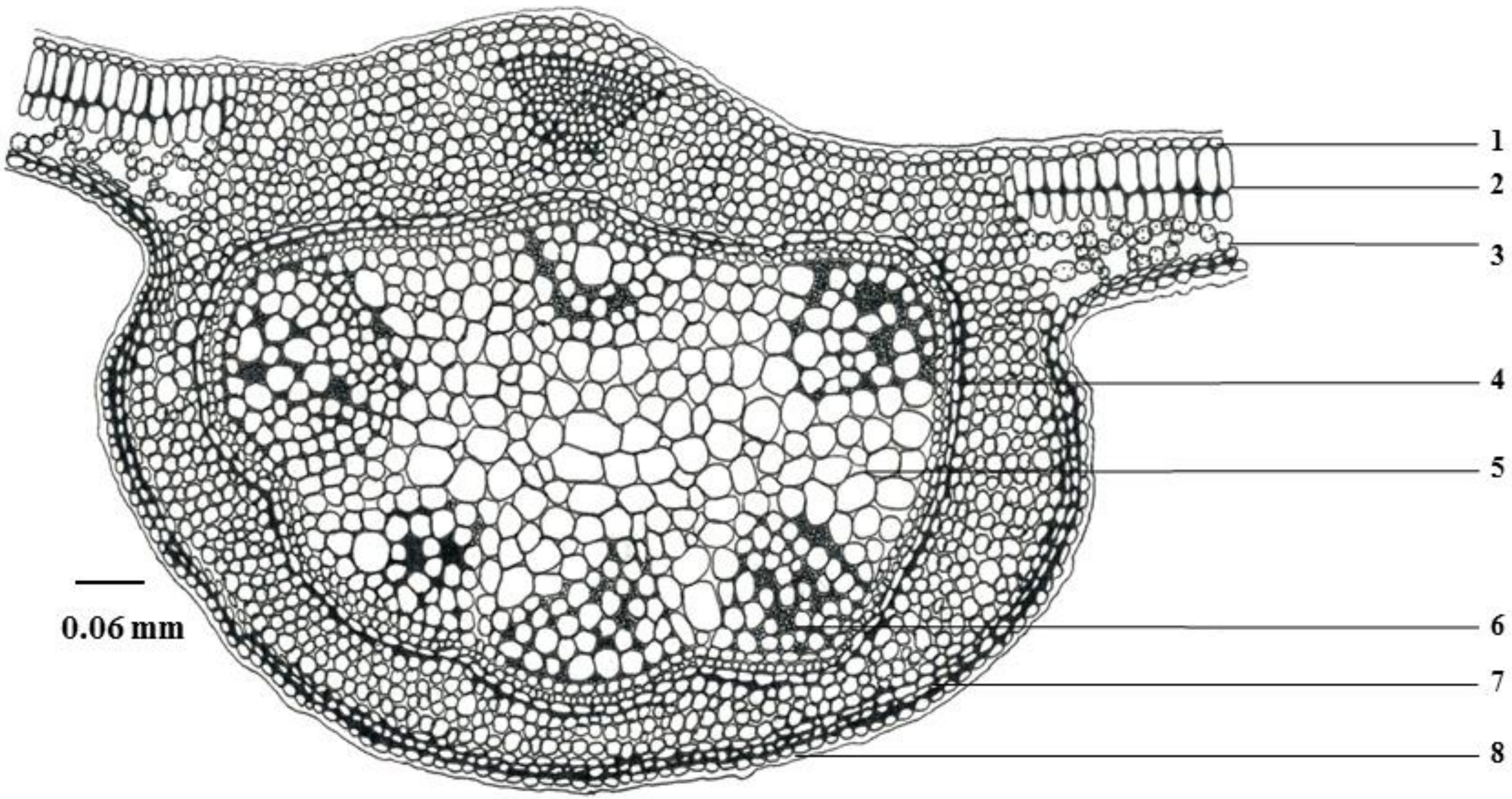


Figure 34 Midrib cross section of *E. indica* leaf

1. Upper epidermis, 2. Palisade cell, 3. Spongy cell, 4. Group of fiber, 5. Phloem tissue, 6. Xylem tissue, 7. Collenchyma and 8. Lower epidermis

Type of stomata

The type of stomata in six *Erythrina* species was classified as a paracytic type which the stoma was surrounded by two subsidiary cells parallel to the long axis of guard cells (Figure 35).

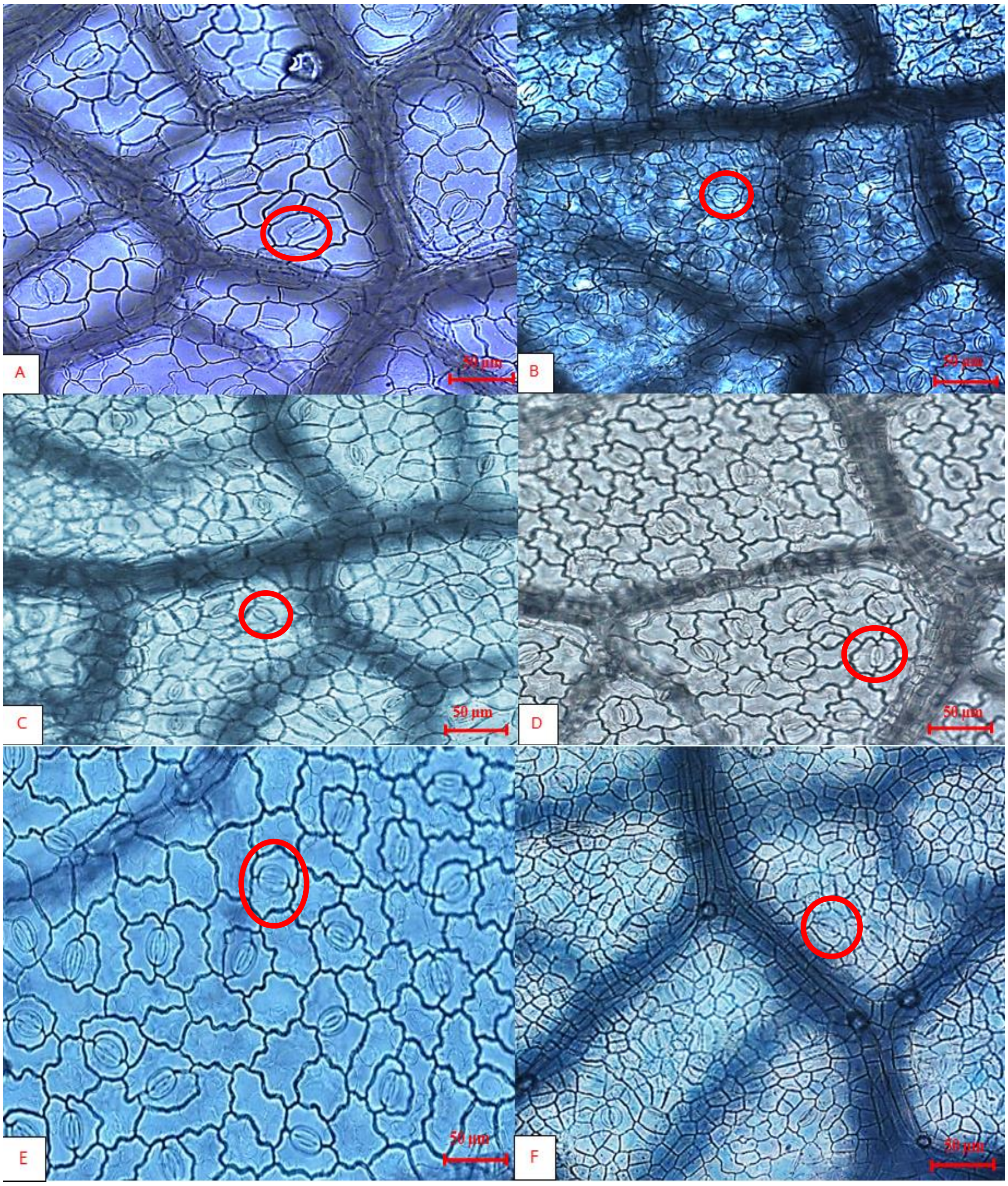


Figure 35 The photograph of paracytic stomata from six *Erythrina* species: (A) *E. fusca*, (B) *E. stricta*, (C) *E. crista-galli*, (D) *E. subumbrans*, (E) *E. variegata* and (F) *E. indica*

Microscopic leaf constant numbers

The results of microscopic leaf constant numbers consisted of stomatal number, epidermal cell number, stomatal index, epidermal cell area, vein islet number and palisade ratio of six *Erythrina* species from three different locations were shown in Table 9-12 and Figure 36-38, respectively.

The raw data of each parameter of microscopic leaf constant numbers from the microscopic analysis were shown in appendix A.

Stomatal number and stomatal index

The result of stomatal number and stomatal index of six *Erythrina* species from three different locations were shown in Table 10. Upper stomata were found only in *E. crista-galli*, *E. subumbrans*, and *E. variegata* especially *E. crista-galli* showed clearly distinct upper stomatal number and stomatal index.

Table 9 The stomatal number and stomatal index of six *Erythrina* species

Erythrina species	Stomatal number* mean ± SD (min - max)		Stomatal index mean ± SD (min - max)	
	Upper epidermis	Lower epidermis	Upper	Lower
E. fusca	-	159±19 (116-200)	-	10.80 ± 1.83 (6.22-14.29)
E. stricta	-	149±21 (84-192)	-	18.50 ± 2.23 (11.29-23.33)
E. crista-galli	92.44 ± 14.60 (60-136)	159±17 (112-184)	7.82 ± 1.26 (5.38-11.63)	10.47 ± 1.35 (6.62-13.68)
E. subumbrans	24.53 ± 6.76 (12-44)	135±18 (104-176)	1.74 ± 0.51 (0.78-3.06)	7.93 ± 1.88 (4.68-11.93)
E. variegata	10.13± 4.95 (4-28)	140±19 (88-180)	2.09 ± 0.94 (0.76-5.30)	14.37 ± 1.64 (10.90-19.72)
E. indica	-	178±46 (108-269)	-	22.41 ± 8.28 (13.74-39.87)

- = absent, * = number per mm^2

Epidermal cell number and epidermal cell area

The epidermal cell number and epidermal cell area of six *Erythrina* species from three different locations were shown in Table 10, and the epidermal cell characteristics were shown in Figure 36. *E. stricta*, *E. variegata*, and *E. indica* showed the area of upper epidermal cell over than 1200 μm^2.

Table 10 The epidermal cell number and epidermal cell area of six *Erythrina* species

Erythrina species	Epidermal cell number* mean ± SD (min - max)		Upper epidermal cell area (μm^2) mean ± SD (min - max)
	Upper epidermis	Lower epidermis	
E. fusca	1213.78 ± 166.32 (880-1604)	1336.98 ± 198.41 (1000-1808)	839.99 ± 112.52 (623.44-1136.36)
E. stricta	693.82 ± 37.97 (624-792)	657.73 ± 64.51 (540-776)	1445.52 ± 78.32 (1262.63-1602.56)
E. crista-galli	1095.38 ± 105.24 (792-1432)	1369.16 ± 152.06 (1060-1820)	849.13±81.98 (652.74-1152.07)
E. subumbrans	1405.25 ± 171.81 (1080-1820)	1636.18 ± 336.37 (1060-2340)	711.94 ± 89.08 (538.79-915.75)
E. variegata	468.18 ± 30.80 (404-532)	839.16 ± 86.80 (496-952)	2100.57±145.41 (1824.82-2118.64)
E. indica	553.82 ± 76.30 (404-708)	648.75 ± 143.99 (376-876)	1841.99 ± 269.36 (1412.43-2475.25)

* = number per mm^2

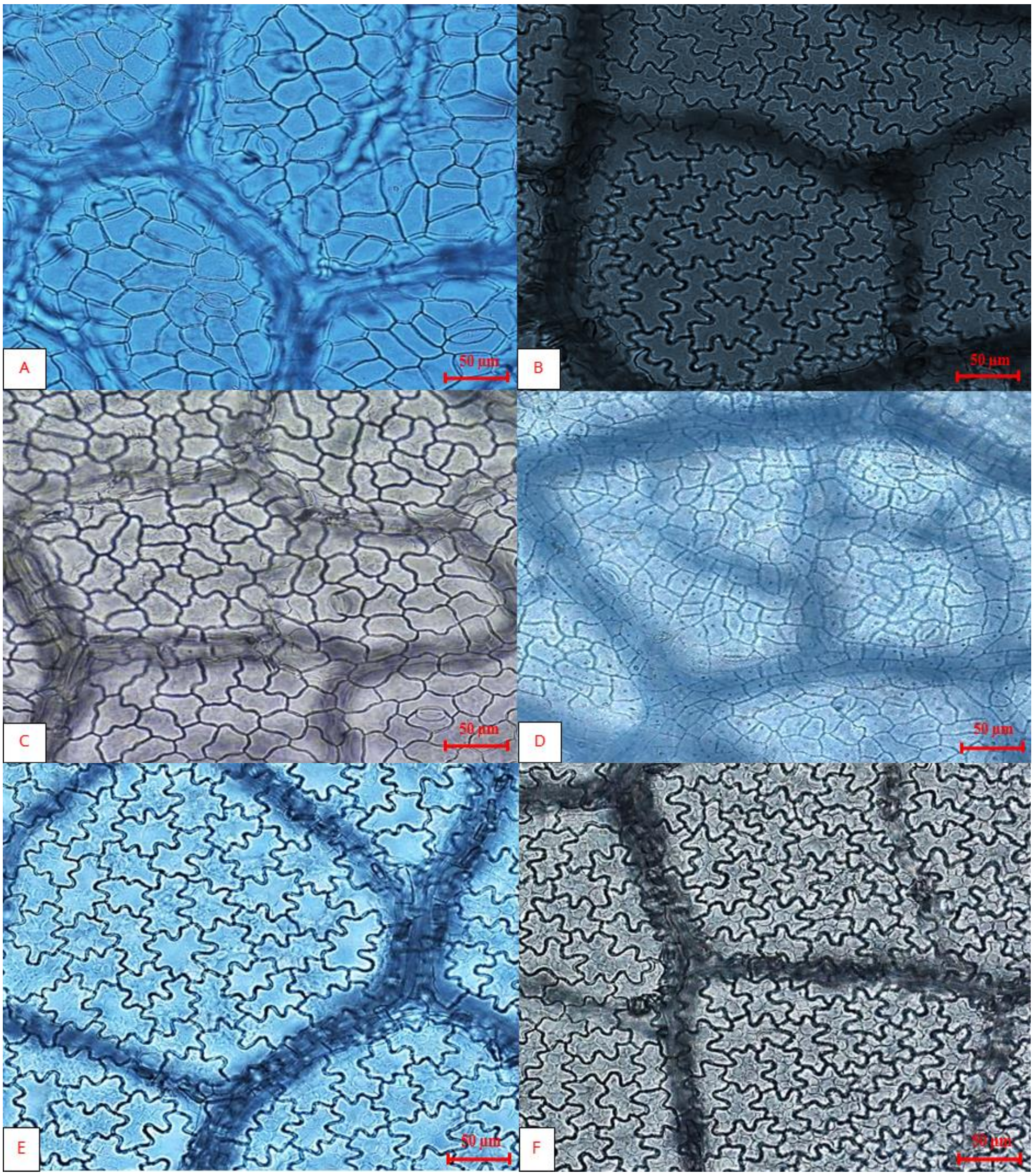


Figure 36 The photograph of upper epidermal cells from six *Erythrina* species (A) *E. fusca*, (B) *E. stricta*, (C) *E. crista-galli*, (D) *E. subumbrans*, (E) *E. variegata* and (F) *E. indica*

Palisade ratio

The Palisade ratio of six *Erythrina* species from three different locations were shown in Table 11, and the palisade cells characteristics were shown in Figure 37. The study revealed the overlapping palisade ratio among these six *Erythrina* species.

Table 11 The palisade ratio of six *Erythrina* species

***Erythrina* species**	**Palisade ratio mean ± SD (min - max)**
E. fusca	7.69 ± 1.11 (4.00-10.00)
E. stricta	6.21 ± 6.21 (3.50-8.75)
E. crista-galli	6.00 ± 1.15 (3.75-9.25)
E. subumbrans	5.13 ± 0.85 (3.50-7.25)
E. variegata	9.74 ± 1.34 (7.25-13.50)
E. indica	8.02 ± 1.68 (4.50-12.50)

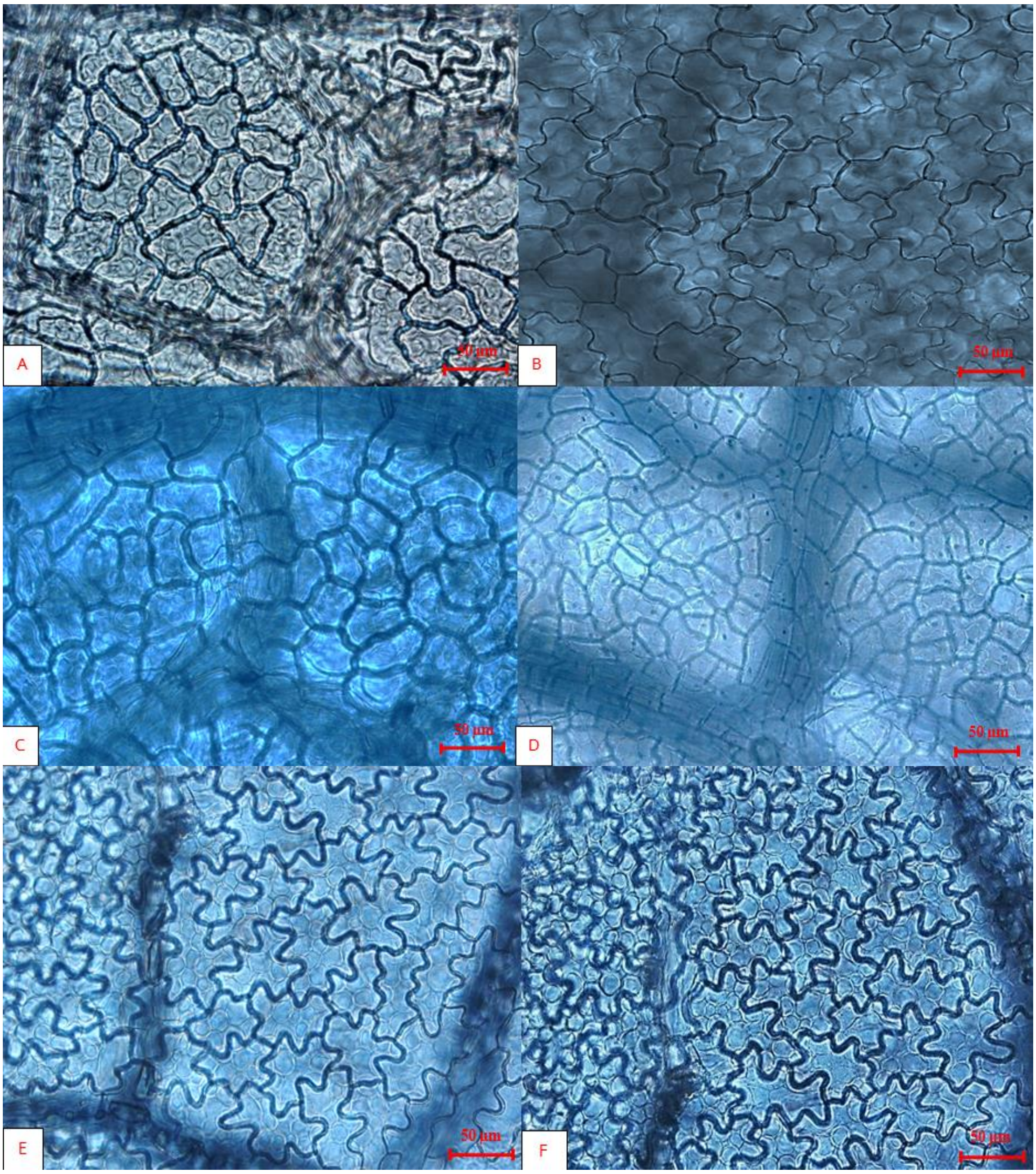


Figure 37 The photograph of palisade cells from six *Erythrina* species (A) *E. fusca*, (B) *E. stricta*, (C) *E. crista-galli*, (D) *E. subumbrans*, (E) *E. variegata* and (F) *E. indica*

Vein islet number

Vein islet numbers of six *Erythrina* species from three different locations were shown in Table 12, and the vein islet characteristics were shown in Figure 38. Vein islet number of *E. subumbrans* (12.75-20.75 cell/mm^2) were clearly separated from other species (3.50-11.00 cell/mm^2).

Table 12 The vein islet numbers of six *Erythrina* species

Erythrina species	**Vein islet number*** **mean ± SD** **(min - max)**
E. fusca	6.24 ± 0.77 (4.00-7.75)
E. stricta	6.45 ± 0.76 (4.50-8.25)
E. crista-galli	6.57 ± 0.94 (5.00-8.50)
E. subumbrans	16.58 ± 1.82 (12.75-20.75)
E. variegata	6.68 ± 1.17 (3.80-11.00)
E. indica	6.16 ± 1.61 (3.50-9.75)

* = number per mm^2

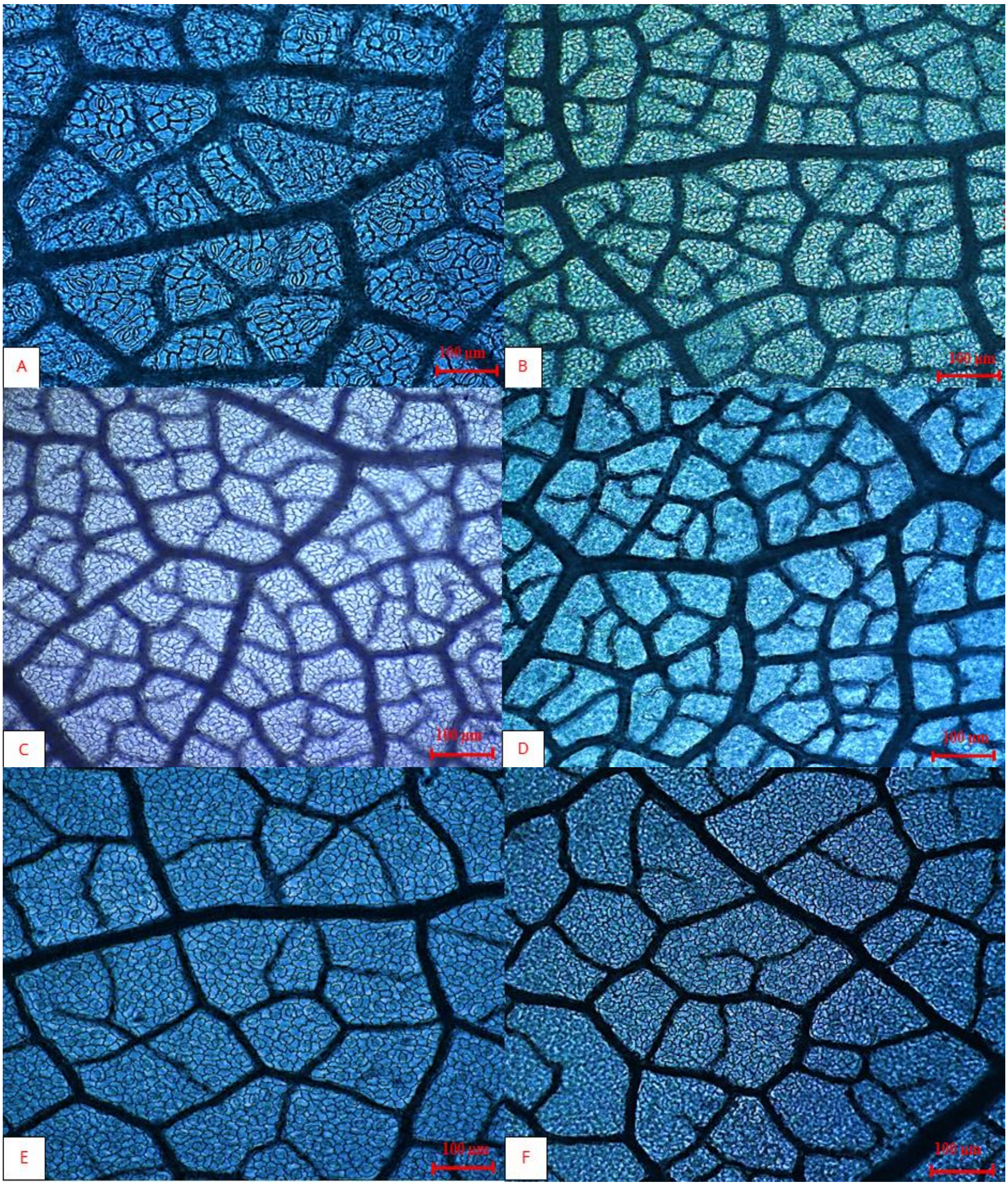


Figure 38 The photograph of vein islet from six *Erythrina* species
(A) *E. fusca*, (B) *E. stricta*, (C) *E. crista-galli*, (D) *E. subumbrans*, (E) *E. variegata* and
(F) *E. indica*

Part II Molecular identification

DNA isolation

Genomic DNA was isolated from whole fresh young leaves of 6 *Erythrina* species and 2 outgroup plants, *Millingtonia hortensis* and *Pterocarpus indicus*, which were collected from different location in Thailand (Table 4). The detection of genomic DNA was on 1% agarode gel shown in Figure 39-40.

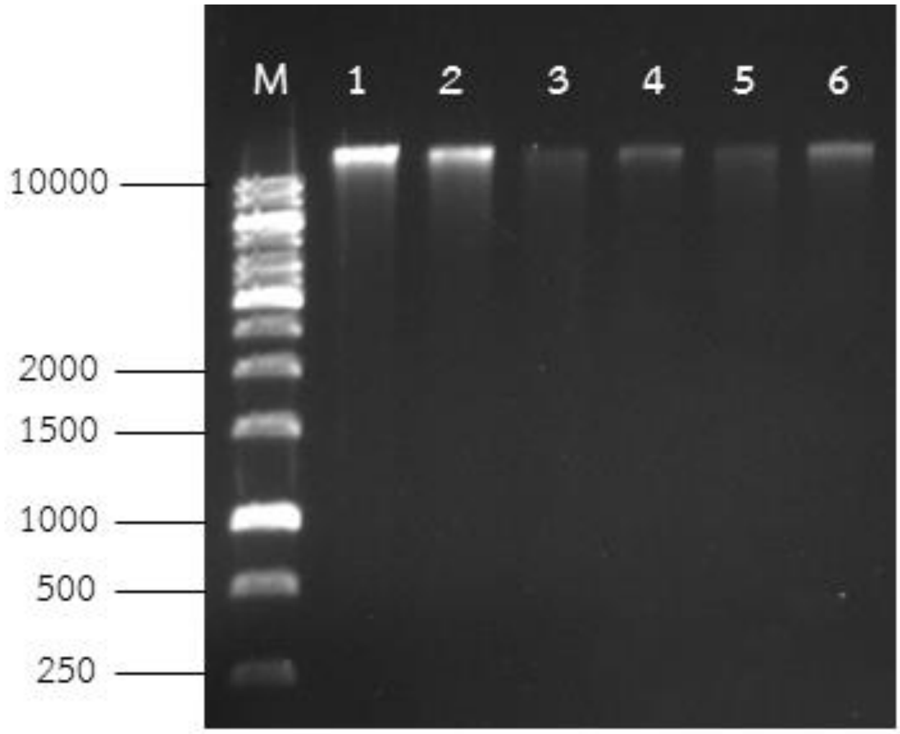


Figure 39 The detection of genomic DNA of 6 *Erythrina* species in 1% agarose gel electrophoresis

Lane M = 1 Kb DNA ladder

Lane 1 = *E. fusca*

Lane 2 = *E. stricta*

Lane 3 = *E. crista-galli*

Lane 4 = *E. subumbrams*

Lane 5 = *E. variegata*

Lane 6 = *E. indica*

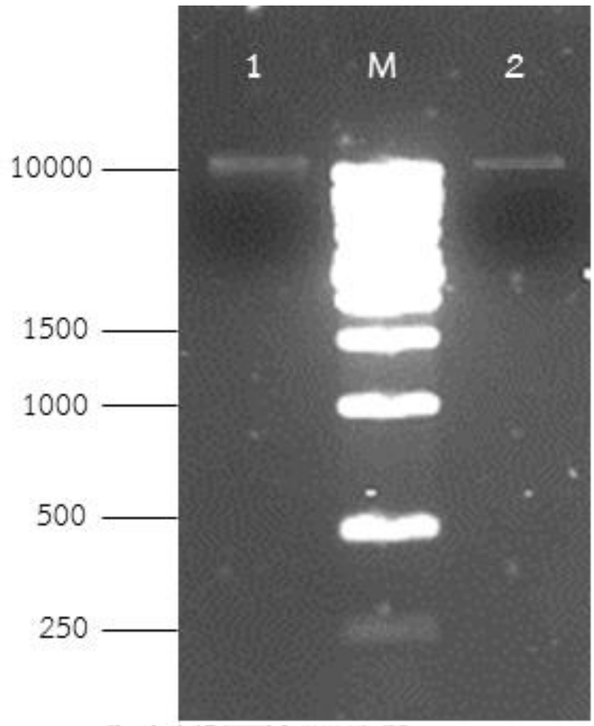


Figure 40 Genomic DNA of 2 outgroup plants in 1% agarose gel electrophoresis

Lane M = 1 kb DNA ladder

Lane 1 = *M. hortensis*

Lane 2 = *P. indicus*

PCR amplification

DNA amplification of six *Erythrina* species and two outgroups were performed using 5 pairs of primers for ITS, *mat*K, *rpo*C, *psb*A_*trn*H and *ycf*1 region. The success of each PCR reaction was verified by electrophoresis of 5 µl of the reaction products which mix with bromophenol blue on 1% agarose gels and stained with SYBR safe DNA stain. Fragment patterns were analyzed under UV transilluminator and photographed. The fragment size was also estimated using GeneRuler 1 Kb DNA ladder. PCR product of six *Erythrina* species obtained by each primer were shown in Figure 41-45, respectively. The total of the nucleotide fragments was about 750, 1300, 500, 750, 1000 bp in length, respectively.

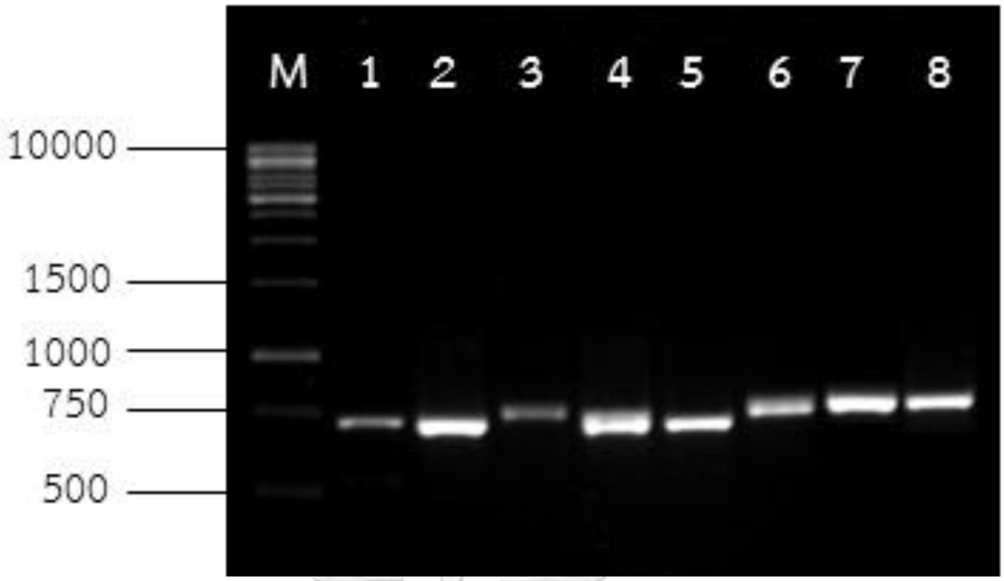


Figure 41 ITS amplification product in 1% agarose gels electrophoresis

Lane M = 1 Kb DNA ladder

Lane 1 = *E. fusca*

Lane 2 = *E. stricta*

Lane 3 = *E. crista-galli*

Lane 4 = *E. subumbrams*

Lane 5 = *E. variegata*

Lane 6 = *E. indica*

Lane 7 = *M. hortensis*

Lane 8 = *P. indicus*

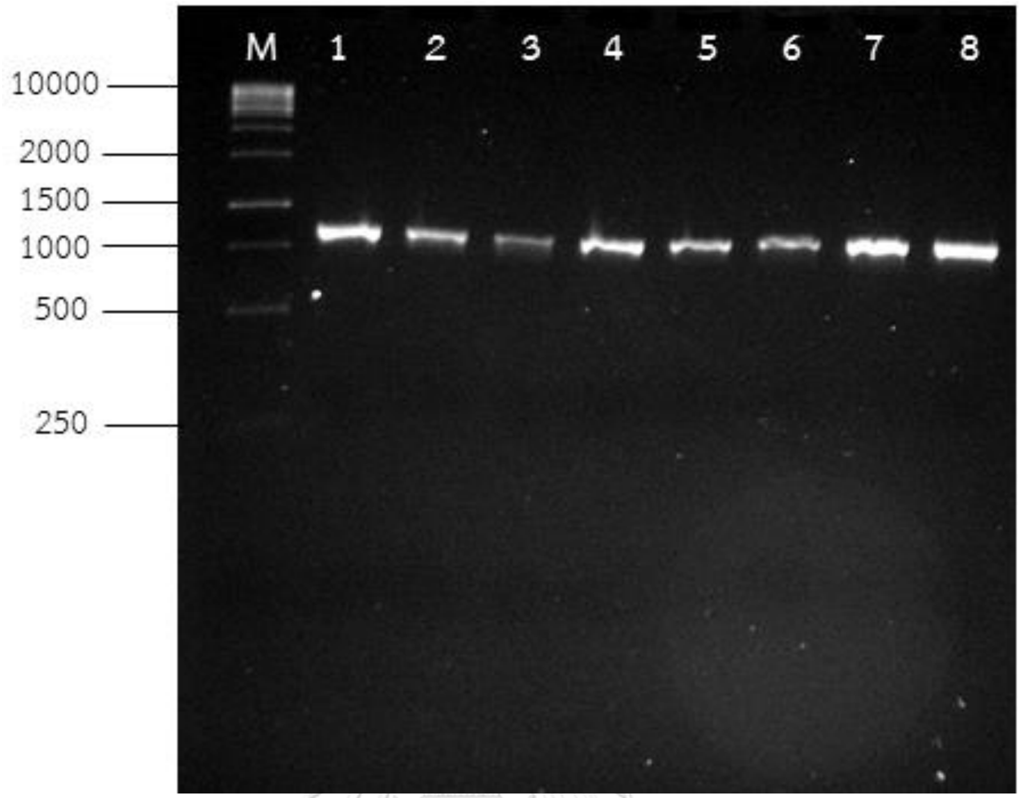


Figure 42 *mat*K amplification product in 1% agarose gels electrophoresis

Lane M = 1 Kb DNA ladder

Lane 1 = *E. fusca*

Lane 2 = *E. stricta*

Lane 3 = *E. crista-galli*

Lane 4 = *E. subumbrams*

Lane 5 = *E. variegata*

Lane 6 = *E. indica*

Lane 7 = *M. hortensis*

Lane 8 = *P. indicus*

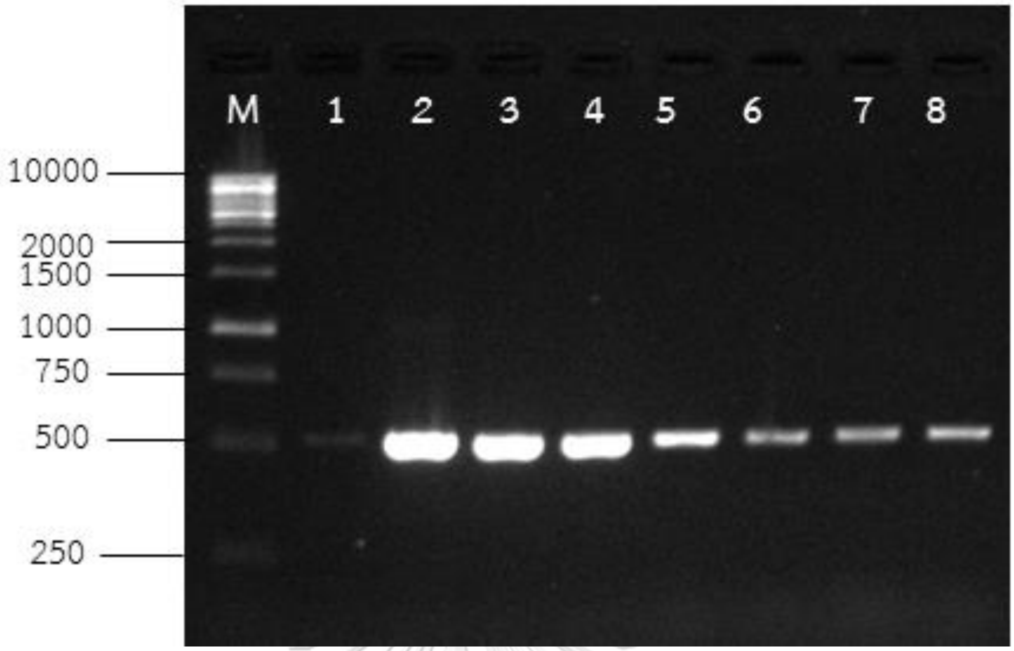


Figure 43 *rpo*C amplification product in 1% agarose gels electrophoresis

Lane M = 1 Kb DNA ladder

Lane 1 = *E. fusca*

Lane 2 = *E. stricta*

Lane 3 = *E. crista-galli*

Lane 4 = *E. subumbrams*

Lane 5 = *E. variegata*

Lane 6 = *E. indica*

Lane 7 = *M. hortensis*

Lane 8 = *P. indicus*

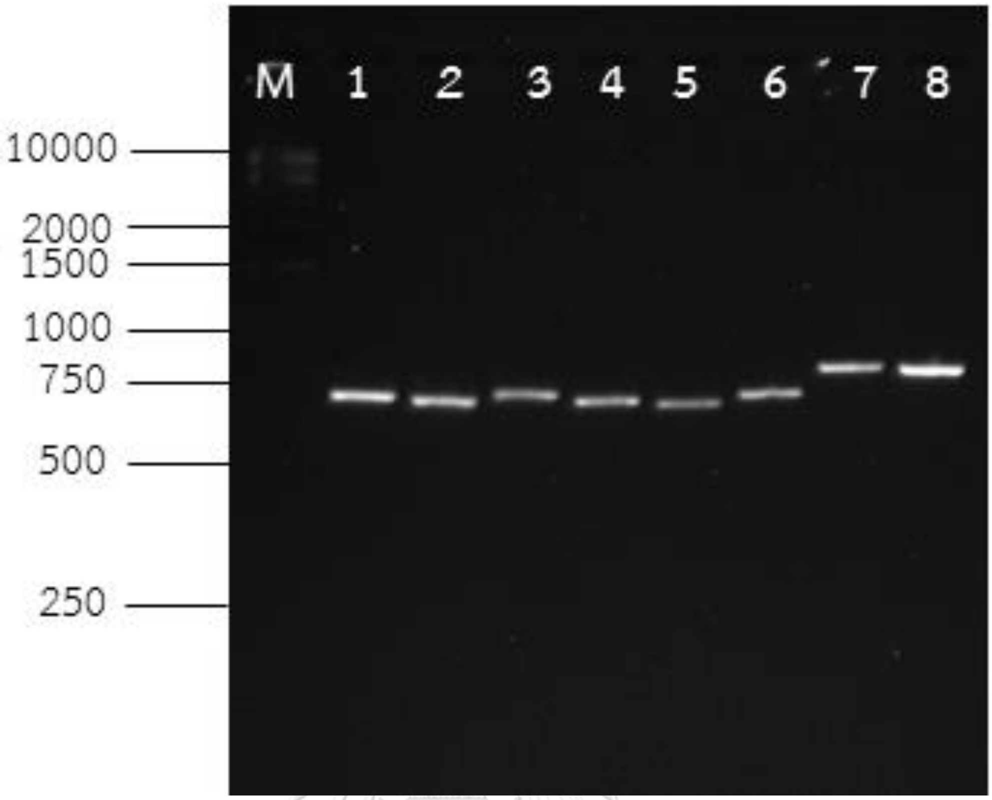


Figure 44 *psb*A_*trn*H amplification product in 1% agarose gels electrophoresis

Lane M = 1 Kb DNA ladder

Lane 1 = *E. fusca*

Lane 2 = *E. stricta*

Lane 3 = *E. crista-galli*

Lane 4 = *E. subumbrams*

Lane 5 = *E. variegata*

Lane 6 = *E. indica*

Lane 7 = *M. hortensis*

Lane 8 = *P. indicus*

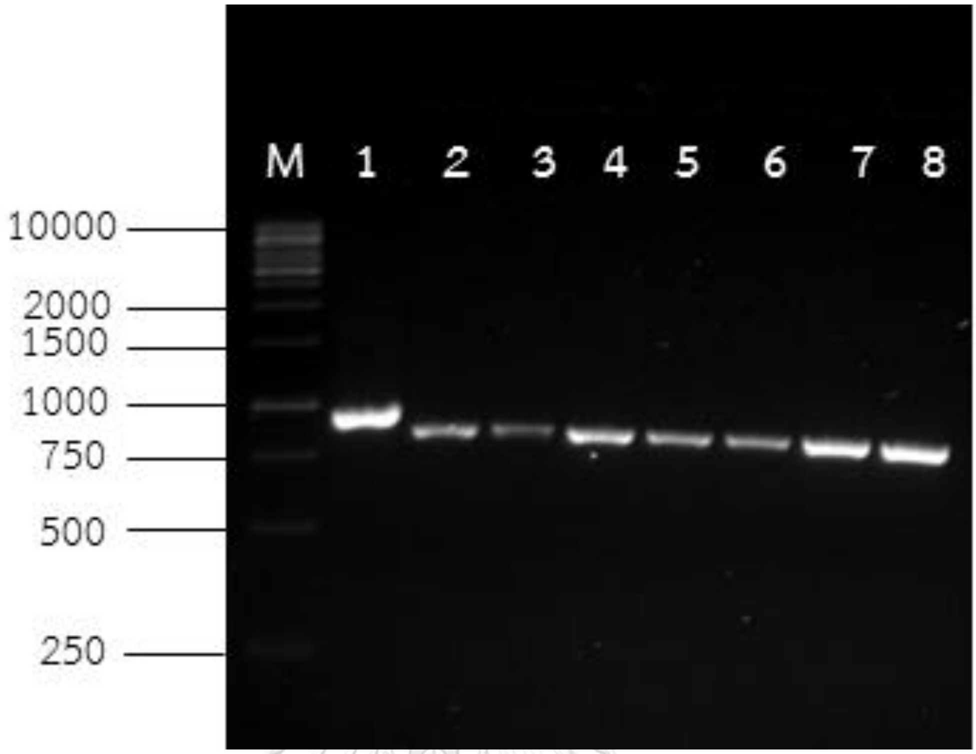


Figure 45 *ycf*1 amplification product in 1% agarose gels electrophoresis

Lane M = 1 Kb DNA ladder

Lane 1 = *E. fusca*

Lane 2 = *E. stricta*

Lane 3 = *E. crista-galli*

Lane 4 = *E. subumbrams*

Lane 5 = *E. variegata*

Lane 6 = *E. indica*

Lane 7 = *M. hortensis*

Lane 8 = *P. indicus*

DNA sequencing analysis

The sequence was assembled and analyzed using the Multalin program. The raw data of DNA sequences of ITS, *mat*K, *psb*A_*trn*H, *rpo*C and *ycf*1 from *E. fusca*, *E. stricta*, *E. crista-galli*, *E. subumbrans*, *E. variegata, E. indica, M. hortensis* and *P. indicus* were shown in Figure 46-50. The ITS, *mat*K, *psb*A_*trn*H, *rpo*C and *ycf*1 sequences among six *Erythrina* species were about 677, 794, 278, 375 and 656 bp in length, respectively.

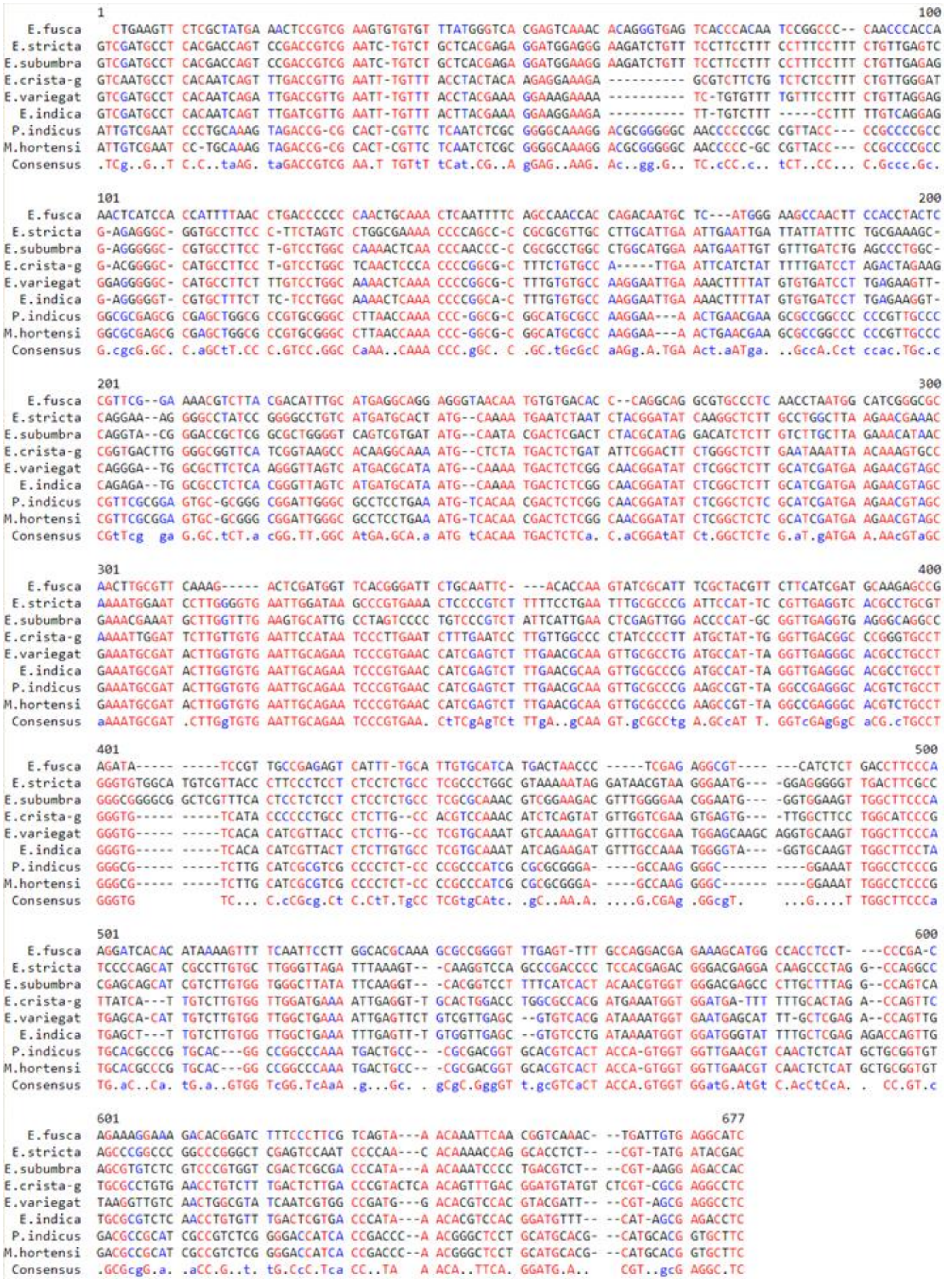


Figure 46 The ITS multiple sequence alignment of six *Erythrina* species and outgroup plants

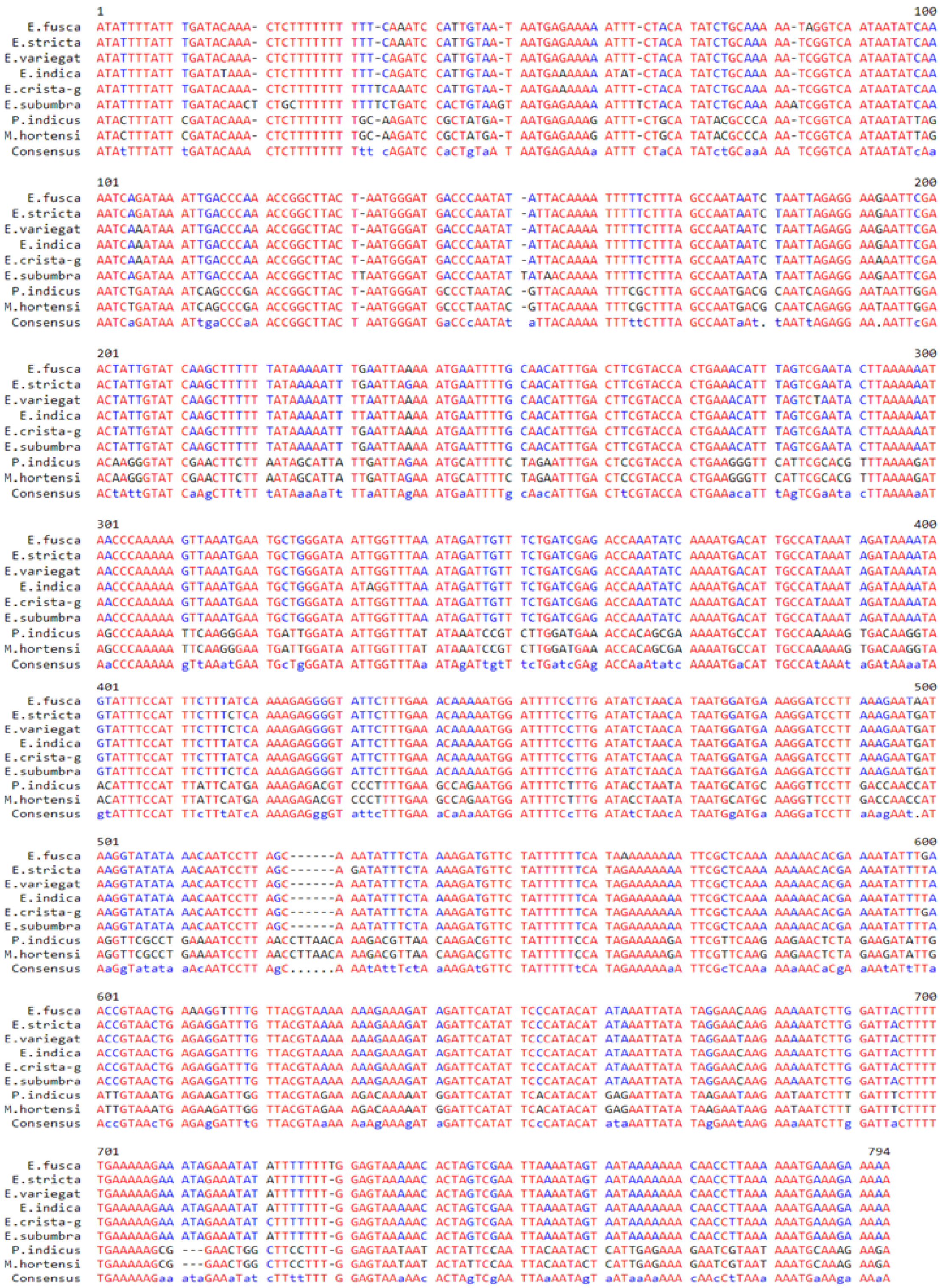


Figure 47 The *mat*K multiple sequence alignment of six *Erythrina* species and outgroup plants

Figure 48 The *psb*A_*trn*H multiple sequence alignment of six *Erythrina* species and outgroup plants

```
             1                                                                                                    100
    E.fusca  GGTCGTTCGG TCATTGTCGT AGGTCCATCA CTTTCATTAC ATAGATGTGG ATTGCCTCGT GAAATAGCAA TAGAACTTTT CCAGACATTT CTAATTCGTG
 E.variegat  GGTCGTTCGG TCATTGTCGT AGGTCCATCA CTTTCATTAC ATAGATGTGG ATTGCCTCGT GAAATAGCAA TAGAACTTTT CCAGACATTT CTAATTCGTG
   E.indica  GGTCGTTCGG TCATTGTCGT AGGTCCATCA CTTTCATTAC ATAGATGTGG ATTGCCTCGT GAAATAGCAA TAGAACTTTT CCAGACATTT CTAATTCGTG
  E.stricta  GGTCGTTCGG TCATTGTCGT AGGTCCATCA CTTTCATTAC ATAGATGTGG ATTGCCTCGT GAAATAGCAA TAGAACTTTT CCAGACATTT CTAATTCGTG
 E.subumbra  GGTCGTTCGG TCATTGTCGT AGGTCCATCA CTTTCATTAC ATAGATGTGG ATTGCCTCGT GAAATAGCAA TAGAACTTTT CCAGACATTT CTAATTCGTG
  P.indicus  GGGCGTTCCG TCATTGTCGT AGGTCCTTCA CTTTCATTAC ATCGATGTGG ATTGCCGCGC GAAATAGCAA TAGAGCTTTT CCAGACATTT GTAATTCGTG
 M.hortensi  GGGCGTTCCG TCATTGTCGT AGGTCCTTCA CTTTCATTAC ATCGATGTGG ATTGCCGCGC GAAATAGCAA TAGAGCTTTT CCAGACATTT GTAATTCGTG
 E.crista-g  GGTCGTTCGG TCATTGTCGT AGGTCCATCA CTTTCATTAC ATAGATGTGG ATTGCCTCGT GAAATAGCAC TAGAACTTTT CCTTACATTT TTATTTCGTG
  Consensus  GGtCGTTCgG TCATTGTCGT AGGTCCaTCA CTTTCATTAC ATaGATGTGG ATTGCCtCGt GAAATAGCAA TAGAaCTTTT CCAGACATTT .TAATTCGTG

             101                                                                                                  200
    E.fusca  GTCTAATTCG AAAACATTTT GCTTCGAACA TAGGAATTGC TAAGAGTAAA ATTCGGGAAA AAGAACCGAT TGTATGGGAA ATACTTCAAG AAGTTATGCA
 E.variegat  GTCTAATTCG AAAACATTTT GCTTCGAACA TAGGAATTGC TAAGAGTAAA ATTCGGGAAA AAGAACCGAT TGTATGGGAA ATACTTCAAG AAGTTATGCA
   E.indica  GTCTAATTCG AAAACATTTT GCTTCGAACA TAGGAATTGC TAAGAGTAAA ATTCGGGAAA AAGAACCGAT TGTATGGGAA ATACTTCAAG AAGTTATGCA
  E.stricta  GTCTAATTCG AAAACATTTT GCTTCGAACA TAGGAATTGC TAAGAGTAAA ATTAGGGAAA AAGAACCGAT TGTATGGGAA ATACTTCAAG AAGTTATGCA
 E.subumbra  GTCTAATTCG AAAACATTTT GCTTCGAACA TAGGAATTGC TAAGAGTAAA ATTAGGGAAA AAGAACCGAT TGTATGGGAA ATACTTCAAG AAGTTATGCA
  P.indicus  GTCTAATTAG ACAACATCTT GCTTCGAACA TAGGAGTTGC GAAGAGTCAA ATTCGGGAAA AAGAACCGAT TGTATGGGAA ATACTTCAGG AAGTTATGCA
 M.hortensi  GTCTAATTAG ACAACATCTT GCTTCGAACA TAGGAGTTGC GAAGAGTCAA ATTCGGGAAA AAGAACCGAT TGTATGGGAA ATACTTCAGG AAGTTATGCA
 E.crista-g  GTGTAATTCT AAAACATTTT TTTTTTAACA TAGGATTTGT TAAGAGTATA ATTCGGGGAA AAGAGCCGCT TTTATGTGGA ATACTTCAAG AGGTTTTGCA
  Consensus  GTCTAATTcG AaAACATtTT GCTTCGAACA TAGGA.TTGC tAAGAGTaAA ATTCGGGAAA AAGAACCGAT TGTATGGGAA ATACTTCAaG AAGTTATGCA

             201                                                                                                  300
    E.fusca  GGGATATCCC GTATTGCTGA ATAGAGCGCC TACTCTGCAT AGATTAGGTA TACAGGCATT CCAACCTATT TTAGTAGAAG GACGTGCTAT TTGTTTGCAT
 E.variegat  GGGATATCCC GTATTGCTGA ATAGAGCGCC TACTCTGCAT AGATTAGGTA TACAGGCATT CCAACCTATT TTAGTAGAAG GACGTGCTAT TTGTTTGCAT
   E.indica  GGGATATCCC GTATTGCTGA ATAGAGCGCC TACTCTGCAT AGATTAGGTA TACAGGCATT CCAACCTATT TTAGTAGAAG GACGTGCTAT TTGTTTGCAT
  E.stricta  GGAATATCCC GTATTGCTGA ATAGAGCGCC TACTCTGCAT AGATTAGGTA TACAGGCATT CCAACCTATT TTAGTAGAAG GACGTGCTAT TTGTTTGCAT
 E.subumbra  GGAATATCCC GTATTGCTGA ATAGAGCGCC TACTCTGCAT AGATTAGGTA TACAGGCATT CCAACCTATT TTAGTAGAAG GACGTGCTAT TTGTTTGCAT
  P.indicus  GGGGCATCCT GTATTGCTGA ATAGAGCACC CACTCTGCAT AAATTAGGCA TACAGGCATT CCAGCCCGTT TTAGTGGAGG GGCGTGTTAT TTGTTTACAT
 M.hortensi  GGGGCATCCT GTATTGCTGA ATAGAGCACC CACTCTGCAT AAATTAGGCA TACAGGCATT CCAGCCCGTT TTAGTGGAGG GGCGTGTTAT TTGTTTACAT
 E.crista-g  GGGATATCCC GTATTGTTGA ATAGAGCGCC TATTCTGCAT ATATTAGGTA TACAGGCATT CCACCCTATT TTAGTGTAAG GACGTGCTAT TTGTTTGCAT
  Consensus  GGGatATCCc GTATTGCTGA ATAGAGCgCC tACTCTGCAT A.ATTAGGtA TACAGGCATT CCA.CCtaTT TTAGTGGAaG GaCGTGcTAT TTGTTTgCAT

             301                                                                  375
    E.fusca  CCATTAGTTT GTAAGGGATT CAATGCAGAC TTTGATGGGG ATCAAATGGC TGTTCATGTG CCTTTATCTT TAGAA
 E.variegat  CCATTAGTTT GTAAGGGATT CAATGCAGAC TTTGATGGGG ATCAAATGGC TGTTCATGTG CCTTTATCTT TAGAA
   E.indica  CCATTAGTTT GTAAGGGATT CAATGCAGAC TTTGATGGGG ATCAAATGGC TGTTCATGTG CCTTTATCTT TAGAA
  E.stricta  CCATTAGTTT GTAAGGGATT CAATGCAGAC TTTGATGGGG ATCAAATGGC TGTTCATGTG CCTTTATCTT TAGAA
 E.subumbra  CCATTAGTTT GTAAGGGATT CAATGCAGAC TTTGATGGGG ATCAAATGGC TGTTCATGTG CCTTTATCTT TAGAA
  P.indicus  CCATTAGTTT GTAAGGGATT CAATGCAGAT TTTGATGGGG ATCAAATGGC TGTTCATGTA CCTTTATCTT TGGAG
 M.hortensi  CCATTAGTTT GTAAGGGATT CAATGCAGAT TTTGATGGGG ATCAAATGGC TGTTCATGTA CCTTTATCTT TGGAG
 E.crista-g  CCATTAGTTT TTAAGGGATT CTTTGCAGAG TTTGATGGGG ATCAAATGGC TGTTCATGTG CGTTTATCTT TAGAG
  Consensus  CCATTAGTTT GTAAGGGATT CAATGCAGA. TTTGATGGGG ATCAAATGGC TGTTCATGTg CCTTTATCTT TaGAG
```

Figure 49 The *rpoC* multiple sequence alignment of six *Erythrina* species and outgroup plants

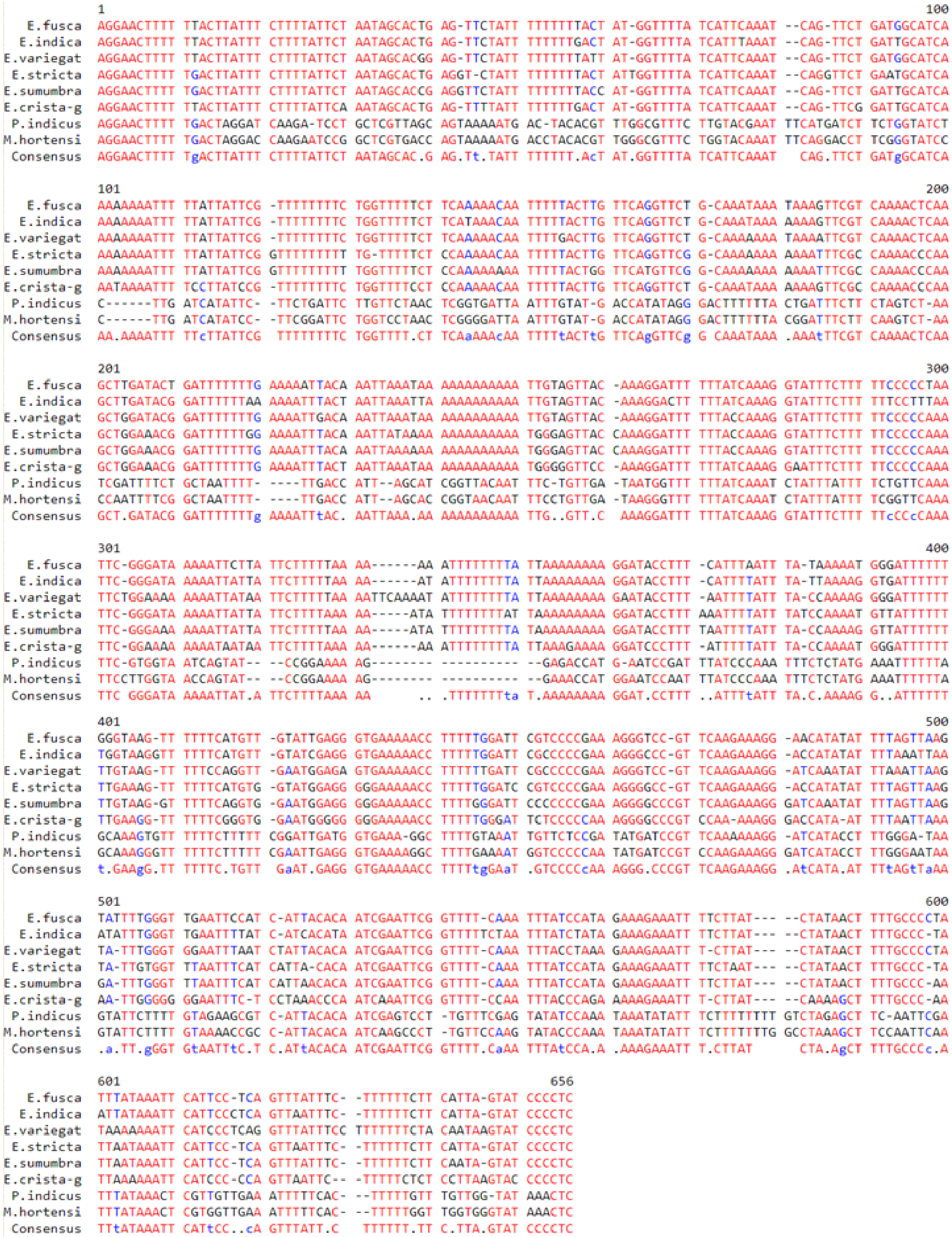


Figure 50 The *ycf1* multiple sequence alignment of six *Erythrina* species and outgroup plants

Phylogenetic analysis

The dendrogram showed that the high efficiency of these DNA sequencing data could clearly distinguish each species including the outgroup was generated by maximum parsimony method with a number of bootstrap 1000 replications using the computer program MEGA7 as shown in Figure 51-55.

Based on the ITS sequences determination of the six *Erythrina* species, the parsimony analysis was performed to produce parsimonious trees. By comparison of 50%, the majority-rule consensus tree divided *Erythrina* into two major clades and six groups. The first group was composed of *E. fusca,* which was separated from other species (100% bootstrap). The second group was composed of *E. indica* (60% bootstrap). The third group included *E. crista-galli* (59% bootstrap). The fourth group belonged to *E. variegata* (57% bootstrap). The fifth group was composed of *E. stricta* and *E. subumbrans* with a bootstrap value of 62%. While *P. indicus* and *M. hortensis*, which were outgroups in this current study, were clearly separated from six *Erythrina* species with 100% bootstrap support. The species with accession numbers were referred from the GenBank (KF186436.1, FN825780.1, KJ419281.1, KJ419280.1, JX856454.4, and JX56570.1, respectively) to compare with the analytical samples as shown in Figure 51.

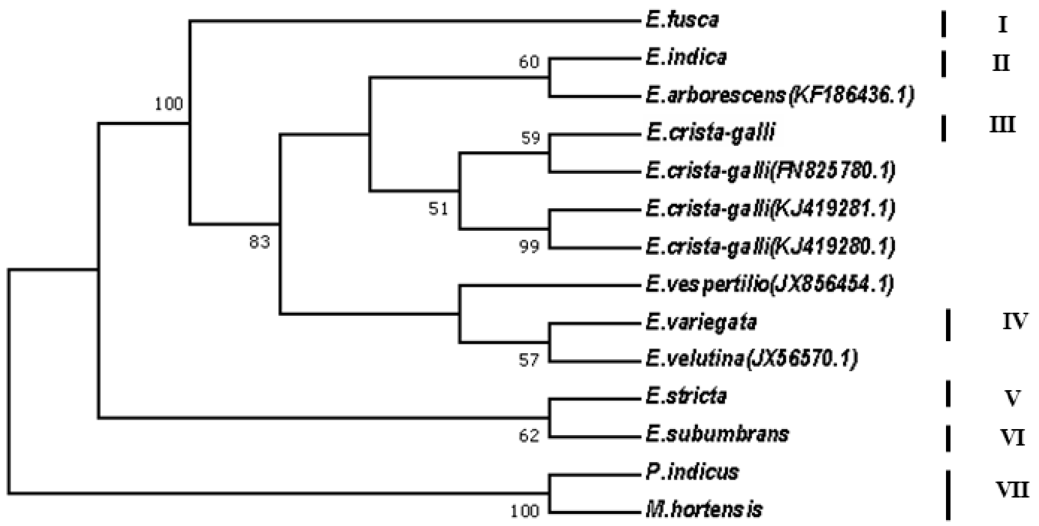


Figure 51 The phylogenetic tree based on parsimony analysis among six *Erythrina* species and outgroup ITS sequences; Tree length of 585 with a number of bootstrap 1000 replications

Based on the determined *mat*K sequences of the six *Erythrina* species, the parsimony analysis was performed to produce parsimonious trees. By comparison of 50%, the majority-rule consensus tree divided *Erythrina* into two major clades and six groups. The first group was composed of *E. indica*, which was separated from other species (100% bootstrap). The second group was divided into two subgroups including *E. stricta*, *E. subumbrans* (89%). The third group was *E. variegata* with a bootstrap value of 60%. The fourth group was composed of *E. crista-galli* (89% bootstrap). The five group was *E. fusca* with a high bootstrap value of 91%. *P. indicus* and *M. hortensis*, which were outgroups in this current study, were clearly separated from six *Erythrina* species with 100% bootstrap support. The species with accession numbers were referred from the GenBank (KF147397.1, AY3863869.1, KX816364.1, KY118274.1, KJ772770.1, and KJ012577.1, respectively) to compare with the analytical samples as shown in Figure 52.

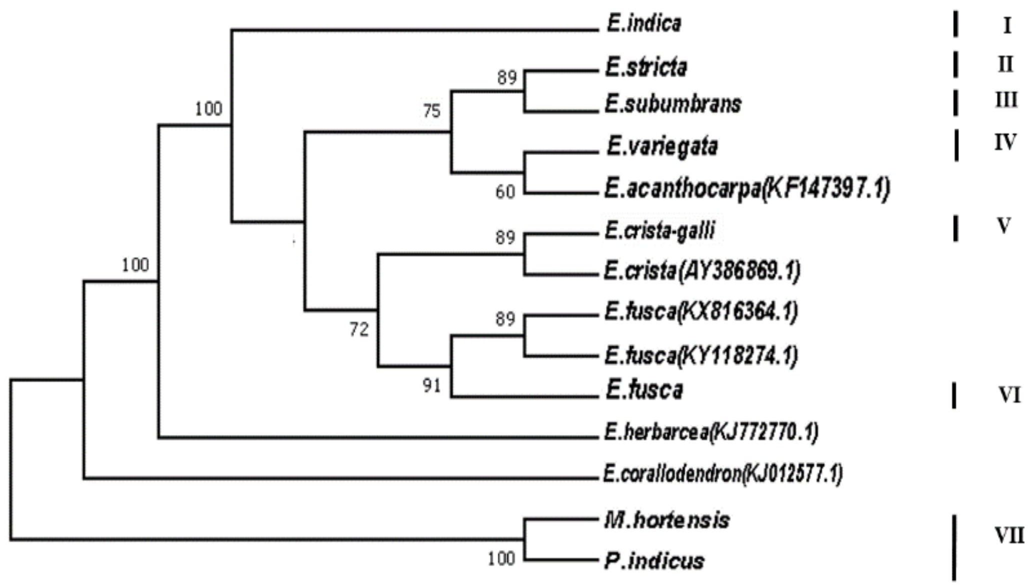


Figure 52 The phylogenetic tree based on parsimony analysis among six *Erythrina* species and outgroup *mat*K sequences; Tree length of 607 with a number of bootstrap 1000 replications

Based on the determined *psb*A_*trn*H sequences of the six *Erythrina* species, the parsimony analysis was performed to produce parsimonious trees. Comparison of 50%, the majority-rule consensus tree divided *Erythrina* into two major clades and five groups. *E. fusca* was in group 1 and was separated from other species with a bootstrap value (98%). The second group contained *E. crista-galli* and *E. variegata* having 56% and 94% of bootstrap value, respectively. The third group was divided into two subgroups with the first subgroup being composed of *E. stricta* (99%) and the second one containing *E. subumbrans* (69%). Next, *E. indica* was in group 4 and was separated from other species (81%). Finally, the outgroup plants, *P. indicus* and *M. hortensis*, were clearly separated from six *Erythrina* species with 100% bootstrap support. The species with accession numbers were referred from the GenBank (GQ982212.1, HG963644.1, KM895353.1, KR534091.1, JQ279722.1, and JX856884.1, respectively) to compare with the analytical samples as shown in Figure 53.

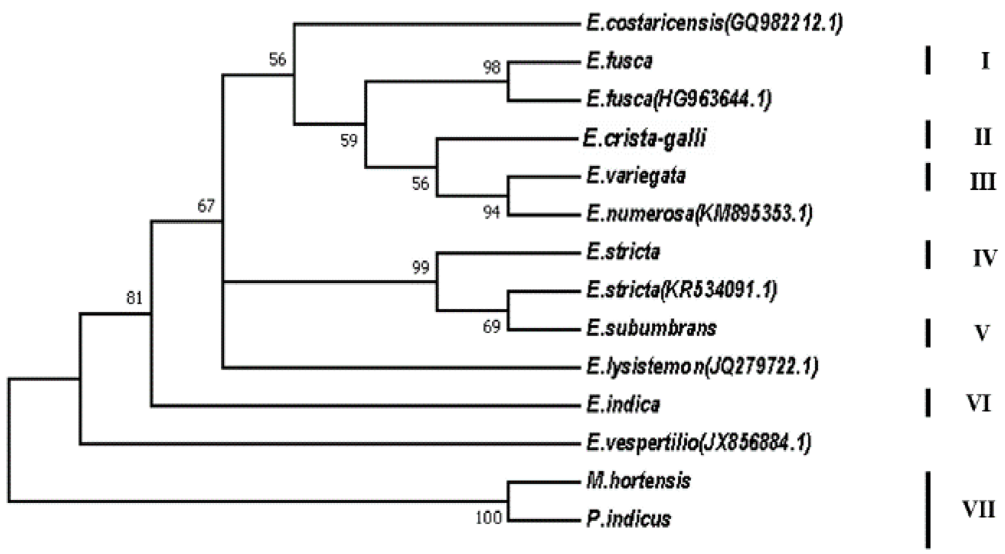


Figure 53 The phylogenetic tree based on parsimony analysis among six *Erythrina* species and outgroup *psb*A_*trn*H sequences; Tree length of 206 with a number of bootstrap 1000 replications

Based on the determined *rpo*C sequences of the six *Erythrina* species, the parsimony analysis was performed to produce parsimonious trees. By comparison of 50%, the majority-rule consensus tree divided *Erythrina* into two major clades and five groups. *E. stricta* and *E. subumbrans* was in the first group with a 87% bootstrap value. For the second group, the bootstrap value of 75% was found in *E. fusca*. The third group was composed of *E. indica*, and *E. variegata* (90% bootstrap). The fourth group belonged to *E. crista-galli* (73% bootstrap). Lastly, *P. indicus* and *M. hortensis*, which were outgroups in this current study, were clearly separated from six *Erythrina* species with 100% bootstrap support (Figure 49). The species of accession numbers have been no reported in the database.

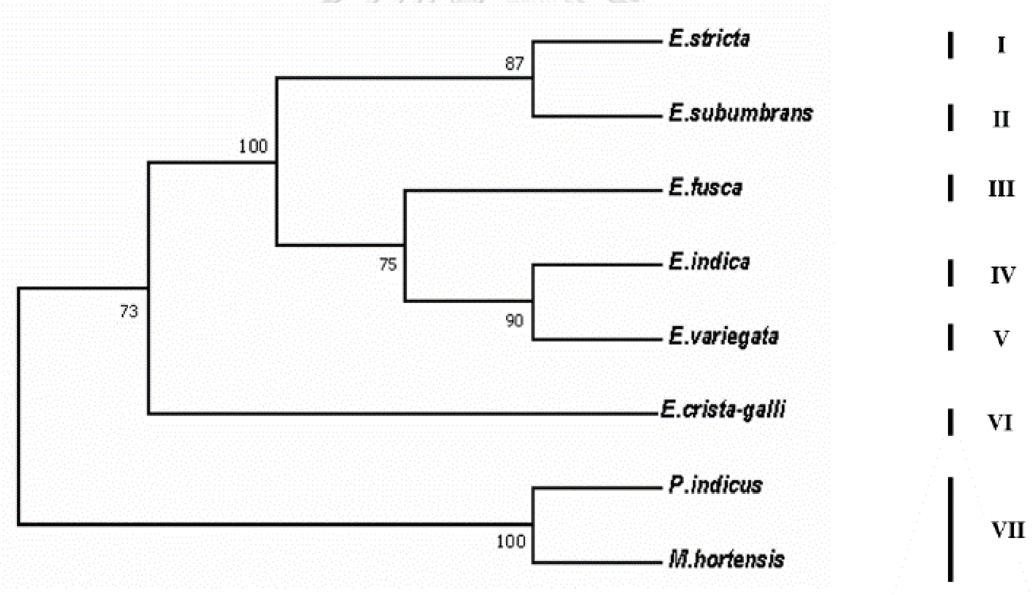


Figure 54 The phylogenetic tree based on parsimony analysis among six *Erythrina* species and outgroup *rpo*C sequences; Tree length of 294 with a number of bootstrap 1000 replications

Based on the determined *ycf*1 sequences of the six *Erythrina* species, the parsimony analysis was performed to produce parsimonious trees. Comparison of 50%, the majority-rule consensus tree divided *Erythrina* into two major clades and four groups. The first group was divided into two subgroups with the first subgroup being composed of *E. fusca* and *E. indica* (83%) and the second one containing *E. variegata* (54%). The second group was composed of *E. crista-galli,* which was separated from other species (77% bootstrap). The third group consisted of *E. stricta* and *E. subumbrans* (71% bootstrap). Finally, the outgroup plants, *P. indicus* and *M. hortensis*, were clearly separated from six *Erythrina* species, with a bootstrap value of 100% (Figure 50). The species of accession numbers have been no reported in the database.

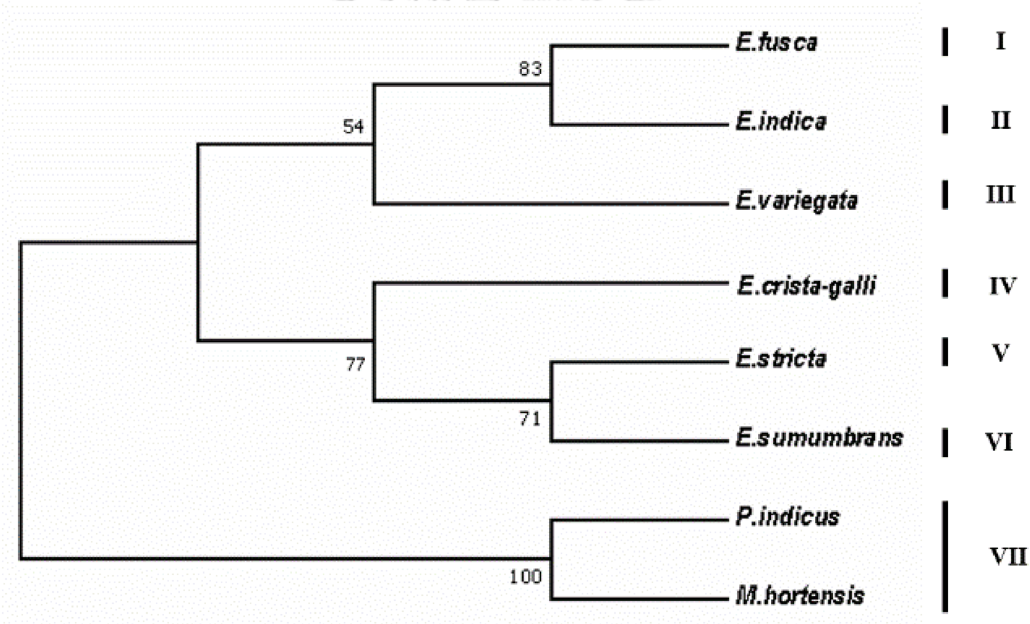


Figure 55 The phylogenetic tree based on parsimony analysis among six *Erythrina* species and outgroup *ycf*1 sequences; Tree length of 495 with a number of bootstrap 1000 replications

CHAPTER V
DISCUSSION AND CONCLUSION

At present, herbs and herbal extracts are popularly used in the Pharmaceutical manufacturing and cosmetics. The quality of herbal materials in important for the effectiveness of herbal medicine. The medicinal plant authentication methods are set by macroscopic, microscopic, chemical constituents and molecular genetic identification. These techniques have been accepted as the standard techniques by WHO guideline for quality control of plant materials [138].

In Thailand, *Erythrina* spp. have been used as anti-inflammatory, analgesic, headache, broken bone healing, anti-bacterial and anti-fungal agents [22, 25, 31, 50, 53, 54]. This study aimed to identify six *Erythrina* species distributed in Thailand using macroscopic, microscopic analyses and DNA analysis. The macroscopic characteristics of *E. fusca, E. stricta, E. crista-galli*, *E. subumbrans, E. variegata* and *E. indica* was observed on leaf shape, leaf apex, leaf base, leaf margin, flower and fruit shape which clearly reported in Table 9.

Microscopic analysis of leaf constant numbers involved leaf surface tissue preparation for transparent thin tissue. Because of *Erythrina* leaf thickness, the leaf could not be exfoliated by nail polish (formaldehyde). The pieces of leaf was clearing by soaking with Haiter solution for 24 hours, following with chloral hydrate solution until the leaf became clear that could observe the plant cells.

Microscopic analysis of the type of stomata in six *Erythrina* species was classified as paracytic type in which the stoma was surrounded by two subsidiary cells parallel to the long axis of guard cells (Figure 30). Generally, there is only one stomatal type found in each genus, hence consistently supporting the results of six *Erythrina* species in this study.

Summary of microscopic leaf constant numbers including stomatal number, epidermal cell number, stomatal index, epidermal cell area, vein islet number and palisade ratio were demonstrated in Table 20. Determination of stomatal number is one of the useful parameters in order to distinguish plants in species level [139]. In this study the stomata in upper epidermis were found only in *E. crista-galli*, *E. subumbrans* and *E. variegata* (60-136, 12-44, and 4-28 stomata/mm^2 respectively). *E. crista-galli* demonstrated the highest number of upper stomata which could be used as characteristics for identification. The stomatal numbers and stomatal indices in lower epidermis among these six species were overlapping. The stomatal number in lower epidermis among *Erythrina* species in Thailand were found to be less than *E. velutina* in Brazil (264.60±16.83) [91].

The variation in stomata usually depend on the genetic and geographical factors. For example, CO_2 content can affect the amount of stomata because CO_2 plays a role in photosynthesis and helps build internal plant structures. Plants normally take in CO_2 during the day and O_2 at night [140]. For *E. subumbrans* and *E. variegata*, whose upper stomatal numbers were in the same range, exhibited distinct upper epidermal cell number (1080-1820 and 404-532 cell/mm^2 respectively). *E. fusca,* could be distinguished from *E. stricta* and *E. indica* by lower epidermal cell number (1000-1808, 540-776 and 376-876 cell/mm^2 respectively).

Epidermal cell area was relatively constant within a narrow range for each species that allows a correct identification although some had degrees of overlapping with closely related species. This value was used as a taxonomic tool for identification of plant materials [141]. In this study, the epidermal cell areas among *E. fusca, E. crista-*

galli and *E. subumbrans* were found to be less than 1200 μm^2 whereas the epidermal cell area among *E. variegata, E. stricta* and *E. indica* were more than 1200 μm^2.

The other important microscopic leaf constant parameters are palisade ratio and vein islet number. The palisade ratio has been used as a diagnostic value for differentiating of plant species. Both values can be affected by geographical variation but different from species to species. However, the study among six *Erythrina* species revealed overlapping of the palisade ratio and vein islet number. Nevertheless, both values could be used to identify *E. subumbrans* from other species. In this study, *E. subumbrans* showed the highest vein islet number (12.75-20.75 cell/mm^2) which was less than previously reported of 23.2 ± 1.16 cell/mm^2 in India [142].

Table 13 Summary of microscopic leaf constant numbers including of stomatal number, epidermal cell number, stomatal index, epidermal cell area, vein islet number and palisade ratio among six *Erythrina* species distributed in Thailand (n=90)

Erythrina species	Stomatal number* mean±SD (min-max)		Epidermal cell number* mean SD (min-max)		Stomatal index mean±SD (min-max)		Upper epidermal cell area (μm^2) mean±SD (min-max)	Palisade ratio mean±SD (min-max)	Vein islet number* mean±SD (min-max)
	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis	Upper	Lower			
E. fusca	-	159±19 (116-200)	1213.78±166.32 (880-1604)	1336.98±198.41 (1000-1808)	-	10.80±1.83 (6.22-14.29)	839.99±112.52 (623.44-1136.36)	7.69±1.11 (4.00-10.00)	6.24±0.77 (4.00-7.75)
E. stricta	-	149±21 (84-192)	693.82±37.97 (624-792)	657.73±64.51 (540-776)	-	18.50±2.23 (11.29-23.33)	1445.52±78.32 (1262.63-1602.56)	6.21±6.21 (3.50-8.75)	6.45±0.76 (4.50-8.25)
E. crista-galli	92.44±14.60 (60-136)	159±17 (112-184)	1095.38±105.24 (792-1432)	1369.16±152.06 (1060-1820)	7.82±1.26 (5.38-11.63)	10.47±1.35 (6.62-13.68)	849.13±81.98 (652.74-1152.07)	6.00±1.15 (3.75-9.25)	6.57±0.94 (5.00-8.50)
E. subumbrans	24.53±6.76 (12-44)	135±18 (104-176)	1405.25±171.81 (1080-1820)	1636.18±336.37 (1060-2340)	1.74±0.51 (0.78-3.06)	7.93±1.88 (4.68-11.93)	711.94±89.08 (538.79-915.75)	5.13±0.85 (3.50-7.25)	16.58±1.82 (12.75-20.75)
E. variegata	10.13±4.95 (4-28)	140±19 (88-180)	468.18±30.80 (404-532)	839.16±86.80 (496-952)	2.09±0.94 (0.76-5.30)	14.37±1.64 (10.90-19.72)	2100.57±145.41 (1824.82-2118.64)	9.74±1.34 (7.25-13.50)	6.68±1.17 (3.80-11.00)
E. indica	-	178±46 (108-269)	553.82±76.30 (404-708)	648.75±143.99 (376-876)	-	22.41±8.28 (13.74-39.87)	1841.99±269.36 (1412.43-2475.25)	8.02±1.68 (4.50-12.50)	6.16±1.61 (3.50-9.75)

- = absent, * = number per mm^2

Qualitative microscopic investigation also provides the supporting evidence for plant identification [143]. The cross sections of midrib of six investigated *Erythrina* species revealed distinguishing characteristics especially xylem and phloem tissues (Figure 29-34).

Nowadays, molecular genetic identification has been developed and increasingly used as contemporary techniques, such as polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) [144], Random Amplified Polymorphic DNA (RAPD) [145], Amplified Fragment Length Polymorphism (AFLP) [146], Inter Simple Sequence Repeat (ISSR) [147], and DNA barcode [137]. In regard to this research, molecular identification supports the efficiency and reliability applied for estimating the genetic information of *Erythrina* species.

This is the first report on sequences of the ITS, *mat*K, *psb*A_*trn*H, *rpo*C and *ycf*1 region of *Erythrina* species distributed in Thailand. Molecular markers using ITS region of ribosomal DNA may vary because they are fast evolving and high level of interspecific divergence [101]. Chloroplast DNA has also been used for plant species identification and discrimination. As a consequence, analysis of the *mat*K, *psb*A_*trn*H, *rpo*C and *ycf*1 have been widely used for organismal identification and taxonomic clarification [148].

Mitochondrial genome, however, is not popularly used in plant molecular analysis because the mitochondrial genome of each plant species is large, varied in size and structure, making it difficult to be analyzed [115]. The mitochondrial genome in plant differs from that in animal in terms of structure and rate of nucleotide substitution. The rate of nucleotide substitution in plant mitochondrial genome is 40-100 times slower than that of animal, 12 times and 3-4 times lesser than that of nuclear and chloroplast genomes, respectively. Nonetheless, the lower rate of nucleotide substitution in plant is advantageous in analyzing the evolutional relationship in higher levels of species and genus. For example, coxI gene (cytochrome C oxidase subunit I) is most widely used in molecular identification of herbal plants because of its role in cellular respiratory and phosphorylation systems in plant with 1,592 bp. Apart from coxI, there are COII, COIII, *atp* (ATP synthase), *nad* (NADH dehydrogenase) and *cob* (apocytochrome *b*) [149, 150].

The phylogenetic relationship of six *Erythrina* species in Thailand constructed in the present study base on ITS, *mat*K, and *psb*A_*trn*H (Figure 46-48) was consistent with that obtained by NCBI (Genbank) database [151]. According to the phylogenetic tree, each primer in this study classified selected *Erythrina* species into 6 groups. However, the relationship among species were different except *E. stricta* and *E. subumbrans*. *E. stricta* and *E. subumbrans* were demonstrated close relationship due to sharing the same node from all primer used (62, 89, 99, 87, and 71% with 1000 replications of bootstrap of ITS, *mat*K, *psb*A_*trn*H, *rpo*C and *ycf*1 region respectively).

Previously, Bruneau [152] studied morphological characteristics and chloroplast DNA restriction site characters among 51 *Erythrina* species and constructed phylogenetic tree. It was found that *E. stricta* and *E. subumbrans* were more related than *E. fusca, E. crista-galli*, and *E. variegata*.

In conclusion, the macroscopic and microscopic characteristics, both qualitatively and quantitatively, based on midrib cross section and the microscopic leaf constant numbers, including stomatal number, stomatal index, palisade ratio, vein islet number and epidermal cell area, of six *Erythrina* species can be used as a tool for these plants authentication. The phylogenetic relationship regarding DNA sequencing data of the ITS, *mat*K, *rpo*C, *psb*A_*trn*H and *ycf*1 is able to provide valuable information to evidently support the identification of six *Erythrina* species, in which *E. stricta* and *E. subumbrans* has closest affinity of all.

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APPENDICES

Appendix A

Microscopic evaluation

The raw data of microscopic leaf constant numbers

Table 14 Stomatal number, stomatal index, palisade ratio, epidermal cell number, epidermal cell area and vein islet numbers of *E. fusca*, samples were collected from Chaiyaphum province

Field	Stomatal number (number per mm^2)		Epidermal cell number (number per mm^2)		Stomatal index		Upper epidermal cell area (μm^2)	Palisade ratio	Vein islet number (number per mm^2)
	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis			
1	0	200	1008	1200	0	14.29	992.06	4.00	4.00
2	0	168	1056	1184	0	12.43	946.97	8.00	5.25
3	0	172	996	1120	0	13.31	1004.02	8.75	6.25
4	0	176	1064	1160	0	13.17	939.85	8.25	6.50
5	0	192	1204	1192	0	13.87	830.56	5.25	5.75
6	0	140	1024	1212	0	10.36	976.56	4.75	6.25
7	0	168	1084	1560	0	9.72	922.51	9.00	6.00
8	0	168	1244	1588	0	9.57	803.86	9.50	6.00
9	0	164	1412	1268	0	11.45	708.22	6.50	6.00
10	0	160	1080	1552	0	9.35	925.93	7.00	6.50
11	0	156	1200	1572	0	9.03	833.33	8.25	7.50
12	0	164	1340	1556	0	9.53	746.27	8.00	6.00
13	0	164	1012	1204	0	11.99	988.14	7.75	6.25
14	0	172	1004	1568	0	9.89	996.02	5.50	7.25
15	0	180	1040	1560	0	10.34	961.54	7.00	7.75
16	0	156	1156	1588	0	8.94	865.05	7.50	7.75
17	0	116	1120	1148	0	9.18	892.86	7.25	5.75
18	0	160	1128	1188	0	11.87	886.52	7.75	6.50
19	0	168	1152	1576	0	9.63	868.06	7.50	6.50
20	0	192	1124	1216	0	13.64	889.68	7.00	6.00
21	0	144	1000	1340	0	9.70	1000.00	7.00	6.50
22	0	168	1080	1200	0	12.28	925.93	6.00	7.00
23	0	156	1060	1212	0	11.40	943.40	9.00	7.25
24	0	188	1032	1204	0	13.51	968.99	8.00	6.50
25	0	172	1340	1192	0	12.61	746.27	9.00	6.25
26	0	168	1216	1100	0	13.25	822.37	6.75	4.00
27	0	168	1172	1220	0	12.10	853.24	8.25	6.25
28	0	188	1060	1152	0	14.03	943.40	9.50	6.00
29	0	176	1244	1244	0	12.39	803.86	8.75	6.50
30	0	144	1020	1288	0	10.06	980.39	7.50	5.25
Min	0.00	**116**	996.00	**1100.00**	0.00	**8.94**	708.22	**4.00**	**4.00**
Max	0.00	**200**	1412.00	**1588.00**	0.00	**14.29**	1004.02	**9.50**	**7.75**
Mean	0.00	**166.38**	1122.40	**1312.13**	0.00	**11.43**	898.86	**7.48**	**6.24**
SD	0.00	**17.13**	111.44	**176.88**	0.00	**1.75**	82.86	**1.37**	**0.87**

Table 15 Stomatal number, stomatal index, palisade ratio, epidermal cell number, epidermal cell area and vein islet numbers of *E. fusca,* samples were collected from Rayong province

Field	Stomatal number (number per mm^2)		Epidermal cell number (number per mm^2)		Stomatal index		Upper epidermal cell area (μm^2)	Palisade ratio	Vein islet number (number per mm^2)
	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis			
1	0	120	1360	1188	0	9.17	735.29	5.50	5.00
2	0	148	1296	1160	0	11.31	771.60	7.50	6.50
3	0	136	1080	1060	0	11.37	925.93	7.25	5.25
4	0	168	1116	1296	0	11.48	896.06	8.50	5.50
5	0	156	1016	1100	0	12.42	984.25	6.75	6.75
6	0	116	1120	1000	0	10.39	892.86	5.00	6.25
7	0	164	1160	1560	0	9.51	862.07	9.25	5.00
8	0	156	1020	1188	0	11.61	980.39	8.00	6.25
9	0	160	1088	1268	0	11.20	919.12	8.25	6.70
10	0	148	1480	1552	0	8.71	675.68	8.25	6.50
11	0	168	1376	1172	0	12.54	726.74	8.00	6.25
12	0	140	1340	1120	0	11.11	746.27	7.50	4.50
13	0	128	1408	1200	0	9.64	710.23	7.00	7.25
14	0	188	1008	1152	0	14.03	992.06	7.50	7.00
15	0	184	1200	1140	0	13.90	833.33	7.25	6.75
16	0	172	1384	1132	0	13.19	722.54	8.50	6.00
17	0	152	1360	1080	0	12.34	735.29	6.50	6.75
18	0	140	1400	1188	0	10.54	714.29	7.75	6.50
19	0	124	1440	1176	0	9.54	694.44	7.50	7.50
20	0	144	1052	1216	0	10.59	950.57	7.50	7.00
21	0	176	1360	1212	0	12.68	735.29	8.75	6.00
22	0	164	1520	1208	0	11.95	657.89	6.25	5.50
23	0	156	1324	1212	0	11.40	755.29	7.25	7.25
24	0	160	1040	1204	0	11.73	961.54	7.75	6.50
25	0	180	1344	1228	0	12.78	744.05	8.75	6.25
26	0	168	1160	1216	0	12.14	862.07	7.00	5.75
27	0	156	1604	1184	0	11.64	623.44	7.50	6.00
28	0	132	1288	1148	0	10.31	776.40	8.50	6.50
29	0	168	1100	1204	0	12.24	909.09	7.75	6.00
30	0	156	1432	1196	0	11.54	698.32	8.25	6.75
Min	0.00	**116**	1360	**1000.00**	0.00	**8.71**	623.44	**5.00**	**4.50**
Max	0.00	**188**	1296	**1560.00**	0.00	**14.03**	992.06	**9.25**	**7.50**
Mean	0.00	**154.13**	1080	**1198.67**	0.00	**11.43**	806.41	**7.57**	**6.26**
SD	0.00	**18.72**	1116	**113.91**	0.00	**1.33**	110.88	**0.94**	**0.72**

Table 16 Stomatal number, stomatal index, palisade ratio, epidermal cell number, epidermal cell area and vein islet numbers of *E. fusca,* samples were collected from Nakhon Pathom province

Field	Stomatal number (number per mm^2)		Epidermal cell number (number per mm^2)		Stomatal index		Upper epidermal cell area (µm^2)	Palisade ratio	Vein islet number (number per mm^2)
	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis			
1	0	120	1036	1808	0	6.22	965.25	7.50	6.00
2	0	128	1540	1388	0	8.44	649.35	8.75	5.25
3	0	132	1112	1760	0	6.98	899.28	8.00	6.25
4	0	160	1200	1604	0	9.07	833.33	7.25	5.75
5	0	140	1020	1800	0	7.22	980.39	8.25	5.25
6	0	152	1284	1460	0	9.43	778.82	7.75	5.00
7	0	164	1380	1440	0	10.22	724.64	7.50	5.00
8	0	164	1248	1356	0	10.79	801.28	8.75	6.00
9	0	160	1420	1448	0	9.95	704.23	7.75	6.25
10	0	176	1488	1392	0	11.22	672.04	9.25	6.00
11	0	140	1080	1640	0	7.87	925.93	7.50	7.50
12	0	156	1040	1300	0	10.71	961.54	6.75	5.25
13	0	172	1200	1288	0	11.78	833.33	8.25	7.00
14	0	132	1280	1432	0	8.44	781.25	8.00	7.28
15	0	180	1364	1516	0	10.61	733.14	7.00	6.00
16	0	164	1192	1544	0	9.60	838.93	8.75	6.50
17	0	120	1500	1608	0	6.94	666.67	6.00	5.75
18	0	116	1280	1588	0	6.81	781.25	7.75	6.50
19	0	192	1244	1576	0	10.86	803.86	7.50	5.75
20	0	184	1336	1616	0	10.22	748.50	7.25	7.25
21	0	144	1512	1600	0	8.26	661.38	7.50	6.50
22	0	168	1416	1280	0	11.60	706.21	7.50	6.75
23	0	148	1200	1720	0	7.92	833.33	9.25	7.00
24	0	164	1192	1204	0	11.99	838.93	10.00	6.50
25	0	180	1080	1228	0	12.78	925.93	8.00	6.00
26	0	156	1032	1360	0	10.29	968.99	8.75	5.50
27	0	164	880	1584	0	9.38	1136.36	8.25	6.75
28	0	156	1460	1592	0	8.92	684.93	9.75	7.75
29	0	168	1480	1276	0	11.63	675.68	9.25	6.50
30	0	180	1196	1596	0	10.14	836.12	7.50	5.50
Min	0.00	**116**	880.00	**1204.00**	0.00	**6.22**	649.35	**6.00**	**5.00**
Max	0.00	**192**	1540.00	**1808.00**	0.00	**12.78**	1136.36	**10.00**	**7.75**
Mean	0.00	**155.88**	1256.40	**1500.13**	0.00	**9.54**	811.70	**8.04**	**6.21**
SD	0.00	**20.31**	174.75	**169.08**	0.00	**1.74**	118.83	**0.91**	**0.74**

Table 17 Stomatal number, stomatal index, palisade ratio, epidermal cell number, epidermal cell area and vein islet numbers of *E. stricta*, samples were collected from Nakhon Ratchasima province

Field	Stomatal number (number per mm^2)		Epidermal cell number (number per mm^2)		Stomatal index		Upper epidermal cell area (µm^2)	Palisade ratio	Vein islet number (number per mm^2)
	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis			
1	0	168	760	740	0	18.50	1315.79	6.00	7.50
2	0	192	700	680	0	22.02	1428.57	5.50	7.00
3	0	184	780	720	0	20.35	1282.05	4.75	6.25
4	0	156	780	740	0	17.41	1282.05	5.00	6.50
5	0	160	712	748	0	17.62	1404.49	7.50	6.25
6	0	168	704	712	0	19.09	1420.45	7.25	7.25
7	0	160	680	740	0	17.78	1470.59	8.00	6.50
8	0	160	700	732	0	17.94	1428.57	6.50	6.00
9	0	164	692	700	0	18.98	1445.09	7.00	4.75
10	0	176	648	708	0	19.91	1543.21	7.25	6.50
11	0	152	660	724	0	17.35	1515.15	3.75	7.25
12	0	168	668	700	0	19.35	1497.01	5.25	6.50
13	0	176	640	704	0	20.00	1562.50	6.00	7.00
14	0	180	740	704	0	20.36	1351.35	5.50	6.75
15	0	172	736	720	0	19.28	1358.70	5.50	6.50
16	0	168	728	700	0	19.35	1373.63	7.00	6.50
17	0	156	720	712	0	17.97	1388.89	6.50	6.75
18	0	176	740	756	0	18.88	1351.35	6.50	5.50
19	0	164	680	768	0	17.60	1470.59	6.50	7.50
20	0	180	664	776	0	18.83	1506.02	6.75	8.25
21	0	156	792	752	0	17.18	1262.63	7.50	8.00
22	0	160	672	768	0	17.24	1488.10	7.00	7.50
23	0	160	660	704	0	18.52	1515.15	8.75	7.50
24	0	184	740	728	0	20.18	1351.35	8.00	6.25
25	0	168	700	672	0	20.00	1428.57	7.50	7.25
26	0	168	680	640	0	20.79	1470.59	6.25	5.50
27	0	160	716	760	0	17.39	1396.65	5.75	7.00
28	0	156	724	748	0	17.26	1381.22	6.50	7.75
29	0	140	708	708	0	16.51	1412.43	5.00	6.75
30	0	160	740	692	0	18.78	1351.35	7.50	6.00
Min	0.00	**140**	640.00	**640.00**	0.00	**16.51**	1262.63	**3.75**	**4.75**
Max	0.00	**192**	792.00	**776.00**	0.00	**22.02**	1562.50	**8.75**	**8.25**
Mean	0.00	**166.38**	708.80	**721.87**	0.00	**18.75**	1415.14	**6.46**	**6.75**
SD	0.00	**11.15**	39.98	**31.09**	0.00	**1.32**	79.03	**1.12**	**0.77**

Table 18 Stomatal number, stomatal index, palisade ratio, epidermal cell number, epidermal cell area and vein islet numbers of *E. stricta,* samples were collected from Prachinburi province

Field	Stomatal number (number per mm^2)		Epidermal cell number (number per mm^2)		Stomatal index		Upper epidermal cell area (µm^2)	Palisade ratio	Vein islet number (number per mm^2)
	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis			
1	0	132	660	568	0	18.86	1515.15	3.50	4.50
2	0	128	656	560	0	18.60	1524.39	5.00	6.00
3	0	136	640	620	0	17.99	1562.50	4.50	5.50
4	0	160	676	644	0	19.90	1479.29	6.00	5.50
5	0	144	708	660	0	17.91	1412.43	7.25	5.75
6	0	124	680	680	0	15.42	1470.59	7.00	6.50
7	0	140	684	668	0	17.33	1461.99	7.50	5.50
8	0	128	700	640	0	16.67	1428.57	7.75	6.25
9	0	144	740	640	0	18.37	1351.35	7.50	7.75
10	0	140	680	636	0	18.04	1470.59	7.50	5.75
11	0	156	696	540	0	22.41	1436.78	4.00	6.25
12	0	168	660	552	0	23.33	1515.15	5.00	5.00
13	0	160	756	564	0	22.10	1322.75	8.00	6.25
14	0	164	700	560	0	22.65	1428.57	7.00	7.50
15	0	168	648	600	0	21.88	1543.21	7.50	6.25
16	0	160	656	580	0	21.62	1524.39	7.75	6.50
17	0	144	716	688	0	17.31	1396.65	6.50	7.00
18	0	152	660	716	0	17.51	1515.15	6.75	6.00
19	0	140	640	620	0	18.42	1562.50	7.00	6.25
20	0	136	648	676	0	16.75	1543.21	5.00	7.00
21	0	144	692	720	0	16.67	1445.09	4.75	6.00
22	0	144	680	680	0	17.48	1470.59	5.00	6.25
23	0	156	700	652	0	19.31	1428.57	4.25	7.00
24	0	120	660	660	0	15.38	1515.15	8.75	6.50
25	0	120	728	608	0	16.48	1373.63	8.75	6.25
26	0	128	624	668	0	16.08	1602.56	6.00	5.00
27	0	140	656	616	0	18.52	1524.39	5.00	5.75
28	0	152	724	672	0	18.45	1381.22	5.75	7.00
29	0	156	692	640	0	19.60	1445.09	7.75	6.25
30	0	156	640	700	0	18.22	1562.50	7.50	6.00
Min	0.00	**120**	624.00	**540.00**	0.00	**15.38**	1322.75	**3.50**	**4.50**
Max	0.00	**168**	756.00	**720.00**	0.00	**23.33**	1602.56	**8.75**	**7.75**
Mean	0.00	**144.63**	680.00	**634.27**	0.00	**18.64**	1473.80	**6.38**	**6.17**
SD	0.00	**13.98**	32.58	**50.60**	0.00	**2.18**	69.44	**1.47**	**0.72**

Table 19 Stomatal number, stomatal index, palisade ratio, epidermal cell number, epidermal cell area and vein islet numbers of *E. stricta,* samples were collected from Saraburi province

Field	Stomatal number (number per mm^2)		Epidermal cell number (number per mm^2)		Stomatal index		Upper epidermal cell area (µm^2)	Palisade ratio	Vein islet number (number per mm^2)
	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis			
1	0	92	720	544	0	14.47	1388.89	6.75	7.00
2	0	120	736	564	0	17.54	1358.70	4.50	7.25
3	0	128	736	560	0	18.60	1358.70	4.75	7.25
4	0	132	708	580	0	18.54	1412.43	5.00	6.50
5	0	140	700	584	0	19.34	1428.57	5.50	6.50
6	0	144	660	596	0	19.46	1515.15	6.25	5.00
7	0	156	680	588	0	20.97	1470.59	6.25	6.50
8	0	152	700	580	0	20.77	1428.57	6.25	6.75
9	0	160	692	580	0	21.62	1445.09	7.00	6.75
10	0	144	684	556	0	20.57	1461.99	6.75	6.00
11	0	128	740	560	0	18.60	1351.35	6.25	6.00
12	0	136	728	564	0	19.43	1373.63	6.00	6.00
13	0	164	740	564	0	22.53	1351.35	6.00	6.00
14	0	160	720	580	0	21.62	1388.89	6.50	7.50
15	0	168	672	580	0	22.46	1488.10	4.75	7.75
16	0	160	708	640	0	20.00	1412.43	5.25	5.25
17	0	148	680	640	0	18.78	1470.59	5.25	6.00
18	0	140	700	640	0	17.95	1428.57	6.25	6.50
19	0	144	720	712	0	16.82	1388.89	5.75	6.75
20	0	144	624	616	0	18.95	1602.56	5.50	7.00
21	0	144	640	660	0	17.91	1562.50	4.50	7.50
22	0	136	644	620	0	17.99	1552.80	4.75	7.00
23	0	148	656	632	0	18.97	1524.39	5.00	7.00
24	0	100	636	680	0	12.82	1572.33	5.00	6.25
25	0	84	652	660	0	11.29	1533.74	5.25	6.25
26	0	116	752	696	0	14.29	1329.79	6.25	4.75
27	0	120	696	676	0	15.08	1436.78	6.25	6.50
28	0	128	740	648	0	16.49	1351.35	6.50	6.50
29	0	152	648	720	0	17.43	1543.21	7.00	6.75
30	0	96	668	692	0	12.18	1497.01	7.00	6.50
Min	0.00	**84**	624.00	**544.00**	0.00	**11.29**	1329.79	**4.50**	**4.75**
Max	0.00	**168**	752.00	**720.00**	0.00	**22.53**	1602.56	**7.00**	**7.75**
Mean	0.00	**135.50**	692.67	**617.07**	0.00	**18.12**	1447.63	**5.80**	**6.51**
SD	0.00	**21.77**	36.53	**52.07**	0.00	**2.92**	77.28	**0.79**	**0.70**

Table 20 Stomatal number, stomatal index, palisade ratio, epidermal cell number, epidermal cell area and vein islet numbers of *E. crista-galli,* samples were collected from Bangkok province

Field	Stomatal number (number per mm^2)		Epidermal cell number (number per mm^2)		Stomatal index		Upper epidermal cell area (µm^2)	Palisade ratio	Vein islet number (number per mm^2)
	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis			
1	76	180	792	1608	8.76	10.07	1152.07	5.00	5.50
2	60	172	880	1316	6.38	11.56	1063.83	6.25	6.00
3	88	184	1000	1588	8.09	10.38	919.12	5.50	6.50
4	96	184	892	1376	9.72	11.79	1012.15	6.25	7.75
5	80	184	1068	1400	6.97	11.62	871.08	6.00	7.00
6	88	172	1152	1320	7.10	11.53	806.45	5.00	5.25
7	100	160	1020	1400	8.93	10.26	892.86	5.25	8.00
8	120	180	1040	1388	10.34	11.48	862.07	7.50	6.25
9	104	148	1160	1368	8.23	9.76	791.14	7.00	5.00
10	68	172	1092	1536	5.86	10.07	862.07	6.25	7.50
11	80	168	1088	1340	6.85	11.14	856.16	5.00	8.50
12	100	144	1088	1340	8.42	9.70	841.75	5.50	6.00
13	100	140	884	1280	10.16	9.86	1016.26	5.25	7.25
14	100	152	928	1396	9.73	9.82	972.76	6.50	8.50
15	100	172	1032	1660	8.83	9.39	883.39	6.50	7.00
16	104	172	1144	1720	8.33	9.09	801.28	5.50	5.25
17	92	172	1140	1720	7.47	9.09	811.69	5.25	6.00
18	88	160	1020	1820	7.94	8.08	902.53	7.25	5.35
19	64	160	992	1272	6.06	11.17	946.97	4.75	5.50
20	72	156	992	1720	6.77	8.32	939.85	4.75	8.00
21	76	120	1152	1692	6.19	6.62	814.33	3.75	7.25
22	104	120	1164	1640	8.20	6.82	788.64	5.50	6.50
23	76	168	1088	1336	6.53	11.17	859.11	5.75	7.00
24	80	164	808	1460	9.01	10.10	1126.13	5.50	7.00
25	100	160	1032	1348	8.83	10.61	883.39	7.25	6.50
26	120	172	912	1796	11.63	8.74	968.99	6.25	5.50
27	80	172	1040	1280	7.14	11.85	892.86	5.25	7.00
28	84	184	1120	1320	6.98	12.23	830.56	6.75	5.25
29	100	160	1160	1200	7.94	11.76	793.65	5.00	7.50
30	92	144	1024	1204	8.24	10.68	896.06	5.00	5.75
Min	**60.00**	**120.00**	**792.00**	**1200.00**	**5.86**	**6.62**	788.64	**3.75**	**5.00**
Max	**120.00**	**184.00**	**1164.00**	**1820.00**	**11.63**	**12.23**	1152.07	**7.50**	**8.50**
Mean	**89.73**	**163.20**	**1030.13**	**1461.47**	**8.05**	**10.16**	901.97	**5.74**	**6.58**
SD	**15.03**	**17.05**	**106.26**	**185.46**	**1.40**	**1.44**	95.97	**0.88**	**1.03**

Table 21 Stomatal number, stomatal index, palisade ratio, epidermal cell number, epidermal cell area and vein islet numbers of *E. crista-galli,* samples were collected from Nakhon Pathom 1 province

Field	Stomatal number (number per mm^2)		Epidermal cell number (number per mm^2)		Stomatal index		Upper epidermal cell area (µm^2)	Palisade ratio	Vein islet number (number per mm^2)
	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis			
1	100	148	1052	1456	8.68	9.23	868.06	3.75	7.00
2	112	168	960	1272	10.45	11.67	932.84	7.25	6.25
3	96	180	1024	1184	8.57	13.20	892.86	7.00	6.00
4	80	172	1036	1372	7.17	11.14	896.06	5.75	5.25
5	112	160	1080	1420	9.40	10.13	838.93	4.00	6.50
6	96	136	1156	1312	7.67	9.39	798.72	5.25	6.50
7	104	140	1272	1388	7.56	9.16	726.74	4.75	6.50
8	128	172	1080	1360	10.60	11.23	827.81	5.00	6.00
9	108	152	1168	1360	8.46	10.05	783.70	5.75	5.50
10	92	180	1096	1136	7.74	13.68	841.75	5.50	5.00
11	84	164	1088	1324	7.17	11.02	853.24	4.75	5.50
12	108	148	1156	1296	8.54	10.25	791.14	5.25	6.50
13	100	132	1432	1276	6.53	9.38	652.74	5.25	7.25
14	76	144	1116	1392	6.38	9.38	838.93	6.75	8.00
15	100	164	1032	1372	8.83	10.68	883.39	4.25	6.25
16	112	160	1144	1288	8.92	11.05	796.18	7.00	7.50
17	92	120	1140	1320	7.47	8.33	811.69	6.75	7.00
18	96	148	1252	1348	7.12	9.89	741.84	4.75	6.50
19	88	176	1136	1272	7.19	12.15	816.99	5.50	7.50
20	112	156	1136	1184	8.97	11.64	801.28	4.75	8.00
21	76	172	1152	1308	6.19	11.62	814.33	4.75	6.00
22	112	168	1164	1328	8.78	11.23	783.70	5.75	5.25
23	72	148	1088	1340	6.21	9.95	862.07	5.50	7.75
24	100	164	1208	1400	7.65	10.49	764.53	5.50	6.00
25	100	156	1140	1324	8.06	10.54	806.45	7.25	5.50
26	136	180	1128	1340	10.76	11.84	791.14	6.25	5.50
27	76	160	1052	1288	6.74	11.05	886.52	5.25	6.00
28	88	172	1132	1420	7.21	10.80	819.67	6.75	7.00
29	88	160	1024	1184	7.91	11.90	899.28	5.00	6.00
30	100	156	1196	1324	7.72	10.54	771.60	6.00	5.75
Min	72.00	120.00	960.00	1136.00	6.19	8.33	652.74	3.75	5.00
Max	136.00	180.00	1432.00	1456.00	10.76	13.68	932.84	7.25	8.00
Mean	98.13	158.53	1128.00	1319.60	8.02	10.75	819.81	5.57	6.38
SD	15.10	14.91	89.70	75.14	1.23	1.19	58.47	0.96	0.98

Table 22 Stomatal number, stomatal index, palisade ratio, epidermal cell number, epidermal cell area and vein islet numbers of *E. crista-galli,* samples were collected from Nakhon Pathom 2 province

Field	Stomatal number (number per mm^2)		Epidermal cell number (number per mm^2)		Stomatal index		Upper epidermal cell area (µm^2)	Palisade ratio	Vein islet number (number per mm^2)
	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis			
1	80	168	1052	1580	7.07	9.61	883.39	6.25	6.00
2	100	156	960	1288	9.43	10.80	943.40	8.50	7.00
3	92	112	1024	1192	8.24	8.59	896.06	6.25	7.75
4	76	160	1036	1200	6.83	11.76	899.28	6.00	7.00
5	72	160	1080	1384	6.25	10.36	868.06	7.25	6.50
6	80	168	1156	1240	6.47	11.93	809.06	4.75	6.00
7	88	128	1272	1328	6.47	8.79	735.29	7.25	5.50
8	80	168	1080	1204	6.90	12.24	862.07	5.00	7.75
9	108	160	1168	1224	8.46	11.56	783.70	5.75	6.00
10	92	164	1096	1260	7.74	11.52	841.75	5.25	8.00
11	84	160	1088	1612	7.17	9.03	853.24	5.00	8.50
12	84	156	1156	1460	6.77	9.65	806.45	5.00	7.00
13	88	140	1432	1448	5.79	8.82	657.89	6.00	6.25
14	76	156	1116	1440	6.38	9.77	838.93	6.00	6.25
15	72	120	1032	1356	6.52	8.13	905.80	5.00	6.25
16	88	112	1144	1200	7.14	8.54	811.69	8.00	7.50
17	88	128	1140	1220	7.17	9.50	814.33	7.75	7.00
18	96	140	1252	1416	7.12	9.00	741.84	9.25	6.25
19	88	168	1136	1244	7.19	11.90	816.99	8.50	7.75
20	100	160	1136	1468	8.09	9.83	809.06	6.50	8.00
21	88	156	1152	1400	7.10	10.03	806.45	8.75	7.25
22	92	160	1164	1060	7.32	13.11	796.18	6.75	7.00
23	76	156	1088	1160	6.53	11.85	859.11	6.25	6.50
24	104	168	1208	1248	7.93	11.86	762.20	7.25	7.75
25	104	156	1140	1280	8.36	10.86	803.86	7.75	5.50
26	120	180	1128	1340	9.62	11.84	801.28	7.00	5.50
27	112	160	1052	1424	9.62	10.10	859.11	7.25	6.00
28	100	176	1132	1400	8.12	11.17	811.69	7.75	6.00
29	88	180	1024	1520	7.91	10.59	899.28	5.50	8.00
30	68	168	1196	1196	5.38	12.32	791.14	7.25	5.00
Min	68.00	112.00	960.00	1060.00	5.38	8.13	657.89	4.75	5.00
Max	120.00	180.00	1432.00	1612.00	9.62	13.11	943.4	9.25	8.50
Mean	89.47	154.80	1128.00	1326.40	7.37	10.50	825.62	6.69	6.76
SD	12.54	18.38	89.70	132.95	1.05	1.37	58.75	1.26	0.92

Table 23 Stomatal number, stomatal index, palisade ratio, epidermal cell number, epidermal cell area and vein islet numbers of *E. subumbrans,* samples were collected from Chiang Mai 1 province

Field	Stomatal number (number per mm^2)		Epidermal cell number (number per mm^2)		Stomatal index		Upper epidermal cell area (µm^2)	Palisade ratio	Vein islet number (number per mm^2)
	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis			
1	36	132	1696	1856	2.08	6.64	577.37	4.25	16.50
2	28	140	1440	1984	1.91	6.59	681.20	5.50	15.00
3	20	136	1548	1780	1.28	7.10	637.76	3.75	15.00
4	20	120	1512	2120	1.31	5.36	652.74	5.00	17.00
5	20	124	1544	1740	1.28	6.65	639.39	4.25	13.75
6	36	112	1444	2060	2.43	5.16	675.68	5.00	20.25
7	28	104	1240	1972	2.21	5.01	788.64	3.75	19.50
8	20	124	1664	2108	1.19	5.56	593.82	6.00	12.75
9	20	140	1520	2196	1.30	5.99	649.35	5.25	15.00
10	28	156	1664	2184	1.65	6.67	591.02	4.75	15.25
11	20	132	1584	2272	1.25	5.49	623.44	4.25	19.50
12	12	116	1520	2204	0.78	5.00	652.74	5.00	16.25
13	28	128	1352	1952	2.03	6.15	724.64	6.00	17.50
14	32	140	1480	2072	2.12	6.33	661.38	4.50	15.25
15	12	112	1260	1760	0.94	5.98	786.16	4.75	14.00
16	28	120	1484	1960	1.85	5.77	661.38	5.00	18.00
17	28	112	1428	1860	1.92	5.68	686.81	5.00	14.50
18	24	136	1440	2000	1.64	6.37	683.06	5.25	15.25
19	28	152	1200	2240	2.28	6.35	814.33	3.75	15.50
20	20	116	1548	2340	1.28	4.72	637.76	4.25	15.00
21	20	132	1500	2320	1.32	5.38	657.89	6.25	18.25
22	24	172	1680	2120	1.41	7.50	586.85	3.50	18.50
23	16	116	1400	2048	1.13	5.36	706.21	5.00	16.00
24	28	152	1540	1792	1.79	7.82	637.76	4.25	20.75
25	44	140	1560	1620	2.74	7.95	623.44	3.50	17.50
26	40	140	1420	1600	2.74	8.05	684.93	4.25	16.50
27	28	120	1380	2208	1.99	5.15	710.23	4.00	16.00
28	28	116	1556	2280	1.77	4.84	631.31	4.50	18.75
29	28	108	1808	1812	1.53	5.63	544.66	4.75	16.00
30	36	108	1820	2200	1.94	4.68	538.79	5.50	18.50
Min	12.00	**104**	1200.00	**1600.00**	0.78	**4.68**	538.79	**3.50**	**12.75**
Max	44.00	**172**	1820.00	**2340.00**	2.74	**8.05**	814.33	**6.25**	**20.75**
Mean	26.00	**129.13**	1507.73	**2022.00**	1.70	**6.03**	659.18	**4.69**	**16.58**
SD	7.63	**16.30**	147.04	**208.78**	0.50	**0.96**	65.00	**0.73**	**2.02**

Table 24 Stomatal number, stomatal index, palisade ratio, epidermal cell number, epidermal cell area and vein islet numbers of *E. subumbrans*, samples were collected from Chiang Mai 2 province

Field	Stomatal number (number per mm^2)		Epidermal cell number (number per mm^2)		Stomatal index		Upper epidermal cell area (μm^2)	Palisade ratio	Vein islet number (number per mm^2)
	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis			
1	20	164	1420	1560	1.39	9.51	683.06	5.50	14.00
2	24	160	1440	1644	1.64	8.87	814.33	5.50	16.25
3	28	156	1200	1600	2.28	8.88	654.45	5.00	16.00
4	28	140	1500	1600	1.83	8.05	649.35	6.00	17.50
5	20	128	1520	1648	1.30	7.21	694.44	5.00	14.00
6	40	120	1400	1500	2.78	7.41	811.69	5.25	15.50
7	32	144	1200	1420	2.60	9.21	647.67	6.25	18.00
8	24	144	1520	1592	1.55	8.29	877.19	6.50	16.25
9	20	152	1120	1528	1.75	9.05	862.07	5.00	15.00
10	20	148	1140	1500	1.72	8.98	909.09	5.25	15.50
11	20	120	1080	1820	1.82	6.19	833.33	7.25	20.00
12	16	120	1184	1804	1.33	6.24	841.75	4.75	17.25
13	28	136	1160	1552	2.36	8.06	816.99	7.00	16.50
14	28	144	1196	1524	2.29	8.63	809.06	5.00	16.25
15	28	120	1208	1360	2.27	8.11	865.05	5.25	15.00
16	20	160	1136	1580	1.73	9.20	791.14	5.50	16.00
17	20	152	1244	1660	1.58	8.39	757.58	6.50	18.00
18	20	144	1300	1664	1.52	7.96	811.69	5.50	16.25
19	32	140	1200	1676	2.60	7.71	708.22	7.00	17.50
20	12	120	1400	1812	0.85	6.21	690.61	6.00	16.00
21	20	116	1428	1536	1.38	7.02	692.52	7.25	19.25
22	24	160	1420	1584	1.66	9.17	809.06	4.50	17.50
23	16	172	1220	1640	1.29	9.49	853.24	5.00	15.50
24	32	112	1140	1536	2.73	6.80	621.89	4.75	20.00
25	16	156	1592	1632	1.00	8.72	850.34	5.00	16.50
26	24	156	1152	1600	2.04	8.88	819.67	5.50	17.00
27	20	140	1200	1732	1.64	7.48	793.65	6.00	15.75
28	12	140	1248	1460	0.95	8.75	819.67	6.00	16.00
29	20	120	1200	1400	1.64	7.89	850.34	5.00	16.00
30	36	168	1140	1400	3.06	10.71	915.75	5.50	15.50
Min	12.00	**112**	1080.00	**1360.00**	0.85	**6.19**	621.89	**4.50**	**14.00**
Max	40.00	**172**	1592.00	**1820.00**	3.06	**10.71**	915.75	**7.25**	**20.00**
Mean	23.33	**141.75**	1276.93	**1585.47**	1.82	**8.24**	784.14	**5.65**	**16.53**
SD	6.73	**17.22**	145.25	**117.41**	0.57	**1.08**	83.60	**0.78**	**1.48**

Table 25 Stomatal number, stomatal index, palisade ratio, epidermal cell number, epidermal cell area and vein islet numbers of *E. subumbrans,* samples were collected from Chiang Rai province

Field	Stomatal number (number per mm^2)		Epidermal cell number (number per mm^2)		Stomatal index		Upper epidermal cell area (μm^2)	Palisade ratio	Vein islet number (number per mm^2)
	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis			
1	16	160	1600	1192	0.99	11.83	618.81	3.75	13.75
2	20	152	1560	1476	1.27	9.34	632.91	5.00	14.00
3	28	132	1520	1380	1.81	8.73	645.99	4.00	15.00
4	28	116	1480	1412	1.86	7.59	663.13	4.50	15.00
5	20	128	1520	1060	1.30	10.77	649.35	4.75	12.75
6	28	120	1440	1600	1.91	6.98	681.20	5.50	18.50
7	24	112	1288	1432	1.83	7.25	762.20	4.50	19.00
8	16	160	1252	1584	1.26	9.17	788.64	6.50	13.00
9	32	156	1240	1200	2.52	11.50	786.16	6.25	18.50
10	28	160	1264	1200	2.17	11.76	773.99	5.00	16.75
11	20	160	1552	1200	1.27	11.76	636.13	4.75	18.50
12	16	140	1440	1352	1.10	9.38	686.81	6.00	16.50
13	16	144	1376	1392	1.15	9.38	718.39	5.50	18.00
14	20	140	1504	1260	1.31	10.00	656.17	5.00	16.25
15	24	116	1320	1200	1.79	8.81	744.05	6.50	14.50
16	32	116	1284	1208	2.43	8.76	759.88	5.00	14.75
17	28	120	1604	1460	1.72	7.59	612.75	5.50	15.00
18	20	128	1464	1400	1.35	8.38	673.85	4.25	16.50
19	20	140	1460	1440	1.35	8.86	675.68	4.50	16.50
20	28	120	1180	1140	2.32	9.52	827.81	5.50	18.00
21	24	124	1516	1192	1.56	9.42	649.35	5.00	16.25
22	20	176	1624	1320	1.22	11.76	608.27	4.00	17.50
23	16	104	1464	1300	1.08	7.41	675.68	5.00	15.00
24	24	152	1520	1232	1.55	10.98	647.67	4.50	20.25
25	28	120	1288	1224	2.13	8.93	759.88	5.00	18.50
26	32	156	1204	1228	2.59	11.27	809.06	5.50	17.50
27	32	168	1360	1240	2.30	11.93	718.39	4.50	18.75
28	28	120	1340	1244	2.05	8.80	730.99	4.00	18.75
29	36	116	1688	1260	2.09	8.43	580.05	5.00	17.00
30	24	128	1580	1204	1.50	9.61	623.44	6.25	18.50
Min	16.00	**104**	1180.00	**1060.00**	0.99	**6.98**	580.05	**3.75**	**12.75**
Max	36.00	**176**	1688.00	**1600.00**	2.59	**11.93**	827.81	**6.50**	**20.25**
Mean	24.27	**136.38**	1431.07	**1301.07**	1.69	**9.53**	693.89	**5.03**	**16.63**
SD	5.75	**19.64**	138.87	**130.58**	0.47	**1.51**	66.99	**0.75**	**1.97**

Table 26 Stomatal number, stomatal index, palisade ratio, epidermal cell number, epidermal cell area and vein islet numbers of *E. variegata*, samples were collected from Bangkok province

Field	Stomatal number (number per mm^2)		Epidermal cell number (number per mm^2)		Stomatal index		Upper epidermal cell area (μm^2)	Palisade ratio	Vein islet number (number per mm^2)
	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis			
1	12	156	488	804	2.40	16.25	2000.00	7.75	6
2	16	168	464	848	3.33	16.54	2083.33	9.75	7
3	12	160	424	832	2.75	16.13	2293.58	9.50	8
4	12	164	500	812	2.34	16.80	1953.13	11.50	7
5	4	156	440	876	0.90	15.12	2252.25	7.50	5
6	16	152	436	888	3.54	14.62	2212.39	9.50	8
7	8	128	412	884	1.90	12.65	2380.95	7.50	8
8	8	128	416	780	1.89	14.10	2358.49	9.25	7
9	12	148	480	804	2.44	15.55	2032.52	7.75	8
10	8	132	460	800	1.71	14.16	2136.75	9.75	8
11	12	136	488	772	2.40	14.98	2000.00	7.75	8
12	8	152	448	852	1.75	15.14	2192.98	8.75	8
13	8	144	440	868	1.79	14.23	2232.14	9.50	9
14	4	124	484	796	0.82	13.48	2049.18	9.00	7
15	4	152	420	916	0.94	14.23	2358.49	10.50	9
16	4	140	464	840	0.85	14.29	2136.75	9.75	7
17	8	164	468	900	1.68	15.41	2100.84	10.00	7
18	16	136	512	948	3.03	12.55	1893.94	11.25	7
19	8	148	492	904	1.60	14.07	2000.00	9.75	7
20	12	132	488	872	2.40	13.15	2000.00	10.00	6
21	12	160	404	908	2.88	14.98	2403.85	9.25	7
22	28	144	500	852	5.30	14.46	1893.94	8.25	8
23	20	156	496	856	3.88	15.42	1937.98	9.50	8
24	8	172	440	952	1.79	15.30	2232.14	8.25	8
25	8	132	432	856	1.82	13.36	2272.73	9.00	7
26	4	160	460	908	0.86	14.98	2155.17	8.75	8
27	12	148	480	840	2.44	14.98	2032.52	10.00	8
28	4	148	448	896	0.88	14.18	2212.39	9.50	8
29	8	128	496	900	1.59	12.45	1984.13	11.25	11
30	8	128	464	828	1.69	13.39	2118.64	8.75	8
Min	4.00	124.00	404.00	772.00	0.82	12.45	1893.94	7.50	5
Max	28.00	172.00	512.00	952.00	5.30	16.80	2403.85	11.50	11
Mean	10.13	146.53	461.47	859.73	2.12	14.56	2131.53	9.28	7.63
SD	5.33	13.68	30.18	47.54	1.02	1.14	148.64	1.07	1.07

Table 27 Stomatal number, stomatal index, palisade ratio, epidermal cell number, epidermal cell area and vein islet numbers of E. *variegata,* samples were collected from Pathum Thani province

Field	Stomatal number (number per mm^2)		Epidermal cell number (number per mm^2)		Stomatal index		Upper epidermal cell area (µm^2)	Palisade ratio	Vein islet number (number per mm^2)
	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis			
1	8	116	464	644	1.69	15.26	2118.64	9.00	6.5
2	4	168	520	684	0.76	19.72	1908.40	8.75	5.5
3	12	96	464	496	2.52	16.22	2100.84	9.75	5.3
4	16	124	448	632	3.45	16.40	2155.17	10.25	5.5
5	12	128	484	716	2.42	15.17	2016.13	9.75	4.8
6	8	120	472	632	1.67	15.96	2083.33	10.50	5.8
7	4	120	484	548	0.82	17.96	2049.18	11.00	5.0
8	20	152	512	720	3.76	17.43	1879.70	9.50	6.0
9	8	88	460	572	1.71	13.33	2136.75	7.25	5.0
10	12	100	480	800	2.44	11.11	2032.52	10.75	6.0
11	8	120	460	772	1.71	13.45	2136.75	10.50	6.3
12	12	128	488	852	2.40	13.06	2000.00	10.75	6.0
13	12	132	524	868	2.24	13.20	1865.67	11.00	5.5
14	12	100	456	796	2.56	11.16	2136.75	11.00	6.5
15	16	144	532	916	2.92	13.58	1824.82	11.50	6.0
16	20	120	516	840	3.73	12.50	1865.67	10.00	6.5
17	8	140	500	900	1.57	13.46	1968.50	10.75	7.3
18	8	116	496	948	1.59	10.90	1984.13	10.75	6.8
19	4	116	460	904	0.86	11.37	2155.17	11.00	6.5
20	8	112	448	872	1.75	11.38	2192.98	13.25	7.0
21	12	128	456	908	2.56	12.36	2136.75	13.50	6.5
22	8	140	448	852	1.75	14.11	2192.98	10.25	6.8
23	12	112	504	856	2.33	11.57	1937.98	12.25	3.8
24	8	140	440	952	1.79	12.82	2232.14	10.75	6.3
25	4	144	480	856	0.83	14.40	2066.12	11.25	6.5
26	4	136	452	908	0.88	13.03	2192.98	11.75	5.8
27	8	132	456	840	1.72	13.58	2155.17	11.75	5.3
28	12	116	532	896	2.21	11.46	1838.24	12.25	6.3
29	12	124	524	900	2.24	12.11	1865.67	10.75	5.3
30	12	128	488	828	2.40	13.39	2000.00	10.00	5.0
Min	**4.00**	**88.00**	**440.00**	**496.00**	**0.76**	**10.90**	1824.82	**7.25**	**3.8**
Max	**20.00**	**168.00**	**532.00**	**952.00**	**3.76**	**19.72**	2232.14	**13.50**	**7.25**
Mean	**10.13**	**124.67**	**481.60**	**796.93**	**2.04**	**13.72**	2040.19	**10.72**	**5.87**
SD	**4.30**	**17.07**	**28.45**	**126.41**	**0.80**	**2.21**	123.25	**1.26**	**0.77**

Table 28 Stomatal number, stomatal index, palisade ratio, epidermal cell number, epidermal cell area and vein islet numbers of *E. variegata*, samples were collected from Prachin Buri province

Field	Stomatal number (number per mm^2)		Epidermal cell number (number per mm^2)		Stomatal index		Upper epidermal cell area (µm^2)	Palisade ratio	Vein islet number (number per mm^2)
	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis			
1	16	160	488	800	2.40	16.67	2000.00	7.50	6.25
2	12	180	464	892	3.33	16.79	2083.33	10.00	5.00
3	12	160	424	836	2.75	16.06	2293.58	9.25	6.25
4	4	132	500	816	2.34	13.92	1953.13	11.25	7.00
5	16	136	440	880	0.90	13.39	2252.25	7.25	6.50
6	8	160	436	884	3.54	15.33	2212.39	8.75	5.25
7	8	140	412	880	1.90	13.73	2380.95	8.00	5.50
8	12	132	416	800	1.89	14.16	2358.49	8.50	6.00
9	8	152	480	804	2.44	15.90	2032.52	7.75	5.75
10	12	140	460	800	1.71	14.89	2136.75	10.00	7.50
11	8	144	488	776	2.40	15.65	2000.00	8.00	8.50
12	8	156	448	844	1.75	15.60	2192.98	9.00	6.00
13	4	148	440	860	1.79	14.68	2232.14	9.50	7.25
14	4	120	484	788	0.82	13.22	2049.18	9.00	8.50
15	4	160	420	900	0.94	15.09	2358.49	10.50	6.25
16	8	140	464	860	0.85	14.00	2136.75	10.00	7.50
17	16	176	468	880	1.68	16.67	2100.84	11.00	6.25
18	8	140	512	952	3.03	12.82	1893.94	10.75	6.25
19	12	156	492	904	1.60	14.72	2000.00	9.25	7.50
20	12	140	488	872	2.40	13.83	2000.00	9.75	8.00
21	28	168	404	908	2.88	15.61	2403.85	9.00	7.25
22	20	148	500	852	5.30	14.80	1893.94	7.75	6.50
23	8	156	496	856	3.88	15.42	1937.98	8.00	7.00
24	8	168	440	952	1.79	15.00	2232.14	8.75	7.00
25	4	136	432	856	1.82	13.71	2272.73	9.25	5.00
26	12	180	460	908	0.86	16.54	2155.17	8.50	5.50
27	4	152	480	840	2.44	15.32	2032.52	9.75	7.00
28	8	152	448	896	0.88	14.50	2212.39	8.75	5.25
29	8	132	496	900	1.59	12.79	1984.13	10.75	7.50
30	16	132	464	828	1.69	13.75	2118.64	11.00	5.75
Min	**4.00**	**120.00**	**404.00**	**776.00**	**0.82**	**12.79**	1893.94	**7.25**	**5.00**
Max	**28.00**	**180.00**	**512.00**	**952.00**	**5.30**	**16.79**	2403.85	**11.25**	**8.50**
Mean	**10.13**	**149.87**	**461.47**	**860.80**	**2.12**	**14.82**	2131.53	**9.22**	**6.57**
SD	**5.33**	**15.29**	**30.18**	**46.00**	**1.02**	**1.15**	148.64	**1.13**	**0.98**

Table 29 Stomatal number, stomatal index, palisade ratio, epidermal cell number, epidermal cell area and vein islet numbers of *E. indica*, samples were collected from Chiang Rai province

Field	Stomatal number (number per mm^2)		Epidermal cell number (number per mm^2)		Stomatal index		Upper epidermal cell area (µm^2)	Palisade ratio	Vein islet number (number per mm^2)
	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis			
1	0	233	488	508	0	31.44	2049.18	4.50	3.75
2	0	262	464	520	0	33.50	2155.17	6.25	4.50
3	0	226	424	464	0	32.75	2358.49	5.25	5.00
4	0	252	500	500	0	33.51	2000.00	6.50	5.25
5	0	242	440	440	0	35.48	2272.73	5.25	5.50
6	0	231	436	480	0	32.49	2293.58	6.75	4.00
7	0	223	412	376	0	37.23	2427.18	6.00	5.25
8	0	210	416	436	0	32.51	2403.85	5.75	6.00
9	0	213	480	452	0	32.03	2083.33	5.75	6.00
10	0	269	460	480	0	35.91	2173.91	5.75	4.00
11	0	256	488	420	0	37.87	2049.18	5.75	4.00
12	0	246	448	448	0	35.45	2232.14	7.50	5.00
13	0	264	440	500	0	34.55	2272.73	7.25	5.25
14	0	232	484	452	0	33.92	2066.12	6.50	5.50
15	0	232	420	428	0	35.15	2380.95	6.00	4.00
16	0	228	464	476	0	32.39	2155.17	6.25	3.75
17	0	204	468	416	0	32.90	2136.75	6.75	5.00
18	0	224	512	448	0	33.33	1953.13	6.00	3.75
19	0	216	492	396	0	35.29	2032.52	6.00	4.25
20	0	236	488	496	0	32.24	2049.18	7.25	3.50
21	0	240	404	452	0	34.68	2475.25	6.50	4.00
22	0	236	500	496	0	32.24	2000.00	5.50	5.75
23	0	252	496	476	0	34.62	2016.13	6.25	4.25
24	0	256	440	488	0	34.41	2272.73	7.50	3.75
25	0	224	432	452	0	33.14	2314.81	6.50	3.50
26	0	252	460	476	0	34.62	2173.91	7.25	3.50
27	0	232	480	500	0	31.69	2083.33	5.75	4.25
28	0	244	448	480	0	33.70	2232.14	7.25	4.50
29	0	236	496	452	0	34.30	2016.13	6.50	4.25
30	0	216	464	444	0	32.73	2155.17	6.50	4.00
Min	0.00	**204.00**	**404.00**	**376.00**	0.00	**31.44**	**1953.13**	**4.50**	**3.50**
Max	0.00	**269.00**	**512.00**	**520.00**	0.00	**37.87**	**2475.25**	**7.50**	**6.00**
Mean	0.00	**236.23**	**461.47**	**461.73**	0.00	**33.87**	**2176.16**	**6.28**	**4.50**
SD	0.00	**16.67**	**30.18**	**34.00**	0.00	**1.58**	**145.15**	**0.72**	**0.78**

Table 30 Stomatal number, stomatal index, palisade ratio, epidermal cell number, epidermal cell area and vein islet numbers of *E. indica*, samples were collected from Chaiyaphum province

Field	Stomatal number (number per mm^2)		Epidermal cell number (number per mm^2)		Stomatal index		Upper epidermal cell area (μm^2)	Palisade ratio	Vein islet number (number per mm^2)
	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis			
1	0	168	608	688	0	19.63	1644.74	8.50	6.25
2	0	136	656	784	0	14.78	1524.39	8.00	6.25
3	0	132	592	720	0	15.49	1689.19	9.25	5.25
4	0	176	676	864	0	16.92	1479.29	8.50	5.00
5	0	152	708	792	0	16.10	1412.43	7.50	4.75
6	0	140	624	812	0	14.71	1602.56	7.50	5.25
7	0	160	684	760	0	17.39	1461.99	8.00	5.00
8	0	160	628	788	0	16.88	1592.36	9.25	6.00
9	0	164	612	868	0	15.89	1633.99	8.25	6.75
10	0	180	680	752	0	19.31	1470.59	9.25	5.50
11	0	156	568	772	0	16.81	1760.56	8.00	7.00
12	0	160	540	756	0	17.47	1851.85	7.00	5.00
13	0	172	608	804	0	17.62	1644.74	8.50	7.25
14	0	184	604	768	0	19.33	1655.63	8.25	7.00
15	0	188	644	760	0	19.83	1552.80	7.00	6.75
16	0	164	584	788	0	17.23	1712.33	8.50	6.25
17	0	156	564	748	0	17.26	1773.05	6.25	5.75
18	0	168	608	788	0	17.57	1644.74	7.50	5.50
19	0	160	640	776	0	17.09	1562.50	7.50	6.50
20	0	184	648	816	0	18.40	1543.21	6.75	7.25
21	0	136	560	812	0	14.35	1785.71	9.25	7.00
22	0	168	600	808	0	17.21	1666.67	7.00	6.50
23	0	152	560	812	0	15.77	1785.71	9.00	7.25
24	0	180	636	804	0	18.29	1572.33	9.75	6.00
25	0	160	544	828	0	16.19	1838.24	9.00	6.25
26	0	164	608	816	0	16.73	1644.74	6.75	4.75
27	0	160	572	784	0	16.95	1748.25	8.25	6.00
28	0	168	512	748	0	18.34	1953.13	9.50	6.75
29	0	172	684	876	0	16.41	1461.99	8.75	6.00
30	0	188	632	796	0	19.11	1582.28	7.75	5.25
Min	0.00	**132**	512.00	**688.00**	0.00	**14.35**	1412.43	**6.25**	**4.75**
Max	0.00	**188**	708.00	**876.00**	0.00	**19.83**	1953.13	**9.75**	**7.25**
Mean	0.00	**163.38**	612.80	**789.60**	0.00	**17.17**	1641.73	**8.14**	**6.07**
SD	0.00	**14.94**	48.14	**40.80**	0.00	**1.44**	130.64	**0.93**	**0.80**

Table 31 Stomatal number, stomatal index, palisade ratio, epidermal cell number, epidermal cell area and vein islet numbers of *E. indica*, samples were collected from Nakhon Ratchasima province

Field	Stomatal number (number per mm^2)		Epidermal cell number (number per mm^2)		Stomatal index		Upper epidermal cell area (μm^2)	Palisade ratio	Vein islet number (number per mm^2)
	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis			
1	0	140	548	640	0	17.95	1824.82	8.00	8.25
2	0	120	636	668	0	15.23	1572.33	9.50	8.00
3	0	148	604	688	0	17.70	1655.63	12.25	6.50
4	0	144	596	684	0	17.39	1677.85	10.00	6.00
5	0	116	612	728	0	13.74	1633.99	9.00	6.75
6	0	124	568	696	0	15.12	1760.56	10.75	7.50
7	0	120	572	624	0	16.13	1748.25	9.00	6.25
8	0	140	544	768	0	15.42	1838.24	8.75	8.25
9	0	152	584	660	0	18.72	1712.33	8.50	8.00
10	0	124	556	700	0	15.05	1798.56	10.25	8.75
11	0	132	560	760	0	14.80	1785.71	12.25	8.00
12	0	144	560	744	0	16.22	1785.71	8.75	9.75
13	0	140	548	744	0	15.84	1824.82	9.00	9.00
14	0	148	548	716	0	17.13	1824.82	9.25	7.00
15	0	120	608	692	0	14.78	1644.74	9.25	8.00
16	0	152	616	648	0	19.00	1623.38	7.50	7.00
17	0	144	536	704	0	16.98	1865.67	10.25	7.75
18	0	132	612	760	0	14.80	1633.99	11.25	7.50
19	0	136	600	668	0	16.92	1666.67	10.00	7.75
20	0	140	648	724	0	16.20	1543.21	9.25	8.25
21	0	108	540	660	0	14.06	1851.85	9.75	9.25
22	0	128	588	688	0	15.69	1700.68	8.75	8.50
23	0	136	592	660	0	17.09	1689.19	8.50	8.25
24	0	128	624	612	0	17.30	1602.56	9.25	8.25
25	0	128	560	640	0	16.67	1785.71	9.00	8.25
26	0	132	616	704	0	15.79	1623.38	11.75	8.25
27	0	120	608	696	0	14.71	1644.74	11.50	8.75
28	0	152	580	784	0	16.24	1724.14	8.75	7.75
29	0	148	648	708	0	17.29	1543.21	10.00	7.25
30	0	128	604	680	0	15.84	1655.63	9.00	8.50
Min	0.00	**108**	**536.00**	**612.00**	0.00	**13.74**	**1543.21**	**7.50**	**6.00**
Max	0.00	**152**	**648.00**	**784.00**	0.00	**19.00**	**1865.67**	**12.25**	**9.75**
Mean	0.00	**133.88**	**587.20**	**694.93**	0.00	**16.19**	**1708.08**	**9.63**	**7.91**
SD	0.00	**11.96**	**32.64**	**43.55**	0.00	**1.31**	**94.70**	**1.21**	**0.86**

Appendix B

Molecular evaluation

The ITS, *mat*K, *psb*A_*trn*H, *rpo*C, and *ycf*1 sequence of six *Erythrina* species distributed in Thailand and outgroup plants

The ITS sequence of six *Erythrina* species and outgroup plants

>*E. fusca*

CTGAAGTTCTCGCTATGAAACTCCGTCGAAGTGTGTGTTTATGGGTCACGAGTCAAACAC
AGGGTGAGTCACCCACAATCCGGCCCCAACCCACCAAACTCATCCACCATTTTAACCTGA
CCCCCCCAACTGCAAACTCAATTTTCAGCCAACCACCAGACAATGCTCATGGGAAGCCAA
CTTCCACCTACTCCGTTCGGAAAACGTCTTACGACATTTGCATGAGGCAGGAGGGTAACA
ATGTGTGACACCCAGGCAGGCGTGCCCTCAACCTAATGGCATCGGGCGCAACTTGCGTTC
AAAGACTCGATGGTTCACGGGATTCTGCAATTCACACCAAGTATCGCATTTCGCTACGTT
CTTCATCGATGCAAGAGCCGAGATATCCGTTGCCGAGAGTCATTTTGCATTGTGCATCAT
GACTAACCCTCGAGAGGCGTCATCTCTGACCTTCCCAAGGATCACACATAAAAGTTTTCA
ATTCCTTGGCACGCAAAGCGCCGGGGTTTGAGTTTTGCCAGGACGAGAAAGCATGGCCAC
CTCCTCCCGACAGAAAGGAAAGACACGGATCTTTCCCTTCGTCAGTAAACAAATTCAACG
GTCAAACTGATTGTGAGGCATC

>*E. stricta*

GTCGATGCCTCACGACCAGTCCGACCGTCGAATCTGTCTGCTCACGAGAGGATGGAGGGA
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TGAATCTAATCTACGGATATCAAGGCTCTTGCCTGGCTTAAGAACGAAACAAAATGGAAT
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TCCTCTGCCTCGCCCTGGCGTAAAAATAGGATAACGTAAGGGAATGGGAGGGGGTTGACT
TCGCCTCCCCAGCATCGCCTTGTGCTTGGGTTAGATTTAAAGTCAAGGTCCAGCCCGACC
CCTCCACGAGACGGGACGAGGACAAGCCCTAGGCCAGGCCAGCCCGGCCCGGCCCGGGCT
CGAGTCCAATCCCCAACACAAAACCAGGCACCTCTCGTTATGATACGAC

>*E. crista-galli*

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GCACTAGACCAGTTCTGCGCCTGTGAACCTGTCTTTGACTCTTGACCCGTACTCAACAGT
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>*E. subumbrans*
GTCGATGCCTCACGACCAGTCCGACCGTCGAATCTGTCTGCTCACGAGAGGATGGAAGGA
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CGACTCGCGACCCATAAACAAATCCCCTGACGTCTCGTAAGGAGACCAC

>*E. variegata*
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>*E. indica*
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GGATGTTTCATAGCGAGACCTC

>*P. indicus*

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CTGCATGCACGCATGCACGGTGCTTC

>*M. hortensis*

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CCCAAATGACTGCCCGCGACGGTGCACGTCACTACCAGTGGTGGTTGAACGTCAACTCTC
ATGCTGCGGTGTGACGCCGCATCGCCGTCTCGGGGACCATCACCGACCCAACGGGCTCCT
GCATGCACGCATGCACGGTGCTTC

The *mat*K sequence of six *Erythrina* species and outgroup plants

>*E. fusca*

ATATTTTATTTGATACAAACTCTTTTTTTTTTCAAATCCATTGTAATAATGAGAAAAATT
TCTACATATCTGCAAAAATAGGTCAATAATATCAAAATCAGATAAATTGACCCAAACCGG
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A

>*E. stricta*

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>*E. crista-galli*

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A

>*E. subumbrans*
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>*E. variegata*
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>*E. indica*
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>*P. indicus*
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AGA

>*M. hortensis*
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AGTAATAATACTATTCCAATTACAATACTCATTGAGAAAGAATCGTAATAAATGCAAAGA
AGA

The *psb*A_*trn*H sequence of six *Erythrina* species and outgroup plants

>*E.fusca*

TCTAGCTGTGATCGAAGTTCCATCTATAAATGGATAAATTTCGGATCTTACATTACAGAT
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CTATAAGGGCG

>*E.stricta*

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TTTTACTATTTTACTA

>*E.crista-galli*

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CCTCCTTTACTTTTCTTTTTCTTGACATACGTTTTTTGATTTTTTTCAGGATCTTTTAGC
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TTTTACTATAAGGGCG

>*E.subumbrans*

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>*E.variegata*

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TTTTACTATAAGGGCG

>*E.indica*

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>*P.indicus*

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>*M.hortensis*

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GATCCAAATGCAATCTTTTGTTTTACAATTTCT

The *rpo*C sequence of six *Erythrina* species and outgroup plants

>*E.fusca*

GGTCGTTCGGTCATTGTCGTAGGTCCATCACTTTCATTACATAGATGTGGATTGCCTCGT
GAAATAGCAATAGAACTTTTCCAGACATTTCTAATTCGTGGTCTAATTCGAAAACATTTT
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CCTTTATCTTTAGAA

>*E.stricta*

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CCTTTATCTTTAGAA

>*E.crista-galli*

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CGTTTATCTTTAGAG

>*E.subumbrans*

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CCTTTATCTTTAGAA

>*E.variegata*

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CCTTTATCTTTAGAA

>*E.indica*

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>*P.indicus*

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>*M.hortensis*

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CCTTTATCTTTGGAG

The *ycf*1 sequence of six *Erythrina* species and outgroup plants

>E.fusca

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>E.stricta

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>E.crista-galli

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>*E.sumumbrans*
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>*E.variegata*
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>*E.indica*
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TCTTTTTTCTTCATTAGTATCCCCTC

>*P.indicus*
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>*M.hortensis*
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TATAAACTC

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Mr. Kitipan Khaonim was born on March 29, 1992, in Trang, Thailand. He received his Bachelor's degree in Sciences (Oriental Medicine) from Rangsit University in 2014. He applied to study Master of Sciences Program in Public Health Sciences in 2015 at College of Public Health Sciences, Chulalongkorn University, Thailand.

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