Genetic and Morphological Diversity and Distribution of *Etlingera littoralis* (König) Giseke (Zingiberaceae) in Southern Thailand

Wassana Chongkraijak

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science Program in Ecology and Biodiversity Walailak University

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Author	Wassana Chongkraijak
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Thesis Title	ความหลากหลายทางพันธุกรรมและสัณฐานวิทยา และการกระจายพันธุ์ของปุดคางคก (*Etlingera littoralis*) ในภาคใต้ของประเทศไทย
Author	วาสนา จงไกรจักร์
Major Program	นิเวศวิทยาและความหลากหลายทางชีวภาพ
Academic Year	2554

บทคัดย่อ

ปุดคางปก (*Etlingera littoralis*) เป็นพืชในวงศ์ขิงข่า มีการกระจายพันธุ์อย่างกว้างขวางทั้งฝั่งอ่าวไทยและฝั่งทะเลอันดามัน ในภาคใต้ของประเทศไทย การศึกษาลักษณะสัณฐานวิทยาและอนุวิทยาของปุดคางคงกับปุดชนิดอื่นที่มีความใกล้ชิดกัน (*E. megalocheilos* และ *Etlingera* sp.) พบว่า ปุดคางคกมีความแตกต่างทางพันธุกรรมกับ *E. megalocheilos* อย่างชัดเจน ถึงแม้ว่า *E. megalocheilos* ยังไม่มีรายงานการค้นพบในการศึกษาครั้งนี้ การศึกษาครั้งนี้ได้มีการค้นพบปุดชนิดใหม่ที่ไม่สารถระบุสายพันธุ์ได้ (*Etlingera* sp.) ซึ่งมีลักษณะทางสัณฐานวิทยาคล้ายกับปุดคางคก และจากการศึกษาลักษณะทางสัณฐานวิทยาด้วยวิธีทางสถิติโดยโปรแกรมอาร์ (R Program) ผลการศึกษาพบว่าส่วนอวัยวะสืบพันธ์ของทั้งสองชนิดมีความแตกต่างอย่างชัดเจน (รูปแบบของช่อดอก, ความยาวของกลีบดอก, สัดส่วนความยาวของเกสรตัวผู้กับกลีบดอก และการทำมุมของละอองเรณูกับก้านชูดอก) นอกจากนี้การ ศึกษาความใกล้ชิดทางพันธุกรรมโดยใช้ข้อมูลดีเอ็นเอจากนิวเครียส (ITS1 และ ITS2) และ คลอโรพลาสต์ (*mat*K) ของปุดคางคก 11 ตัวอย่าง *Etlingera* sp. 17 ตัวอย่าง และ *E. araneosa* 2 ตัวอย่าง ด้วยวิธีการวิเคราะห์ข้อมูลทางพันธุกรรมแบบมัธยัสต์ (Maximum Parsimony), แบบวิเคราะห์ความเป็นไปได้มากที่สุด (Maximum likelihood) และการวิเคราะห์แบบเบย์ (Bayesian analysis) พบว่า ปุดคางคก และ *Etlingera* sp. มีความแตกต่างทางพันธุกรรมอย่างชัดเจนด้วยค่าสนับสนุนทางสถิติ (Bootstrap support) ที่สูง ดังนั้นจากหลักฐานทั้งทางสัณฐานวิทยาและอนุวิทยาแสดงให้เห็นว่าปุดคางคงกับ *Etlinera* sp. เป็นปุดต่างชนิดกัน

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Abstract

Etlingera littoralis is a common ground species in Zingiberaceae. It is widely distributed in Peninsular Thailand both Gulf of Thailand and Andaman Sea. Morphological characters and molecular data of *E. littoralis* and two related species, *E. megalocheilos* and *Etlingera* sp. were studied. Although, *E. megalocheilos* have not yet been found in this study, but its morphological characters from previous studies indicated that *E. littoralis* and *E. megalocheilos* are different species. Interestingly, an unknown *Etlingera* sp. and *E. araneosa* were found instead. The morphological character of *Etlingera* sp. which is superficially similar to *E. littoralis* were studied using R statistic. The results showed that *E. littoralis* and *Etlingera* sp. can be clearly separated by reproductive parts (inflorescence color pattern, labellum length, labellum and stamen length ratio, and angle of anther). Eleven samples of *E. littoralis*, seventeen samples of *Etlingera* sp., and two samples of *E. araneosa* were sequenced for the nuclear internal transcribed spacer (ITS1 and ITS2) loci and the partial plastid *mat*K region. Maximum parsimony (MP) analyses of both individual and combined data sets identified two different clades; *E. littoralis* and *Etlingera* sp. clades with high bootstrap values. The two clades were also supported by maximum likelihood (ML) and Bayesian analyses with high bootstrap values. Both morphology and molecular evidences strongly support that *E. littoralis* and *Etlingera* sp. may be classified as two different species.

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I wish to express my deep gratitude to Asst. Prof. Dr. Chatchai Ngamraibsakul, my advisor, for his guidance and encouragement throughout my study. He paid a lot of time to help me collecting sample, supporting my field work, and especially answering my entire question, reading and correcting my English.

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Wassana Chongkraijak

Table of Contents

Table of Contents (cont'd)

List of Tables

List of Figures

List of Figures (Cont'd)

List of Figures (Cont'd)

Chapter 1

Introduction

1.1 Background and Rationale

The Southern Thailand or Peninsula Thailand is divided into west and east sides by north-south lying mountain ranges. There is not much different of the temperature in the Peninsula among seasons. There are many characters of the geography; shore, island and mountain range e.g. Phuket mountain, Nakhon Si Thammarat mountain and San Kala Kiri mountain ranges. The weather character is tropical. It is usually rainy and hot that it is alternated with dry period in a very short-term. The temperature on the average is from 26 to 28 degree celsius. Thus, the weather and geographical characters are suitable for plants, such as *Etlingera,* which many species, can be found.

Etlingera littoralis (König) Giseke is widely distributed in Malay Peninsula, and Southern Thailand. The type specimen of *E. littoralis* was from Phuket province, Thailand, but lost in the sea (Burtt and Smith, 1986). Later researchers who studied this species used only its description as a basis for its morphological characters. From this study, *E. littoralis* has a median red with yellow lateral labellum. Interestingly, *E. megalocheilos* (Griff.) A.D. Poulsen is morphologically similar to *E. littoralis*. They must be studied, if they are the same or different. *Etlingera araneosa* Baker, which widely spread in Myanmar, but it is also distributed in Western Thailand. Their inflorescence quite similar to *E. littoralis* but peduncle very short, bract ovate densely matted on the edge, lip rather longer than the corolla segment.

Type specimen of *E. littoralis* was collected by König in 1779. It was named, *Amomum littorale*. In 1972 Giseke used König's description and placed it in a new genus which is named *Etlingera* (Burtt and Smith, 1986; Pederson, 2004). Because

the type specimen was lost, so later researcher used only König's description to give *Etlingera* in different names. In 1986, Smith reviewed Bornean Zingiberaceae using only morphological characters and placed *E. megalocheilos* synonym with *E. littoralis* using only three points in Konig's description (Burtt and Smith, 1986).

Smith (1986) mentioned that *E. littoralis* is commonly distributed in Malay Peninsula and extends to Southern Thailand. Their flowers were no yellow on the labellum but bright red at ground level.

In 2004-2005, Poulsen visited Phuket Island making new collections of *E. littoralis*, which the morphology of this species is not the same as *E. megalocheilos* (Bornean materials) (Poulsen, 2006). Even Burtt and Smith (1986) emphasized three points in König's description to justify their placement of *E. megalocheilos* in the synonymy of *E. littoralis*, but there are other points to be considered.

This study aims to answer the questions related to the two species (*E. littoralis* and *E. megalocheilos*) and verify the true *E. littoralis* by using morphological data, and molecular data. The distributions and ecological data of the studied species will be also investigated.

Figure 1 The inflorescence of *E. littoralis* in Southern Thailand.

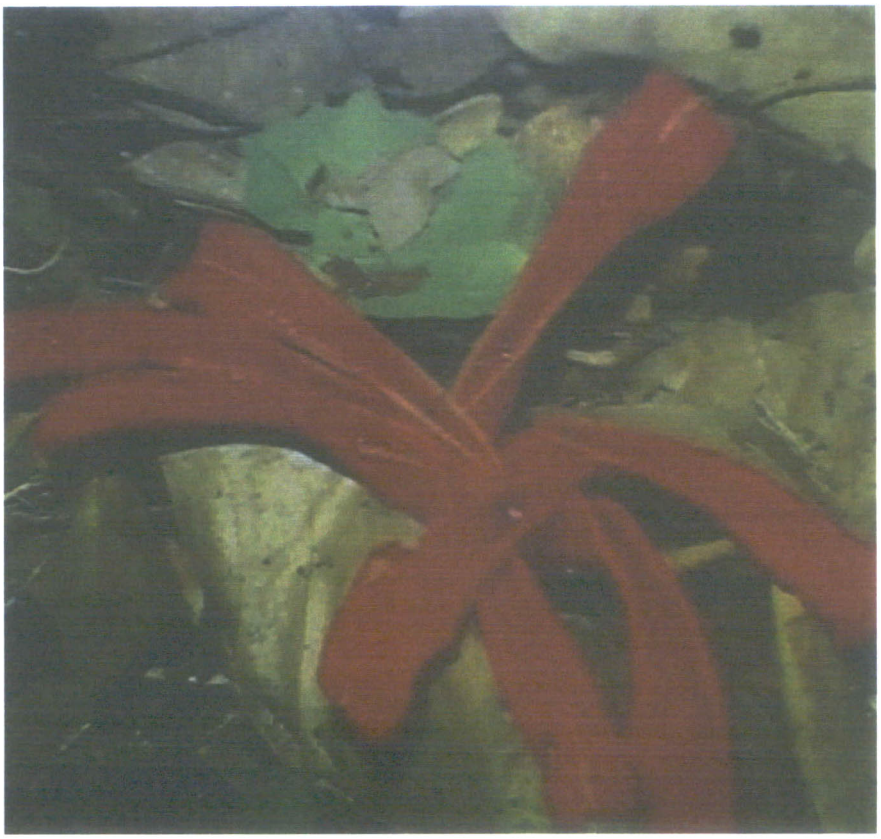

Figure 2 The inflorescence of *E. megalocheilos* in Borneo. (Poulsen, 2006)

1.2 Research Objectives

1.2.1 To study distribution ranges of *E. littoralis* and *E. megalocheilos* in Southern Thailand for database.

1.2.2 To study genetic relationships of *E. littoralis* populations and between *E. littoralis* and *E. megalocheilos*.

Chapter 2

Literature review

2.1 Geography of Southern Thailand

Southern Thailand is located geographically between the latitude of 11 degrees 42 minutes north and the latitude of 5 degrees 37 minutes north covering a distance of 592 kilometers. It is 750 kilometers in length and 50 to 220 kilometers in width.

Southern Thailand is divided into 2 sides by north-south lying mountain ranges. The west side is flanked by Andaman Sea of Indian Ocean while the east side is flanked by Gulf of Thailand of South China Sea. There is much topography in Southern Thailand e.g. basins, beaches, waterfalls, caves, lakes and many islands. Plains are found in central of the region and along the coasts. Important mountains in Southern Thailand are Tanao Wa Si mountain range, Nakhon Si Thammarat mountain range and San Kala Kiri mountain range. (Charoenphong, 1991)

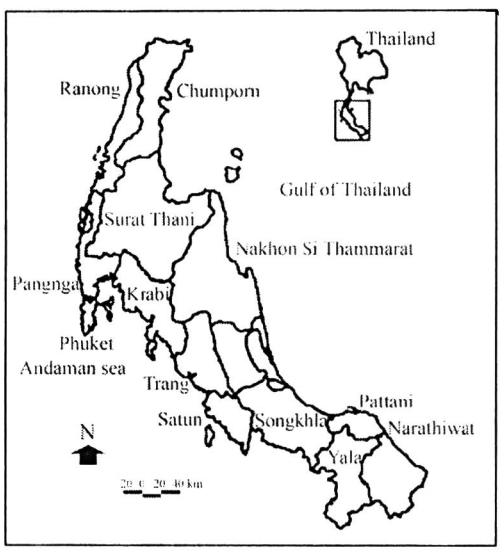

Figure 3 Southern Thailand map.

2.2 The family Zingiberaceae

Zingiberaceae is a family of flowering plants consisting of aromatic perennial herbs, especially on the ground flora of Malaysian tropical forest (Ibrahim, 1998). The family Zingiberaceae or ginger is well known for its foods, medicines, spices, dyes, perfume, vegetable, economic, condiments and aesthetics (Sirirugsa, 1998; Ngamriabsakul, 2001; Kaewsri *et al.*, 2007). The best known of these are ginger (*Zingiber officinale* Rosc.), turmeric (*Curcuma rotunda* L.) and cardamoms (species of *Amomum* and *Elettariopsis*). The family of Zingiberaceae is a large and important monocot family and it is conspicuous throughout South to Southeast Asia with a few species extending to China, Australia, and the South Pacific, but the highest diversity is concentrated in India and Thailand (Skornickova *et al.*, 2007). Thailand has one of the richest ginger floras in the world. About 50 genera of the Zingiberaceae are at present known to science. In Thailand, 26 genera of more than 300 species are found. This is due to Thailand have a suitable zone for species distribution (Larsen and Larsen, 2006). The family Zingiberaceae in Thailand was first studied by Kai Larsen (1980), who is a taxonomist and proposed the key to genera of Thai Zingiberaceae. The Zingiberaceae form a monophyletic group together with Cannaceae, Marantaceae, and Costaceae (sister family to Zingiberaceae) (Pederson, 2004).

The Zingiberaceae is the largest family in the order Zingiberales which is a tropical group of monocotyledons that includes bananas, gingers, and their relatives (Kress *et al.*, 2001). The first classification was proposed in 1889 and refined by subsequent scientists. Previously, the family had been divided into four tribes (Globbeae, Hedychieae, Alpinieae, and Zingibereae) base on morphology (Kress *et al.*, 2002). New phylogenetic analyses base on DNA sequences of the molecular internal transcribe spacer (ITS) and plastid *matK* regions suggests new classification of the Zingiberaceae which divided the family into 4 subfamilies and 6 tribes: Siphonochilideae W. J. Kress (*Siphonochilus* only), the Tamijioideae W. J. Kress (*Tamijia* only), Alpinioideae Link (most of the former Alpinieae), and the Zingiberaceae (including the former tribes Hedychiae, Zingibereae, and Globbeae) (Table 1) (Kress *et al.*, 2002; Pederson, 2004).

2.2.1 Characteristics

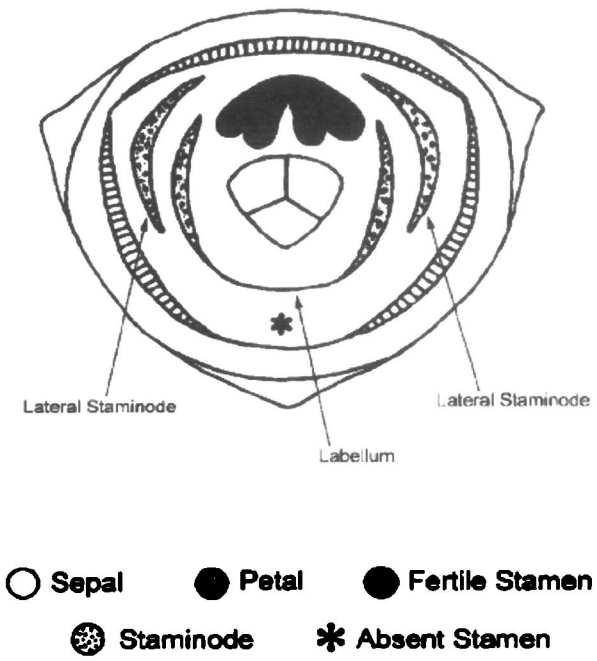

Figure 4 Floral diagram of the Zingiberaceae with perianth whorls, fertile stamen, lateral staminodes, and labellum indicated. (modified from Kress *et al.*, 2002)

Zingiberaceae is perennial, terrestrial, rarely epiphytic herbs, with fleshy, tuberous or non tuberous rhizomes, often with tuber-bearing roots. Stem is usually short or replaced by pseudostems which are formed by leaf sheaths. Leaves are always distichously, quite simple, those toward base of plant usually bladeless and reduced to sheaths; leaf sheath open; ligules usually present; petiole present or absent, located between leaf blade and sheath. Inflorescence is terminal on pseudostems or on separate, short; sheaths covered shoots arising from rhizome, cylindric or fusiform, sometime globose, lax to dense, few to many flowered, sometime with bracteolate cincinni in bract axils and then a thyrse, sometime a raceme or spike. Flower is bisexual, epigynous, zygomorphic. Calyx is usually tubular, thin, split on one side, apex 3-toothed or lobed. Corolla is proximally tubular; lobes varying in size and shape. Stamens are 6 but consist only one fertile stamen. Lateral staminodes of outer

whorl are petaloid or foming small teeth at base of labellum (Figure 4), or adnate to labellum, or absent. Median staminode of outer whorl is always reduced. Labellum is formed from lateral 2 staminodes of inner whorl. Fertile stamen is median of inner whorl; filament long or short; anther locules 2, introrse, dehiscing by slit or occasionally pores; connective often extended basally into spurs and apically into a crest. Ovary is inferior, 3-loculed initially, 1- or 3-loculed. Developed style 1, very thin placed in a furrow in filament and between anther locules; stigma appearing above anther, margin often ciliate. Stylodes 2, reduced to nectarines at apex of ovary. Fruit is a capsule, fleshy or dry, dehiscent. Seeds are few too many, arillate; aril often lobed or lacerate (Ke *et al.*, 2000).

2.3 The genus *Etlingera*

The most well known species of *Etlingera* is *E. elatior* (Jack) Smith, commonly known as "Torch Ginger" in the floral world. This species are known for their long flower stems and thick, waxy, brightly colored bracts. Nevertheless, the majority of species are shad plants of the rain forest of evergreen tropical regions, but some grow mainly on forest in clearings, or on riverbanks.

Kress *et al.* (2002) placed *Etlingera* in subfamily Alpinioidea Link (most of the Alpinieae). The subfamily is divided into two tribes; the first is the Alpinieae A. Rich. i.e. *Aframomum, Alpinia, Amomum, Aulotandra, Cyphostigma, Elettaria, Elettariopsis, Etlingera, Geocharis, Geostachys, Honstedtia, Leptosolena, Paramomum, Plagiostachys, Renealmia*, and *Vanoverbernia.* The second is the Riedelieae W. J. Kress i.e. *Burbidgea, Pleuranthodium, Riedelia,* and *Siamanthus.* The Alpinieae is defined by having fleshy or indehiscent fruits and lacking extrafloral nectaries. The Riedeliae are characterized by the presence of extrafloral nectaries as well as a silique like capsule opening by a longitudinal slit (Pedersen, 2004).

Smith (1986) and Pedersen (2004) classified *Etlingera* into four groups (group A, B, C, and D) by using morphological characteristics (Table 2).

When *Etlingera* was established, it has only one species, *E. littoralis* (König) Giseke (Pedersen, 2004). The type specimen was collected from Phuket Island, Thailand, but it was lost in the sea. So Giseke used only König's description for

character description of *Etlingera*. Later researchers also used the same description for *E. littoralis*, while new species of *Etlingera* were also described.

2.4 *Etlingera littoralis* (König) Giseke.

Etlingera littoralis was first discovered by König on Phuket Island, Thailand in 1779 and he gave a name, *Amomum littorale*, but the type specimens were lost in the sea. After that Giseke used König's description as the basis for the establishment of several new genera (Burtt and Smith, 1986; Pederson, 2004).

***Amomum littorale*:** König's description (Burrt and Smith, 1986)

Rhizomes nodes, articulate, with filiform fibrous roots, aromatic.

Stems very numerous, quite simple, terete, erect, leafless for one third, nodding in upper part, taller than a man, clavate above the rhizome, globose, glabrous, included within a single sheath, at length wrapped in three or five alternate, distant, sheaths closely appressed to the stem. In the upper part of the stem sheath oblong, marginate, ciliate, appressed, green.

Leaves distichous, alternate, petiolate, spreading, oblong; acute, quite entire lightly striate as is usual in all Scitamineae. Lower leaves more distant, very small.

Petioles spreading, glabrous, compressed, short, and woody.

Flowers near the rhizome, scarcely above ground, numerous, crowded into dense fascicles, surrounded by numerous involucral bracts, the size of the swan's egg.

Peduncles arising from the rhizome below ground, short, erect, clothed with small scales, white, scarcely as thick as the little finger.

Outer involucral bracts sessile, imbricately appressed, orbicular-cordate, acute, quite entire, the tips slightly keeled on the back, lightly striate outside, smooth within, alternately striate with white pellucid longitudinal lines, subcoriaceous, rigid, the margin thinner, brownis.

Bract solitary to each flower adnate to the receptacle of the flower below the ovary, linear-lanceolate, quite entire, at the apex rather acute incurved and ciliate, concave, outside glabrous slightly striate with sparse scattered hairs towards the tips, white semi-transparent, inside smooth, a little longer than the spathes [bracteole and calyx] of the flower, of an equal breadth.

Calyx double [bracteole and cylyx]:

exterior [bracteole]: spathe monophyllous, on both sides a little inflated and keeled, compressed, broader than the tube of the flower, membranous, white, bifid at the tip; lociniae with their tips appressed to the flower, ciliate, acute, pink, scareely longer than the tube of the flower.

interior [calyx] monophyllus, tubular at the base, ovate-lanceolate, appressed to the very large lower lip of the flower, quite entire, acute, less concave, membranous, pink, especially towards the tip, narrower than the larger lip of the flower, a little shorter.

Corolla gamopetalous, tubular at the base.

Tube erect, slightly curved, glabrous, white and inch long, occasionally somewhat longer. Limb double [petals and staminodes]:

outer [petals] small, irregular, united above the tube with the very large interior lip, tripartite. Upper segment incumbent on the anther, oblong ovate, quite entire, somewhat acute, very thin, membranous, most elegantly silky-scarlet, rather short. Lower two approximate to the lower lip and appressed to the very large interior one, lanceolate, acute, very thin, a little shorter.

inner and lower lip opposite the stamen, cordate, margins delicately undulate-crispate, very elegantly coloured with orange colour, recurved at the tip, distichously bidentate, concolorous.

Disc [throat] of the flower silky-scarlet on both sides.

Stamen opposite of the lower lip.

Filament broad, flat, fleshy, stiff, short, coloured.

Anther ascending, oblong, broadly truncate at tip, emarginate, necked on the smooth back, flattish, coloured; on the other side divided by deepish longitudinal groove. Thecae fertile towards the margins, whitish, opposite the lower lip, shorter by half and much narrower.

Ovary inferior, small, white, glabrous, rather compressed.

Style within the tube slender, glabrous, white, outside the tube ascending in the groove of the anther and a little longer than it.

Stigma clavate, with a dorsal rather acute somewhat prominent callus, pink, almost cup-shaped, concave, with very thin ciliate whitish margin.

Pericarp. Capsule oblong, obsoletely triangular, evanescent in decay.

Seeds very numerous, angular.

Table 1 Placement of genera in the new classification of family based on phylogenetic analysis (Kress *et al.*, 2002)

Subfamily Siphonochiloidea W.J.Kress	**Subfamily Tamijioideae W.J. Kress**	**Subfamily Alpiniodeae Link**	**Subfamily Zingiberoide ae Haask.**
Tribe Siphonochileae W.J. Kress	Tribe Tamijieae W.J. Kress	Tribe Alpinieae A. Rich.	Tribe Zingibereereae Meisn.
Siphonochilus	*Tamijia*	*Aframomum*	*Boesenbergia*
		Alpinia	*Amandra*
		Amomum	*Cautleya*
		Cyphostgma	*Cornukaempferia*
		Elettariopsis	*Curcuma*
		Etlingera	*Curcumorpha*
		Geostachris	*Distichochlamys*
		Geostachy	*Hniffia*
		Honstedtia	*Haplochorema*
		Leptosolena	*Hedychium*
		Paramomum	*Hitchenia*
		Plagiostachys	*Laosanthus*
		Renealmia	*Parakaempferia*
		Vanoverberghia	*Pommereschea*
			Pygrophyllum
		Tribe Riedelieae W.J. Kress	*Rhynchanthus*
			Roscoea
			Scaphochlamys
			Smithatris
		Burbidgia	*Stadiochilus*
		Pleuranthodium	*Stahlianthus*
		Riedelia	*Zingiber*
		Siamanthus	
			Tribe Globbeae Meisn.
		Incertae Sedis	*Gangepainia*
		Siliquamomum	*Globba*
			Hemorchis
			Mantisia
			Incertae Sedis
			Caulokeampferia

Table 2 Morphological characteristics of *Etlingera* in different groups (Smith, 1986 and Pedersen, 2004)

Characters	***Nicolaia* Horan.**	***Achasma* Griff.**			***Geanthus* Valeton**
		***Etlingera* Group B**			
	***Etlingera* group A**	**B (i)**	**B (ii)**	***Etlingera* Group C**	***Etlingera* Group D**
1. Peduncle	Long (60-130 cm.), held erect	Very short, almost subterranean		Very short, almost subterranean	Very short, almost subterranean
2. Involucral bracts	Spreading and very showy	Partly embedded in the soil		Partly embedded in the soil	Partly embedded in the soil-sometimes much reduced
3. Central lobe of labellum	Short	Expanded		Short	Short
4. Anther held	More or less erect	At an angle to the free part of the filament		At an angle	Erect or slightly angled
5. Thecae dehiscing	In upper ½-2/3	In upper ½-2/3	More or less throughout the length	More or less throughout the length	less throughout the length or not
6. Flowers	Numerous	Numerous	4-many	Many	Few to numerous
7. Lateral lobes of labellum	Not folded over the anther	Not folded over the anther	Folded over the anther	Not folded over the anther	Not folded over the anther
8. Petals	c. the same length as the sepals	the same length as the sepals	Longer than the sepals	Longer than the sepals	Longer than the sepals

Table 2 Morphological characteristics of *Etlingera* in different groups (Smith, 1986 and Pedersen, 2004)) (Cont'd.)

Characters	***Nicolaia* Horan.**	***Achasma* Griff.**			***Geanthus* Valeton**
		***Etlingera* Group B**			
	***Etlingera* group A**	**B (i)**	**B (ii)**	***Etlingera* Group C**	***Etlingera* Group D**
Species placed by Smith	*E. elatior* *E. pyramidosphaera*	*E. triorgyalis* *E. metriocheilos* *E. littoralis*	*E. nasuta* *E. punicea*	*E. sessilanthera*	*E. brevilabris* *E. pubescens* *E. sanguinea* *E. longipetiolata* *E. brachychila* *E. fimbriobracteata* *E. muluensis*
Species placed by Pedersen	*E. hemisphaerica* *E. maingayi* *E. elatior* *E. aff. pyramidosphaera* *E. corneri* *E. venusta*	*E. littoralis* *E. metriocheilos* *E. australasica* *E. triorgyalis* *E. yunnanensis* *E. velutina*	*E. aff. pauciflora* *E. punicea* *E. rubromarginata* *E. belalongensis*	*E. sessilanthera*	*E. brevilabris* *E. aff. muluensis* *E. fimbriobracteata* *E.* sp. nov. 'albiflora' *E. corrugate* *E.* cf. *brachychila*

2.4.1 Comparison of the name of *E. littoralis* with the other names

Achasma megalocheilos Griff. (Holttum, 1950)

Basionym: *Hornstedtia megalocheilos* Ridl.

Amomum megalocheilos Baker

Leafy shoots 3-6 m tall, the sheaths on basal part of stem green. **Leaves** to about 90 by 12 cm, apex very shortly tipped (usually about 1 cm) base often unequal, broadly cuneate to truncate slightly decurrent on petiole; petiole 3-4.5 cm long, blade softly short-hairy beneath, or on midrib only, or glabrous; ligule to about 2 cm long, glabrous or short-hairy.

Inflorescence with basal 1/4-1/2 of involucre immersed in earth, usually near a leafy stem (sometimes to 50 cm away); peduncle to about 10 cm long (often much less) covered with overlapping sheaths in 2 ranks, the upper grading to the involucral bracts; 4-12 flowers open at once.

Involucral bracts about 8, to about 6 by 3 cm, where underground white or pale pink, where exposed crimson, shining, the outer ones at least with a short stiff point.

Floral bracts: outer ones to 7 by 2.8 cm (their tips seen above sterile ones), inner gradually narrower.

Bracteoles c. 5-6 cm long. Calyx c. 7-8 cm long, pale pink, or with deeper coloured tips.

Corolla about same length as calyx, the tube white, the lobes pink, about 3 cm long and 5 mm wide, rounded at the tips slightly hairy at tips.

Lip 5-6 cm. long, the blade about 2 cm wide, entire or more or less cleft at the apex, flame colour or scarlet with the edges towards the base yellow, orange or concolourous with the rest, the yellow edges sometimes extended as a narrow border on to the midlobe.

Stylode flat, 6 mm long, shortly pointed, cream, quite free to the base, not enclosing base of style.

Stamen: filament white or pale pink, anther rose-pink, about 8 mm long, as long as free part of filament.

Stigma bright carmine, large, bent back above the anther, the narrow aperture facing forwards.

Fruit: head usually of 12-0 fruits close together, the whole 8 cm diameter; each fruit unevenly many-sided due to lateral pressure, the apex broadly rounded, smooth and slightly short-hairy, not ridged c. 2.5 cm diameter.

The distinctive features are: usually large size, leaves with long stalks, never pink beneath; inflorescence with fairly long involucral bracts; calyx and collora about equal; lip rather large with usually (not always) yellow margin towards base. Lip of various colour are found on plants near together.

***Etlingera megalocheilos* (Griff.) Poulsen (Poulsen, 2006)**

Basionym: *Achasma megalocheilos* Griff.
Amomum megalocheilos (Griff.) Baker
Hornstedtia megalocheilos (Griff.) Ridl.

Rhizome long-creeping, subterranean, stout, > 2 cm in diameter, cream, scales to 6 cm, brown, pubescent at base. **Leafy shoot** 2-8 m, leafless 1.5-3 m, with up to 28 leaves; base 5-8 cm in diameter, dark green, basal sheath pubescent at base. Sheath striate with some cross bars, especially in upper part of the shoot, glabrous, green when flesh. Ligule 10-25 mm, entire, green or tinged reddish brown, glabrous or with a few scattered hairs, margin ciliate. Petiole 12-40 mm, glabrous. Laminar to 104×14-17 cm, oblong, broadest above the middle, mid- to dark green, pale beneath, glabrous; average length to width ratio c. 7; base+unequal; apex acute. **Inflorescence** (including peduncle) 10-20 cm, embedded in the soil, often some distance from base of leafy shoot, with 11-15 flowers, 2-10 open at time. Peduncle 2-12 cm, subterranean, peduncular bracts to 8.5×3 cm (usually smaller), acute, shiny, glabrous. Spike to, 10-12×2.5 cm, cylindrical, flowers extended 3-4 cm above the bracts, length of spike only including bracts 5-8 cm. sterile bracts c. 5, loosely and spirally arranged, to 4-7×1.5-3.5 cm (upper longest and narrowest), ovate to broadly spathulate (widest above the middle), rigid, mucronate, cream-white, densely pubescent at least in lower half. Fertile bracts 5-8.5×0.6-1.9 cm, linear to spathulate, semitransparent, white, pubescent at least in lower 3/4; apex cucullate, ciliate. Bracteole 4.5-7 cm, white,

membranous, with two fissures of 1.5-2.5 cm, pubescent at least in the lower half, apex 2-toothed, ciliate. **Flower:** Calyx 6.1-9 cm, almost reaching apex of anther, ± as long as corolla lobes, white to pale red with pinkish apices, fissured 3-3.5 c, pubescent in lower 1/4; apex irregularly 3-toothed, tufted. Corolla tube 5.8-8 cm, white to pale red, with scattered hairs at base, tube hairy inside especially in a 15 mm band ending 12 mm from labellum. Lobes pale red or pink, glabrous, delicately membranous; dorsal lobe 25-30×7-9 mm, reaching near middle of anther (but pushed to the side by the lateral lobes of labellum), elliptic, broadest below middle, apex slightly ciliate; lateral lobes 22-25×5 mm, linear-elliptic, broadest below middle, apex slightly ciliate; insertion oblique, converging, 0-3 mm above dorsal lobe. Stamen tube 12-22 mm; labellum hourglass-shaped, 52-70×22 mm, red, with a longitudinal central ridge, glabrous, lateral erect, adhering to sides of anther, base slightly auriculate, margin membranous pale red, central lobe 40-48×15-17 (measured from apex of anther and when flattened), spathulate, entire or slightly emarginated, margin recurved, apex extended 33-50 mm longer than anther; stamen 13-17 mm; filament 4-7×4-5 mm, slightly hairy on outside, red; anther 10-11.5×5-5.5 mm, broadest at apex, emarginated 1.5-2.5 mm, angled c. 135 degree, red, crest with an irregular narrow, dark purple ridge; thecae dehiscing in upper 1/2-2/3, glabrous with a few hairs at the base. Style 8.5-9.5 cm, glabrous to very sparsely hairy adaxially near apex. Stigma 3.5-4 mm wide, rounded-triangular with a rounded back, pale or dark red; ostiole transverse, 2.5-3 mm, facing downwards or forwards, perhaps flexistylous. Ovary 4-6×4 mm, densely hairy; epigynous gland 5-9 mm, deeply bilobed, apex sometimes hairy.

In 1986, Burtt and Smith recognized *Geanthus*, *Achasma* and *Nicolaia* as synonyms for *Etlingera*. They also translated König's description of *E. littoralis*. However, they noted that the inflorescence size of *E. littoralis* is not the size of swan's eggs as described by König.

In 2004-2005 Poulsen visited Phuket Island, where the type specimen was found and made new collections which correspond König's description. Poulsen found that the *E. littoralis*'s description from Phuket Island by König cannot be applied to Bornean materials because it differs in many characters; the longer lip, the longer corolla tube, the usually longer labellum, the narrower central lobe of the

labellum, the shorter and narrower filament, shorter and narrower stamen, which is less emarginated, the labellum being 3-4 time as long as the stamen, anther dehiscence in upper 1/2-2/3 (2/3-3/4)) and the fruit being rounded and hardly ridge vs. being pyriform, flat-topped and deeply and finely ridge.

The Bornean material matchs *Achasma megalocheilos* and cannot be synonomized with *E. littoralis*. Even if Burtt and Smith (1986) emphasized three points in König' description to justify their placement of *A. megalocheilos* in the synonymy of *E. littoralis*, there are other points to consider. However, *A megalocheilos* from Peninsular Malaysia are also mentioned by Holttum (1950), Khaw (2001) and Lim (2001), which they called *E. littoralis* following Burtt and Smith (1986).

2.4.2 Research on *E. littoralis*

Sirirugsa (1998) reported species of Zingiberaceae in Thailand. There are 20 genera and 200 species. Of these, three species of *Etlingera* were recorded; *Etlingera elatior* (Jack) Smith, *E. littoralis* (König) Giseke and *E. maingayi* (Bak.) Smith.

Pederson (2004) studied phylogenetic analysis of the subfamily Alpinoideae (Zingiberaceae), particularly *Etlingera* Giseke using nuclear and plastid DNA. The result showed that *Etlingera* was placed in subfamily Alpinioideae. The result showed that subfamily division is strongly supported. *Etlingera* is monophyletic with *Hornstedtia* as the sister group.

2.5 Criteria of species identification

2.5.1 Morphological characters

Morphological characters are feature of external form that is used for study of the morphology of plants (Judd *et al.*, 2002). Plant morphologist makes comparisons between structures in many different plants of the same or different species and it can also be used to descriptive science and distinguish the diversity and

identification of plant (Stuessy, 1994). Because of morphological characters are easily observed and find practical use in key and descriptions, so taxonomist have been used many parts of plant for taxonomic evidence data in the plant systematic and phylogeny reconstruction (Judd *et al.*, 2002)

There are two types of morphological characters that can be compared and used for plant identification. The first is quantitative characters, morphological features that can be counted or measured. Using numbers describe the relative size or shape of a structure (e.g. a plant species has flower petals 10-12 mm). The second is qualitative characters, morphological features which described with words short, long, color, present and absent in many part of plants (Wiens, 2001).

Normally, there are variations in their forms and structures of plants. These variations are most easily seen in the many organs of plant, such as inflorescences, stems, leaves, seeds and reproductive parts. Morphological variation mostly depends on seasonal or environmental changes (Gaston, 1996).

Zingiberaceae are placed in the Zingiberales which is supported by morphology and DNA (Kress, 1990; Smith *et al.* 1993; Wood *et al.* 2000). The first classifications of the ginger family is proposed in 1889 based on morphological features, such as number of locules and placentation in the ovary, development of staminodes, modifications of the fertile anther and rhizome-shoot-leaf orientation (Kress *et al.*, 2002).

Boesenbergia classification study using reproductive parts, such as anther crests, labellum and inflorescence position, because their parts play an important role for taxonomy of plant. This study they found *B. plicata* have two forms of inflorescence (yellow and red flowers) but can be placed in the single species (Vanijajiva *et al.*, 2003; Techaprasan *et al.*, 2006).

Baker (1894) considered the species of *Alpinia* that occur from Sri Lanka to Singapore. His account included descriptions of 17 species from a known total of 30 and divided them into two subgenera and two sections according to the presence of an anther crest, the possession of large bracteoles, and the position of the inflorescence (Kress *et al.*, 2005).

2.5.2 Ecological data

Ecology is a tool, which has been always taken for plants and animals classification and studies the affinities between organism that performs similar functions or exhibit parallel responses in contemporary ecosystem. In seeking opportunities to contribute to the development of an ecological classification of organisms, two considerations are, firstly they should recognize morphological, physiological or biochemical traits that are reliable predictors of ecological responses, secondly they are necessary to establish large databases documenting patterns of variation in the selected traits across taxa and throughout the world (Grime, 1998).

a. Habitats

External characteristics of plant, such as flowers, inflorescences color or shape, fruit size and stem height are influenced by a variety of habitats (disturbed and undisturbed) and environmental conditions (Techaprasan *et al.*, 2006). Sometime, two sympatric species may be morphologically similar and misidentified as a single species. On the other hand, allopatric taxa in different habitats may show ecomorphological variation and have questionable species status. However, similarity in species can be changes if there are topographic habitat variations even though the same degree and other environmental conditions between habitats (Valencia *et al.*, 2004).

Sirirugsa (1998) explained the habitat of Zingiberaceae species that they are the ground plants of the tropical forests. Some species stand along logging road, river bank, damp and humid shady places. They are also found infrequently in secondary forest and the gap area. Some species can fully expose to the sun, and grow on high elevation.

Poulsen (2006) mentioned that many species of *Etlingera* play an important role in disturbed habitats that caused by human or nature. The inflorescences, leaves, stems, fruits and other parts of the plants may be adapted to different areas such as disturbed and undisturbed area.

b. Flowering seasons

Flowering season of plants depend upon many environmental factors in their habitat such as climate, soil, temperature and photoperiod although other external stimulation such as light quality and nutrition, these factors can also play a role in particular locations (Cosmulescu and Baciu, 2002). Some species their flowering is in winter but others are in spring period. There are still variations in plant flowering if they bloom in different duration, even they are the single species.

One of the most important environmental factors affecting flowering time is the daily duration of light, the photoperiod. Plant in which flowering occurs or is accelerated in short days or long days. Long day plants often flower in late spring or early summer (when the day length becomes longer) to set seeds in a favorable season. Short day plants generally flower in fall (when photoperiods are getting shorter) to finish reproduction before the cold winter arrives (Lin, 2000).

The flowering times of Zingiberaceae are relatively short and some species show similar floral morphology, but differ in colors and inflorescence positions (Techaprasan *et al.*, 2006). So, it is rather difficult for morphological analysis and species classification.

c. Latitudinal position

Plant populations within an ecosystem often become adapted to their specific latitude via common flowering and maturity characteristics. Population of a species from different latitudinal zone can be different characters that effected from environment around them such as temperature, climate, soil, humidity (Vogel *et al.*, 2005).

Yang (2008) studied flowering pattern of *Boesenbergia longiflora* with compared for three populations of different habitats and latitudes. The results showed that there are different of floral morphology and number of flowering inflorescence in the different habitat.

2.5.3 Molecular Studies

One of the most interesting data that is used for plant identification is molecular data. Because, the broad goals of the molecular data have been used in the fields of systematics, phylogenetic and evolutionary prediction (Soltis *et al.*, 1992). Molecular data was more likely than morphological data because it can be reflected to gene changing level. In many case molecular have been supported the monophyly of living groups which cannot be recognized on morphological data (Judd *et al.*, 2002). Many aspects of morphological phylogenetics are highly controversial in the theoretical systematics, poorly explained in empirical studies because many morphological character variations are described in quantitative traits such as different size, shape, but regardless in qualitative traits (Wiens, 2001).

Many benefits from molecular data are used to genetic analysis, PCR techniques, DNA markers. Those techniques have now become a popular for identification of the plant and animal species. Because of the molecular technologies can be detected both intraspecific and interspecific morphological variations (Techaprasan *et al.*, 2008) and the technique is not want tissue specific, so it can be used at any parts of plant or animal for genetic analysis. Only a small amount of sample is enough for detecting. DNA sequence data are the most informative tool for molecular systematic because of the characters of DNA sequences have the basic units of the information encode in organisms.

In plant, there are many kinds of sources of DNA for genetic diversity and molecular phylogenetic study, such as nuclear DNA (nrDNA), chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA). Particularly, cpDNA and nrDNA have been used as major sources of phylogenetic information. Because of some part of them (*mat*K gene in cpDNA and ITS gene in nrDNA) highly conserved in plant systematic, more slowly evolve in *mat*K gene and rapidly evolve in ITS gene (Selvaraj *et. al.*, 2008; Wicke and Quandt, 2009). The *mat*K gene and Interernal transcribed spacer (ITS) are often used to combine for study of the genetic diversity of plant family; Zingiberaceae (William *et al.*, 2004; Pederson, 2004; Kress *et al.*, 2005),

Sonchinae (Kim *et al.*, 2007), Orchidacae (Gravendeel and Vogle, 1999), Valerianaceae (Hidalgo *et al.*, 2004) and Asterceae (Lee *et al.*, 2005)

a. Internal Transcribed spacer (ITS) gene

Overview and function of the ITS gene

Internal transcribed spacer (ITS) of nuclear ribosomal DNA (rDNA) are one of the most extensively sequenced molecular markers and are components of rDNA cistron, which consist of 18s, ITS1, 5.8s, ITS2, and 28s sequence ITS exist of several hundred copies in most eukaryotes. They are located in one or several loci and are distributed in one or several chromosome. The nuclear rDNA copies within a genome can be highly homogeneous due to conserved evolution of intra and inter chromosomal loci. Both ITS1 and ITS2 are non-coding regions located in the rDNA between 18s and 5.8s rRNA genes and between 5.8s and 28s rRNA, respectively. Because ITSs sequence show more divergence than their flanking regions and are easily amplified, they are routinely used to distinguish related species and to infer phylogenetic relationship from populations to families and even higher taxonomic levels.

ITS regions are a part of nuclear DNA. It plays an important role in rRNA maturation (Voronuv *et al.,* 2005). It is found between 18s, 5.8s and 26s rDNA, which are subdivided into ITS1, which is placed between 18s and 5.8s (<200 bp), and ITS2 which is placed between 5.8s and 26s (<300 bp). The ITS1 and ITS2 have shown to be appropriate for genetic diversity for a wide range of the plant, particularly, the most widely use ITS region for phylogeny reconstruction of angiosperm, fern, and algae because it 1) is easy to amplify even from small quantities of DNA (due to the highly copy number of rRNA gene) and 2) has a high degree of variation even between closely related species. This can be explained by the relatively low evolutionary pressure acting on such non-functional sequences and 3) the ITS is quite conserved evolutionary history, very high numbers of copies in the genome and highly heterogeneous in size and primary structure (Voronuv *et al.,* 2005 and Ngamriabsakul *et al.*, 2004.).

The ITS region is now perhaps the most widely sequenced DNA region in fungi. It has typically been most useful for molecular systematics at the species level and even within species. Because of its higher degree of variation than other genic regions of rDNA, variation among individual rDNA repeats can sometimes be observed within both the ITS and IGS regions.

Application of ITS gene for plant systematic

Won and Renner (2005) studied structure of the internal transcribed spacers ITS1 and ITS2 of the nuclear ribosomal DNA in the gymnosperm *Gnetum*, using a phylogenetic framework derived mainly from an intron in the nuclear low-copy *LEAFY* gene. The result showed that ITS functionality were highly divergent nucleotide substitution, GC content, secondary structure, and incongruent phylogenetic placement of presumed paralogs. The length of ITS1 ranged from 225 to 986 bp and that of ITS2 from 259 to 305 bp. *Gnetum* ITS1 contains two informative sequence motifs, *Gnetum* ITS2 contains two structural motifs. The strict consensus tree showed two clades of them, ITS1 of one clade and ITS2 of another.

Qian *et al.* (2009) studied the origin and evolution of the A, B, and D genomes in common wheat (*Triticum aestivum* L.) with evidence on ribosomal DNA (rDNA) internal transcribed spacer (ITS) sequences. The result showed that the sequence of wheat ITS region was 602 bp in length, of which ITS1 and ITS2 had 8 and 20 variation sites, respectively. The genetic distance among the ITS sequences ranged from 0 to 0.038 with the mean value of 0.021. A dendrogram was constructed with *Bromus tectorum* as the out-group. Common wheat had the ITS sequences highly similar to a few of its wild relatives, which indicated that the formation of common wheat genome was a relatively recent event and the concerted evolution in its genome is incomplete.

Wood *et al.* (2000) studied phylogenetic tree of *Hedychium* and related genera (Zingiberaceae) based on Internal Transcribed Spacer (ITS) sequences data. The phylogenetic tree was constructed from 29 taxa of *Hedychium* sequences and one species in each of other genera of Zingiberacae (*Hedychieae*, *Globbeae*, *Zingibereae*, and *Alpinieae*). The cladistic result showed that *Hedychium* are monophyly, which

they were highly supported by bootstrap and *Hedychium* can be divided into four major clades with a moderate support. However, the relationships of *Hedychium* and other genera are poorly supported.

Harris *et al.* (2000) studied rapid radiation in 42 accessions of *Aframomum* (*Zingiberaceae*) baased on Internal Transcribed Spacer (ITS) sequences data. The result showed that *Aframomum* sequences varied from 187 bp to 190 bp (ITS1) and 251 bp to 216 bp (ITS2). Parsimony analysis of the ingroup and outgroup taxa supports the monophyly of the genus *Aframomum.* However, the species sampled vary greatly invegetative, floral and fruit characters, so the morphological variation is not refracted in the ITS sequences data.

Kress *et al.* (2002) studied the phylogeny and a new classification of the gingers (Zingiberaceae) based on molecular data (*mat*K and ITS gens). Previously, family had been divided into four tribes (Globbeae, Hedychieae, Alpinieae, and Zingibereae) based on morphology. But, new phylogenetic analyses based on internal transcribe spacer (ITS) and plastid *matK* regions showed that Zingiberaceae family can be divided into 4 subfamilies and 6 tribes: Siphonochilideae W. J. Kress (*Siphonochilus* only), the Tamijioideae W. J. Kress (*Tamijia* only), Alpinioideae Link (most of the former Alpinieae), and the Zingiberaceae (including the former tribes Hedychiae, Zingiberaceae, and Globbeae).

Julius *et al.* (2008) studied 111 taxa of Bornean *Plagiostachys* (Zingiberaceae), including 25 taxa of *Plagiostachys* based on Internal Transcribed Spacer (ITS). The strict consensus tree showed that *Plagiostachys* comprised a strong supported (bootstrap 96%) clade with some *Alpinia* species. *Plagiostachys* clade can be divided into three subclades and each subclade was moderately to strongly support with relatively high bootstrap values.

b. The *mat*K gene

Overview and function of the *mat*K gene

The *mat*K gene was first identified by Sugita and team in 1985. They found a 509 codon major open reading frame (ORF) in the intron of the *trn*K

gene, which encoding the $tRNA^{Lys}$ (UUU) of the chloroplast. When the chloroplast genomes code for all components of $rRNA_s$ and probably complete set of $tRNA_s$. The $tRNA_s$ are for the protein synthesis in chloroplast (Sugita *et al.*, 1985).

In 1986. Ohyama and his team studied chloroplast gene of the non-vascular plant. They found that, there is actually the open reading frame in those plants. The open reading frame is flanked by two exons of *trn*K gene in all land plants.

MaturaseK (*mat*K gene) or formerly known as Open Reading Frame K (ORF K) (Hilu and Liang, 1997). The *mat*K gene, a chloroplast genome encode locus located within the intron of the chloroplast gene and approximately 1500 base pairs (bp) that are flanked by two exons of the *trn*K gene (Selvaraj *et. al.*, 2008; Ince *et al.*, 2005). The *mat*K gene has been proposed to play an assential role in RNA processing by acting as putative general muturase for plastid introns. The genic region coding for the lysine transfer RNA (tRNA) is divided into two exons, which are separated by group-II intron (Wicke and Quandt, 2009). The matK gene has been used effective in addressing systematic question in the many families; Zingiberaceae (Selvaraj *et al.*, 2008). Rosa (Matsumoto *et al.*, 1998), Polemoniaceae (Steele and Vilgalys, 1994; Johnson and Soltis, 1995), and Poaceae (Liang and Hilu, 1996).

The *mat*K gene is well used for evolutionary and phylogenetic studies particularly above the species level because of it is relatively abundant component of plant total DNA, containing primary single copy gene, and conservative rate of nucleotide substitution. In addition the *mat*K gene has ideal size, high rate of substitution, large of proportion of variation at nucleotide acid level at first and second position, low transition/transversion ratio and the presence of mutationally conserved sectors (Selvaraj *et al.*, 2008). There is several advantages of the *mat*K gene in the chloroplast DNA because of the chloroplast DNA has evolved at a higher rate than several other genes; *rbc*L (widely used for inferring phylogeny above the genus level) for two time in Saxifragaceae and Polemoniaceae (Mutsumoto *et. al.*, 1998). The fast evolved region, especially *mat*K not only tend to provide the highest phylogenetic structure they also offer the desired phylogenetic information even at deeper nodes (Wicke and Quandt, 2009).

Application of *mat*K gene for plant systematic

Gravendeel and Vogel (1999) revised the section *Speciosae* Pfitzer and Kraenzl. of the gunus *Coelogyne* Lindl. using morphological and molecular characters (ITS and *mat*K gene). The sequence data showed monophyly of the section and the section can be divided into two clade, the species of Peninsular Malysia, Sumatra, Java, Borneo, Sulawesi and Malaccas were placed into the first clade and the species of Sulawesi, New Guinea and Pacific Island were put in the second clade.

Hilu *et al.* (1999) studied phylogeny of Poaceae using *mat*K sequences. Nine subfamily of Paceae were used for phylogenetic relationships. The strict consensus tree showed that the phylogenetic clade was divided into three clades. Firstly, subfamily Bambusoideae (excluding *Brachyelytrum*) plus Pooideae. Secondly, Oryzoideae, and thirdly, subfamilies panicoideae, Arundinoideae, Centothecoideae and Chloridoideae. However, the relationships among subfamilies are unresolved or weakly supported.

Sogo *et al.* (2001) studied the molecular phylogeny of Casuarinaceae base on two chloroplast genes (*rbc*L and *mat*K). The fifteen species of Casuarinaceae were taken for studies. They found that analyzing of combined two genes are better resolution than analysis base on *rbc*L gene sequence alone. The cladogram showed that Casuarinaceae are monophyletic comprinsing four distinct genera.

Ge *et al.* (2002) studied phylogeny of the rice tribe Oryzeae (Poaxeae) base on *mat*K sequence data. The nucleotide sequence of the *mat*K gene from 11 genera of the tribe Oryzeae and three outgroup species were used to construct the phylogenetic tree. The results showed that species of Oryzeae form a strongly supported monophyletic group and the tribe Oryzeae can be divided into two monophyletic lineages. But the *mat*K sequence data did not support the close affinities of the monoecious genera in Oryzeae.

Chuang and Hu (2004) studied the evolution and classification of new homologus from *Ophioglossum petiolatum*, two *Lycophytes* and other green algae using *mat*K gene. They found that *mat*K gene is expressed in *Ophioglossum petiolatum* and *Lycopodiella cernua* but no signal detected in the green algae. From

the studies, the phylogenetic clade showed *Pinus*, *Gingko* and *Cycas* formed a monophyletic group and sister group to angiosperm.

Ince *et al.* (2005) studied phylogeny of some important plants using chloroplast *mat*K gene. The 142 plant species belong to the families of 26 plants were conducted to study the evolutionary relationships among the studied plant orders, families, genus and species. The results indicated that the chloroplast *mat*K gene sequences ranking from 730-1545 nucleotides. The consensus tree showed that gymnosperm were different from the monocotledons and dicotyledons, the C_4 plant were improved from common ancestors, and other cereals were evolved from another or similar ancestors.

Specht (2006) studied systematics and evolution of the tropical monocot family Costaceae (Zingiberales) which was collected from South America, Asian and African-neotropical using molecular technique (ITS, *trn*L-F, *trn*K and *mat*K) to construct the phylogenetic tree. The results indicated that the Malanesian genus Tapeinochilos is monophyletic and included within the Asian clade, Monocotus and Dimerocostus are sister taxa and form part of the South Amercan clade. But the African-neotropical showed only the genus Costus within the clade.

Kim *et al.* (2007) studied genetic relationships among genera of subtribe Sonchinae (11 genera and ca. 130 species) and Dendroseridinae (2 genera and 12 species) using ITS and *mat*K gene sequences. The results showed that, the Sonohinae is strongly supported as paraphyletic and can be divided to ten major clades, but subtribe of Dendroseridinae is poorly supported. The phylogentic tree showed monotypic of Acetheorhiza is more closely related to Sonchus than to Launaea and Sonchus is highly polyphyletic.

Bloch *et al.* (2010) studied molecular phylogeny of the edelweiss (*Leontopodium*, *Asteraceae-Gnaphalieae*), which are collected from the Himalayan/Tibetan and Europe using sequences of nuclear ribosomal (ITS and ETS) and plastid (*mat*K and *trn*L/F) DNA. The results indicated that the *Leontopodium* and *Sinoleontopodium* were monotypic. On the other hand *Leontopodium alpinum* and *L. nivale* showed surprisingly little divergence from its Asian relatives.

Chapter 3

Methodology and Material

3.1 Sample collection

Etlingera samples were collected from all provinces in Southern Thailand, except three provinces (Yala, Narathiwat and Pattani) in the lowest part of Southern Thailand). The inflorescences and fruits of *Etlingera* samples were preserved in 70% alcohol, and the vegetative parts were collected for voucher specimens.

3.2 Morphological study

All morphological characters of the samples collected from the fields were made both qualitative and quantitative measurements. The lists of the characters are shown in Table 3. Those measured characters were converted to "0" and "1" and the data were grouped by Cluster Analysis using R program version 2.11.1 (R Development Core Team, 2010)

3.3 Molecular genetic studies

3.3.1 Plant materials

Thirty accessions of *Etlingera* were collected from Southern Thailand (Table 4) for morphological characters, Internal Transcribed Spacer (ITS) and *mat*K study, comprising thirty of *Etlingera* samples; eleven *E. littoralis*, two *E. araneosa* and seventeen of *Etlingera* sp. In addition one samples; *Honstedtia leonurus* (accession AB097237.1) was used as outgroup for ITS analysis.

3.3.2 Genomic DNA Isolation

Total genomic DNA was extracted from the fresh young leaves and silica dried samples using a modification of CTAB method of Doyle and Doyle (1987). Genomic DNA was precipitated by the cold Isopropanol or 95% ethanol and then DNA was air dried and resuspended in 0.1x TE (10 mM Tris-HCl, pH 8, 0.1 mM EDTA). Purity and concentration of DNA was monitored spectrophotometrically at wavelength of 260 and 280 nm using NanoDrop ND-1000 Spectrophotometer (Rabbani *et al.*, 2008). DNA samples were also electrophoresed in 0.8 % agarose gel.

3.3.3 PCR Amplification and Sequencing

ITS regions in each genomic DNA were amplified by Polymerase Chain Reaction method (PCR) with ITS 5p and ITS 8p (Moller and Cronk, 1997a) used as primers. The primer sequences were (5' to 3'), ITS 5p = GGAGG AGA AGT CGT AAC AAG G and ITS 8p = CAC GCT TCT CCA GAC TAC A. The PCR conditions were conducted in 50 µl of total volume, contained 5.0 µl of 10X reaction buffer (1x: 10 mM Tris-HCl, 50 mM KCl, and 1.5 mM $MgCl_2$), 1.0 µl of a mix of each dNTP at 10 mM (final concentration 200 µl) (BioLabs, England), 1.0 µl of each primer at 10 µM (Pacific Science), 0.4 µl (5 U) of DNA polymerase (BioLabs, England), and 1.0 µl (500 ng/µl) of genomic DNA. PCR amplification of the ITS region was carried out in 0.2 ml microcentrifuge tubes in the Perkin Elmer thermal cycler for 30 cycles of denaturation at 94' for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min, with an initial denaturation of 5 min at 94°C before cycling and a final extension of 10 min at 72 °C after cycling. Each set of reactions was monitored by the inclusion of a negative (no DNA template) control.

For amplification of the *mat*K gene, the Polymerase Chain Reaction (PCR) amplification were performed in a total volume of 20 µl containing 100-200 ng of total DNA template, 1.0 µM of *trn*K-3914F and *trn*K-2R primers (Johnson and Soltis, 1995), 10x PCR buffer, 1.0 µl of a mix of each dNTP at 10 mM and 0.4 µl (5 U) of DNA polymerase. Amplification were carried out with an initial denaturation

step at 94°C for 4 min. followed by 35 cycles of 94°C for 30 sec, 48°C for 60 sec, and 72°C for 60 sec, and finished with a final elongation step at 72°C for 10 min.

A portion of a PCR product (5 µl) were electrophoresed in 1.5 % agarose gel comparing with 1 kb DNA marker, using 0.5 x TBE as the gel buffer. The presence of a single bright band of ethidium bromide was showed under the UV Box of Gel Document, for check the successful PCR amplification. The PCR product was purified using the Qiagen PCR purification kit (Qiagen Ltd, Dorking, and Surrey, UK). The fragments obtained were directly sequenced using the same primers that were used for amplification. Sequencing was conducted under BigDyeTM terminator cycling conditions. The PCR products purified using Ethanol Precipitation and run using Automatic Sequencer 3730xl (Macrogen, Korea).

3.3.4 Sequence alignment and phylogenetic analysis

Sequences were assembled the complementary strands and edited nucleotide by BioEdit program, version 7.0.9.0 (Hall, T.A., 1999). Sequences were aligned by multiple sequence alignment using ClustalX2 program (Thompson *et al.*, 1997), using default parameters for sequence alignment.

Molecular data were evaluated using maximum parsimony (MP), maximum likelihood (ML), and Bayesian methods for each data set individually (ITS, *mat*K, and combined data set). MP and ML were performed using PAUP* 4.0b1 (Swofford, 2002). Bayesian method were performed using MRBAYES, version 3.1.2 (Huelsenbeck and Ronquist, 2001)

MP analyses of ITS, *mat*K and combined data set were conducted by PAUP* 4.0b1 (Swofford, 2002) with unweighted characters, saving all shortest tree, with the options tree bisection-reconnection (TBR) branch swapping to find the most parsimonious trees (Kress *et al.*, 2005). Branch support values were obtained using heuristic bootstrap. Bootstrap analyses (Felsenstein, 1985; Mort *et al.*, 2000) were also conducted using PAUP* with ten random addition replicates. The bootstrap was carried out with 1,000 replicates to examine the relative level of support for individual clade on the phylograms of each search. The following scheme of support was applied: 50-74% weak support, 75-84% moderate, and 85-100% strong support

(Sarkinen *et al.*, 2007; Williams *et al.*, 2004). As measures of the amount of homoplasy and the structure in the data, consistency index (CI) and retention index (RI) are used (Farris, 1898) with autapomorphies excluded (Poulsen, 2006)

Maximum likelihood analysis of the ITS, *mat*K and combined data set were conducted and based on using the Hasegawa-Kishino-Yano (HKY) model of molecular evolution (Hasegawa *et al.*, 1985; Alexander *et al.*, 2002; Burbrink, 2002). Gap was treated as missing data. Bootstrap analyses of 1,000 replicates were conducted to evaluate the relative support for individual clade (Felsenstein, 1985). The default transition (ti)/transversion (ts) ratio of 2 was initially used (Gastony and Ungerer, 1997).

A Bayesian analysis using MRBAYES, version 3.1.2 (Huelsenbeck and Ronquist, 2001) was performed using the same parsimony data matrix. The Bayesian of each data set was run under the GTR model with rate variation among site (Boykin *et al.*, 2010; Kumaria *et al.*, 2010). The parameter (rate = gamma, nst = 6) was used for ITS, *mat*K, and combined data. All data set were partitioned (using "lset apply to" command) in order to accommodate differing evolutionary rate for the respective data set. Markov chain Monte Carlo (MCMC) chains were performed on 1,000,000 generations.

Trees were sample every 100^{th} cycle from the chain. All samples point that occurred before stationary of negative log likelihood (-lLn) scores was achieved were discarded as part of the burn-in period (Huelsenbeck and Ronquist, 2001). Nodes with posterior probability values $\geq$ 95% were retained in the 50% majority rule consensus tree (Kress *et al.*, 2005).

Table 3 Morphological characters of *Etlingera* sample collection in Southern Thailand for R statistic analysis.

Morphological characters	0	1
1. Leaf forms	simple	compound
2. Leaf shape	oblong	lanceolate
3. Leaf length and width ratio	1-5 time	>5 time
4. Leaf base	oblique	rounded
5. Leaf margin	entire	not entire
6. Leaf apex	acuminate	acute
7. Leaf length	50-100 cm	>100 cm
8. Leaf width	1-10 cm	>10 cm
9. Leaf abaxial	hairs	glabrous
10. Leaf adaxail	hairs	glabrous
11. Number of leaf	1-20	>20
12. Leafy shoot tall	1-5 m	5.1-10 m
13. Leafless (the base to the first leaf)	1-2 m	2.1-3 m
14. Leafy shoot	hairs	glabrous
15. Inflorescence color	entire red labellum	yellow edge labellum
16. Inflorescence length	1-10 cm	>10 cm
17. Total number of flower	10-20	21-30
18. Number of flower open at a time	1-10	>10
19. Bract length	1-5 cm	>5.0 cm
20. Bract width	1-3 cm	3.1-5 cm
21. Bract length and width ratio	1-3 time	>3 time
22. Bract tip	acuminate	acute
23. Flower length	1-10 cm	>10 cm
24. Dorsal corolla lobe length	1-3 cm	3.1-5 cm
25. Labellum length	1-5 cm	5.1-10 cm
26. Labellum width (middle of the labellum)	0.1-1 cm.	>1.0 cm
27. Labellum tip	emarginated	rounded
28. Corolla tube length	1-5 cm	>5 cm
29. Stamen length	0-1.5 cm	>1.5 cm
30. Labellum and stamen length	1-3 time	>3 time

Chapter 4

Results

4.1 Distribution

Thirty *Etlingera* samples were collected from all provinces in Southern Thailand, except for three provinces (Yala, Pattani and Narathiwat) in the lowest part of Southern Thailand. (see Table 4 and Figure 5). The samples were collected in flowering saeson; March to July. They are divided into *E. littoralis* (11 samples), *Etlingera* sp. (17 samples) and *E. araneosa* (2 samples)

Distribution map shows that *Etlingera* species are widely spreaded in Southern Thailand, both Gulf of Thailand and Andaman Sea sides (Figure 6). The collected data showed that the habitats of *E. littoralis* and *Etlingera* sp. are not overlaps. *Etlingera littoralis* is mostly distributed in the upper part of Southern Thailand; Krabi, Phuket, Nakhon Si Thammarat, Phang Nga, Ranong, and Surat Thani provinces. While, *Etlingera* sp. is generally distributed in the lower part of Southern Thailand; Trang, Phattalung, Satun, Songkhla, Surat Thani, and Nakhon Si Thammarat provinces. However, both species in Surat Thani and Nakhon Si Thammarat provinces were found, and *E. araneosa* were found only in Chumporn and Surat Thani provinces.

Table 4 *Etlingera*'s sample were collected from Southern Thailand for morphological and molecular (ITS and *mat*K) analyses.

No.	Sample code	Species Name	Location (District, Province)	GPS locality	Ass. No.
1.	RN1_Etlingera	*Etlingera littoralis*	Kra Buri, Ranong	N 010° 30' 43.1" E 098° 53' 27.3"	WU11
2.	PNG1_Etlingera	*Etlingera littoralis*	Ta Kua Pa, Phang Nga	N 08° 49' 56.8" E 098° 26' 49.8"	WU36
3.	PNG2_Etlingera	*Etlingera littoralis*	Tai Muang, Phang Nga	N 08° 29' 49.4" E 098° 17' 0"	WU37
4.	KB1_Etlingera	*Etlingera littoralis*	Khlong Tom, Krabi	N07° 55' 27.2" E099° 15' 38.8"	WU29
5.	KB2_Etlingera	*Etlingera littoralis*	Khlong Tom, Krabi	N07° 55' 502.6" E099° 12' 22"	WU30
6.	NST2_Etlingera	*Etlingera littoralis*	Lan Saka, Nakhon Si Thammarat	N08° 22' 56.4" E099° 44' 12"	WU40
7.	NST6_Etlingera	*Etlingera littoralis*	Si Chon, Nakhon Si Thammarat	N09° 1' 18.9" E099° 46' 22.3"	WU68
8.	NST7_Etlingera	*Etlingera littoralis*	Tha Sala, Nakhon Si Thammarat	N07° 5' 6.6" E099° 47' 54.2"	WU87
9.	PK1_Etlingera	*Etlingera littoralis*	Tha Lang, Phuket	N08° 1' 51" E098° 22' 27.1"	WU31
10.	PK2_Etlingera	*Etlingera littoralis*	Tha Lang, Phuket	N08° 2' 37.3" E098° 16' 43.4"	WU34
11.	SRT1_Etlingera	*Etlingera littoralis*	Pa Nom, Surat Thani	N08° 54' 10.5" E098° 37' 19.1"	WU38
12.	SRT2_Etlingera	*Etlingera* sp.	Wipa Wadee, Surat Thani	N09° 9' 42.1" E098° 53' 5.9"	WU88
13.	TR1_Etlingera	*Etlingera* sp.	Yan Ta Koaw, Trang	N07° 24' 45.2" E099° 49' 20.7"	WU55

Table 4 *Etlingera*'s sample were collected from Southern Thailand for morphological and molecular (ITS and *mat*K) analyses. (Cont'd)

No.	Sample code	Species Name	Location (District, Province)	GPS locality	Ass. No.
14.	TR3_Etlingera	*Etlingera* sp.	Yan Ta Koaw, Trang	N07° 26' 27.6" E099° 48' 56.3"	WU61
15.	NST1_Etlingera	*Etlingera* sp.	Lan Saka. Nakhon Si Thammarat	N08° 22' 56.2" E099° 44' 12"	WU39
16.	NST3_Etlingera	*Etlingera* sp.	Lan Saka, Nakhon Si Thammarat	N08° 22' 56.2" E099° 44' 12.1"	WU43
17.	NST4_Etlingera	*Etlingera* sp.	Si Chon, Nakhon Si Thammarat	N09° 5' 26.4" E099° 53' 50.8"	WU63
18.	NST5_Etlingera	*Etlingera* sp.	Sichon, Nakhon Si Thammarat	N09° 5' 19.7" E099° 53' 20.6"	WU66
19.	ST1_Etlingera	*Etlingera* sp.	Kuan Ka Lhung, Satun	N06° 54' 43.1" E0100° 7' 47.4"	WU70
20.	ST2_Etlingera	*Etlingera* sp.	Kaun Don,Satun	N06° 45' 28.9" E100° 9' 18.6"	WU75
21.	ST3_Etlingera	*Etlingera* sp.	Kuan Don, Satun	N06° 43' 39" E100° 9' 45.1"	WU76
22.	SKL1_Etlingera	*Etlingera* sp.	Boripat, Songkhla	N07° 0' 31.2" E100° 18' 45.4"	WU78
23.	SKL2_Etlingera	*Etlingera* sp.	Had Yai, Songkhla	N07° 0' 5.3" E100° 14' 5.9"	WU79
24.	SKL3_Etlingera	*Etlingera* sp.	Had Yai, Songkhla	N06° 59' 40.1" E0100° 8' 57"	WU80
25.	SKL4_Etlingera	*Etlingera* sp.	Natawee, Songkhla	N06° 35' 13.7" E100° 34' 33.7"	WU82

Table 4 *Etlingera*'s sample were collected from Southern Thailand for morphological and molecular (ITS and *mat*K) analyses. (Cont'd)

No.	Sample code	Species Name	Location (District, Province)	GPS locality	Ass. No.
26.	SKL5_Etlingera	*Etlingera* sp.	Natawee, Songkhla	N06° 36' 1.3" E100° 35' 15.9"	WU85
27.	PTL1_Etlingera	*Etlingera* sp.	Si Banphot, Phatthalung	N07° 40' 38.2" E098° 53' 27.3"	WU46
28.	PTL2_Etlingera	*Etlingera* sp.	Si Banphot, Phatthalung	N07° 42' 20.3" E099° 48' 51.6"	WU50
29.	CP1_Etlingera	*Etlingera araneosa*	Muang, Chumporn	N010° 43' 26.3" E099° 7' 23.6"	WU09
30.	SRT3_Etlingera	*Etlingera araneosa*	Khoa Sok, Surat Thani	N08° 53' 53.7" E098° 44' 29.1"	WU90

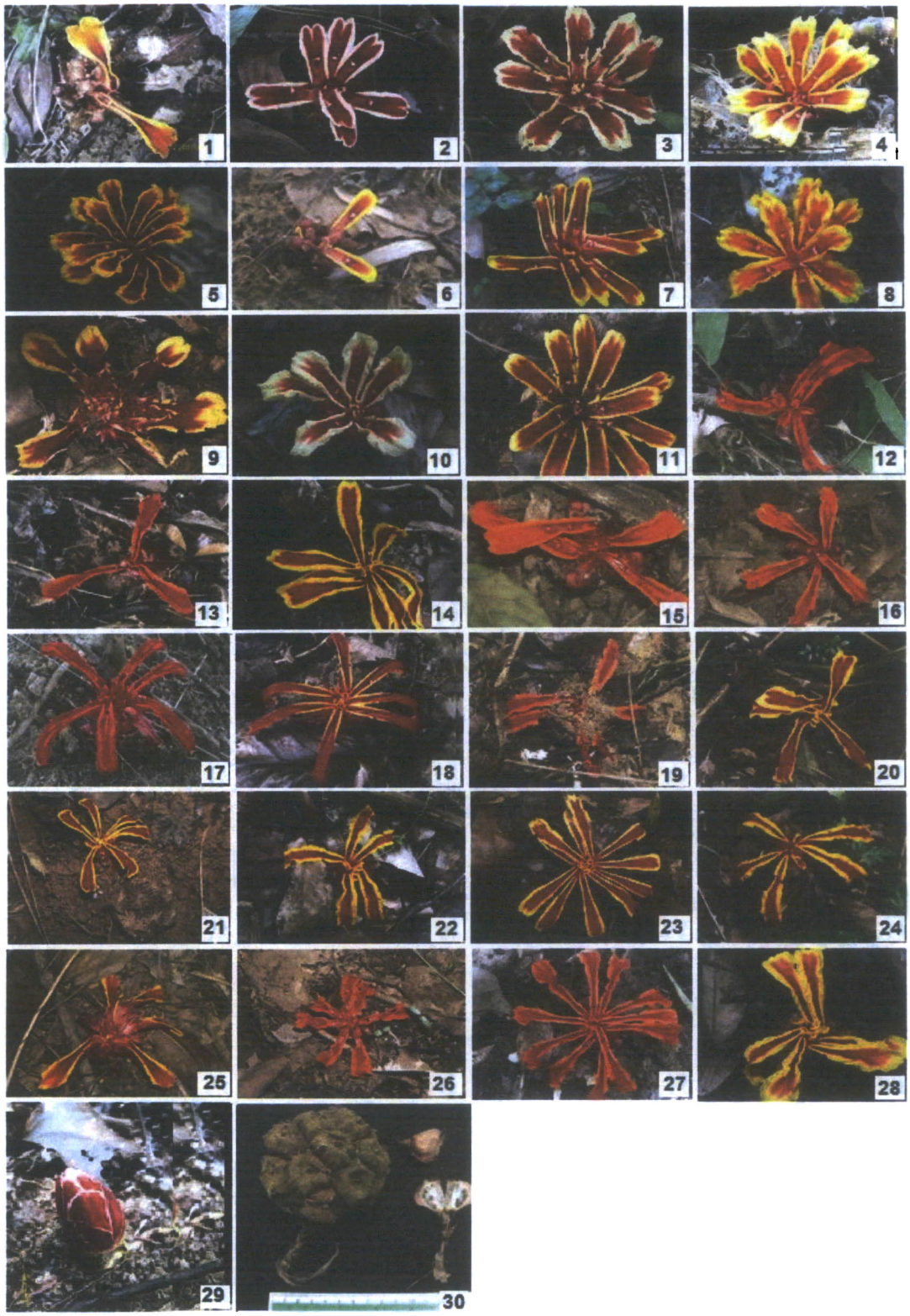

Figure 5 *Etlingera* samples collected in this study. 1-11 *E. littoralis*, 12-28 *Etlingera* sp. and 29-30 *E.araneosa*

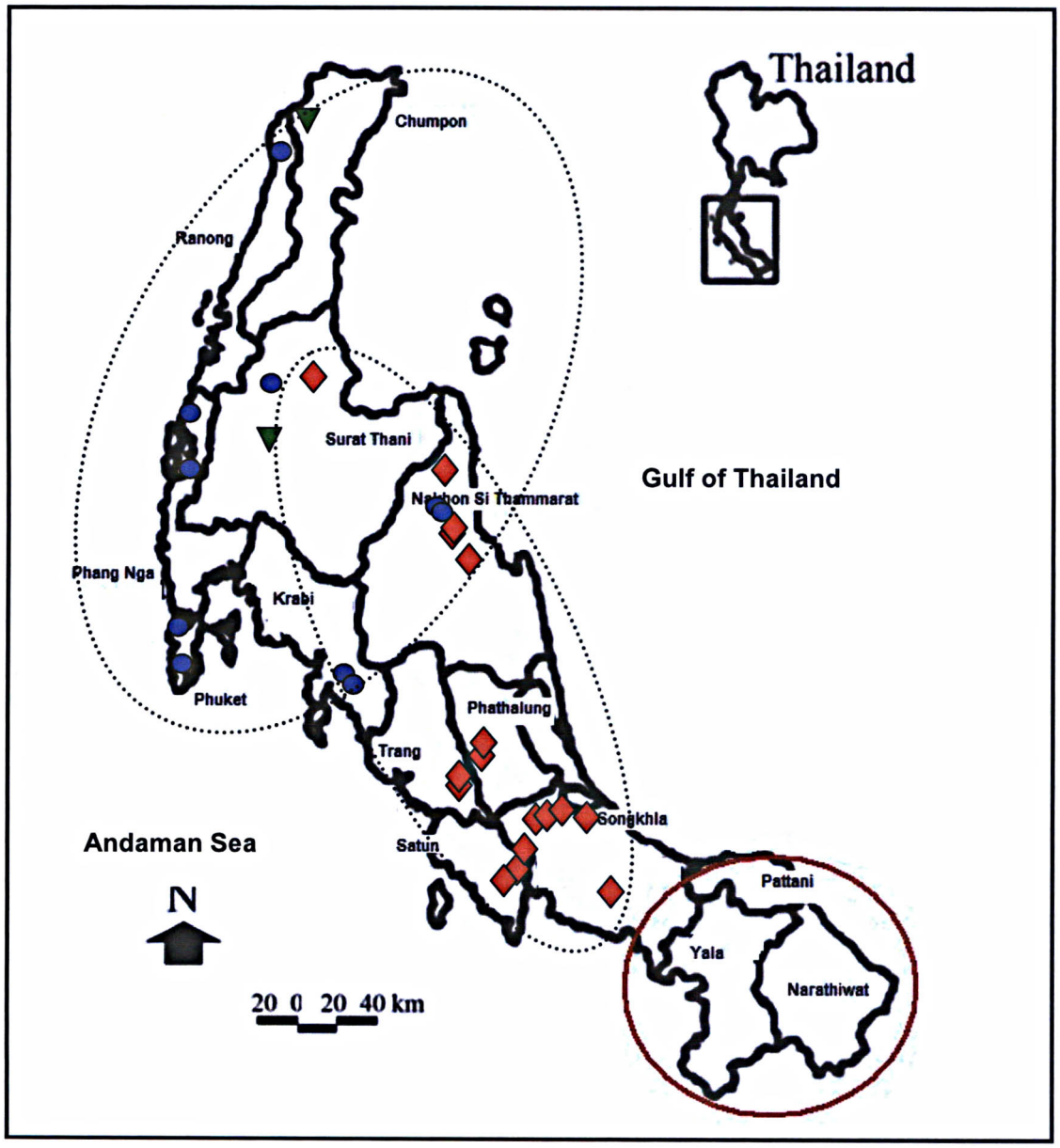

Figure 6 Geographical distributions of all *Etlingera* samples collected from all provinces in Southern Thailand, except for three provinces in the lowest part. ● *E. litttoralis*,◆ *Etlingera* sp., ▼ *E. areneosa*.

4.2 Morphological studies

4.2.1 Inflorescences and Infructescences

There are three *Etlingera*'s inflorescence forms. *Etlingera littoralis*'s form, inflorescence embedded in the soil. The flowers have a median red with yellow lateral labellum. The labellum length means 4.92 cm, shorter than the other forms. The middle of the labellum width means 1.7 cm, broadest below middle. Stamen 1.5-2.0×0.7-1.1 cm. Anther 1.0-1.2×0.5-0.6 cm, quite erect with filament or a bit angled *ca.* 10-15 C°, broadest at apex, emarginated 0.1-0.2 cm, thecae dehiscing in upper 1/2-2/3. Infructescence embedded in the soil, brown, deeply ridged and densely pubescent (Figure 7).

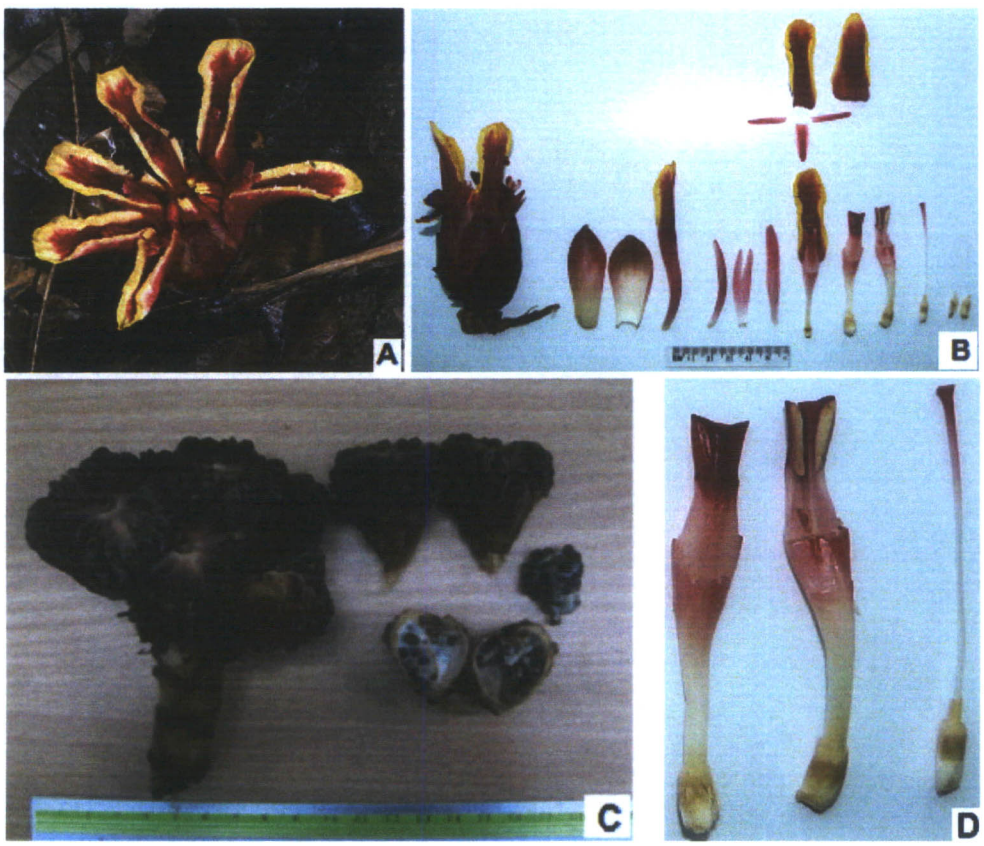

Figure 7 *Etlingera littoralis*; **A.** a whole inflorescence, **B.** dissected parts of the inflorescence, **C.** Fruits and **D.** Stamen and stigma.

Inflorescence form of *Etlingera* sp. is divided into two forms; a median red with yellow lateral labellum and entirely red labellum. Inflorescences are also embedded in the soil. The labellum length means 6.8 cm, the middle of the labellum quite narrow 0.8-1.0 cm, broader apex 1.0-1.7 cm, emarginated and broadest below the middle 1.9-2.5 cm. Stamen 0.5-1×0.4-0.6 cm, emarginated, narrower than stamen of *Etlingera littoralis*. Anther 1.0-1.2×0.3-0.5 cm. It is much angled *ca.* 40-65 C° with filament, emarginated, thecae dehiscing in upper 1/2-2/3. Infructescence is very similar to *E. littoralis*, embedded in the soil, brown, deeply ridged and densely pubescent (Figure 8 and Figure 9).

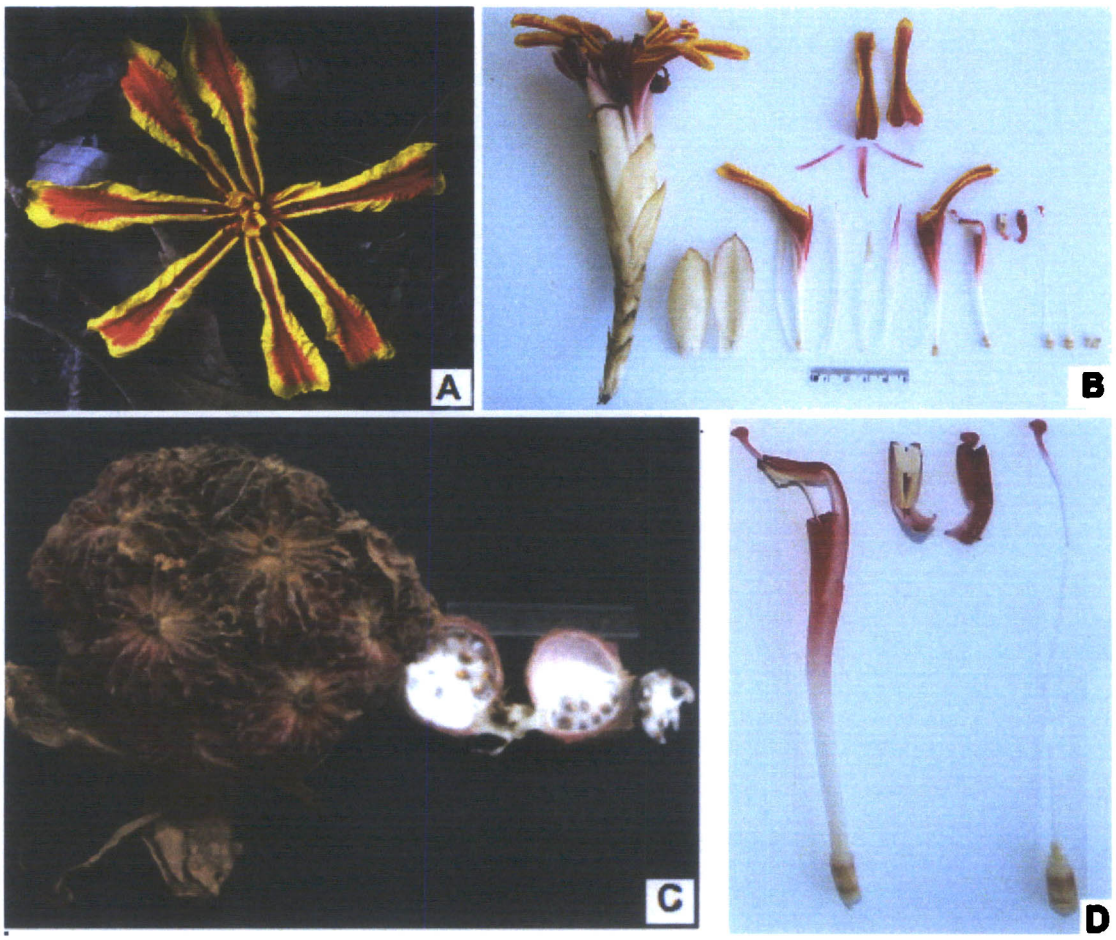

Figure 8 *Etlingera* sp. (yellow lateral labellum); **A.** a whole inflorescence, **B.** dissected parts of the inflorescence, **C.** Fruits and **D.** Stamen and stigma.

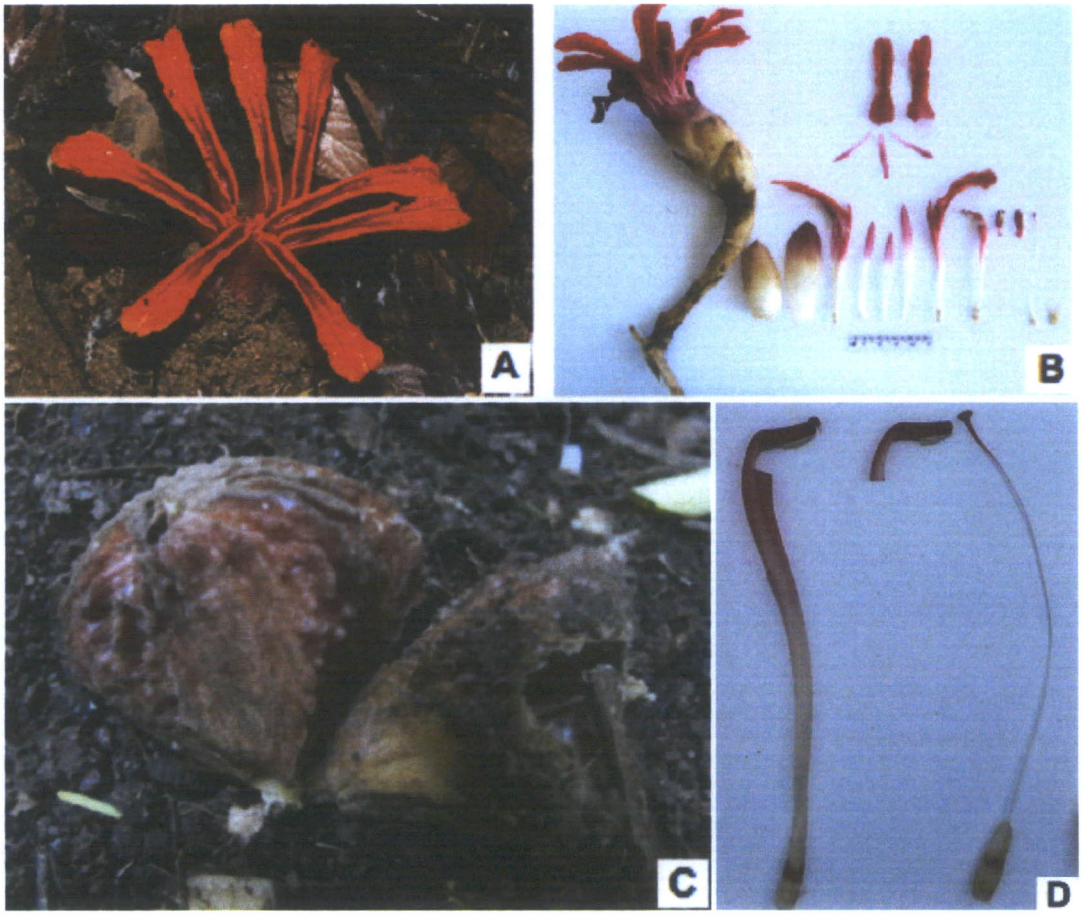

Figure 9 *Etlingera* sp. (entirely red labellum); **A.** a whole inflorescence, **B.** dissected parts of the inflorescence, **C.** Fruits and **D.** Stamen and stigma.

4.2.2 Cluster analysis of morphological characters

In this study, R statistic v.2.11.1 (R Development Core Team, 2010) was used for cluster analysis of *Etlingera* sample. All of the morphological characters of the samples, both qualitative and quantitative characters, were considered and measured respectively. Those characters were converted to the symbol ("0" and "1") (Table 3) for analysis by R program. The morphological character analysis were studied in three patterns (only vegetative character, only reproductive character, and together reproductive and vegetative characters) (Figure 10-12 respectively). The results showed that the morphological character of only reproductive part, and together reproductive and vegetative parts analyses separated the collected samples

into two groups; *E. littoralis* group and *Etlingera* sp. group, with R value from ANOSIM statistic analysis = 0.55 and 0.79 respectively (Figure 11 and Figure 12).

While, the morphological character from the only vegetative part cannot be grouped to *E. littoralis* or *Etlingera* sp. There are four groups, which they were mixed between *E. littoralis* and *Etlingera* sp. (Figure 10).

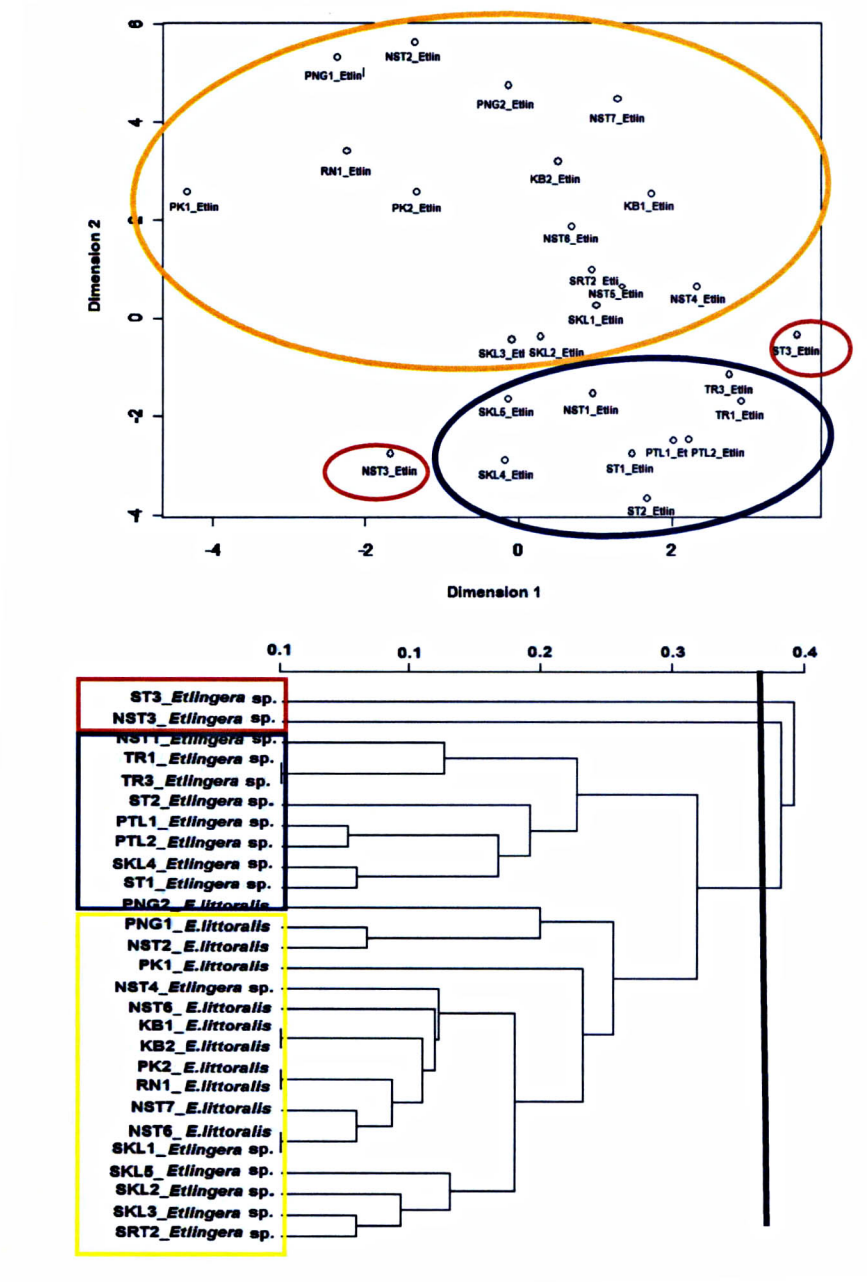

Figure 10 A cluster analysis pattern of vegetative characters. The samples are not clearly separated into *E. littoralis* or *Etlingera* sp. groups.

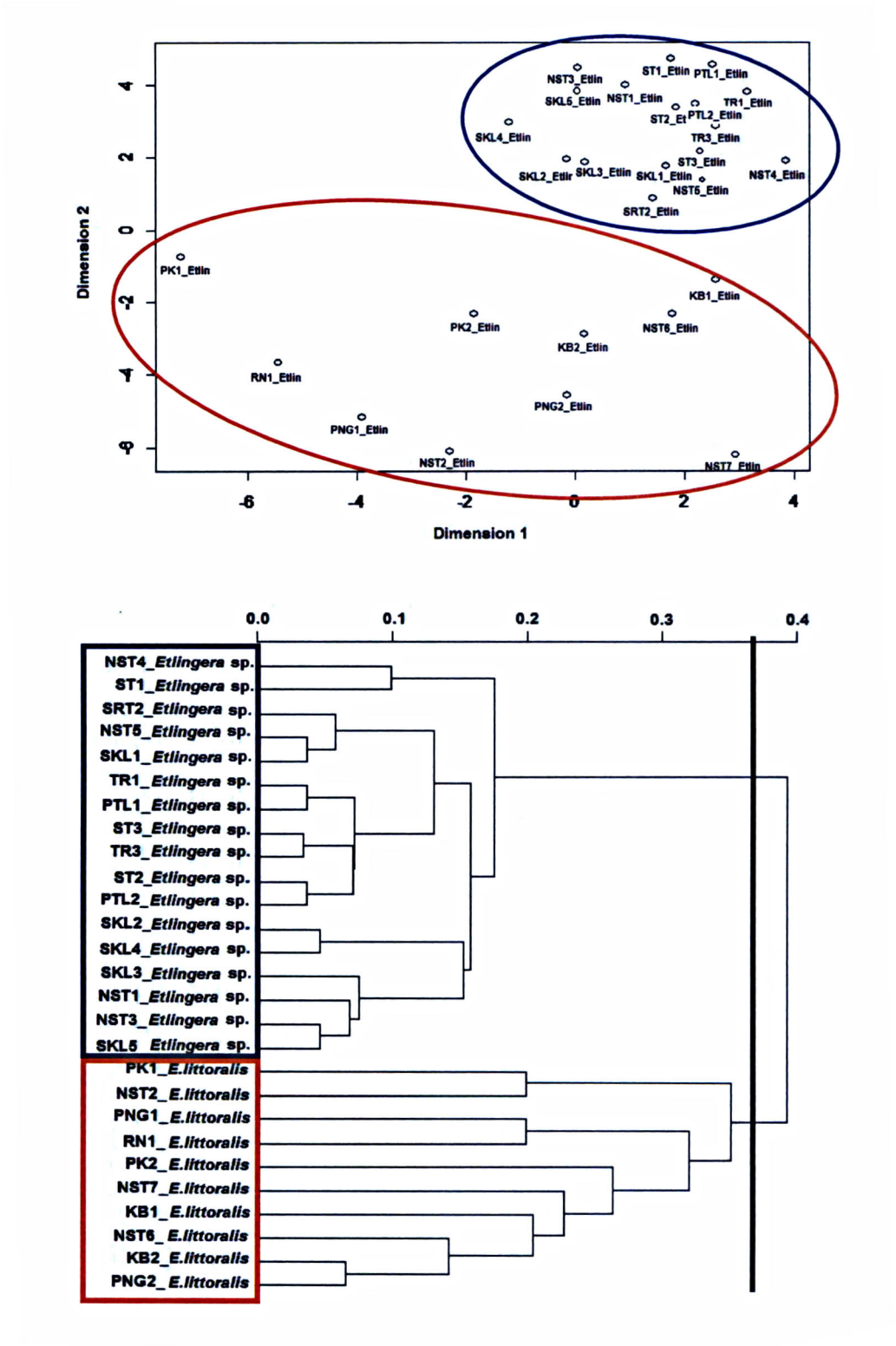

Figure 11 A cluster analysis pattern of only reproductive characters. The samples were separated into two groups by R statistic, R value = 0.79.

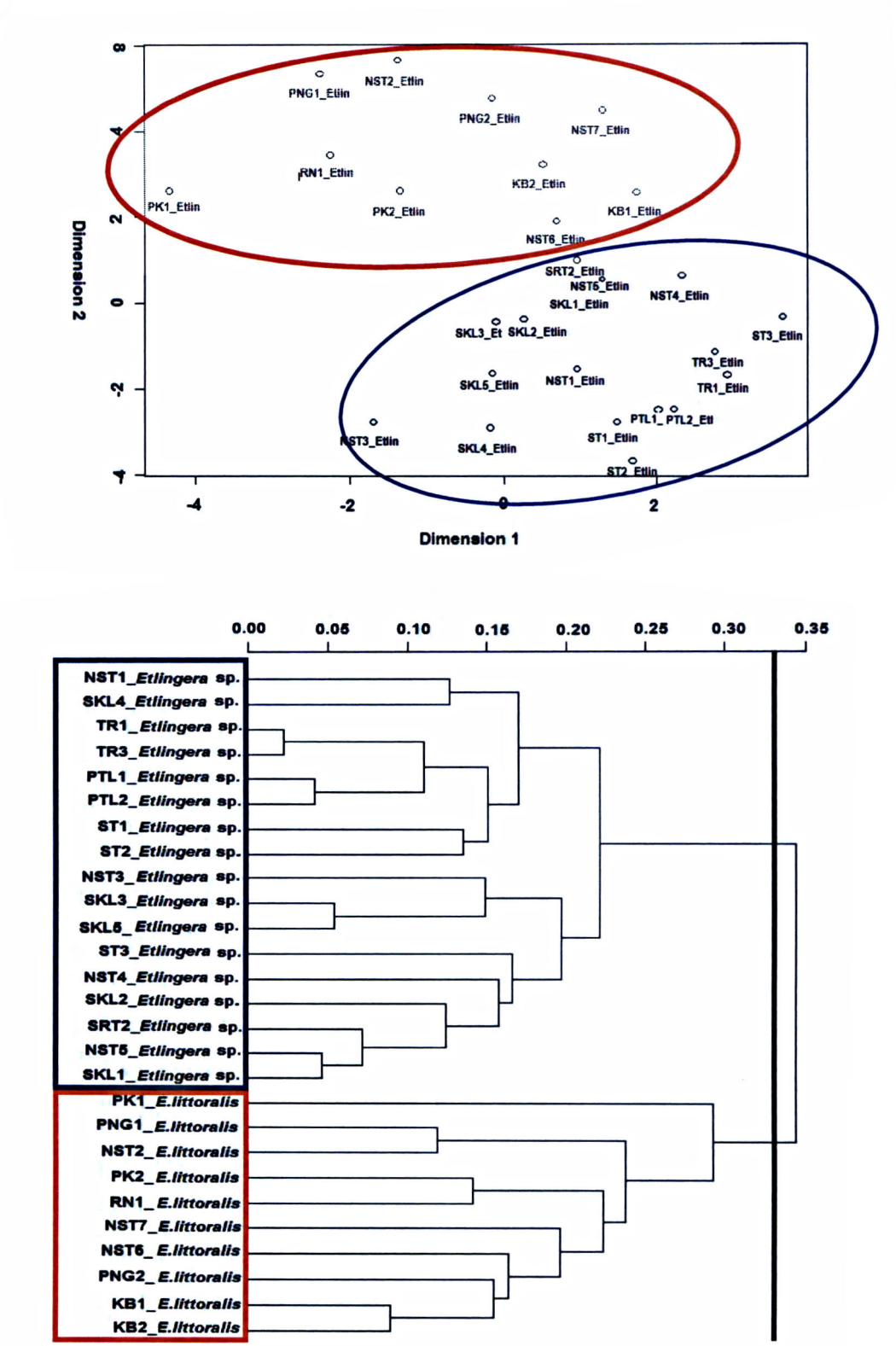

Figure 12 A cluster analysis pattern of reproductive and vegetative characters. The samples were separated into two groups by R statistic, R value = 0.55.

4.3 Molecular genetic studies

4.3.1 Internal Transcribed Spacer (ITS) analyses

There are 30 taxa in the ITS data matrix. Ten samples of *Etlingera listtoralis*, 17 samples of *Etlingera* sp., two samples of *E. araneosa* and one sample of *Honstedtia leonurus* from the GeneBank (accession AB097237.1) was also included as outgroup.

a. Sequence analyses

Alignments of ITS sequences were set with default values (i.e., gap opening and extension penalties) in Claustal X version 2.0.3 (Thompson *et al.*, 1997). The ITS sequences alignment resulted in 709 bp in length and its characteristics are shown in Table 5.

The length of complete ITS sequences were on average 676.6 bp. The length of aligned ITS1, 5.8s and ITS2 were 200, 148 and 349 bp respectively. Of these aligned guanine-cytosine (GC) content mean 57.1%. The sequences divergence of ITS1, 5.8s and ITS2 among ingroup species ranged from 0.00% to 3.72% while sequences divergence between the ingroup and outgroup species ranged from 1.49% to 3.77%. The maximum sequence variation among ingroup species was 3.72% between PK2_*E. littoralis* and NST5_*Etlingera* sp. The maximum sequence variation between ingroup and outgroup species was 3.77% between NST6_*E. littoralis* and *Hornstedtia leonurus*.

A total of 709 manually aligned characters were used for phylogenetic analyses. The results showed 638 (90%) constant characters, 71 (10%) variable parsimony uninformative characters, 37 (5.2%) parsimony informative characters between ingroup and outgroup and 34 (4.8%) parsimony autapomorphic characters.

The sequence of PK2_*E. littoralis* is the longest in this study (688 bp) and the shortest of the sequence belong to TR1_*Etlingera* sp. (667 bp).

Table 5 Sequence characteristics of ITS1, 5.8s, ITS2 (nuclear ribosomal DNA)

Parameter	ITS1,5.8s, ITS2
Length range (total) (bp)	615-688
Length mean (total) (bp)	676.6
Length range (ingroup) (bp)	676-688
Length mean (ingroup) (bp)	678.8
Length range (outgroup) (bp)	615-678
Length mean (outgroup) (bp)	657
Aligned length (bp)	709
G+C content range (%)	56.6-58.0
G+C content mean (%)	57.1
Sequence divergence (ingroup) (%)	0.00-3.72
Sequence divergence (in/outgroup) (%)	0.00-3.77
Number of variable sites (%)	71 (10)
Number of constant sites (%)	638 (90)
Number of informative sites (%)	37 (5.2)
Number of autapomorphic sites (%)	34 (4.8)
Transition/Transversion	1.36
Tree length	119
Average number of steps per character	0.17

b. Phylogenetic analyses

Parsimony analyses

Phylogenetic tree of *Etlingera* samples, with a total of 30 taxa, including 3 outgroup taxa and 27 taxa of ingroup, were reconstructed by PAUP* version 4.0b10 (Swofford, 2002). The analysis of the ITS sequence data resulted in two hundred most parsimonious trees, tree length of 119, consistency index (CI) = 0.698, retention index (RI) = 0.746 and rescaled consistency index (RC) = 0.521.

The 50% majority rule consensus tree showed two major clades (Figure 13), the first clade is the *Etlingera littoralis* clade with strong support (bootstrap value = 91%) and the other clade is the *Etlingera* sp. clade, with weak support (bootstrap value = 54%). The two samples, PK1 and PK2 from Phuket province, where a type specimen of *E. littoralis* was described and collected by Konig, were placed in the clade of *E. littoralis*.

Maximum likelihood analyses

Analysis of the ITS data set under the optimality criterion of maximum likelihood with the HKY85 model, which were examined by jModelTest 0.1.1 (Posada, 2003) with standard value for the model parameters. The resulting phylogram is given in Figure 14.

Analysis under the optimality criterion of maximum likelihood with the HKY85 model yielded the same result whether the analysis was conducted by parsimony by PAUP, and the topologies of the ingroup portion of the resulting trees were essentially identical to the single topology found under parsimony analysis. Bootstrap values computed under the maximum likelihood criterion (100 replicates) are similar to those determined under parsimony criterion, ranging from 51 to 100%.

The resulting phylogram is given in figure 14, and the best tree was 1697.6741, estimated parameters are -log L = 1649.846, transition/transversion ratio = 0.568, nucleotide A 0.282, C = 0.198, G = 0.236, T = 0.283 and gamma shape parameter alpha = 0.0138. The maximum likelihood tree, when compared to the strict consensus tree of the parsimony analysis, is very similar. *Etlingera littoralis* and *Etlinger* sp. are separated into two different clades. The *E. littoralis* clade is separated from *Etlingera* sp. with strong support (bootstrap value = 89%).

Bayesian analyses

In addition, the ITS data were also generated under the criterion of Bayesian using MrBayes 2.0 (Huelsenbeck and Ronquist, 2001), with a posterior probability distribution using Metropolis-couple MCMC under the GRT model. The

results from the Bayesian analysis are very similar to the ML and MP analyses, although the Bayesian posterior probabilities are generally higher than the ML and MP bootstrap values. *E. littoralis* clade was clearly separated from the *Etlingera* sp. clade, with strong support (bootstrap value = 100%) (Figure 15).

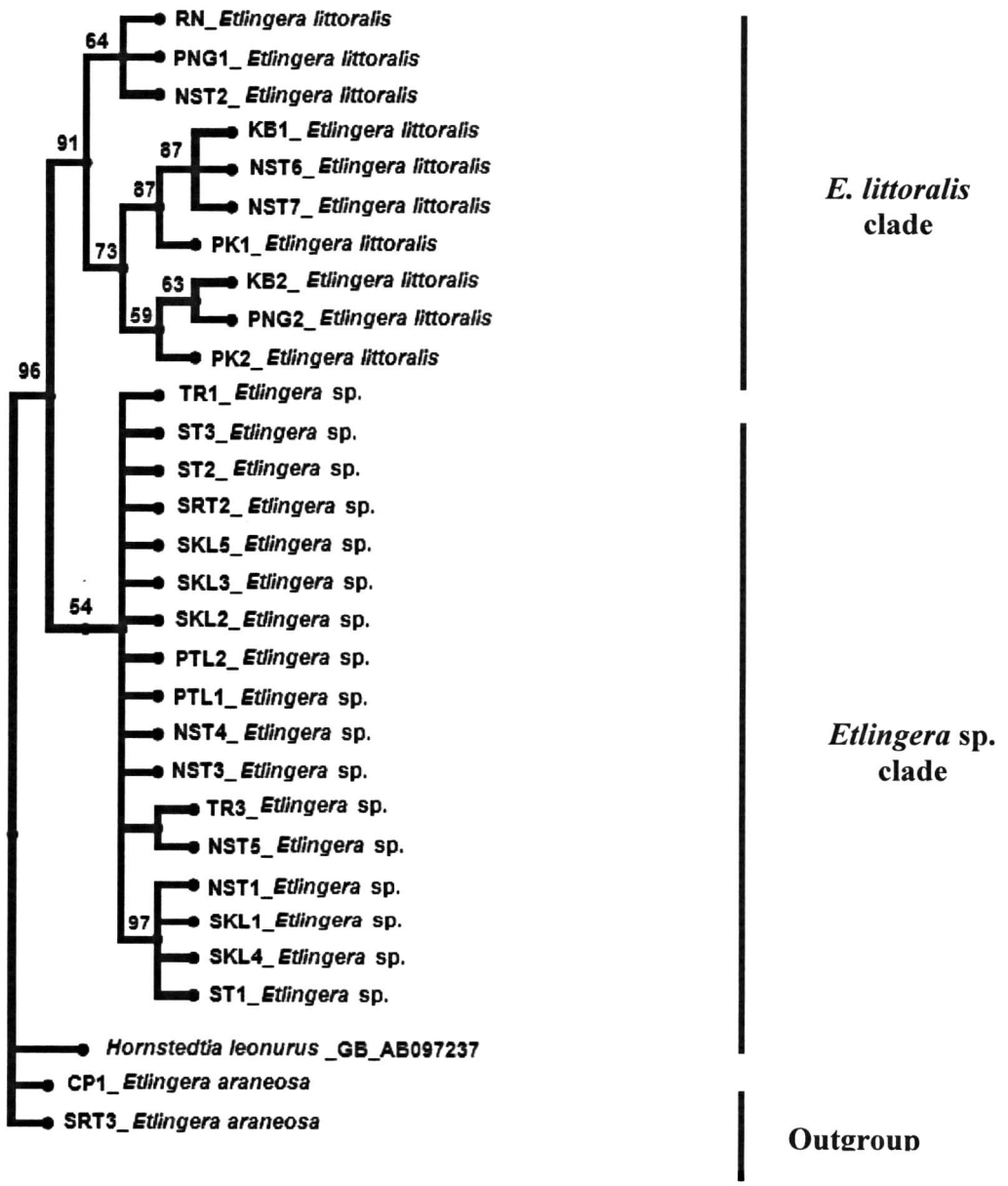

Figure 13 The 50% majority rule consensus tree of the parsimonious trees resulting from the analysis of 30 taxa based on ITS sequences. Upper numbers are bootstrap values (>50% are shown) of 1,000 replicates. (CI=0.698, RI=0.746, RC=0.521).

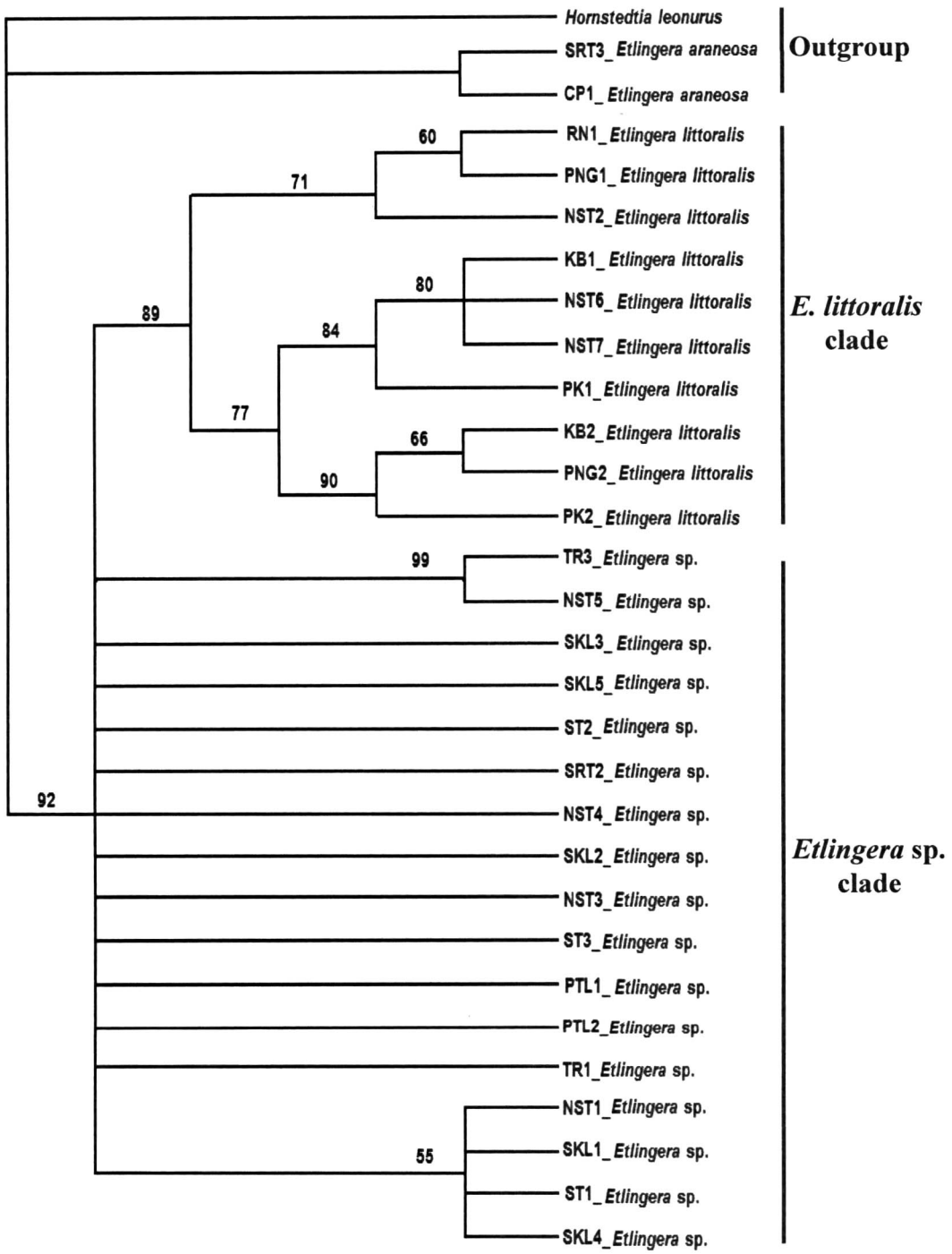

Figure 14 The maximum likelihood tree inferred from the ITS data based on the HKY85 model of molecular evolution Upper numbers are bootstrap values (>50% are shown) of 1,000 replicates, (-log L = 1649.846).

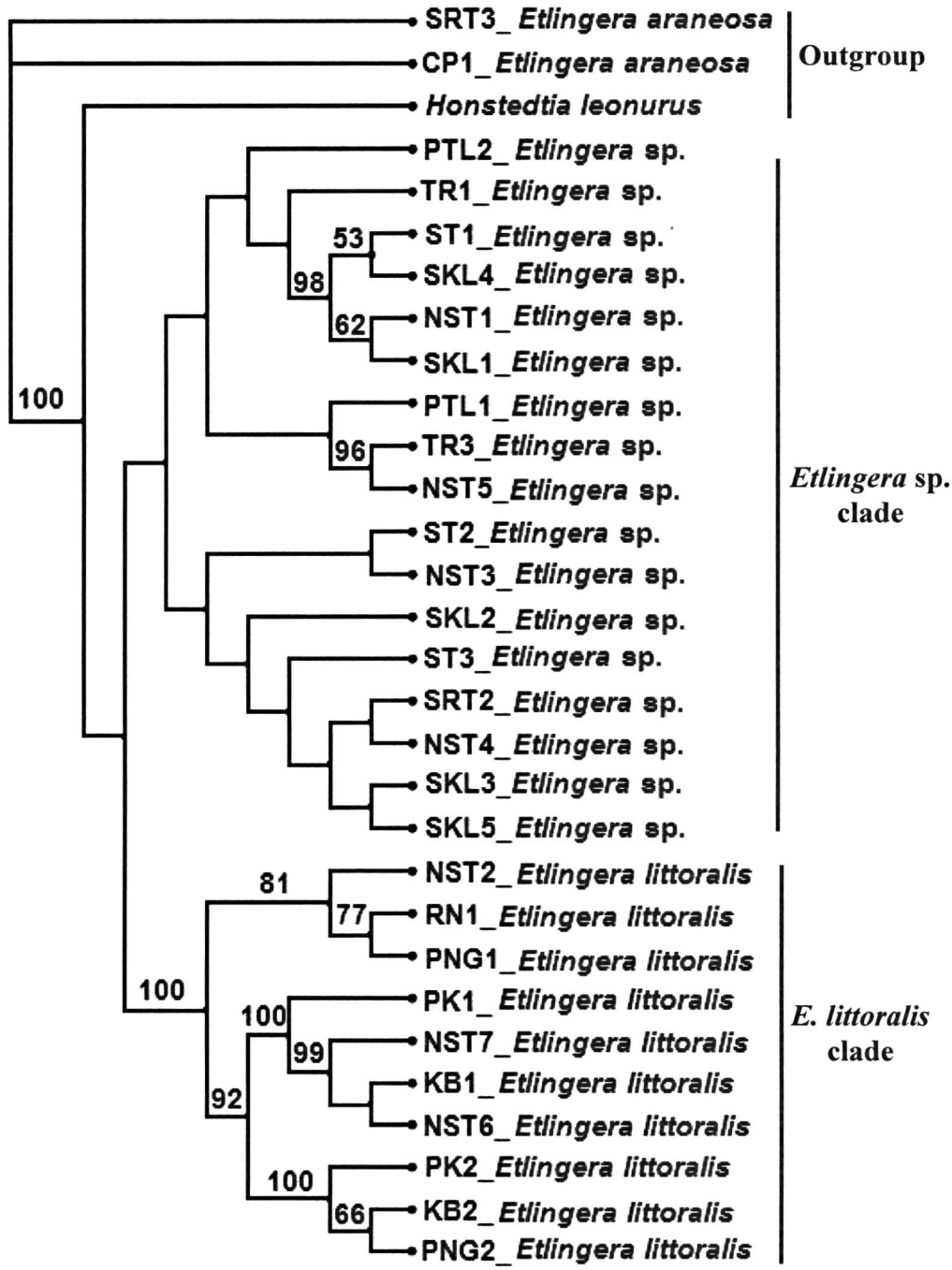

Figure 15 A 50% majority rule consensus tree of the Bayesian tree inferred from the ITS data set data based on the GRT model of molecular evolution. Upper numbers are bootstrap values (>50% are shown) of 1,000 replicates.

4.3.2 *mat*K analyses

Etlingera in Southern Thailand were collected for *mat*K analysis, comprising twenty-three samples (9 samples as *Etlingera listtoralis* and 12 samples as *Etlingera* sp.) and two samples of *E. araneosa*, which they were used as the outgroup.

a. Sequence analysis

The partial of *mat*K sequence alignment of twenty-three (including two outgroups) samples were analyzed by Claustal X version 2.0.3 (Thompson *et al.*, 1997). The total aligned lengths of sequence were 810 bp, unaligned sequences ranged from 803-807 bp, with a mean GC content of 32.18%. The aligned sequences of the 23 taxa showed that among the 810 nucleotides, 652 (80.49%) were constant, 158 (19.51%) were variable and 147 (18.15%) were phylogenetically informative (Table 6).

The sequences divergence of *mat*K among ingroup species ranged from 0.25%-12.72% while sequences divergence between the ingroup and outgroup species ranged from 3.49%-10.97%. The maximum sequence variation among ingroup species was 12.72% between PTL1_*Etlingera* sp. and KB1_*E. littoralis*. The maximum sequence variation between ingroup and outgroup species was 10.97% between PTL1_*Etlingera* sp. and SRT3_*E. araneosa*.

b. Phylogenetic tree analyses

Parsimony analyses

The phylogenetic tree of twenty-three *mat*K sequences were analyzed by PAUP* version 4.0b10 (Swofford, 2002). Parsimony analysis of aligned partial *mat*K sequences provided 200 most parsimonious trees, with a tree length (TL) of 455, consistency index (CI) of 0.462), a retention index (RI) of 0.722, and rescaled consistency index (RC) of 0.334 (Figure 16).

The consensus tree showed that among *E. littoralis* clade are strongly supported as a monophyletic group, which they are separated from the *Etlingera* clade by bootstrap value = 85%. However, one species of *Etlingera* sp. (NST1_*Etlingera* sp.) appeared to be a stem lineage of *E. littoralis* clade with strong support (bootstrap value = 85%).

Maximum likelihood analyses

A maximum likelihood analysis of the *mat*K data sets of twenty-three sequences was conducted using the HKY85 model of molecular evolution (Hasegawa *et al.*, 1985). Rate variation among sites following gamma parameter (Jin and Nei, 1990) was incorporated into the models.

The phylogenetic trees under the HKY85 model were retained and shown in figure 17. The score of the best tree found by PAUP was 3675.181. The value of the gamma shape parameter alpha with four discrete rate categories = 0.0126 and the estimated parameters were -log L = 3275.715, transition/transversion ratio was 0.511, with the following nucleotide frequencies: A = 0.333, C = 0.142, G = 0.179, T = 0.345. The topology of 50% majority rule consensus tree was clearly similar with that of the parsimony analysis. The *E. littoralis* clade are separated from the other clade with high support (bootstrap value = 98%).

Bayesian analyses

There are twenty-three of partial *mat*K alignment sequences, which were analyzed by Bayesian method. The phylogenetic tree was reconstructed under the GRT model. The tree topology is very similar to that of the analyses of MP and ML of *mat*K data by PAUP*. *E. littoralis* clade, with NST1_*Etlingera* sp. as a basal taxon was high support (bootstrap value = 97%). In addition, the bootstraps of the Bayesian tree, both *E. littoralis* and *Etlingera* sp. clades are higher than those of MP and ML trees (Figure 18).

Table 6 Sequence characteristics of partial *mat*K gene (chloroplast genome)

Parameter	Partial *mat*K
Length range (total) (bp)	803-807
Length mean (total) (bp)	805
Length range (ingroup) (bp)	803-805
Length mean (ingroup) (bp)	804
Length range (outgroup) (bp)	805-807
Length mean (outgroup) (bp)	806
Aligned length (bp)	810
G+C content range (%)	29.96-34-35
G+C content mean (%)	32.18
Sequence divergence (ingroup) (%)	0.25-12.72
Sequence divergence (in/outgroup) (%)	3.49-10.97
Number of variable sites (%)	158 (19.51)
Number of constant sites (%)	652 (80.49)
Number of informative sites (%)	147 (18.15)
Number of autapomorphic sites (%)	11 (1.36)
Transition/Transverion	0.511
Tree length	455
Average number of steps per character	0.56

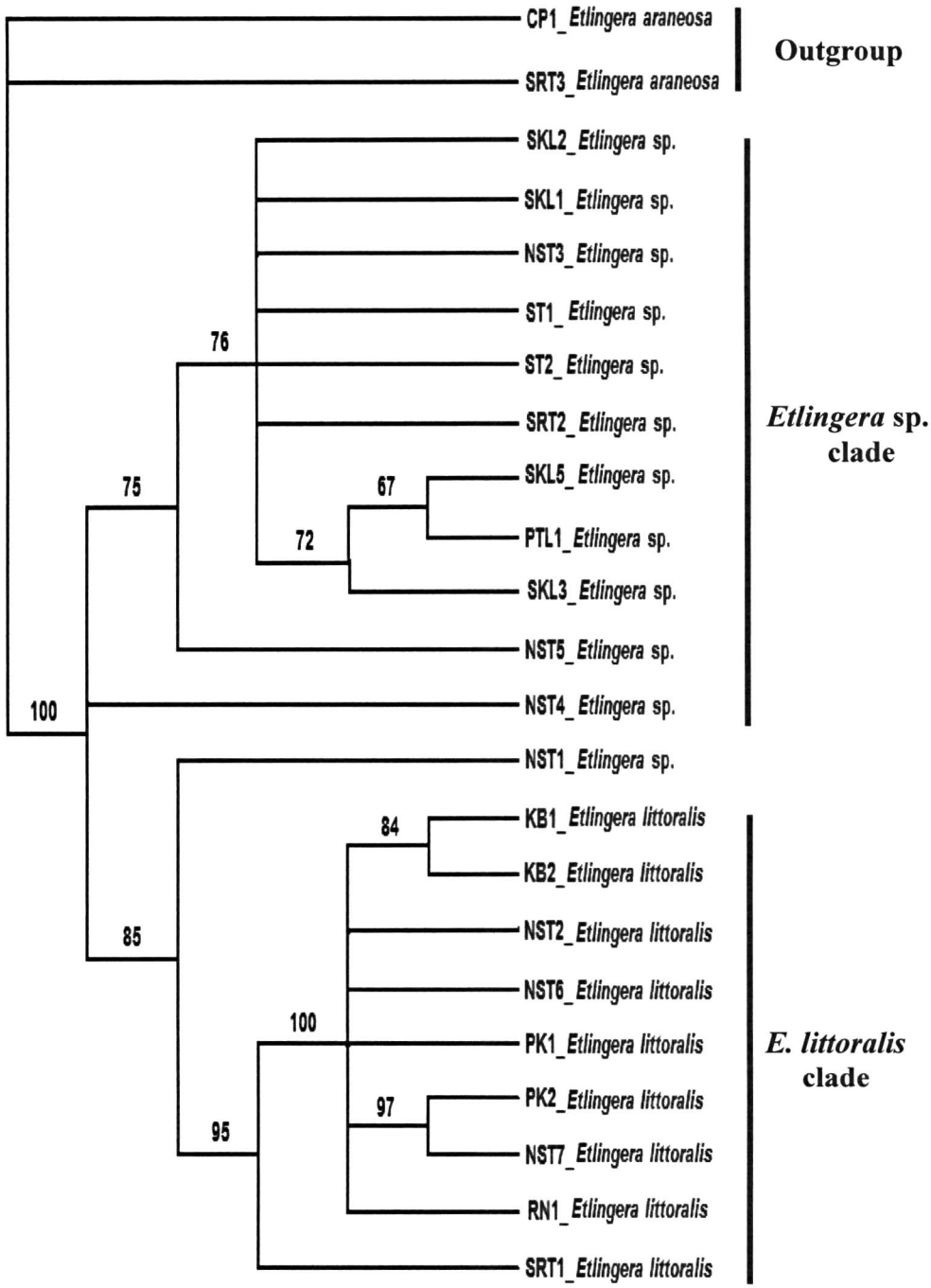

Figure 16 The 50% majority rule consensus tree of the parsimonious trees resulting from the analysis of 23 taxa based on *mat*K sequences. Upper numbers are bootstrap values (>50% are shown) of 1,000 replicates. (CI = 0.462, RI = 0.722, RC = 0.334).

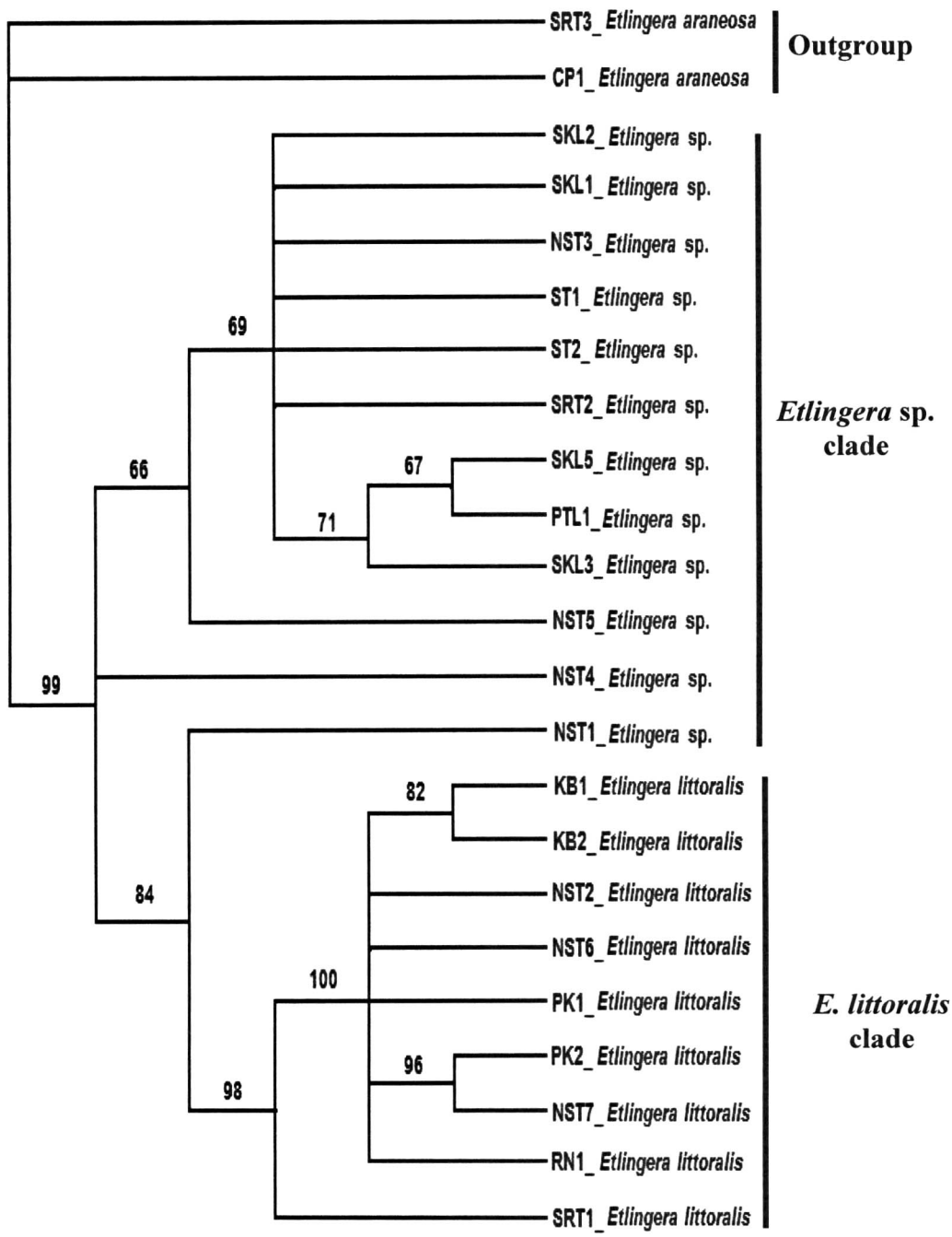

Figure 17 The maximum likelihood tree inferred from the *mat*K data based on the HKY85 model of molecular evolution. Upper numbers are bootstrap values (>50% are shown) of 1,000 replicates (-log L = 3275.715).

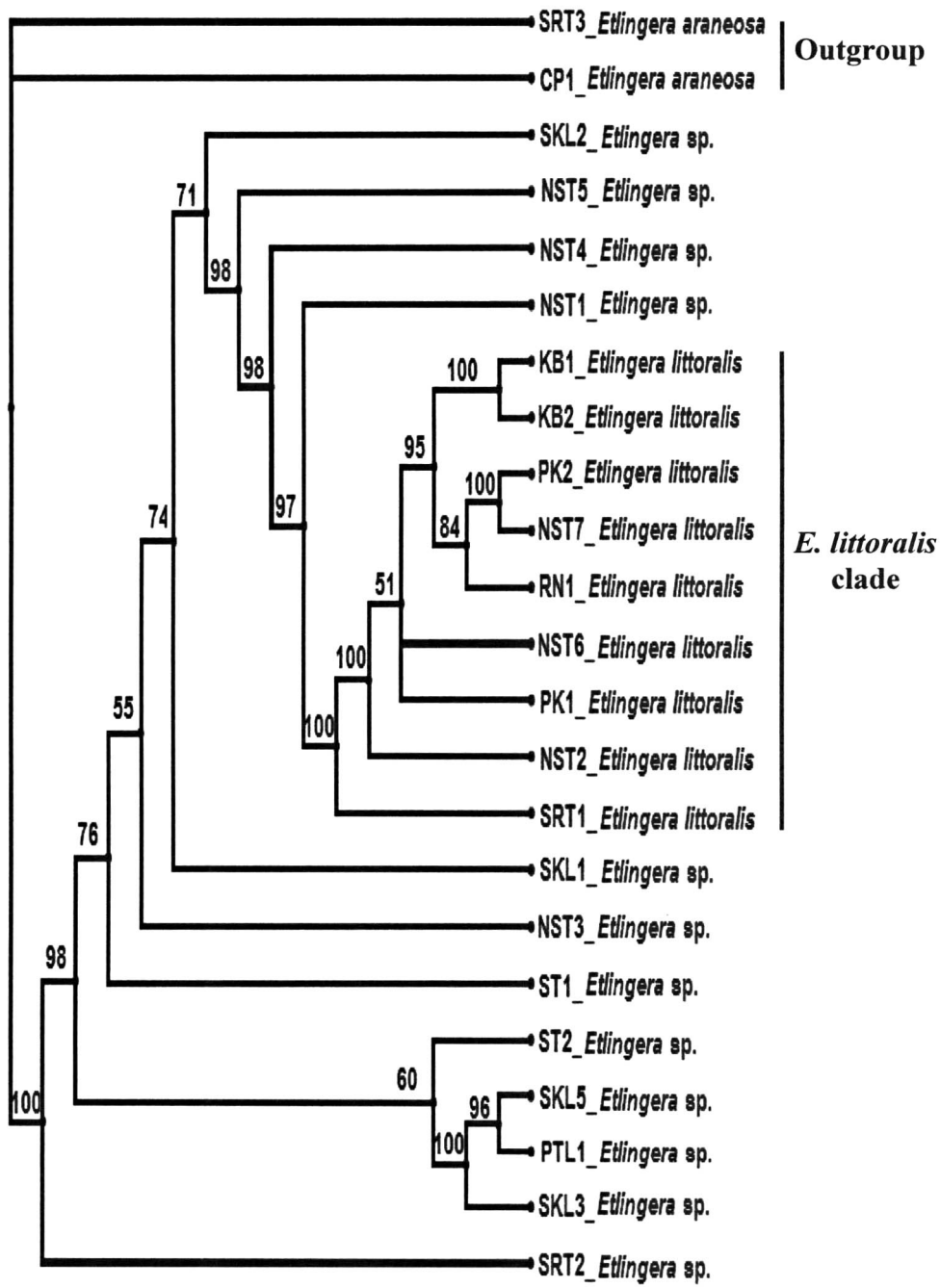

Figure 18 A 50% majority rule consensus tree of the Bayesian tree inferred from the *mat*K data set data based on the GRT model of molecular evolution. Upper numbers are bootstrap values (>50% are shown) of 1,000 replicates.

4.3.3 Combined ITS and partial *mat*K data set analysis

Etlingera samples in Southern Thailand were collected for combined ITS and *mat*K, comprising twenty-two samples (8 samples as *Etlingera littoralis* and 12 samples as *Etlingera* sp.) and two samples of *Etlingera araneosa*, which they were used as the outgroup.

a. Sequence analyses

The aligned matrix of the combined ITS and partial *mat*K data of twenty-two samples (including two outgroups) were analyzed by Claustal X version 2.0.3 (Thompson *et al.*, 1997). The total aligned lengths of sequence were 1,480 bp (unaligned sequences ranged from 1,440bp to 1,451bp) with a mean GC content of 48.13%. The aligned sequences of the 22 taxa showed that among the 1480 nucleotides 1,307 (88.31%) were constant, 173 (11.69%) were variable and 157 (10.61%) were phylogenetically informative (Table 7).

Table 7 Sequence characteristics of combined ITS and partial *mat*K gene.

Parameter	combined *mat*K and ITS data
Length range (total) (bp)	1440-1451
Length mean (total) (bp)	1445.59
Length range (ingroup) (bp)	1440-1451
Length mean (ingroup) (bp)	1445.40
Length range (outgroup) (bp)	1446-1449
Length mean (outgroup) (bp)	1447.50
Aligned length (bp)	1480
G+C content range (%)	42.61-49.22
G+C content mean (%)	48.13
Sequence divergence (ingroup) (%)	0.28-7.63

Table 7 Sequence characteristic of combined ITS and partial *mat*K gene. (Cont'd)

Parameter	combined *mat*K and ITS data
Sequence divergence (in/outgroup)(%)	2.00-6.50
Number of variable sites (%)	173 (11.69)
Number of constant sites (%)	1307 (88.31)
Number of informative sites (%)	157 (10.61)
Number of autapomorphic sites (%)	16 (1.08)
Transition/Transversion	0.568
Tree length	437
Average number of steps per character	0.29

The sequences divergence of combined data set among ingroup species ranged from 0.28-7.63 while sequences divergence between the ingroup and outgroup species ranged from 2.00-6.50. The maximum sequence variation among ingroup species was 7.63 between PTL1_*Etlingera* sp. and KB1_*E. littoralis*.

a. Phylogenetic tree analysis

Parsimony analysis

Parsimony analysis was carried out using a Phylogenetic Analysis Using Parsimony (PAUP*) software, version 4.0b10 (Swofford, 2002). The most parsimony trees were obtained through the heuristic search option. Bootstrapping (1000 replicates) was performed to assess levels of support for individual clade using the heuristic search with random sequence addition.

The analysis of the combined ITS and partial *mat*K sequence data resulted in 40,198 equally parsimonious trees of 437 steps (number of parsimony-informative characters = 157; CI = 0.462; RI = 0.722; RC = 0.334). A 50% majority rule consensus tree of these 40,198 shortest trees provided highly similar tree

topologies to the topology of consensus tree, resulting from individual ITS and partial *mat*K.

The 50% majority rule consensus tree resolves two major clades; the *E. littoralis* clade and the *Etlingera* sp. clade. The *E. littoralis* clade is clearly separated from the *Etlingera* sp. clade with strong support (bootstrap value = 100%). Whereas the *Etlingera* sp. clade is also highly supported (bootstrap value = 91%) (Figure 19).

Maximum likelihood analyses

Analysis under the optimality criterion of maximum likelihood with the HKY85 model yielded the same topologies with that of the parsimony analysis by PAUP*. The topologies of the ingroup portions of the resulting trees were essentially identical to the single topology found under maximum likelihood analysis. Bootstrap values computed under the maximum likelihood criterion (1000 replicates) are similar to those determined under parsimony criterion, ranging from 51 to 100%.

The resulting phylogram is given in Figure 20, and the best tree was 1697.6741, estimated parameters are -log L = 4488.145, transition/transversion ratio = 0.568, nucleotide parts are A = 0.282, C = 0.198, G = 0.236, T = 0.283 and gamma shape parameter alpha = 0.0138. The maximum likelihood tree, in comparison to the strict consensus tree of the parsimony analysis, is very similar in topology. *E. littoralis* and *Etlingera* sp. are separated into two different clades. The *E. littoralis* clade is separated from the *Etlingera* sp. clade with strong support (bootstrap value = 100%).

Bayesian analyses

Bayesian analyses of combined data were studied by MrBayes program version 3.1.2 under the GRT model. Each search was run for 1,000,000 generations and every 100^{th} tree was sampled. Burn-in, or the time for each parameter to reach stationary, was determined when visual inspection indicated that the log-likelihood values reach an asymptote over a large number of generations.

The 50% majority rule consensus tree from the Bayesian analysis resolves topology that is very similar to that of the analyses of MP+MLcombined data set. The phylogram showed two highly supported clades; the *E. littoralis* clade (bootstrap value = 100%) and *Etlingera* sp. clade (bootstrap value = 94%) (Figure 21).

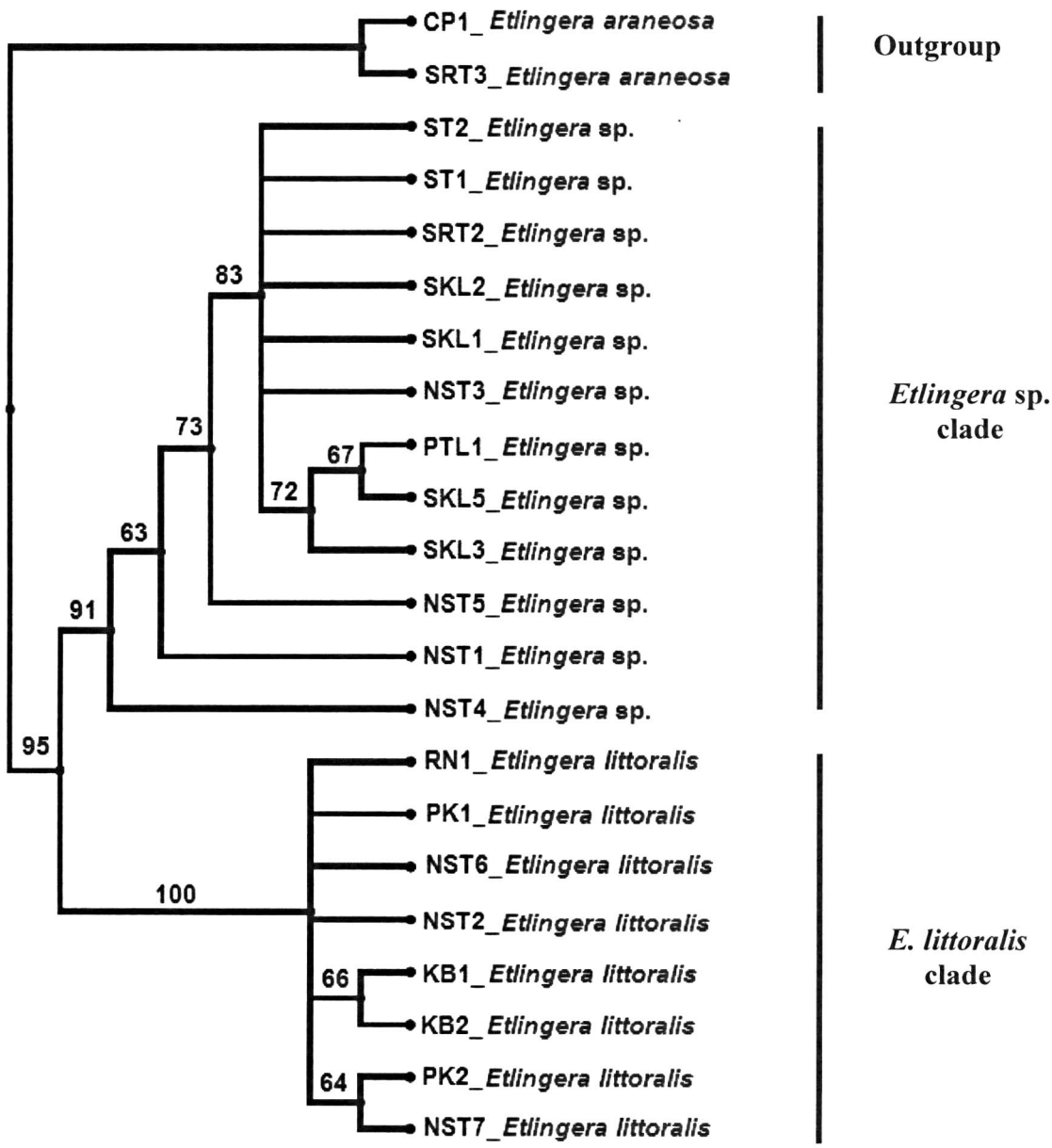

Figure 19 The 50% majority rule consensus tree of the parsimonious trees resulting from the analysis of 22 taxa based on combined ITS and partial *mat*K region sequences data. Upper numbers are bootstrap values (>50% are shown) of 1,000 replicates. (CI = 0.462; RI = 0.722; RC = 0.334).

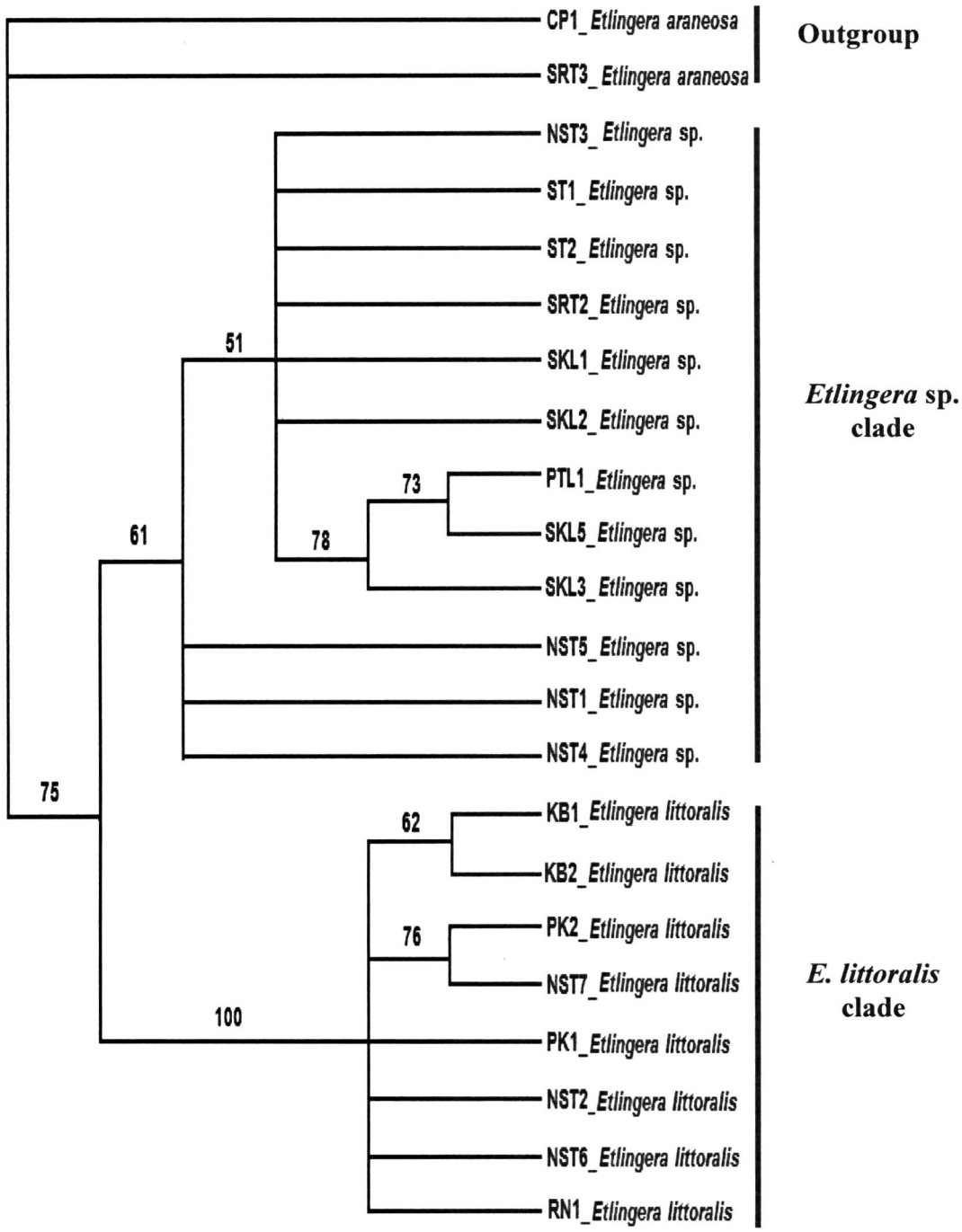

Figure 20 The maximum likelihood tree inferred from the combined ITS and *mat*K data based on the HKY85 model of molecular evolution. Upper numbers are bootstrap values (>50% are shown) of 1,000 replicates, (-log *L* = 4488.145).

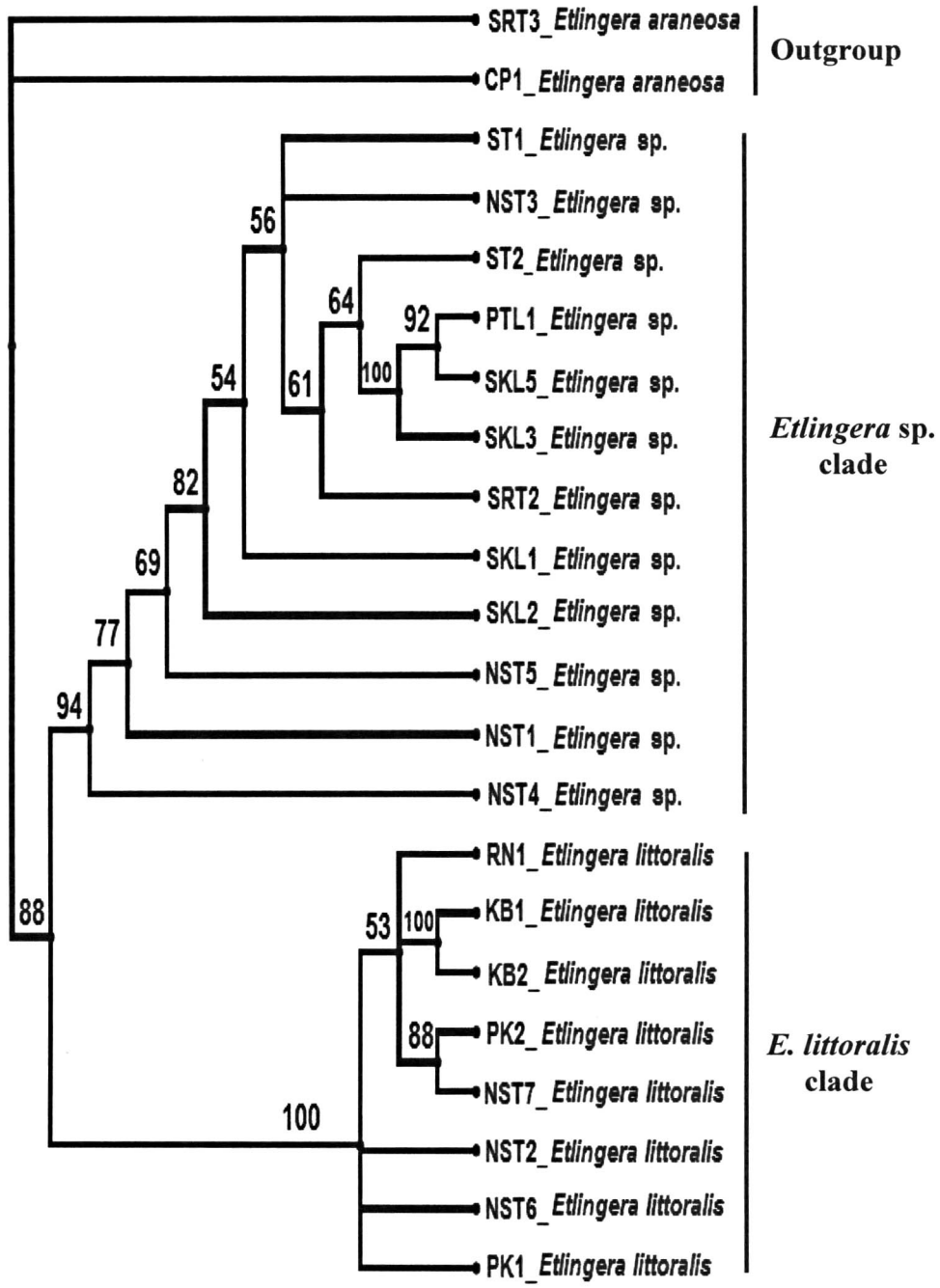

Figure 21 A 50% majority rule consensus tree of the Bayesian tree inferred from the combined ITS and *mat*K data set data based on the GRT model of molecular evolution. Upper numbers are bootstrap values (>50% are shown) of 1,000 replicates,

Chapter 5

Discussion

From this study, *E. littoralis* (König) Giseke and *E. megalocheilos* (Griff.) A.D. Poulsen have been confirmed that they actually are different, even though *E. megalocheilos* has not been found in Southern Thailand yet. However, there is one collection in Peninsular Malaysia which photos of both inflorescences and fruits were taken (Forest Research Institute Malaysia (FRIM), pers. comm.). Comparison between the two species showed that the inflorescences of *E. littoralis* and *E. megalocheilos* are quite analogous, except the fruits are rather different, *E. littoralis*'s fruits are deeply ridge, but *E. megalocheilos* smooth (Figure 22).

Interestingly, *E.* sp. is another species which was collected from Southern Thailand. *Etligera* sp. cannot be identified to both *E. littoralis* and *E. megalocheilos*, even though the external morphology, including their infructescences and fruits look very like to *E. littoralis*'s (Figure 23) but other morphological characters are quite different, particularly reproductive characters such as inflorescence color pattern, labellum length, labellum and stamen length ratio, and angle of anther.

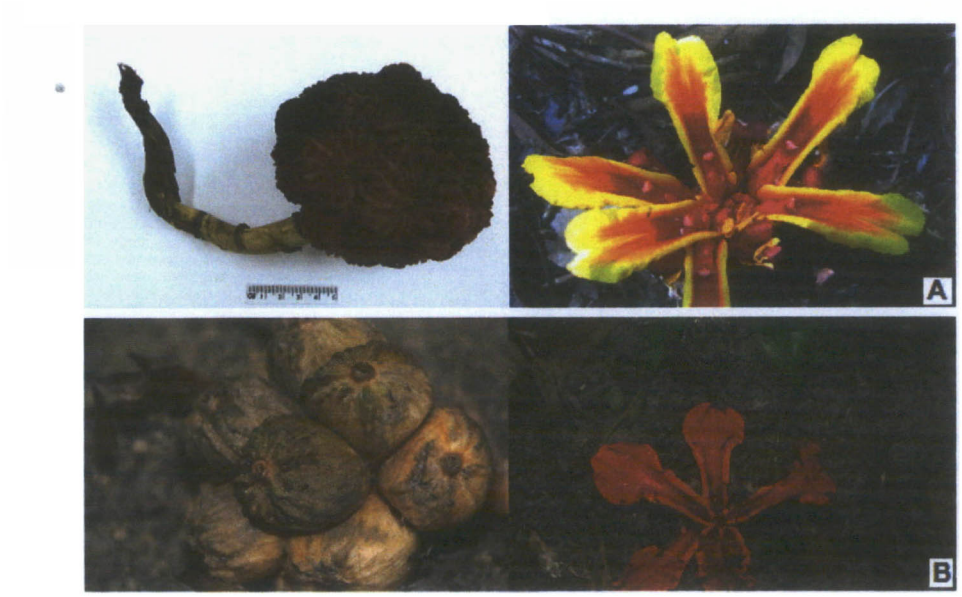

Figure 22 The infructescence and inflorescences of *E. littoralis* (A) and *E. megalocheilos* (B).

Figure 23 The infructescence and inflorescences of *Etlingera* sp. (A) and *E.littoralis* (B).

5.1 Morphological studies of *Etlingera littoralis* and *Etlingera* sp.

5.1.1 External morphology

External morphologies of *Etlingera* sp. and *E. littoralis* samples, which were collected from Southern Thailand are very similar in terms of vegetative part e.g. leaf (green blade color), leaf margin (most often ciliate to pubescent), leafy shoot tall (up to 8-10 m, the sheath is often striate or variously reticulate) etc. Considering floral morphology, on the other hand, it is superficially similar. The inflorescence of *E. littoralis* is short and compact. Each flower shows bright red and yellow labellum (Kittipanangkul and Ngamriabsakul, 2006). Differently, the floral morphology of *Etlingera* sp. is varying. There are two different inflorescence color forms; absolute red and median red with yellow edge labellum (Figure 24).

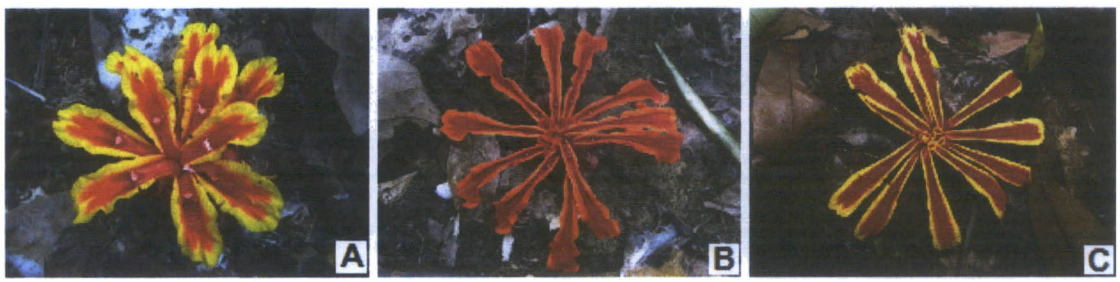

Figure 24 Inflorescence of *E. littoralis* (A), *Etlingera* sp. (entirely red) (B), and *Etlingera* sp. (median red with yellow edged labellum) (C)

5.1.2 Morphological character analysis using R statistic

Thirty one morphological characters (Table 3); fifteen characters of vegetative part and fifteen characters of reproductive part were selected for morphological species identification using R statistic (R Development Core Team, 2010). The data analysis was divided into three patterns; only vegetative characters, only reproductive characters, and combined vegetative and reproductive characters.

From the results, the characters of vegetative part cannot be used for species identification because those characters are very morphologically similar.

Considering sixteen characters of reproductive part, the cluster analysis showed that *E. littoralis* and *Etlingera* sp. were completely separated into two groups; *E. littoralis* group and *Etlingera* sp. group. Morphologically, *E. littoralis* is distinguished from related species, *Etlingera* sp. by many floral characters, such as inflorescence pattern color, labellum length, labellum and anther length ratio, and the angle of anther.

5.1.3 Species distributions and their ecology

Etlingera samples in this study; *E. littoralis* and *Etlingera* sp. were found in both Gulf of Thailand and Andaman Sea coasts (Figure 6). It is because both coasts are quite similar in weather and topographic characters, which are suitable for the plant growth.

Peninsular Thailand comprises 14 provinces covering an area of 70,715 km, approximately 14% of the country. About 40% of the region is hilly or mountainous and the highest peak, Khao Luang (1,835 m), lies in Nakhon Si Thammarat province. The peninsula has a tropical monsoon type climate and, in its south and west, the natural conditions resemble tropical rainforest. The peninsula experiences higher temperatures, heavier rainfall and more frequent precipitation than other areas of Thailand during the Northeast monsoon. The greatest contrast occurs from November to January when the peninsula is hot, humid and rainy while the mainland is relatively cool and dries (Charoenpong, 1991).

Etlingera samples, which were collected from Southern Thailand, can grow in different areas. They stand along logging road, river bank, damp and humid shady places (Sirirugsa, 1989) (Figure 25). They are also found infrequently in secondary forest, gap area, lowlands to the highest elevations in secondary and primary forests, respectively. Some species can fully expose to the sun (Kittipanangkul and Ngamriabsakul, 2006). The *E. littoralis* is mostly found in upper part, while *Etlingera* sp. is mostly found in lower part of Southern Thailand. There are only two provinces; Surat Thani and Nakhon Si Thammarat provinces, which both

plant species were found concurrently. The lower part of Southern Thailand has environmental conditions such as temperature, humidity, rainfall, etc. better than upper part of Southern Thailand. So, it is a possible condition for *Etlingera* sp. but not *E. littoralis*. However, *Etlingera* sp. may be widely distributed in Malay Peninsula and just early extend to Southern Thailand. In addition, some species of the Flora of Thailand has encouraged collaboration with Flora Malesiana because of the considerable overlap in the floras (65% of Thai species are also found in Malesia) (Pendry *et al.*, 2009). Peninsular Thailand includes the important biogeographic transition between Thai seasonal dry evergreen forest and the extremely diverse mixed dipterocarp forest (Van Steenis, 1950; Whitmore, 1984) characteristic of much of western Malesia. This transition has never been quantitatively described but it is clear that the Isthmus of Kra, The northern limit for Flora Malesiana accounts, is much further north than the edge of this forest type. However, there are areas of it in the southern Thai provinces right on the Malaysian border so one would expect many more of the Malaysian elements to be found in this area if they were better collected. This increased collecting would have benefits: firstly that taxa found there could be incorporated into the ongoing Flora of Thailand, and secondly that biogeographic studies would have a more accurate pool of data to use in describing this transition zone (Woodruff, 2003; Middleton, 2003).

In addition, *E. araneosa*, which were used as outgroup, found in two provinces; Chumporn and Surat Thani provinces. *E. araneosa* was first described from Myanmar and commonly found along border areas in northern Thailand. In this study, in addition, *E. araneosa* are also found in Southern Thailand; Chumporn and Suratthani provinces.

Figure 25 *Etlingera* samples were found in different habitats in Southern Thailand.

5.2 Molecular genetics analyses

5.2.1 Sequence characteristic

Normally, the total lengths of ITS1, 5.8s and ITS2 regions in Zingiberaceae range from 576 to 704 bp (Zhao *et al.*, 2001; Kress *et a*l., 2002; Takano and Okada, 2002; Williams *et al.*, 2004). In this study, the unaligned length of the *Etlingera* sample range from 615 to 688 bp. These range are within the ITS length variation of Zingiberaceaae (Li *et al.*, 2002). Whereas, the sequences of *mat*K gene are 1,534 to1600 bp in length of the Zingiberaceae (Cheng *et al.*, 2000; Li *et al.*, 2002; Nyffeler, 2002). However, partial *mat*K sequences analysis of *Etlingera* samples in this study range from 803 to 807 bp.

5.2.2 Phylogenetic tree analyses

The results of the phylogenetic analyses using ITS, *mat*K and combined data regions by Maximum Parsimony (MP), Maximum Likelihood (ML), and Bayesian Inference (BI) showed two different clades of *Etlingera littoralis* and *Etlingera* sp.

Internal Transcribed Spacer (ITS)

The ITS sequences alignment of thirty *Etlingera* samples were constructed and submitted to phylogenetic analyses. The data matrix was produced MP using PAUP* 4.1b10 (Swofford, 2002). The results of parsimony reveal that the *Etlingera* samples collected from Southern Thailand were divided into two major monophyletic lineages. One comprises the *E.littoralis,* while the other consists of species of *Etlingera* sp. (Figure 13). The *Etlingera ittoralis* is strongly supported to be monophyletic clade (bootstrap = 91%) and under conditions of MP this clade is strongly supported by 37 informative sites. Nonetheless, the *Etlingera* sp. clade is weakly supported (bootstrap = 54%) and some taxa lacked bootstrap support. However, it is clear that *E. littoralis* and *Etlingera* sp. are different species, even though sequences divergence between *E. littoralis* and *Etlingera* sp. is low. ML analyses were performed based on the general time reversible nucleotide substitution model (HKY model), which allows different substitution frequencies for each type of nucleotide substitution, with rate variation among sites model using discrete gamma distribution with four categories (Yang, 1994) and a separate category for the percentage of invariable sites. The ML analyses yielded very similar topologies to MP tree topologies.

matK

The plastid *mat*K gene has been among the most useful single loci for plant phylogenetic at both shallow and deep levels of evolution (Soltis and Soltis,

1998; Hilu *et al.*, 2003; Hilu *et al.*, 2008). The *matK* gene stands out among genes used in plant systematics in its substantially greater number of: (1) nucleotide substitutions, (2) nonsynonymous mutations, and (3) insertion/deletion events or indels (Johnson and Soltis, 1994; Olmstead and Palmer, 1994; Hilu and Liang, 1997; Soltis and Soltis, 1998). The gene also exhibits a relatively high proportion of transversions, with the transition/transversion ratio (ts/tv) approaching unity (Olmstead and Palmer, 1994; Hilu and Liang, 1997).

The partial *mat*K data set, consisting of 810 aligned site and 147 informative characters provide to be somewhat successful in resolving relationships among the major clades in the *Etlingera* samples. The *mat*K region can be established the effectiveness for phylogenetic studies at higher taxonomic levels and those robust phylogenies can be generated from partial sequences. The entire *mat*K gene might not be as informative or necessary as the use of sections of the gene because some sectors of the gene might provide phylogenetic noise (Hilu and Liang, 1997)

MP analysis of *Etlingera* in the partial *mat*K data set result in two hundred parsimoneous trees of length 455 steps. The topology of MP tree of *mat*K is similar to MP tree in ITS analysis, with two different clades of *Etlingera* sp. and *E. littoralis*. However, bootstrap values of *mat*K are higher than those in ITS phylogenetic tree. *E. littoralis* clade was supported by 100% bootstrap and *Etlingera* sp. clade was also highly supported by bootstrap values (85%). The study confirms this result and resolves two separate groups of species of *Etlingera* samples.

ML analyses were carried out under the HKY model, because the model had an optimal fit to the original data and was the most commonly selected model for the bootstrap replicates (Gastony and Ungerer, 1997). Bootstrap values computed under the ML criterion are very similar to those determined under MP ranking from 67 to 100% (Figure 16). The ts/tv of *mat*K analyses were 0.511 and the genetic divergence ranked from 3.49 to 10.97.

Though *mat*K has provided adequate information to resolve species relationships in some taxa, it offers less resolution at lower taxonomic levels (Shaw *et al.* 2005). However, it can be used in this study to identify specific taxa via nucleotide polymorphisms and to understand relationships between *E. littoralis* and *Etlingera* sp.

Combined ITS and matK data set

The combined ITS and *mat*K data had an aligned length of 1480 bp in the taxa surveyed. The data set were combined for comparison of the potential phylogenetic information between ITS and *mat*K (Hilu *et al.*, 2008). Parsimony informative sites in combined data are very similar to *mat*K more than ITS. MP analysis of the combined ITS and *mat*K data resulted in 40,198 shortest trees of length 437 steps. The MP analysis consisted of a heuristic tree search that used TBR branch swapping from 1,000 random stepwise addition replicate starting trees. The bootstrap values were higher than in those trees analysis individuals of ITS or *mat*K. The *E. littoralis* clade was separated from the *Etlingera* sp. clade by 100% bootstrap value. In the same way, the *Etlingera* sp. clade was highly supported by bootstrap values, ranking form 63% to 91% (Figure 17).

ML analysis of the combined ITS and *mat*K data set were carried out using PAUP* software, version 4.0b10 (Swofford, 2002). The tree topologies from ML analysis are similar to MP tree topologies (Figure 18), but the bootstrap support values were slightly higher in MP. However, the combined data analysis provided the strongest support for phylogenetic tree in *Etlingera* sample both MP and ML analyses.

In addition, a Bayesian analysis was also performed for the ITS, *mat*K and combined ITS and *mat*K data sets using MrBayes, with GRT model of evolution. Alternatively, several authors have used Bayesian inference to generate support for phylogenetic relationships (Burbrink, 2002; Steane *et al.*, 2003; Guzman and Vargas, 2005; Rex *et al.*, 2009; Boykin *et al.*, 2010). Markov chain Monte Carlo (MCMC) with the Metropolis-Histing algorithm was used to sample posterior probability space by these authors. These methods have several advantages over traditional bootstrapping method (Geyer, 1991; Laget and Samon, 1999). Using the Metropoloiscopled MCMC allows user to run multiple chains simultaneously. Additionally, these chains can swap states which potentially minimizes the chance of any chain becoming struck on local optima (Huelsenbeck and Ronquist, 2001; Burbrink, 2002). Consequently, these attractive features of Bayesian inference lend themselves to analyzing this molecular data set, which is composed of many closely related samples.

The results from the Bayesian analysis are very similar to the MP and ML analysis, though analyses of the data set provided strong bootstrap support and overall bootstrap support was higher in MP and ML analyses. However, all phylogenetic trees, which were analyzed by MP, ML and Bayesian method showed that *E. littoralis* and *Etlingera* sp. were grouped in different clades, with strong bootstrap support.

Chapter 6

Conclusion

The results indicated that *Etlingera littoralis* widely distributes in Southern Thailand, both Gulf of Thailand and Andaman Sea coasts, particularly the upper part of Southern Thailand. Normally, *E. littoralis* can grow in different habitats from lowland to high elevation. They stand along logging road, river bank, damp and humid shady places (Figure 25). They are also found frequently in secondary forests, gap areas secondary and primary forests. Some species can fully expose to the sun.

Morphologically, the inflorescence of *E. littoralis* is short and compact. Each flower shows bright red and yellow labellum. The labellum length means 4.92 cm broadest below middle. Stamen (length x width) 1.5-2.0×0.7-1.1 cm. Anther (length x width) 1.0-1.2×0.5-0.6 cm, quite erect with filament or a bit angled *ca.* 10-15 C°, broadest at apex, emarginate 0.1-0.2 cm, thecae dehiscing in upper 1/2-2/3. The fruit is rounded and hardly ridge.

From this study, *E. megalocheilos* was not found. This species is widely distributed in Borneo and Malay Peninsula. Its characters are different from *E. littoralis*, i.e. the longer lip, the longer corolla tube, the longer labellum, the narrower central lobe of the labellum, shorter and narrower stamen (Poulsen, 2006). So, *E. megalocheilos* cannot be synonym to *E. littoralis*.

Morphological characters and ecological habitat of *Etlingera* sp. are very similar to *E. littoralis* were found. It is mainly distributed in the lower part of Southern Thailand. Morphological characters showed that *Etlingera* sp. is not *E. megalocheilos*. *Etlingera* sp. is also not *E. littoralis*, even though their morphological characters, both vegetative and reproductive parts, are very similar. Cluster analysis using R statistic program showed that *Etlingera* sp. was clearly separated from *E. littoralis* (Table 8). There are two flowers forms of *Etlingera* sp.; a median red with yellow lateral labellum and entirely red labellum. Inflorescences are

median red with yellow lateral labellum and entirely red labellum. Inflorescences are also embedded in the soil. Labellum is more elongate than that of *E. littoralis*. The middle of the labellum is quite narrow, broader at apex, emarginated and broadest below the middle. Stamen is emarginated and narrower than stamen of *E. littoralis*.

Table 8 Floral morphological characters to be used for *E. littoralis* and *Etlingera* sp. identification.

Characteristics	***E. littoralis***	***Etlingera* sp.**
Inflorescence pattern color	- red median with yellow edge labellum	- red median with yellow edge labellum - entire red flower
Labellum length	1-5 cm	5.1-10 cm
Labellum and anther length ratio	1-3 time	>3 time
The angle of anther	10-15 C°	40-65 C°

Anther is highly angled *ca.* 40-65 degree with filament, emarginate, thecae dehiscing in upper 1/2-2/3. Infructescence and fruits are very similar to *E. littoralis*, embedded in the soil. Fruit is brown, deeply ridged and densely pubescent. In summary, some clear external morphological characters can be used to identify *Etlingera* sp., *E. megalocheilos* and *E. littoralis*.

The molecular genetics between *Etlingera* sp. and *E. littoralis* were analyzed to confirm the reparation between these three species. Phylogenetic tree both ITS and *mat*K regions indicated that *Etlingera* sp. and *E. littoralis* are different species by strong bootstrap support.

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