ดีเอ็นเอบาร์โคดและรูปแบบทางเคมีของพืชสกุล *Aristolochia* สำหรับการตรวจสอบสมุนไพรไคร้เครือ

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DNA BARCODES AND CHEMICAL PROFILES OF *ARISTOLOCHIA* PLANTS FOR EXAMINATION OF KRAI-KRUA HERBS

Miss Piroonrat Dechbumroong

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Pharmacognosy
Department of Pharmacognosy and Pharmaceutical Botany
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พืชสกุล *Aristolochia* จัดอยู่ในวงศ์ *Aristolochiaceae* หรือวงศ์กระเช้าสีดา สารหลักที่พบในทุกส่วนของพืชสกุลนี้คือ aristolochic acid I (AAI) และ AAII ซึ่งเป็นสารก่อมะเร็งในมนุษย์ ในปี พ.ศ. 2556 คณะกรรมการยาจึงมีมติให้ตัดเครื่องยาที่ได้จากพืชสกุลนี้ออกจากทะเบียนตำรับยาที่ได้ขึ้นทะเบียนไว้แล้ว รากแห้งของพืชสกุลนี้ 3 ชนิดถูกใช้เป็นแหล่งของเครื่องยา “ไคร้เครือ” ได้แก่ กระเช้าถุงทอง หนอนตาย และกระเช้าผีมด อย่างไรก็ตามไคร้เครือยังได้จากรากแห้งของข้าวสารดอกใหญ่ พืชสกุล *Jasminum* และรากขี้กาขาวขี้กาแดงเช่นกัน การพิสูจน์เอกลักษณ์ของไคร้เครือด้วยสัณฐานวิทยาเป็นไปได้ค่อนข้างยากจึงอาจทำให้เกิดความสับสนในการใช้สมุนไพรชนิดนี้ ดังนั้นเพื่อความปลอดภัยของผู้บริโภค เครื่องมือในการระบุเอกลักษณ์ของวัตถุดิบสมุนไพรที่เชื่อถือได้และมีประสิทธิภาพจึงได้รับการพัฒนาอย่างต่อเนื่อง ในการศึกษานี้ใช้การประเมินทางพันธุกรรมของพืชสกุล *Aristolochia* จำนวน 11 ชนิด โดยอาศัยเทคนิค DNA barcode ของดีเอ็นเอ 4 บริเวณ ได้แก่ *rbc*L *mat*K ITS และ *trn*H-*psb*A ความแตกต่างของลำดับนิวคลีโอไทด์ของดีเอ็นเอทุกบริเวณดังกล่าวสามารถใช้ในการจำแนกชนิดของพืชสกุล *Aristolochia* ทั้ง 11 ชนิดนี้ได้ ข้อมูลลำดับนิวคลีโอไทด์บริเวณ internal transcribed spacers 2 (ITS2) นี้ได้ถูกนำไปใช้ใน multiplex PCR ร่วมกับการประเมินรูปแบบองค์ประกอบทางเคมีโดยใช้วิธี high-performance thin layer chromatography (HPTLC) โดยใช้ AAI เป็นสารมาตรฐาน เพื่อใช้ระบุเอกลักษณ์สมุนไพรไคร้เครือ วิธีการนี้สามารถประยุกต์ใช้เพื่อการทดสอบเบื้องต้นสำหรับสาร AAI ในอุตสาหกรรมสมุนไพรและการบังคับใช้กฎหมายได้ ผลจากการศึกษาในครั้งนี้แสดงให้เห็นว่าการประเมินทางพันธุกรรมร่วมกับการประเมินรูปแบบองค์ประกอบทางเคมีสามารถพิสูจน์เอกลักษณ์ของพืชสกุล *Aristolochia* และจำแนกชนิดสมุนไพรไคร้เครือได้

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The genus *Aristolochia* belongs to the Aristolochiaceae family. The major chemical constituents in the whole part of *Aristolochia* plant are aristolochic acids I (AAI) and AAII, which are classified as human carcinogens. In Thailand, 2013, The National Drug Committee have issued an order that demands the removal of crude drugs derived from *Aristolochia* plants from all registered formulas. Dried roots of *A. pothieri* Pierre ex Lecomte, *A. pierrei* Lecomte and *A. tagala* Cham., have been reported as sources of medicinal crude drugs called “Krai-Krue”. However, Krai-Krue can also be derived from dried roots of *Raphistemma pulchellum* (Roxb) Wall, *Jasminum* spp and *Gymnopetalum integrifolium* Kurz. Authentication of Krai-Krue by morphological examination is quite difficult and can cause confusion. For the protection of consumer’s safety, reliable and effective tools for identification of raw herbal materials have been continuously developed. In this study, genetic assessment of 11 *Aristolochia* plants by DNA barcoding technique was conducted based on four DNA regions including *rbc*L, *mat*K, ITS and *trn*H-*psb*A. The nucleotide variations of the four regions are useful to differentiate the eleven *Aristolochia* species. Multiplex PCR based on nucleotide sequences of ITS2 region combining with HPTLC using AAI as standard substance were used for the identification of Krai-Krue herbs. This method can be used as a preliminary AAI-screening test for safety control by the herbal industries as well as the regulatory authorities. The results from these studies indicated that the combination of genetic and chemical assessment would be useful for the identification and discrimination of *Aristolochia* plants and Krai-Krue herbs.

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LIST OF ABBREVIATIONS

AA	=	aristolochic acid
AAN	=	Aristolochic Acid Nephropathy
AFLPs	=	amplified fragment length polymorphisms
AL(s)	=	aristolactam(s)
ARMS	=	amplification-refractory mutation system
bp	=	base pair
CBOL	=	Consortium for the Barcode of Life
CHN	=	Chinese herb nephropathy
°C	=	degree Celsius
DNA	=	deoxyribonucleic acid
dNTP	=	deoxyribonucleotide triphosphate
GC	=	gas chromatography
GC–MS	=	gas chromatography–mass spectroscopy
H_2O	=	water
HPLC	=	high-performance liquid chromatography
HPLC–MS	=	high-performance liquid chromatography- mass spectroscopy
HPTLC	=	high-performance thin-layer chromatography
IARC	=	International Agency for Research on Cancer
ITS	=	internal transcribed spacer
Kb	=	kilobase
MARMS	=	multiplex amplification refractory mutation system
*mat*K	=	maturase K
min	=	minute(s)
NIR	=	near infrared
PCR	=	polymerase chain reaction
ppm	=	part(s) per million
RAPD	=	random amplified polymorphic DNA
*rbc*L	=	large subunit of ribulose-bisphosphate carboxylase
rDNA	=	ribosomal deoxyribonucleic acid

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RFLP	=	restriction fragment length polymorphism
SCAR	=	sequence characterized amplified region
sp	=	species (singular)
spp	=	species (plural)
SSR	=	simple sequence repeat
TLC	=	thin-layer chromatography
*trn*H-*psb*A	=	trnH-psbA intergenic spacer region
UV	=	ultraviolet
V	=	voltage

CHAPTER I
INTRODUCTION

In recent years, the natural product markets have grown rapidly (Da-cheng, Shi-lin et al. 2010). Due to the high market demands, adulteration and substitution of medicinal materials widely occur in many countries (Ernst 2002, Mitra and Kannan 2007). Therefore, authentication of raw materials plays an important role for efficacy and safety of consumers. After the discovery that consumption of *Aristolochia* containing herbal products causes severe nephropathy and associated malignancies, the intake of botanical products containing *Aristolochia* plants becomes one of global concerns (Arlt, Stiborova et al. 2002).

The genus *Aristolochia*, a member of the family Aristolochiaceae, consists of about 500 species and is widely distributed in tropical and subtropical area including Asia, Africa, Europe and America (Heywood 1993, González 1997). Several *Aristolochia* species have been medicinally used in many traditional drug formulas including Chinese traditional medicines, Indian folk medicines and European medicines. For example, in China, *Aristolochia contorta* Bunge was used as an antitussive and a purgative against rabies (James A. Dook and Ayensu 1985), *A. debilis* Siebold & Zucc as a sedative (James A. Dook and Ayensu 1985), *A. fangchi* Y.C. Wu ex L.D. Chow & S.M. Hwang as an antirheumatic and a diuretic (Heinrich, Chan et al. 2009), *A. manshuriensis* Kom for treating problems relating to the urine and the bladder problems (Heinrich, Chan et al. 2009). *A. gigantea* Mart. et Zucc. was used as abortifacients and in the treatment of wounds and skin diseases in Brazil (Holzbach and Lopes 2010), *A. tagala* Cham. and *A. indica* L. were used as emmenagogue, antirheumatism and anti-snake bite in India (S.K. Jain and DeFilpips 1991).

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In Thailand, there are 22 species of *Aristolochia* (Smitinand 2014) as follows:

1) *A. arenicola* Hance
2) *A. baenzigeri* B.Hansen & Phuph.
3) *A. curtisii* King ex Gamble
4) *A. dinghoui* Favio González & O. Poncy
5) *A. grandis* Craib
6) *A. hansenii* Phuph.
7) *A. helix* Phuph.
8) *A. gigantea* Mart.
9) *A. kerrii* Craib
10) *A. kongkandae* Phuph.
11) *A. labiata* Willd.
12) *A. littoralis* Paradi.
13) *A. longeracemosa* B.Hansen & Phuph.
14) *A. macrophylla* Lam.
15) *A. perangustifolia* Phuph.
16) *A. pierrei* Lecomte
17) *A. poomae* Phuph.
18) *A. pothieri* Pierre ex Lecomte
19) *A. ringens* Vahl
20) *A. tagala* Cham.
21) *A. versicolor* S.M. Hwang
22) *A. yalaensis* Phuph.

However, three species were used in Thai traditional medicine in term of crude drug “Krai-Krue” (Athikomkulchai and Ruangrungri 2001, Sathornviriyapong, Picheansoonthon et al. 2007).

Krai-Krue is a crude drug used as an ingredient in Thai folk medicinal formulas for tonic, muscle relaxant, diuretic, antipyretic, analgesic, anti-rheumatism, immunostimulant, emmenagogue, abortive agent and liver enhancer (Vuthithammavech 1997). It is also one of ingredients in 10 herbal recipes on the Thailand list of Herbal Medicinal Products A.D. 2006, for example, Ya hom Nawakod (ยาหอมนวโกฐ), Ya hom Inthajuk (ยาหอมอินทจักร์), Ya Ummaruekawatee (ยาอำมฤควาที), Ya Tatbunjob (ยาธาตุบรรจบ), Ya Wisumpayayai (ยาวิสัมพยาใหญ่), Ya Munthatat (ยามัณฑธาตุ), Ya Kheawhom (ยาเขียวหอม), Ya Treehom (ยาตรีหอม), Ya Prasaganplu (ยาประสะกานพลู), Ya Prasajettapungkee (ยาประสะเจตพังคี) (Health 2006). According to microscopic, morphological and chemical profiling approaches, Krai-Krue derived from dried roots of the three *Aristolochia* species, *A. pothieri* Pierre ex Lecomte (Vuthithammavech 1997, Athikomkulchai and Ruangrungri 2001), *A. pierrei* Lecomte and *A. tagala* Cham. (Sathornviriyapong, Picheansoonthon et al. 2007). In 2013, the National Drug Committee have legally issued

to manufacturer to remove Krai-Krue from all formulas within one years after April 19th, 2013 (Control 2013, Health 2013). However, despite warnings, Krai-Krue still be bought from local dispensaries.

The genus *Aristolochia* contains aristolochic acids and its derivatives. These compounds are kidney targeting carcinogenic substances and are found in the whole plant through DNA adduct mechanism. The toxicity is known as "Aristolochic Acid Nephropathy (AAN)" (IARC 2012). Therefore identification of *Aristolochia* plants by accurate and efficient analysis method is very important for safety of customer.

Due to some limitations of classical plant authentication methods, for example, microscopic and macroscopic approaches need experts. Recently, many molecular biological technologies have been used as useful tools for DNA analysis of medicinal plants (Li, Cao et al. 2011, Techen, Parveen et al. 2014). For example, SCAR markers to detect adulterants of saffron (Marieschi, Torelli et al. 2012), RAPD for authentication of *Cuscuta reflexa* (Khan, Mirza et al. 2010), RAPD and AFLP for identification of *Withania* species (Mir, Koul et al. 2013), multiplex PCR for discrimination of *Artemisia* herbs (Lee, Doh et al. 2008). They can provide accurate identifications for plant samples that are not distinguishable by morphology or their names.

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In recent years, DNA barcoding is become one of the methods for identification an organism at family, genus and species levels (Li, Cao et al. 2011). DNA barcoding is the latest technique involves using the analysis of short standardized regions of genome. It is not only an effective tool to establish centralized sequence database of organism but also is the gold standard for authentication and identification of organism (Group 2009, Hollingsworth 2011). A Consortium for the Barcode of Life (CBOL) Plant Working Group proposed four DNA regions for land plants. Nucleotide sequence of *mat*K region and *rbc*L region have been proposed to be core barcode to identify angiosperm at species levels and ITS (or ITS2) and *trn*H-*psb*A have been proposed as alternative DNA barcode (Hollingsworth 2011, Fazekas, Kuzmina et al. 2012). The United States Pharmacopoeia and Chinese Pharmacopoeia also include this technique as one of raw material identification (Song, Yao et al. 2009, Li, Cao et al. 2011).

However, the limited number of DNA sequences has restricted the development of rapid molecular identification techniques for these herbs.

Normally, the whole plants in the genus *Aristolochia* produce aristolochic acids (AAs). Aristolochic acids are a family of nitrophenanthrene carboxylic acids. Two major substances found are aristolochic acid I (AAI) and aristolochic acid II (AAII). AAI is commonly found at higher concentration than AAII (NTP 2011). Therefore, the presence of aristolochic acid I is an important chemical marker to identify herbs and herbal products derived from *Aristolochia* species (Blatter and Reich 2004, Li, Au et al. 2012, Phadungrakwittaya, Akarasereenont et al. 2012). High-performance thin layer chromatography (HPTLC) pattern is referred by European Pharmacopoeia and British Pharmacopoeia for screening of aristolochic acids in herbal products since 2012 with the presence of aristolochic acid I at levels equal to or greater than 2 ppm (Commission and Britain 2012, Pereira Sena, Ashton-Prolla et al. 2012).

In the present study, four DNA barcodes (*rbc*L, *mat*K, ITS and *trn*H-*psb*A) of eleven *Aristolochia* species collected in Thailand were successfully generated. The nucleotide sequences of ITS2 was utilized for the discrimination of three *Aristolochia* species used in Thai traditional medicine under the name “Krai-Krue” using multiplex PCR. Application of ITS2 nucleotide polymorphisms, in combination with the presence of aristolochic acid I as chemical marker, was used to investigate seven crude drug samples from various local dispensaries. Combination of multiplex PCR and HPTLC analysis was successful for the identification of Krai-Krue herbs. Randomly purchased 23 Krai-Krue containing formulas were also analyzed by HPTLC.

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CHAPTER II
LITERATURE REVIEWS

2.1 Plant samples

2.1.1 *Aristolochia* spp

2.1.1.1 Morphology of *Aristolochia* plants

Aristolochia plants belong to Aristolochiaceae family or birthwort family or Krachauseeda family in Thai. The taxonomic description of *Aristolochia* was reported by Leena Phuphathanaphong (1987) as follows:

"Prostrate, scrambling, climbing herb or woody climber. Leaves entire or 3-lobed, palmately or pinnately nerved. Flowers solitary, racemose or paniculate, irregular; perianth basally expanded into the utricle, then contracted into a slender tube; limb 1-2 lipped. Ovary slightly 6-angular and 6-locular; ovules many in each cell. Stamens 6, adnate to the style to form a gynostemium. Stigmatic lobes 3 or 6. Capsule septicidal, dehiscing from base upwards, 6-valved. Seeds numerous, flat, winged or not, verrucose."

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According to Tem Smitinand (2014), there are 22 species of *Aristolochia* found in Thailand as shown in Table 1.

Table 1 *Aristolochia* plants found in Thailand.

No.	Scientific name	Local name
1	*Aristolochia arenicola* Hance	กอมก้อยลอดขอน (ตะวันออกเฉียงเหนือ)
2	*A. baenzigeri* B.Hansen & Phuph.	กระทุงบวบเหลี่ยม
3	*A. curtisii* King ex Gamble	กระเช้าภูเก็ต
4	*A. dinghoui* Favio González & O. Poncy	นกกระจิบ (ประจวบคีรีขันธ์)
5	*A. grandis* Craib	นกขมิ้น (เหนือ)
6	*A. hansenii* Phuph.	กระเช้าเชียงราย
7	*A. helix* Phuph.	กระเช้าหนู (ใต้)
8	*A. gigantea* Mart.	ไก่ฟ้าใหญ่ (กรุงเทพ), Brazillian Dutchmant's Pipe, Giant Pelican Flower
9	*A. kerrii* Craib	กระเช้าปากเป็ด (เหนือ), เครือไก่น้อย (เชียงใหม่)
10	*A. kongkandae* Phuph.	กระเช้าคลองพนม
11	*A. labiata* Willd. syn. *A. brasiliensis* Mart. & Zucc.	นกกระทุง (กลาง), Mottled Dutchmant's Pipe, Rooster Flower
12	*A. littoralis* Paradi. syn. *A. elegans* Mast.	นกกระทุง (เชียงใหม่), นกกระทุงปากบาน, เหนียงนกกระทุง (กรุงเทพมหานคร), Calico flower
13	*A. longeracemosa* B.Hansen & Phuph.	ไก่แจ้ (เหนือ)
14	*A. macrophylla* Lam. syn. *A. durior* Hill	นกกระทุงใหญ่ (กลาง), Broadleafed Birthwort
15	*A. perangustifolia* Phuph.	กระเช้าใบแคบ (general)
16	*A. pierrei* Lecomte	กระเช้าผีมด (เชียงใหม่), หนอนตาย (กลาง)
17	*A. poomae* Phuph.	กระเช้านกกระสา (general)
18	*A. pothieri* Pierre ex Lecomte syn. *A. siamensis* Craib	กระเช้าถุงทอง (กลาง)
19	*A. ringens* Vahl	ไก่ฟ้า (กรุงเทพมหานคร), Gaping Dutchman's Pipe, Pelican flower
20	*A. tagala* Cham.	กระเช้าผีมด, กระเช้ามด, กระเช้าสีดา (กลาง), ปุลิง (เชียงใหม่), หาบกะเชอ (ขอนแก่น), Indian Birthwort
21	*A. versicolor* S.M. Hwang	กระทุงขน (เหนือ)
22	*A. yalaensis* Phuph.	กระเช้ายะลา (general)

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Among these *Aristolochia* plants, seven of them including *A. gigantea* Mart., *A. kerrii* Craib, *A. littoralis* Paradi., *A. pierrei* Lecomte, *A. pothieri* Pierre ex Lecomte, *A. ringens* Vahl and *A. tagala* Cham. have been chosen as plant materials in this study because they are widely used as medicinal plants in many countries (Heinrich, Chan et al. 2009). In addition, another three *Aristolochia* species, *A. tentaculata* Schmidt in Fedde, *A. grandiflora* Sw. and *A. anguicida* Jacq., were also included in this investigation. The other species are excluded because they are very rare or completely absent. The botanical descriptions of *A. kerrii* Craib, *A. littoralis* Paradi., *A. pierrei* Lecomte, *A. pothieri* Pierre ex Lecomte, *A. ringens* Vahl and *A. tagala* Cham. were reported by Leena Phuphathanaphong (1987) whereas those of *A. anguicida* Jacq., *A. gigantea* Mart., *A. grandiflora* Sw. and *A. tentaculata* Schmidt in Fedde were reported as follows (Pfeifer 1966):

1) ***A. kerrii*** **Craib** (Aristolochiaceae) is called as “กระเช้าปากเป็ด”.

“Climber, stem glabrous. Leaves: petioles *2.3-3.2* cm, glabrous; lamina ovate-lanceolate, triangular, triangular-ovate to broadly ovate, 5.2-7.4 by *4.5-6.6* cm, base truncate or ± cordate, margin entire, apex acute to acuminate, glabrous above, puberulous below, gland-dotted on both sides, palmately 5-nerved, venation finely reticulate, obscure above, prominent below. Inflorescences axillary, fascicled racemose 1.5-3 cm long, puberulous; bracts lanceolate, 0.5-1.2 by 0.2-0.5 cm longitudinally veined, densely gland-dotted. Flowers with 0.*8-1.1*cm pedicel and ovary 2-3 by 0.5-1 mm. Perianth 1.3-2 cm, purple, inside cream; utricle spherical or ovoid, 2.9-4.7 by 2.7-4.1 mm, tube 3.1-7.5 by 1.3-2 mm, bent on transition from utricle, limb sagittate, 0.7-1.4 by 0.4-1.2 cm, Anthers ellipsoid, 0.5-0.6 by 0.8-1 mm. Gynostemium 0.8-1.8 by 1.2-3.5 mm, stigmatic lobes 6, short, obtuse. Fruit ovoid, 1.8-2.2 by 1.6-1.8 cm;

pedicel 1.8-2.2 cm. Seeds obovate, flat, not winged, 4-4.5 by 2.3-2.6 mm, verrucose on both sides."

2) *A. littoralis* Paradi. (Aristolochiaceae) is called as "เหนียงนกกระทุง".

"Woody climber, stem glabrous. Leaves with pseudo-stipules, leaf-like, amplexicaul, ± 1.2 by 2 cm; petiole 3.5-4 cm, slender, glabrous; lamina triangular 4-5.5 by 6-7 cm, base cordate, margin entire, apex rounded or obtuse, glabrous on both sides, but young leaves pubescent, palmaterly finely 3-nerved, venation reticulate, obscured. Flowers solitary, large; pedicel and ovary 7-8.5 cm. Perianth white or greenish with purple-black mottlings; utricle obliquely ellipsoid, 2.5-4 by 1.2-1.5 cm, tube bent upwards, 1.2-2 by ± 0.5 cm, limb orbicular, 5-7 cm diam. Anthers linear, ± 4 by 1 mm. Gynostemium ± 5.8 by 4.5 mm, stigmatic lobes 6, flattened, outside apically pubescent, with margins recurved outwards. Fruit not seen."

3) *A. pierrei* Lecomte (Aristolochiaceae) is called as "หนอนตาย".

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"Climber, stem glabrous. Leaves: petioles 0.9-1.5 (-3.5) cm, grooved above, hairy along edges, glabrous beneath; lamina lanceolate to broadly lanceolate, 8.5-13.8 by 2.8-4.6 cm, base cordate, margin entire, slightly recurved, hairy above, apex acute or tapering acute, uper surface finely pubescent, lower surface puberulous, with scattered gland-dots on both sides, palmately 5-nerved, lateral nerves 3-4, venation reticulate, conspicuous above, prominent below. Inflorescences racemose, 3.5-7 cm, tomentose; bracts lanceolate or narrowly lanceolate, 0.8-1.2 by 0.35-0.45 cm both sides hairy. Flowers with 0.4-1 cm pedicels, slender, ovary 3.7-4.5 by 0.5-1 mm. Perianth 2-3.5 cm, utricle spherical or ovoid, 3.5-4.5 by 3-4 mm, purple inside, tube 5-8.5 by ± 1 mm, greenish light brown, limb oblong, 1-1.5 by 0.45-0.55 cm, purplish-brown. Anthers

oblong ± 0.4 by 0.2 mm. Gynostemium 1.8-2.1 mm, stigmatic lobes 6, long conical, obtuse. Fruit ovoid, 2-2.5 by 1.8-2 cm; pedicel 3.2-4.5 cm. Seeds broadly obcordate or triangular, winged, 4.7-5.1 by 5.5-6 mm, verrucose on both sides."

4) *A. pothieri* Pierre ex Lecomte (Aristolochiaceae) is called as "กระเช้าถุงทอง".

"Climber, stem laxly adpressed puberulous. Leaves: petiole 3.5-5.2 cm, puberlous; lamina entire or 3-lobed, broadly ovate, lobes less than half the length of the lamina, 11-11.5 by 12-13.6 cm, base deeply cordate, apex acute, acuminate or obtuse, lateral lobes obtuse, laxly pubescent and gland-dotted on both sides, palmately 3-nerved with transverse nerves conspicuous above and prominent beneath. Inflorescences axillary panicles, 6 cm, densely puberulous; bracts ovate or lanceolate, ± 1 by 0.5 mm. Flowers with pedicels 6-7 mm, ovary 6-8 by 1-1.2 mm, densely pubescent. Perianth 2.4-4.4 cm, brown, purplish, reddish, straight; utricle spherical or ovoid, 3.5-7.5 by 3.3-4 mm; tube 0.8-1.6 by 0.1-0.2 cm; limb obovate, oblong or spathulate, 1.3-1.8 by 0.5-0.7 cm. Anthers ellipsoid, 0.7-0.9 by 0.7-0.9 mm. Gynostemium 2-2.2 by 2.7-3 mm, stigmatic lobes 6, conical, obtuse. Fruit ovoid, 4-4.5 by 2.5-3 cm; pedicel 5-6 cm. Seeds triangular-obcordate, thin, winged, 7.5-8 by 6.7-7 mm, verrucose on one side the other side smooth, brown."

5) *A. ringens* Vahl (Aristolochiaceae) is called as "ไก่ฟ้า".

"Climber, stem glabrous; the whole plant gland-dotted. Leaves with pseudo-stipules, leaf like, reniform, unequal, the bigger one 1.5-2.2 by 2-3 cm, the smaller on 9.6-1.5 by 1-2 cm; petiole 3-8 cm, slender, glabrous; lamina reniform 5-10 by 7.5-14.5 cm, base deeply cordate, margin entire, apex rounded, ± cuspidate, glabrous,

but young leaves slightly pubescent, palmately nerved, nerves branched near the margin, venation reticulate. Flowers solitary, large, dark red or dark purple; pedicels ± 6.5 cm, ovary 2.5-4.5 by 0.1-0.2 cm. Perianth 17.5-27 cm, utricle obliquely ellipsoid, 4-7.5 by 3-4 cm; tube bent upwards, 2-2.5 by 0.6-0.8 cm; limb 2 lipped, upper lip spathulate, 4.5-6 cm, reticulate, lower lip oblong-lanceolate, 9-15 cm, apex obtuse. Anthers linear, 5.5 by 0.8 mm. Gynostemium ± 8 by 5 mm, stigmatic lobes 6, long conical, obtuse. Fruit oblong, 7-7.5 by 3-3.5 cm; pedicel 10-12 cm, strongly 6-ribbed. Seeds including wing obovate 9-12 by 5-7.5 mm, seed proper obcordate, 3-3.5 by 2.5-3.5 mm, brown with light brown verrucae, the other side dark brown, wing light brown."

6) *A. tagala* Cham. (Aristolochiaceae) is called as "กระเช้าผีมด".

"Climber, young stem pubescent, glabrescent. Leaves: petioles 3-6 cm, grooved above, pubescent along grooved surface, glabrous below; lamina ovate to ovate-lanceolate, 9.5-16.5 by 5.8-7.6 cm, base deeply cordate, margin entire ± recurved, apex acuminate, mature leaves glabrous on both sides, nerves and veins ± pubescent, laxly gland-dotted; palmately 3-nerved, pinnately 3-4 nerved along the midrib, veins transverse, venation reticulate, obscurely above, prominent below. Inflorescences paniculate or racemose, 6-13.5 cm, pubescent to nearly glabrous; bracts ovate, 1.8-3 by 1-1.5 mm, base cuneate, margin ciliate, apex acute, puberulous on both surfaces. Flowers with pedicel 5-8 mm, ovary 5-9.7 by 1-1.5 mm. Perianth 3.6-5 cm long, greenish, dark purple hairy inside, laxly hairy outside; utricle globular, 5-6.5 by 5-7 mm, tube 0.6-1.2 by 1-1-.5 mm, limb linear-lanceolate, 1.6-2.5 by ± 0.8 cm. Anthers ellipsoid ± 1 by 0.8-1.7 mm. Gynostemium 3-4 by 3.5-4 mm, stigmatic lobes 6, long conical, obtuse. Fruit ovoid, 3.3-5.5 by 2.7-4.3 mm; pedical 2.2-5.8 cm. Seeds broadly oboavate ±

obcordate, winged, 5-7.5 by 7-7.5 mm, laxly verrucose on one side, the other side smooth, light brown."

7) *A. anguicida* Jacq. (Aristolochiaceae)

"Glabrous lianas. Leaves membranous, broadly triangular, acute to obtuse at the apex, basally deeply cordate, 5-7 cm broad, 7-9 cm long, smooth above, beneath with emersed veins. Pseudostipules usually present on strong stems, amplexicaul. Flowers solitary in the leaf axils, ebracteolate, rectilinear, purple, green and yellow, the utricle ovoid, gibbous, 1 cm long, syrinx strongly inequilateral, the tube straight, 1.5 cm long, the limb 1-lobed, narrowly triangular, smooth, tightly revolute after anthesis, 1 cm wide, 1.5-2.0 cm long, uunappendaged. Gynostemium deeply 6-lobed, 3 mm high, 2 mm broad, the anthers 6, equidistant. Fruits short, thick-cylindric, 3 cm long, 2 cm wide, dehiscence acropetal, septifragal, the hypanthium 1.5 mm long. Seeds numerous, flat, 3 mm wide, 4 mm long, 1 mm thick."

8) *A. gigantea* Mart. (Aristolochiaceae)

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"Large strong-growing lianas. Leaves broadly ovate-triangular, acuminate, basally subtruncate, 10-15 cm broad, 12-16 cm long, deep green, glabrous above, beneath white-tomentose. Pseudostipules absent. Flowers cauliflorous, ebracteolate, geniculate, purple and yellow-orange, the utricle sublacrimiform, gibbous, 10 cm long, syrinx absent, the tube not sharply differentiated from the utricle and limb, U-shaperd, ca 4 cm long, annulus absent, the limb 1-lobed, abruptly spreading from the tube, broadly cordate, ca 14 cm wide, 16 cm long, unappendaged. Gynostemium 6-lobed, 1 cm high, 4 cm broad, the anthers 6, equidistant. Fruits large, glaucous, 8 cm long,

2.5-3.0 cm wide, dehiscence acropetal, septifragal, the hypanthium curved, 5 mm long. Seeds numerous, flat, 5 mm wide, 7 mm long, very thin, papery."

9) *A. grandiflora* Sw. (Aristolochiaceae)

"Strong-growing, glabrescent lianas. Leaves triangular-cordate, apex acute to acuminate, basally deeply cordate, 8-15 cm broard, 10.2 cm long, deep green, smooth above, beneath strigose in juvenile leaves, becoming smooth with age, paler. Pseudostipules absent. Flowers solitary in leaf axils, bracteolate, more or less twice-geniculate (once at the tube flexure and again at the annulus), variously blotched with purple, white, yellow, red and green, very variable in size over a vast range, but commonly very large, the utricle lacrimiform, gibbous, 6-18 cm long, syrinx cylindric, as long as 4 cm, directed obliquely into the utricle, the tube bent at its middle, 7-15 cm long, annulus thin, sharp-edged, the limb abruptly spreading from the annulus and tube, 1-lobed, 20-50 cm or more wide, 0.5-3.0 m of the limb. Gynostemium 6-lobed, coroniform, 1.5 cm high, 1.0 cm broad, the anthers 6, equidistant. Fruits cylindric, 10 cm long, 4 cm wide, dehiscence acropetal, septifragal, hypanthium absent. Seeds numerous, triangular, flat, 1 cm wide, 1.2 cm long, 2 mm thick."

10) *A. tentaculata* Schmidt in Fedde (Aristolochiaceae)

"Glabrous small lianas. Leaves cordate-orbiculate, slightly emarginated, cordate-auriculate, 2-6 cm broad, 3-8 cm long, smooth above, beneath paler with emersed veins. Pseudostipules absent. Flowers solitary in the leaf axils, often on small-leafed, short, lateral shoots, bracteolate, subgeniculate, purple, green and yellow, the utricle gibbous-obconic, 1 cm long, syrinx inequilaterl, annular, the tube straight, narrow at first, thence flaring into the limb, 2-3 cm long, the limb broadly lanceolate,

sparsely long-fimbriate along the lateral margins, 2.5 cm wide, 6-8 cm long, unappendaged. Gynostemium 6-lobed, 4 mm high, 4-5 mm broad, the anthers 6. Fruits cylindric, 5 cm long, 2.5 cm wide, dehiscence acropetal, septifragal, the hypanthium straight, 2 mm long. Seeds numerous, 6.5 mm wide, 8 mm long, thin, papery."

2.1.1.2 Bioactivity of *Aristolochia* plants

Aristolochia plants have been used in many countries around the world as important medicinal plants. Seven *Aristolochia* species including *A. indica* L. (Asia), *A. serpentaria* L. (North America), *A. debilis* Sieb & Zucch. (China), *A. acuminata* Lam (including *A. tagala* Cham.) (India), *A. trilobata* L. (Central/South America, Caribbean), *A. clematitis* L. (Europe) and *A. bracteolata* Lam. (Africa) are widely reported for various therapeutic purposes. The most commonly medicinal use is the treatment of gastrointestinal problems, especially diarrhea. The other traditional uses of birthworth species are anti-snake venom, treatment of female reproductive system conditions and sexually transmitted diseases, treatment of central nervous system conditions, dermatological problems and cardiovascular ailments (Heinrich, Chan et al. 2009).

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2.1.1.3 Chemical constituents of *Aristolochia* plants

The plants belonging to the family Aristolochiaceae normally produce aristolochic acids (AAs) such as AAI, AAII, aristolactams (ALs). Aristolochic acids are a family of nitrophenanthrene carboxylic acids. The amount of AAs varies depending on the plant species, habitat, harvesting time and other factors. Concentrations ranged from 3 to 12,980 ppm for AA I and from not detected to 6,325 ppm for AA II (NTP 2008). However, the two major substances found in whole parts of *Aristolochia* plants are aristolochic acid I (aristolochic acid A) and its demethoxylated derivative, aristolochic acid II (aristolochic acid B). The structures of AAI and AAII were shown in Figure 1 (NTP 2011).

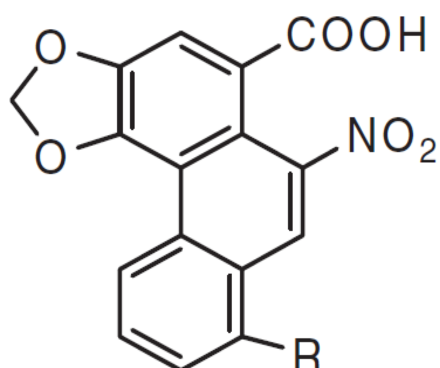

Figure 1 Structure of aristolochic acid I (AAI) (R = OCH_3) and AAII (R = H)

There are other common compounds found in this genus such as alkaloids (AA III, AA IIIa, AA IV, aristolactams) (Holzbach and Lopes 2010, Lu, Wei et al. 2012), mono-, sesqui-, di-, triterpens (ß-caryophyllene, isocaryophyllene, E-caryophyllene, bicyclogermacrene, ß-sitosterol) (Kumar, Prasad et al. 2003, Silva-Brandão, Solferini et al. 2006).

2.1.1.4 Toxicity of *Aristolochia* plants

Exposure to aristolochic acid has been reported throughout the world. (NTP 2008). The first report is a cluster of patients with progressive renal interstitial fibrosis associated with urothelial malignancies after taking pills from slimming clinic in Belgium in 1992. The incident was caused by the substitution of Chinese herb labelled as Han Fang Ji (roots of *Stephania tetrandra*) by Guang Fang Ji (roots of *Aristolochia fangchi*). The disease was called as “Chinese herb nephropathy (CHN)” (Vanherweghem, Tielemans et al. 1993). Since then, many cases from various countries were continuously reported, for instance, four cases in France from intake of slimming pills containing *Aristolochia fangchi* instead of *Stephania tetrandra*, two cases in England after treatment of eczema with Guan Mu tong (stem of *Aristolochia manshuriensis*) instead of Chuan Mu Tong

(stem of *Clematis armandii* and *C. montana*) or Bai Mu Tong (stem of *Akebia quinata* and *A. trifoliata*) and one case in Spain after chronic intake of a tea containing *Aristolochia pistolochia* (IARC 2002).

From many studies and case reports, consumption of *Aristolochia* plants causes severe toxicities including renal fibrosis, irreversible nephropathy, end-stage renal failure and kidney cancer which are due to aristolochic acids and its derivatives through DNA adduct mechanism (Nortier, Martinez et al. 2000, IARC 2002, Chan, Luo et al. 2007, Shibutani, Dong et al. 2007). According to the International Agency for Research on Cancer (IARC) Monographs (2015) (IARC 2015), aristolochic acid and plants containing them are classified as group 1 agent. The group 1 agent is carcinogenic to humans by sufficient evidence of carcinogenicity in humans and/or experiential animals and strong evidence in exposed humans that the agent acts through a relevant mechanism of carcinogenicity (IARC 2015).

2.1.2 Krai-Krue herbs

2.1.2.1 Morphology of Krai-Krue herbs

Krai-Krue herbs is slightly brown hard root derived from many climbers with characteristic odor and bitter taste. It is yellow-brown in powder form. Sources of Krai-Krue have been reported as *Raphistemma pulchellum* (Roxb) Wall (ข้าวสารดอกใหญ่) (Apocynaceae), *Aristolochia indica* L. (กระเช้าสีดา) (Aristolochiaceae), *A. pothieri* Pierre ex Lecomte (กระเช้าถุงทอง) (Aristolochiaceae) (Vuthithammavech 1997, Picheansoonthon, Chawalit et al. 2001) and *Jasminum* spp. (Picheansoonthon, Chawalit et al. 2001) and also can be substituted by dried root of *Gymnopetalum integrifolium* Kurz. (ขี้กาขาว/ขี้กาแดง) (Cucurbitaceae) (Vuthithammavech 2003).

According to microscopic, morphological and chemical profiling approaches, Krai-Krue is derived from dried roots of the three *Aristolochia* species, *A. pothieri*

Pierre ex Lecomte (Athikomkulchai and Ruangrungri 2001), *A. pierrei* Lecomte and *A. tagala* Cham. (Sathornviriyapong, Picheansoonthon et al. 2007).

Therefore, the plant samples were additionally collected in this study including *Raphistemma pulchellum*, *Jasminum sambac*, *J. adenophyllum* and *Gymnopetalum integrifolium*. The botanical description of *Raphistemma pulchellum* was reported in Flora of China (1995). That of *Jasminum sambac* was reported by Chang Mei-chen, Qiu Lian-qing and Peter S. Green (1996), *J. adenophyllum* by Peter Shaw Green (2000) and *Gymnopetalum integrifolium* by Willem J.J.O. De Wilde and Brigitta E.E. Duyfjes (2008) as follows:

1) *Raphistemma pulchellum* (Roxb) Wall (Apocynaceae) is called as “ข้าวสารดอกใหญ่”.

“Stems to 8 m, terete, glabrous. Petiole 4-12 cm, with apical adaxial gland cluster; leaf blade ovate, 6-20 × 4-15 cm, sparsely adpressed pubescent to glabrous, base deeply cordate, apex acute-acuminate; lateral veins 6 or 7 pairs. Peduncle 3.7-13 cm. Pedicel 1.2-4 cm. Sepals ovate-oblong, 3-4 mm, ciliate. Corolla yellowish white; tube 1.2-1.8 cm, limb 3-4 cm in diam.; lobes shorter than tube, oblong, glabrous. Corona lobes white, 1-1.2 cm. Ovaries glabrous. Follicles ca. 16 × 4 cm. Seeds ovate; coma to 4 cm. Fl. Jun-Aug, fr. Sep-Dec.”

2) *Gymnopetalum integrifolium* Kurz. (Cucurbitaceae) is called as “ขี้กาขาว/ขี้กาแดง”.

“Climbing or creeping herb, rooting at the nodes, to 5 m long, stem (densely) grey or brownish hairy. Probact lanceolate, acute, unlobed or (deeply) 2- or 3-lobed, (1-)1.5-2.5 cm long, sometimes absent, green-yellow, late-caducous. Tendrils

unbranched or unequally 2- branched near the base. Leaves: blade circular, or reniform, or broadly ovate in outline, or 5- angular, 2-11 cm diam., subglabrous above, densely coarse-pubescent below, at least on the veins, when fresh bullate above, cystoliths in older leaves present, base deeply cordate, margin entire, finedly dennate-mucronate or ± coarsely lobulate or wavy-dentate, apex rounded or subacute, 5 palmately veined, reticulation distinct below; petiole 1-5 cm long. Male inflorescences: flowers solitary or in bracteates racemes; bracts 1-2 cm long, lobed, base cuneate, sessile. Male flowers: densely grey (or brown) pubescent; pedicel 2-12 cm long for solitary flowers, 1(-2) cm long in the racemes, persistent, at apex faintly articulate; receptacle-tube (strongly) narrowed below insertion of stamens, 15-20 (-30) by 6-7 (at throat) mm, outside and inside pubescent, throat inside yellow; sepals narrowly triangular, lanceolate, entire or ± lobed, recurved, (4-)5-8 mm long, green; petals obovate, ± clawed, ca. 2 by 1.5 cm, distinctly veined; stamens inserted ca. 10 mm below throat; filaments 2-2.5 mm long, ± glabrous, synandrium 8-12 by 2-2.5 mm. connectives not enlarged, apex of synandruim flat, narrow,, hairy, bright yellow when fresh; disc consisting of 3 short linear bodies. Female flowers; solitary, resembling male flowers; pedicel 1-3 cm long; ovary ellipsoid, 8-10 by 6-7 mm, long-pubescent; receptacle-tube cylindrical, ca. 10 by 5 mm; style 7-10 mm long, stigmas erect, ca. 2 mm long, ± included; disc at base of the tube, very low and minute (nectariferous?) or absent. Fruits short ellipsoid or globose, (2-)3-4 cm long, (orange-) red, at first sparsely hairy, later on glabrous; fruiting pedicel 1-3(-5) cm long. Seeds (elliptic) oblong, 6-9 by 2.5-3 by 1.5-2 mm, faces small, almost smooth, demarcated by groove from broad, rounded margin."

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3) ***Jasminum adenophyllum*** **Wall. Ex C.B. Clarke** (Oleaceae) is called as *“มะลิวัลย์”*.

“Woody climber, young shoots glabrous or scattered puberulent. Leaves elliptic to oblong-elliptic, 6-15 cm long, 2.5-7 cm broad, base attenuate onto the grooved petiole; apex slightly aceminate; glabrous; 4 or 5 primary veins on each side of the midrib, raised below, but not reticulate, slightly sunk above, joining to form a distinct submarginal vein; 3-4 tufted domatia in the axils of the primary nerves with the midrib below; petioles 5-20 mm long, glabrous or pilose above. Inflorescence axillary or terminal on short shoots, 1- to 3(-5)-flowered, glabrous; bracts linear, 2 mm long; glabrous; pedicels 1-4 cm long. Calyx tube 2 mm long; lobes 5-14 mm long, somewhat filiform, glabrous. Corolla white, fragrant; tube (9-)15-20 mm long; lobes 8 or 9, 15-20 mm long, 2-3.5 mm broad. Fruit spheroidal, 7x15 mm.”

4) ***Jasminum sambac*** **(L.) Aiton** (Oleaceae) is called as “มะลิลา”.

“Shrubs erect or scandent, to 3 m. Branchlets terete or slightly compressed, sometimes hollow, sparsely pubescent. Leaves opposite, simple; petiole 2-6 mm, articulate, pubescent; leaf blade orbicular to elliptic or obovate, 4-12.5 × 2-7.5 cm, papery, glabrous except for tufted hairs at vein axils abaxially, both ends blunt, sometimes base subcordate; primary veins 4-6 on each side of midrib. Cymes terminal, (1 or)3(or 5)-flowered; bracts subulate, 4-8 mm. Flowers very fragrant. Pedicel 0.3-2 cm. Calyx glabrous or sparsely pubescent; lobes 8-9, linear, 5-7 mm. Corolla white; tube 0.7-1.5 cm; lobes oblong to suborbicular, 5-9 mm broad. Berry purple-black, globose, ca. 1 cm in diam. Fl. May-Aug, fr. Jul-Sep. 2*n* = 26*.”

2.1.2.2 Traditional use of Krai-Krue herbs

Krai-Krue is used as a common ingredient in several Thai folk medicinal formulas for various purposes such as tonic, muscle relaxant, diuretic, antipyretic, analgesic, anti-rheumatism, immunostimulant, emmenagogue, abortion and liver enhancer (Vuthithammavech 1997, มูลนิธิฟื้นฟูส่งเสริมการแพทย์ไทยเดิมอายุรเวทวิทยาลัย(ชีวกโกมารภัจจ์) 2535, พระยาวิชยาธิบดี(กล่อม) 2546). It has been used as an ingredient in 10 herbal recipes on the list of Herbal Medicinal Products A.D. 2006 of Thailand, for example, Ya hom Nawakod (ยาหอมนวโกฐ), Ya hom Inthajuk (ยาหอมอินทจักร์), Ya Ummaruekawatee (ยาอำมฤควาที), Ya Tatbunjob (ยาธาตุบรรจบ), Ya Wisumpayayai (ยาวิสัมพยาใหญ่), Ya Munthatat (ยามัณฑธาตุ), Ya Kheawhom (ยาเขียวหอม), Ya Treehom (ยาตรีหอม), Ya Prasaganplu (ยาประสะกานพลู), Ya Prasajettapungkee (ยาประสะเจตพังคี) (Health 2006).

2.2 Assessment for identification of the medicinal plants

2.2.1 Genetic assessment

Genetic assessment of medicinal plants can be classified into three categories, namely hybridization-based method, polymerase chain reaction-based and sequencing-based. First, the hybridization-based method is a technique that measures the degree of nucleotide similarity when two complementary single-stranded nucleic acids anneal into a double-stranded nucleic acid by hydrogen bonds formation. Second, the PCR-based method is carried out by amplification of the region(s) of interest in the genome. Then gel electrophoresis is performed to size and/or score the amplification products. Examples are sequence characterized amplified regions (SCAR), simple sequence repeat (SSR) analysis, PCR-restriction fragment length polymorphism (PCR-RFLP), amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD). Nucleotide sequences are needed for some techniques of PCR-based method. Third, the sequencing-based method is a technique that obtains

nucleotide sequences of organism and establishes centered database such as GenBank. Searching and comparison to the database are performed for identification of unknown organism (Yip, Chau et al. 2007). The advantages of genetic assessment are that: it can be performed with a small amount of sample, the sample can be analyzed without prior nucleotide sequence, and it can be succeeded within a short time. Besides, the molecular techniques is also performed very well at sensitivity, reliability, reproducibility, and running costs (Heubl 2010).

Application of genetic assessment for accurate identifications for plant samples have been reported, such as randomly amplified polymorphic DNA (RAPD) (Khan, Mirza et al. 2010, Mir, Koul et al. 2013), amplified fragment length polymorphism (AFLP) (Mir, Koul et al. 2013), sequence characterized amplified region (SCAR) (Marieschi, Torelli et al. 2012), multiplex amplification refractory mutation system (MARMS) (Wang, Kwon et al. 2011), polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) (Suwanchaikasem, Phadungcharoen et al. 2013) and DNA barcode (Ogata, Uchiyama et al. 2013, Chen, Zhu et al. 2014).

Currently, genetic assessment by sequencing-based techniques has been widely developed for the identification and authentication of medicinal plant species. The latest focused approach is DNA barcoding which is DNA sequencing of standardized DNA region(s) for each organism (Hollingsworth, Graham et al. 2011).

DNA sequencing is a powerful process involving the order of four bases, adenine (A), thymine (T), cytosine (C), and guanine (G), in DNA samples that can be used to identify species, analyze phylogenetic relationships, population genetics, and evolutionary processes. It is also widely used in many other fields such as archaeology, anthropology, genetics, biotechnology, molecular biology, and forensic sciences (França, Carrilho et al. 2002). In addition, this technique can be used to determine the sequence of individual genes, clusters of genes or entire genomes (Shendure and Ji 2008).

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DNA barcoding is one of sequencing-based techniques that involve using the analysis of short standardized regions of genome and establishment of centralized sequence database of organism. It has been proposed for identifying and authenticating unknown biological material to species level (Group 2009). Up to the present, there is no universal DNA region for species discrimination among all organisms (Stoeckle 2003). A Consortium for the Barcode of Life (CBOL) Plant Working Group proposed four DNA regions including large subunit of ribulose-bisphosphate carboxylase *(rbc*L) and maturase K (*mat*K) as the core barcode, and nuclear internal transcribed spacer (ITS or ITS2) and *trn*H-*psb*A spacer as the additional barcode for standard land plant barcode (Hollingsworth 2011). This technique is included in the one of raw material identification steps in The United States Pharmacopoeia and Chinese Pharmacopoeia (Song, Yao et al. 2009, Li, Cao et al. 2011).

Multiplex PCR is a modified PCR-based technique that is based on the amplification of two or more DNA targets. The process uses multiple primers in a single reaction mixture (Da-cheng, Shi-lin et al. 2010). It has been widely applied for species identification of several organisms, including microorganisms (Oliveira and de Lencastre 2002, Lucignano, Ranno et al. 2011), genetically modified crops (Forte, Di Pinto et al. 2005), and medicinal plants (Lee, Doh et al. 2008, Jigden, Wang et al. 2010). However, studies of multiplex PCR for the authentication of *Aristolochia* Krai-Krue have not been reported. Recently, nucleotide polymorphisms based on ITS sequences have been widely used for the development of molecular markers for medicinal plant identification (Lee, Kim et al. 2012).

2.2.2 Chemical assessment

Many chromatographic and spectroscopic techniques have been used for both authentication and quality control purposes of herbal materials, for instance, thin layer chromatography (TLC), near infrared (NIR), gas chromatography (GC), high-performance

liquid chromatography (HPLC), gas chromatography–mass spectroscopy (GC–MS), high-performance liquid chromatography- mass spectroscopy (HPLC–MS) (Liang, Xie et al. 2004, Xie, Chen et al. 2006).

HPTLC is a method of choice because it is a simple, reliable, and rapid analytical technique of chromatography. It has been widely used as a screening tool and routinely used for qualitative assessment of chemical constituents of botanical materials by detection of specific peaks or zones due to known or unknown components of the extract. (Reich and Schibli 2007). HPTLC is recognized as one of chromatographic fingerprint in herbal standardization (Kulkarni, Patil et al. 2014). The technique has been successfully applied in many fields of research such as pharmaceutical analysis, environmental analysis, food and clinical laboratories (Shewiyo, Kaale et al. 2012). With retard to *Aristolochia* plants or suspected *Aristolochia*-containing products, this method is recommended as a preliminary screening test for aristolochic acids (Blatter and Reich 2004, Li, Au et al. 2012, Phadungrakwittaya, Akarasereenont et al. 2012, Li, Au et al. 2014).

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CHAPTER III

DNA BARCODES OF ELEVEN *ARISTOLOCHIA* SPECIES

3.1 Introduction

At present, biomolecular technologies are popular tools for plant identification. DNA barcode is a latest focused technique. The objective of DNA barcoding is to find one or a few DNA regions that can distinguish among the species existing on the earth and to obtain the DNA information to produce a large-scale database of creatures of the world (Hollingsworth 2011). In recent years, DNA barcoding of land plants using four standard regions (*rbc*L, *mat*K, ITS and *trn*H–*psb*A) has been recommended as a tool for identification at species level for land plant (Li, Cao et al. 2011). In this study, the *rbc*L gene, *mat*K gene, ITS region and *trn*H-*psb*A region were studied for the identification and discrimination of different eleven species in the genus *Aristolochia* using the DNA barcoding technique.

3.2 Materials and methods

3.2.1 Plant materials

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Forty-two samples of eleven *Aristolochia* taxa, considered as authentic samples were collected from various locations from Thailand (Table 2). All specimens were identified by Assoc. Prof. Thatree Phadungcharoen at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Science, Chulalongkorn University, Thailand. Herbarium voucher specimens were prepared and kept at the Museum of Natural Medicines, Faculty of Pharmaceutical Science, Chulalongkorn University.

Table 2 Plant materials and their accession numbers for DNA barcodes.

Species	Place of collection (Thailand, Province)	Voucher number	GenBank accession number			
			*rbc*L	*mat*K	ITS	*trn*H-*psb*A
A. anguicida Jacq.	Chiang Mai	MUS-5405	KP903720	KP998777	KP998791	KP998805
	Chiang Mai	MUS-5406	-	-	-	-
A. gigantea Mart. et Zucc.	Nakhon Pathom	MUS-5376	-	-	-	-
	Bangkok	MUS-5377	-	-	-	-
	Chiang Mai	MUS-5396	-	-	-	-
	Bangkok	MUS-5393	KP998764	KP998778	KP998792	KP998806
	Bangkok	MUS-5394	-	-	-	-
	Bangkok	MUS-5395	-	-	-	-
	Chiang Mai	MUS-5397	-	-	-	-
A. grandiflora Sw.	Phitsanulok	MUS-5379	-	-	-	-
	Petchabun	MUS-5390	-	-	-	-
	Lampang	MUS-5392	-	-	-	-
	Lampang	MUS-5391	KP998765	KP998779	KP998793	KP998807
	Phitsnulok	MUS-5380	-	-	-	-

Table 2 Plant materials and their accession numbers for DNA barcodes (continued).

Species	Place of collection (Thailand, Province)	Voucher number	GenBank accession number			
			*rbc*L	*mat*K	ITS	*trn*H-*psb*A
A. kerrii Craib	Chiang Mai	MUS-5415	-	-	-	-
	Chiang Mai	MUS-5413	KP998766	KP998780	KP998794	KP998808
A. littoralis D. Parodi	Bangkok	MUS-5404	KP998767	KP998781	KP998795	KP998809
A. pierrei Lecomte	Sakon Nakhon	MUS-5407	-	-	-	-
	Sakon Nakhon	MUS-5408	-	-	-	-
	Sakon Nakhon	MUS-5409	KP998768	KP998782	KP998796	KP998810
	Sakon Nakhon	MUS-5410	-	-	-	-
	Sakon Nakhon	MUS-5411	-	-	-	-
A. pothieri Pierre ex Lecomte	Bangkok	MUS-5374	KP998769	KP998783	KP998797	KP998811
	Bangkok	MUS-5381	-	-	-	-
	Bangkok	MUS-5382	-	-	-	-
	Bangkok	MUS-5402	-	-	-	-
	Bangkok	MUS-5403	-	-	-	-
	Bangkok	MUS-5416	KP998776	KP998790	KP998804	KP998818

Table 2 Plant materials and their accession numbers for DNA barcodes (continued).

Species	Place of collection (Thailand, Province)	Voucher number	GenBank accession number			
			*rbc*L	*mat*K	ITS	*trn*H-*psb*A
A. ringens Vahl	Bangkok	MUS-5375	KP998770	KP998784	KP998798	KP998812
	Bangkok	MUS-5383	-	-	-	-
	Nakhon Pathom	MUS-5384	-	-	-	-
	Nakhon Pathom	MUS-5385	-	-	-	-
	Chiang Mai	MUS-5387	-	-	-	-
	Chiang Mai	MUS-5388	-	-	-	-
	Bangkok	MUS-5389	-	-	-	-
	Bangkok	MUS-5412	-	-	-	-
A. tagala Cham.	Chiang Mai	MUS-5400	KP998772	KP998786	KP998800	KP998814
	Bangkok	MUS-5401	KP998775	KP998789	KP998803	KP998817
	Bangkok	MUS-5386	KP998774	KP998788	KP998802	KP998816
A. tentaculata Schmidt in Fedde	Bangkok	MUS-5398	KP998773	KP998787	KP998801	KP998815
	Bangkok	MUS-5414	-	-	-	-
A. sp.	Bangkok	MUS-5399	KP998771	KP998785	KP998799	KP998813

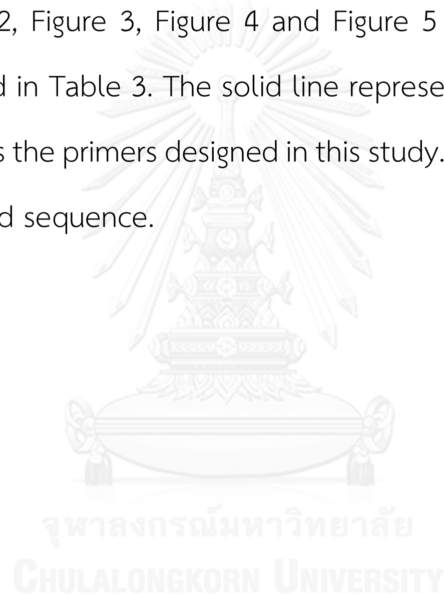
3.2.2 Genomic DNA extraction

Total genomic DNA was extracted from 80 to 100 mg of fresh leaves from each sample. Extraction of the genomic DNA utilized DNeasy™ Plant Mini Kit (Qiagen, Germany) and Genomic DNA Mini Kit (Plant) (Geneaid, Taiwan) according to the manufacturer's protocol. Briefly, fresh leaves were ground into fine powder in liquid nitrogen by a mortar and a pestle, and suspension and lysis buffers were then added. The lysate was applied to a column to remove precipitates and cell debris by centrifugation. The flow-through fraction was applied to a spin column. The spin column was centrifuged, washed and eluted with elution buffer. Genomic DNA quality were determined by 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light. The extracted DNA samples were stored at -20°C until further use.

3.2.3 Primer design

A pair of the universal primers of each target DNA (Table 3), rbcL_af and rbcL_R23, ITS1 (or ITS3) and ITS4 and trnHR and psbAF, positioned on the conserve regions were used to amplify the complete *rbc*L gene (Figure 2), ITS regions (Figure 4) and *trn*H-*psb*A regions (Figure 5) of the genus *Aristolochia*, respectively. In order to amplify and sequence the complete *mat*K gene of *Aristolochia* plants, the *trn*K-*mat*K sequences of related species in the genus *Aristolochia* including *A. pierrei* (accession number DQ296649), *A. grandiflora* (accession number DQ532052), *A. gigantea* (accession number JX485569) and *A. gigantea* (accession number DQ882187) were aligned and flanking conserved regions were selected. Three primers, matK-Aris-96F, matK-Aris-50F and matK-Aris-201R, were designed based on the sequences of the *trn*K-*mat*K regions obtained from GenBank (Figure 3). In some cases of ITS region, the ITS sequences of related species in the genus *Aristolochia*, including *A. cucurbitifolia* (accession number AM501925), *A. kaempferi* (accession number AM501928),

A. mollissima (accession number JQ255433), *A. mollissima* (accession number JQ255434), *A. shimadai* (accession number AM501926), *A. kaempferi* (accession number AM501930), *A. kwangsiensis* (accession number FJ980372), *A. faveolata* (accession number AM501927) and *A. zollingeriana* (accession number AM501929) were aligned and flanking conserved regions were selected to design primer ITS-Aris-371F (Figure 4). The obtained sequences of each region were aligned. The flanking conserved regions were selected to design sequencing primers. The locations of amplification primers and the sequencing primers on *rbc*L, *mat*K, ITS and *trn*H–*psb*A are shown in Figure 2, Figure 3, Figure 4 and Figure 5 respectively. Details of these primers are presented in Table 3. The solid line represents the universal primers. The dotted line represents the primers designed in this study. The length of lines represents the length of obtained sequence.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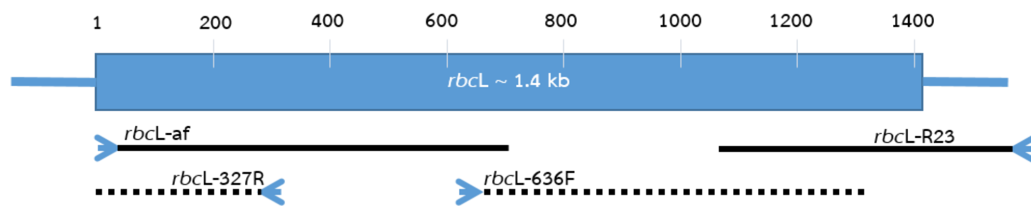

Figure 2 Schematic diagram of the chloroplast *rbc*L gene and relative positions of the PCR amplification primers and sequencing primers used in this study. The arrows represent the directions of the primers.

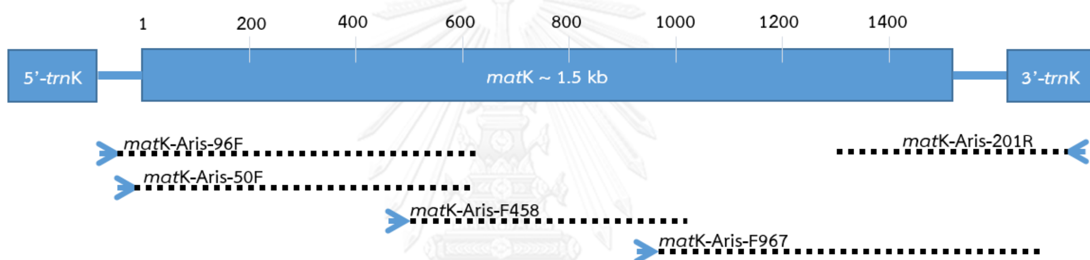

Figure 3 Schematic diagram of the chloroplast *mat*K gene and relative positions of the PCR amplification primers and sequencing primers used in this study. The arrows represent the directions of the primers.

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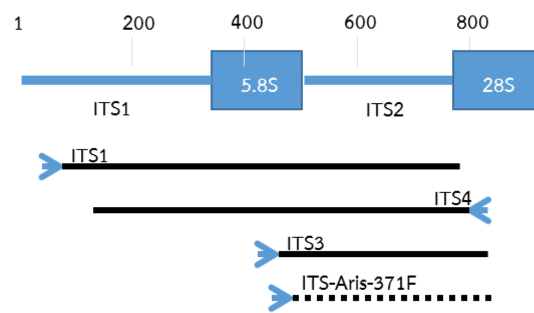

Figure 4 Schematic diagram of ITS region and relative positions of the PCR amplification primers and sequencing primers used in this study. The arrows represent the directions of the primers.

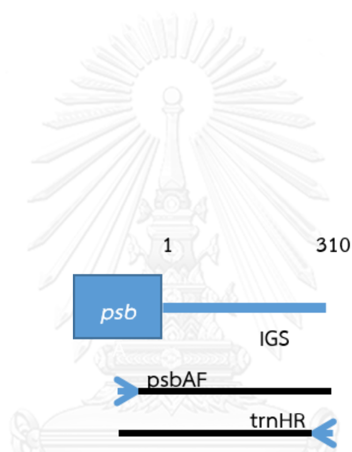

Figure 5 Schematic diagram of the chloroplast intergenic spacer *trn*H-*psb*A and relative positions of the PCR amplification primers and sequencing primers used in this study. The arrows represent the directions of the primers.

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Table 3 Primers used for the generation of DNA barcodes.

Barcode	Primer	Sequence (5'⟶3')	References
*rbc*L	rbcL_aF	ATG TCA CCA CAA ACA GAG ACT AAA GC	(Kress and Erickson 2007)
	rbcL-Aris-327R	TTC AAA AAG GTC TAA AGG GTA AGC	-
	rbcL_636F	GCG TTG GAG AGA TCG TTT CT	(Ohi-Toma, Sugawara et al. 2006)
	rbcL_R23	TTT TAG TAA AAG ATT GGG CCG	(Ohi-Toma, Sugawara et al. 2006)
*mat*K	matK-Aris-96F	ATC CCC TAT TCC TTC AGT TCA A	-
	matK-Aris-50F	CCT TGT TTT GAC TGT ATC GCA C	-
	matK-Aris-F458	ATA CCC CAC CCC ATC CAT CTG	-
	matK-Aris-F967	CAC TTG TGG TCT CAA CCG GG	-
	matK-Aris-201R	GCA CAC GGC TTT CCC TAT G	-
ITS	ITS1	TCC GTA GGT GAA CCT GCG G	(White, Bruns et al. 1990)
	ITS3	GCA TCG ATG AAG AAC GCA GC	(White, Bruns et al. 1990)
	ITS4	TCC TCC GCT TAT TGA TAT GC	(White, Bruns et al. 1990)
	ITS-Aris-371F	AAT TGC AGA ATC CCG CGA AC	-
*trn*H-*psb*A	psbAF	GTT ATG CAT GAA CGT AAT GCT C	(Li, Ling et al. 2010)
	trnHR	CGC GCA TGG TGG ATT CAC AAA TC	(Li, Ling et al. 2010)

3.2.4 PCR amplification of the barcode regions

The complete *rbc*L regions of the *Aristolochia* samples were amplified with a primer pair of rbcL_aF and rbcL_R23. The complete *mat*K regions of the *Aristolochia* samples were amplified with a primer pair of matK-Aris-50F (or matK-Aris-96F) and matK-Aris-R201. The ITS regions of the *Aristolochia* samples were amplified with a primer pair of ITS1 (or ITS3 or ITS-Aris-371F) and ITS4. The *trn*H-*psb*A of the *Aristolochia* samples regions were amplified with a primer pair of trnHR and psbAF. The primers used in this study are shown in Table 3. The PCR amplification was performed in 50 µL of GoTaq® Flexi DNA Polymerase reaction mixture, consisting of 5X PCR buffer, 25 mM $MgCl_2$, 2.5 mM of each dNTP, 10 mM of each primer, 5U *Taq* polymerase (Promega, USA), and 10–100 ng of total genomic DNA as a template. PCR amplifications were carried out in a C1000™ Thermal Cycler (Bio-Rad, USA) using cycling conditions at 96°C for 3 min, followed by 30 cycles of 96°C for 60 s, 55°C for 1 min and 72°C for 2 min (for *rbc*L and *mat*K) and 45 sec (for ITS and *trn*H-*psb*A), and final extension at 72°C for 10 min. The amplified products were detected by 1.2% agarose gel electrophoresis in 1X TAE buffer were then visualized by ethidium bromide staining under UV light.

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3.2.5 Cloning technique

In some cases, the PCR products of ITS and *trn*H-*psb*A regions were purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA), and were then cloned using the pGEM®-T Easy Vector Systems (Promega). The cloning followed standard procedures with 1 µl vector, 1 µl ligase, 5 µl Buffer (all provided with each kit) and 3 µl PCR product. Colony PCR were performed, and then the sequencing process was determined with the amplification primers after plasmid isolation and purification through automatic DNA isolation system PI-50 (Kurabo, Japan).

3.2.6 DNA sequencing of the barcode regions

The PCR products of the *rbc*L, *mat*K, ITS and *trn*H-*psb*A region were purified and then the sequencing process was performed by capillary sequencing (AIT Biotech, Singapore) with sequencing primers for the region. The sequences were aligned, edited, analyzed and corrected using BioEdit Sequence Alignment Editor Version 7.2.5 (Hall 1999). The obtained sequences were assembled for their consensus sequences using DNASTAR® (Version 8.0.2) program. The sequences were then submitted to DDBJ/EMBL/GenBank nucleotide sequence databases with their accession numbers listed in Table 2.

For phylogenetic analysis, the complete *mat*K sequences of the *Aristolochia* spp. were aligned by ClustalX Version 2.1 (Larkin, Blackshields et al. 2007) and MEGA Version 6 (Koichiro Tamura, Glen Stecher et al. 2013). *Thottea dependens* (DQ882194.1) and *Thottea siliquosa* (JN415679.1) were included as outgroup for matK gene sequences.

3.3 Results

3.3.1 Sequence analysis of the barcode regions of eleven *Aristolochia* species

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The properties of selected DNA loci are shown in Table 4 including accession number, length, GC content (%) and nucleotide variation (%). The degrees of sequence variations between the *Aristolochia* samples were in the order of ITS > *trn*H-*psb*A > *mat*K > *rbc*L. The large insertions/deletions in ITS caused high variations (28.96%) between the eleven *Aristolochia* plants and were much higher than *trn*H-*psb*A (13.35%), *mat*K *(11.22%)* and *rbc*L (3.29%) (Table 4).

The complete *rbc*L gene of eleven *Aristolochia* plants was amplified using the amplification primers rbcL_af and rbcL_23R by PCR technique. The PCR products of *rbc*L gene about 1,500 bp in length were obtained. The purified products were sequenced individually using the sequencing primers listed in Table 3. The complete

*rbc*L gene sequences of eleven *Aristolochia* plants were 1,428 bp in length. Sequence distance (percent divergence) were calculated using the program MEGA Version 6. The *rbc*L sequences from all samples of the same species showed completely identical sequence. Forty-seven different sequence variations were found of 1,428 total aligned sites (3.29%). The sequence divergence among eleven *Aristolochia* plants varied from 0% to 1.92%. A pairwise comparison between *A.* sp and *A. anguicida* showed the lowest nucleotide sequence divergence at 0%. Whereas, a pair of *A. pothierri* and *A. anguicida, A. pothierri and A. gigantea, A. pothieri and A. littoralis* and a pair of *A.* sp *and A. pothieri* showed the highest nucleotide sequence divergence at the same percentage of 1.92%. The nucleotide sequence divergence amoung the eleven *Aristolochia* plants are shown in Table 5. The obtained *rbc*L gene sequences have been deposited in GenBank (Appendix B) and the accession numbers are listed in Table 4.

The complete *mat*K gene of eleven *Aristolochia* plants was amplified using the amplification primers matK-96F (or matK-50F) and matK-201R by PCR technique. The PCR products of *mat*K gene about 1,700 bp in length were obtained. The purified products were sequenced individually using the sequencing primers listed in Table 3. The complete *mat*K gene sequences of the samples were 1,518-1,554 bp in length. The complete *mat*K sequences were aligned by MEGA Version 6. The nucleotide sequences obtained from two samples of *A. pothieri* (KP998811 and KP998818) were completely identical. The sequences obtained from two samples of *A. tagala* (KP998786 and KP998788) were completely identical, but intraspecies variation was found from another one (KP998789) at positions 906 and 1,063 (Appendix B). One hundred and seventy-six different sequence variations were found of 1,569 total aligned sites (11.22%). Sequence distance (percent divergence) were calculated by using the program MEGA Version 6. The sequence divergence among eleven *Aristolochia* plants varied from 0.19% to 4.68%. A pairwise comparison between

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A. tagala and *A. pierrei* showed the lowest nucleotide sequence divergence at 0.19%, whereas, a pair of *A. pothierri* and *A. littoralis* showed the highest nucleotide sequence divergence at 4.68%. The nucleotide sequence divergence amoung eleven *Aristolochia* plants are shown in Table 6. The obtained *mat*K gene sequences have been deposited in GenBank (Appendix B) and the accession numbers are listed in Table 4.

The ITS region of *A. grandiflora* and *A. pothieri* were amplified using the amplification primers ITS*1* and ITS4. A primer pair of ITS-Aris-371F and ITS4 was used for *A. anguicida*, *A. kerrii*, *A. pierrei* and *A. tagala*. The other *Aristolochia* plants were amplified by ITS3 and ITS4. The PCR products of ITS region about 750 bp and 450 bp in length were obtained. The purified products were sequenced individually using the sequencing primers listed in Table 3. Some of the amplified products were purified, cloned in competent *Escherichia coli* cells and then sent for DNA sequencing process. The ITS sequences of the samples were 360-751 bp in length. The ITS sequences were aligned by MEGA Version 6. The nucleotide sequences obtained from two samples of *A. pothieri* were completely identical. However, intraspecies variation was found in the sequences obtained from three samples of *A. tagala* at positions 576, 715 and 737 (Appendix B). Two hundred and forty-six different sequence variations were found of 846 total aligned sites (28.96%*)*. Sequence distance (percent divergence) were calculated using the program MEGA Version 6. The sequence divergence among the eleven *Aristolochia* plants varied from 0.50% to 31.32%. A pairwise comparison between *A.* sp and *A. angucida* showed the lowest nucleotide sequence divergence at 0.50%, whereas, a pair of *A. pothierri* and *A. grandiflora* showed the highest nucleotide sequence divergence at 31.32%. The nucleotide sequence divergence between eleven *Aristolochia* plants are shown in Table 7. The obtained ITS gene sequences have been deposited in GenBank (Appendix B) and the accession numbers are listed in Table 4.

The *trn*H-*psb*A region of eleven *Aristolochia* plants was amplified using the amplification primers psbAF and trnHR by PCR technique. The PCR products of *trn*H-*psb*A region about 300-400 bp in length were obtained. The purified products were sequenced individually using the sequencing primers listed in Table 3. Some of the amplified products were purified, cloned in competent *Escherichia coli* cells and then sent for DNA sequencing process. The *trn*H-*psb*A sequences of the samples were 300-369 bp in length. The *trn*H-*psb*A sequences were aligned by MEGA Version 6. The nucleotide sequences obtained from two samples of *A. pothieri* were completely identical. While the sequences obtained from two samples of *A. tagala* (KP998816 and KP998817) were completely identical, intraspecies variation was found when compared to another sample (KP998814) at positions 140, 309 and 310 (Appendix B). Fifty-three different sequence variations were found of 397 total aligned sites (13.35%*).* Sequence distance (percent divergence) were calculated using the program MEGA Version 6. The sequence divergence among the eleven *Aristolochia* plants varied from 0% to 11.38%. A pairwise comparison between *A. tagala* and *A. anguicida*, *A. tagala* and *A. pierrei*, *A. sp and A. anguicida, A.* sp and *A. tagala* showed the lowest nucleotide sequence divergence at 0%. A pair of *A. grandiflora* and *A. anguicida* showed the highest nucleotide sequence divergence at 11.38%. The nucleotide sequence divergence between eleven *Aristolochia* plants are shown in Table 8. The obtained *trn*H-*psb*A sequences have been deposited in GenBank (Appendix B) and the accession numbers are listed in Table 4.

The pairwise percent sequence divergence in *rbc*L, *mat*K, ITS and *trn*H-*psb*A among three *Aristolochia* species used as Krai-Krue are shown in Table 9.

Table 4 Properties of selected DNA loci (*rbc*L, *mat*K, ITS and *trn*H-*psb*A) of *Aristolochia* plants used in this study.

Barcode	*rbc*L				*mat*K			
Property	Accession no.	Length (bp)	GC content (%)	Variation (%)	Accession no.	Length (bp)	GC content (%)	Variation (%)
A. anguicida	KP903720	1428	44.96		KP998777	1539	34.50	
A. gigantea	KP998764	1428	45.03		KP998778	1554	34.23	
A. grandiflora	KP998765	1428	45.03		KP998779	1524	33.92	
A. kerrii	KP998766	1428	44.40		KP998780	1527	34.51	
A. littoralis	KP998767	1428	45.03		KP998781	1539	34.18	
A. pierrei	KP998768	1428	45.10		KP998782	1518	34.58	
A. pothieri	KP998769	1428	44.82	3.29	KP998783	1524	34.19	11.22
A. pothieri	KP998776	1428	44.82		KP998790	1524	34.19	
A. ringens	KP998770	1428	44.89		KP998784	1548	34.75	
A. tagala	KP998772	1428	44.96		KP998786	1518	34.52	
A. tagala	KP998775	1428	44.96		KP998789	1518	34.39	
A. tagala	KP998774	1428	44.96		KP998788	1518	34.52	
A. tentaculata	KP998773	1428	45.03		KP998787	1524	34.84	
A. sp	KP998771	1428	44.96		KP998785	1539	34.44	

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Table 4 Properties of selected DNA loci (rbcL, matK, ITS and trnH-psbA) of *Aristolochia* plants used in this study (continued).

Barcode	ITS				*trn*H-*psb*A			
Property	Accession no.	Length (bp)	GC content (%)	Variation (%)	Accession no.	Length (bp)	GC content (%)	Variation (%)
A. anguicida	KP998791	399	69.42	28.96	KP998805	300	40.33	13.35
A. gigantea	KP998792	461	65.73		KP998806	305	40.00	
A. grandiflora	KP998793	751	64.98		KP998807	308	38.96	
A. kerrii	KP998794	360	76.39		KP998808	319	39.81	
A. littoralis	KP998795	437	67.73		KP998809	305	39.67	
A. pierrei	KP998796	379	70.71		KP998810	315	40.32	
A. pothieri	KP998797	696	70.69		KP998811	369	34.96	
A. pothieri	KP998804	708	70.48		KP998818	369	34.96	
A. ringens	KP998798	432	68.06		KP998812	305	40.00	
A. tagala	KP998800	378	71.69		KP998814	318	39.94	
A. tagala	KP998803	422	68.96		KP998817	319	40.13	
A. tagala	KP998802	422	68.48		KP998816	319	39.81	
A. tentaculata	KP998801	436	66.97		KP998815	310	40.00	
A. sp	KP998799	431	67.95		KP998813	283	40.28	

Table 5 Pairwise percent sequence divergence in the complete *rbc*L gene among eleven species in the genus *Aristolochia*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1														
2	0.14													
3	1.20	1.20												
4	1.70	1.70	1.56											
5	0.14	0.14	1.13	1.70										
6	1.41	1.41	1.41	1.27	1.41									
7	1.92	1.92	1.49	1.49	1.92	0.63								
8	1.92	1.92	1.49	1.49	1.92	0.63	0.00							
9	0.21	0.21	1.13	1.63	0.21	1.34	1.84	1.84						
10	1.56	1.56	1.27	1.13	1.56	0.14	0.49	0.49	1.49					
11	1.56	1.56	1.27	1.13	1.56	0.14	0.49	0.49	1.49	0.00				
12	1.56	1.56	1.27	1.13	1.56	0.14	0.49	0.49	1.49	0.00	0.00			
13	1.06	1.06	0.99	1.41	1.06	1.27	1.34	1.34	0.99	1.13	1.13	1.13		
14	0.00	0.14	1.20	1.70	0.14	1.41	1.92	1.92	0.21	1.56	1.56	1.56	1.06	

1	*A. anguicida*	5	*A. littoralis*	9	*A. ringens*	13	*A. tentaculata*
2	*A. gigantea*	6	*A. pierrei*	10	*A. tagala*	14	*A.* sp
3	*A. grandiflora*	7	*A. pothieri*	11	*A. tagala*		
4	*A. kerrii*	8	*A. pothieri*	12	*A. tagala*		

Table 6 Pairwise percent sequence divergence in the complete *mat*K gene among eleven species in the genus *Aristolochia*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1														
2	0.66													
3	3.17	3.53												
4	3.30	3.60	3.74											
5	0.80	0.61	3.74	3.96										
6	2.35	2.80	2.87	2.66	2.90									
7	4.21	4.37	4.18	4.17	4.68	2.14								
8	4.21	4.37	4.18	4.17	4.68	2.14	0.00							
9	1.09	1.57	2.76	3.15	1.72	2.10	3.69	3.69						
10	2.24	2.70	2.67	2.46	2.80	0.19	2.04	2.04	2.00					
11	2.29	2.75	2.72	2.51	2.85	0.28	2.14	2.14	2.04	0.09				
12	2.24	2.70	2.67	2.46	2.80	0.19	2.04	2.04	2.00	0.00	0.09			
13	1.30	1.64	3.02	3.22	1.78	2.20	3.80	3.80	1.05	2.10	2.15	2.10		
14	1.68	1.73	3.96	4.10	1.78	2.80	2.99	2.99	2.42	2.69	2.74	2.69	2.60	

1	*A. anguicida*	5	*A. littoralis*	9	*A. ringens*	13	*A. tentaculata*
2	*A. gigantea*	6	*A. pierrei*	10	*A. tagala*	14	*A.* sp
3	*A. grandiflora*	7	*A. pothieri*	11	*A. tagala*		
4	*A. kerrii*	8	*A. pothieri*	12	*A. tagala*		

Table 7 Pairwise percent sequence divergence in ITS region among eleven species in the genus *Aristolochia*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1														
2	5.46													
3	12.66	13.01												
4	22.95	27.01	22.38											
5	3.86	1.86	13.45	24.68										
6	17.35	19.21	19.45	17.47	18.52									
7	22.77	21.64	31.32	21.70	21.36	16.75								
8	22.77	21.64	31.32	21.70	21.36	16.75	0.00							
9	5.20	4.04	13.27	25.07	3.07	17.28	21.18	21.18						
10	15.90	18.47	17.25	16.37	17.43	4.12	15.85	15.85	16.20					
11	15.32	16.00	15.82	15.30	15.09	4.38	13.71	13.71	14.01	0.80				
12	15.32	15.91	15.16	15.99	15.01	3.82	13.92	13.92	13.93	0.27	0.48			
13	8.89	7.85	14.81	25.34	7.56	16.23	21.28	21.28	7.33	15.95	13.78	13.71		
14	0.50	4.54	11.25	22.95	3.08	17.35	20.18	20.18	4.30	15.53	13.43	13.35	7.65	

1	*A. anguicida*	5	*A. littoralis*	9	*A. ringens*	13	*A. tentaculata*
2	*A. gigantea*	6	*A. pierrei*	10	*A. tagala*	14	*A.* sp
3	*A. grandiflora*	7	*A. pothieri*	11	*A. tagala*		
4	*A. kerrii*	8	*A. pothieri*	12	*A. tagala*		

Table 8 Pairwise percent sequence divergence in *trn*H-*psb*A among eleven species in the genus *Aristolochia*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1														
2	2.08													
3	11.38	8.58												
4	6.85	5.56	7.33											
5	2.50	0.36	8.82	5.84										
6	4.67	3.03	6.18	3.68	3.35									
7	8.30	6.35	8.25	7.23	6.61	3.63								
8	8.30	6.40	8.43	7.38	6.66	3.63	0.00							
9	3.78	1.43	9.42	6.34	1.79	3.81	7.14	7.19						
10	0.00	1.83	9.67	5.89	2.16	4.17	7.09	7.14	3.32					
11	5.10	3.27	5.97	3.71	3.58	0.35	4.30	4.39	4.02	4.38				
12	4.67	2.96	5.95	3.56	3.28	0.00	4.19	4.19	3.72	4.08	0.33			
13	2.49	1.07	8.50	5.64	1.41	3.32	6.39	6.43	1.79	2.15	3.46	3.20		
14	0.00	1.83	9.67	5.89	2.16	4.17	7.09	7.14	3.32	0.00	4.38	4.08	2.15	

1	*A. anguicida*	5	*A. littoralis*	9	*A. ringens*	13	*A. tentaculata*
2	*A. gigantea*	6	*A. pierrei*	10	*A. tagala*	14	*A.* sp
3	*A. grandiflora*	7	*A. pothieri*	11	*A. tagala*		
4	*A. kerrii*	8	*A. pothieri*	12	*A. tagala*		

Table 9 Pairwise percent sequence divergence in *rbc*L, *mat*K, ITS and *trn*H-*psb*A among three *Aristolochia* species used as Krai-Krue.

		A. pothieri	*A. pothieri*	*A. pierrei*	*A. tagala*	*A. tagala*	*A. tagala*	
*rbc*L	*A. pothieri*		0.00	2.14	2.04	2.14	2.04	*mat*K
	A. pothieri	0.00		2.14	2.04	2.14	2.04	
	A. pierrei	0.63	0.63		0.19	0.28	0.19	
	A. tagala	0.49	0.49	0.14		0.09	0.00	
	A. tagala	0.49	0.49	0.14	0.00		0.09	
	A. tagala	0.49	0.49	0.14	0.00	0.00		
ITS	*A. pothieri*		0.00	3.63	7.09	4.30	4.19	*trn*H-*psb*A
	A. pothieri	0.00		3.63	7.14	4.39	4.19	
	A. pierrei	16.75	16.75		4.17	0.35	0.00	
	A. tagala	15.85	15.85	4.12		4.38	4.08	
	A. tagala	13.71	13.71	4.38	0.80		0.33	
	A. tagala	13.92	13.92	3.82	0.27	0.48		

3.3.2 Phylogenetic analysis of *Aristolochia* species based on *mat*K sequences

To examine the phylogenetic relationships among the eleven *Aristolochia* taxa in the present study, the complete *mat*K gene sequences of all samples were analyzed. The phylogenetic tree was conducted based on the percent divergence of the complete *mat*K gene from eleven *Aristolochia* plants (Figure 6). A total aligned length of 1,578 bp, including the outgroup sequences, formed the final data set. The monophyly of the genus *Aristolochia* was highly supported in NJ tree with bootstrap value 100 %. The tree revealed that all *Aristolochia* spp. were monophyletically grouped together. The eleven *Aristolochia* species were divided into two groups (Figure 6). The first group consisted of *A. tagala*, *A. pierrei*, *A. pothieri* as one subgroup whereas the bootstrap value is lower than 50 in the other subgroup of *A. grandiflora* and *A. kerrii*. The second group with two subgroups were comprised of *A. tentaculata* and *A. ringens* as one subgroup and *A.* sp, *A. anguicida A. littoralis* and *A. gigantea* all included as the other one subgroup.

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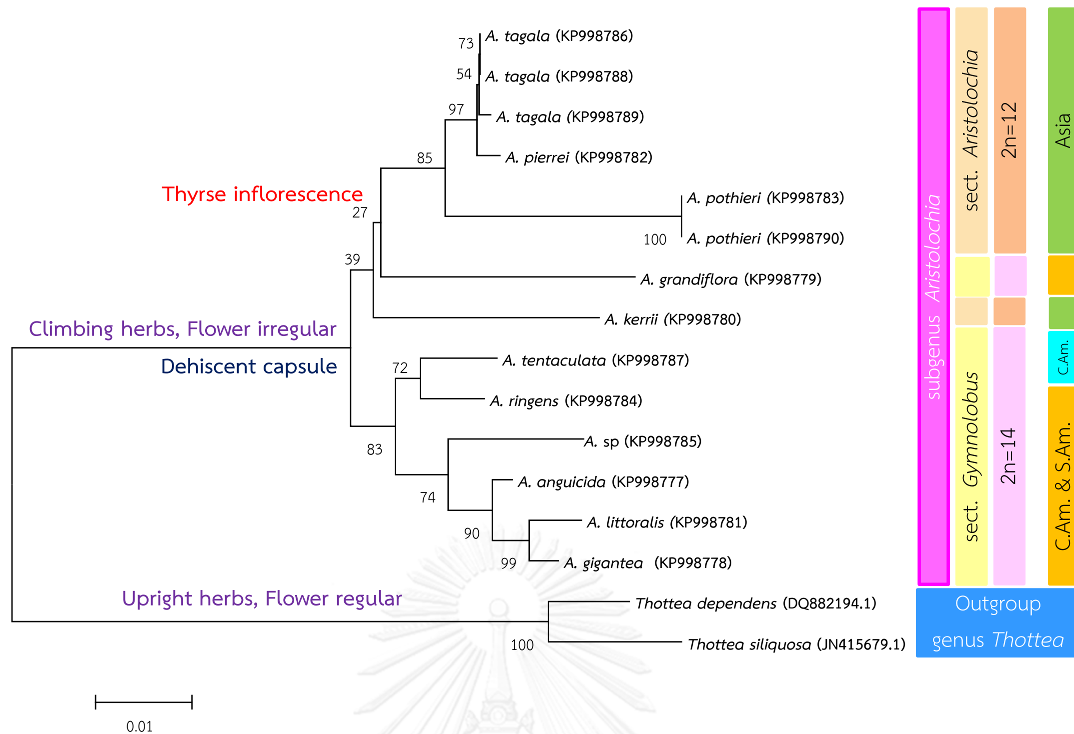

Figure 6 Phylogenetic assessment of eleven *Aristolochia* species constructed with the *mat*K sequences using the Neighbor-joining algorithm (bootstrap values are shown below the branches) with *Thottea dependens* and *T. siliquosa* as the outgroup. The sequence data of the species followed by accession numbers in brackets were retrieved from the GenBank DNA database.

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3.4 Discussion

Currently the molecular identification technique is extremely useful for the identification of medicinal herbs. It is included in the latest edition of the Pharmacopoeia of the People's Republic of China and its online supplementary note (Commission 2010b). Amongst various molecular techniques, nucleotide sequencing of standardized region becomes an alternative approach for herbal material identification, as a complement to other methods for better quality control. The chloroplast coding region, *rbc*L and *mat*K sequences, are proposed to be the core standardized region whereas the intergenic spacer *trn*H-*psb*A region and internal transcribed spacer (ITS) (or ITS2) are proposed as additional region for land plant identification at species level (Hollingsworth 2011, Fazekas, Kuzmina et al. 2012). Interestingly, some *Aristolochia* have very limited DNA sequences available in GenBank database. For instance, a partial *trn*H-*psb*A sequence (HG963744.1) of *A. anguicida*, a partial *rbc*L sequence (AB180121.1) of *A. kerrii*, a partial *mat*K sequence (AB060778.1) and a complete *mat*K sequence (DQ296649.1) of *A. pierrei,* and 4 accessions of partial *mat*K sequences (AB211567.1, AB060780.1, AB060779.1 and KF498584.1) of *A. tagala* are provided. Unfortunately, no nucleotide data for *A. pothieri* are available on the existing database and there is no nucleotide sequence of ITS region of the *Aristolochia* plants for use in this study. The limited number of DNA sequences has restricted the development of rapid molecular identification techniques for these herbs.

DNA barcoding based on five candidate regions (*rbc*L, *mat*K, ITS, *trn*H-*psb*A and *trn*L-*trn*F) have been continuously studied for identification of *Aristolochia* plants used in China, for example, *A. fangchi*, *A. manshuriensis*, *A. contorta* and *A. debilis* (Li, Au et al. 2014), *A. mollissima* (Li, Au et al. 2012), and *A. californica*, *A. championii*, *A. contorta*, *A. debilis*, *A. heterophylla* and *A. kaempferi* (Li, Ling et al. 2010). However, prior to this study, there was no DNA barcoding study in other *Aristolochia* species used in Thailand.

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In this study, DNA barcoding technique was used for identification of eleven *Aristolochia* species. The four DNA regions (*rbc*L, *mat*K, ITS and *trn*H-*psb*A) were used as suitable DNA regions. The results demonstrated that all four candidate DNA barcodes have performed very well in *Aristolochia* plants. The interspecific genetic distances were obviously greater than the intraspecific distance among the eleven *Aristolochia* species as indicated by *mat*K and *rbc*L. On the other hand, the intraspecific variations of ITS and *trn*H-*psb*A regions were greater than the interspecific variations. The degree of sequence variations amoung the *Aristolochia* samples were in the order of ITS > *trn*H-*psb*A > *mat*K > *rbc*L. The large insertions/deletions in ITS caused high variations (28.96%) amoung the eleven *Aristolochia* plants and were much higher than *trn*H-*psb*A (13.35%), *mat*K *(*11.22%*)* and *rbc*L (3.29%*)*.

The complete *rbc*L gene was easily amplified with a universal primer pair and the sequencing result was very good. The obtained fragments were found to be approximately 1,428-1,485 bp in length. The *rbc*L sequence from all samples of the same species showed completely identical sequence. The nucleotide information of the *rbc*L gene among these *Aristolochia* species were highly conservative. *A. anguicida* and *A.* sp could not be distinguished from each other based on the sequence divergences.

The complete *mat*K gene was amplified using primers matK-Aris-96F (or matK-Aris-50F) and matK-Aris-201R based on the sequences of *trn*K-*mat*K region. The *trn*K-*mat*K genes of the eleven *Aristolochia* plants were about 1,610-1,758 bp in length which is consistent with a previous report showing that the *mat*K coding region in the most angiosperms is around 1.5-1.6 kb in length (Neuhaus and Link 1987). As a result, two bases of intraspecies variation were observed in samples of *A. tagala*. collected from different locations. Notably, intra-species polymorphism with *mat*K sequences was generally limited to substitution of one or a few bases (Parmentier, Duminil et al. 2013). According to GenBank database, the variations in the same species were observed

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in *A. tagala* (AB060779, AB060780 and AB211567), *A. indica* (AB060771 and AB211579), *A. ringens* (AB211583, AB211584 and AB211586), *A. gigantea* (DQ882187 and JX485569), *A. acuminata* (DQ296646 and DQ532063), *A. albida* (AB211566, DQ296648 and DQ532064), *A. arborea* (DQ532044 and JX485577), *A. baetica* (DQ296653 and DQ882189), *A. cucurbitifolia* (AB060741 and AB180183), *A. kaempferi* (AB060743, AB180157, AB180159, AB180156, AB180158, AB180161, AB180162, AB180163, AB180160, AB180175, AB180165, AB180172, AB180173, AB180174, AB180167, AB180168, AB180171, AB180169, AB180170, AB180166 and DQ532042), *A. kwangsiensis* (AB060745, AB060746, AB060747, AB180176 and AB180179), A. maxima (AB060782 and AB060783), *A. moupinensis* (AB060750, AB060751, AB211562), *A. odoratissima* (AB060798, AB211561 and AB211585), *A. onoei* (AB060753, AB060754, AB060755, AB060756, AB060757, AB180164, AB189730 and AB353065), *A. pistalochia* (DQ296652 and AF543724), *A. ringens* (AB060800 and AB060801), *A. rotunda* (DQ296665 and DQ532061), *A. saccata* (AB211569 and AB353067), *A. salvadorensis* (JX485576 and DQ882191), *A. shimadai* (AB060760, AB060761, AB060762, AB060763). However, the DNA sequences of the *mat*K gene among these *Aristolochia* species can be used for discrimination of these plants.

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The entire ITS region was amplified using a universal primer pair of ITS1 and ITS4; however, the amplification was not successful in many species. ITS2 is an optional barcode for better amplification and sequencing compared to the entire ITS region (Chen, Yao et al. 2010). ITS3 and ITS-Aris-371F located on 5.8s rDNA was used in the amplification step. The cloning process was also needed for some species. Sequencing analysis failed probably caused by fungal contamination or natural endophytes (Hollingsworth 2011). The entire ITS regions of *A. pothierri* and *A. grandiflora* were observed at 696-751 bp in length, while ITS2 regions of other species were about 360-461 bp in length. The sequencing result indicated that ITS showed the highest

variations (28.96%) because of the large insertions/deletions. It can be used for plant discrimination at species level.

The length of *trn*H-*psb*A region of plant samples were about 300-369 bp. The cloning process was needed to obtain the good quality of sequencing process. The sequencing result indicated that *trn*H-*psb*A is the second region showing high variation (13.35%). It also can be used for plant discrimination at species level.

For unknown species, *A.* sp, could not be identified by morphological characteristics. The BLAST results between *A.* sp and other plant species which are existing on the GenBank database. The closest species referred by *trn*H-*psb*A region are *A. littoralis* (GU135396.2) and *A. ringens* (KP763860.1) at 89% query covery and identity at 97 and 96%, respectively, while the ITS region showed that the closest species are *A. ringens* (KP763867.1 and KP763865.1) at 99% query covery and at 92% identity. From *mat*K BLAST results, the closest species at 100% query covery and identity at 97% are *A. cruenta* (DQ882186.1), *A. cf. cordiflora* (DQ532056.1), *A. nummularifolia* (DQ532053.1), *A. eriantha* (DQ882185.1 and DQ532054.1) and *A. gigantea* (DQ882187.1) and the *rbc*L region indicated that the closed species at 97% query covery and 99% identity are *A. reniformis* (AB205600.1), *A. eriantha* (AB205590.1), *A. grandiflora* (AB205592.1), *A. micrantha* (AB205595.1), *A. maxima* (AB205594.1), *A. gigantea* (AB205591.1), *A. burelae* (AB205587.1), *A. zollingeriana* (AB205599.1), *A. clematitis* (AB205588.1) and *A. pentandra* (AB205596.1). As the BLAST results described above, there is no complete match sequence for *A.* sp through DNA sequences. Although in this study, *A.* sp shows the complete matches to *A. anguicida* by *rbc*L sequences and *A. tagala* by *trn*H-*psb*A sequences, *A.* sp could not be identified by morphological characteristics and nucleotide assessment by now.

The systematic of *Aristolochia* has been based on morphological characters such as perianth tubes, leaves, number of styles and anthers on gynostemium and fruit.

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The genus *Aristolochia* consists of three subgenera, the subgenera *Siphisia*, *Aristolochia* and *Pararistolochia*. The subgenus *Siphisia* is characterized by dehiscent capsules, a trilobed each accompanying two anthers, while the subgenus *Aristolochia* is defined by dehiscent capsules, lobes of the perianth unilatally appressed in the bud and breaking up into one to three segments, six or fewer lobes of the gynostemium and six or fewer anthers and chromosome 2n=12, 14 or 16. The subgenus *Pararistolochia* is distinguished from the others by fleshy indehiscent fruits, trilobed perianth, valvate in bud, sometimes with one or three long tails, six to twelve lobes of the gynostemium, six to twenty-four anthers and chromosome 2n=12 (González 1999, Murata, Ohi et al. 2001, Ohi-Toma, Sugawara et al. 2006).

The morphological features indicated that all samples in this study belong to the subgenus *Aristolochia*. The molecular phylogeny potentially divided the eleven *Aristolochia* species into two clades, following the taxonomic suggestion of González (González 1999): the section *Aristolochia* clade of Asia species (*A. tagala*, *A. pierrei*, *A. pothieri* and *A. kerrii*) and the section *Gymnolobus* clade of central and south America (*A. tentaculata*, *A. ringens*, *A. anguicida*, *A. littoralis* and *A. gigantea*). *A. grandiflora* was the only taxon congruent with section *Aristolochia* and not grouped specifically with section *Gymnobolus*. This result related to morphological features; section *Aristolochia* is distinguished from section *Gymnobolus* by the thyrsoid branching in the inflorescence.

The plants in subsect. *Podanthemum*, sect. *Aristolochia* were all included in a single clade consisting of *A. pierrei* and *A. tagala* which was supported by a relatively high bootstrap (97%). This group was consistent with morphological characteristics which are the unique stipitate utricle of the perianth tube and the chromosome number of 2n=12. While *A. grandiflora* arranged to sect. *Hexandrae* was divided into other close clade. The section *Gymnolous*, subsection *Hexandrae,* ser. *Hexandrae* consisting of *A. tentaculata*, *A. anguicida*, *A. ringens*, *A. littoralis* and *A. gigantea* were all included in a single clade at bootstrap value 83%, which was

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consistented with morphological features which are hexamerous stemium and chromosome number of 2n=14.

The results from phylogenetic analysis were well correlated to morphological criteria, habitats and chromosome numbers. Our results agree well with the previously published *mat*K phylogenetic tree (Murata, Ohi et al. 2001, Ohi-Toma, Sugawara et al. 2006) and *trn*L-*trn*F phylogenetic tree (Neinhuis, Wanke et al. 2004). In addition, this is the first study of *A. pothieri*.

The nucleotide variations of the three *Aristolochia* plants used as Krai-Krue herb are shown in Table 9. The nucleotide sequences of three DNA regions which are *rbc*L, *mat*K and ITS, could be used for identification of these plants at species level except the nucleotide sequences of *trn*H-*psb*A spacer. From phylogenetic analysis related to the habitat, *A. pothieri*, *A. pierrei* and *A. tagala* which are botanical origin of Krai-Krue herbs are Asia species. The results also relate to traditional uses of these herbs, *A. pothieri* and *A. pierrei* is used in Thailand, *A. tagala* is widely used in many countries such as Thailand, India, Philippines and Maleysia (Sathornviriyapong, Picheansoonthon et al. 2007, Heinrich, Chan et al. 2009).

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3.5 Conclusion

The DNA sequencing technique could be used for the establishment of DNA database and the identification of organisms. The *rbc*L, *mat*K, ITS and *trn*H-*psb*A contained diagnostic polymorphic sites and could be used to distinguish the *Aristolochia* plants from other species or its adulterants/substitutes. The *mat*K sequence was chosen for phylogenetic analysis. The ITS region appeared to be suitable DNA regions for the species identification and provided the simple and rapid tools for further study. The DNA information will be helpful for forensic investigation and safety control by the herbal industries and regulatory authorities and also for new plant species discovery.

CHAPTER IV
AUTHENTICATION OF “KRAI-KRUE” DERIVED FROM THREE ARISTOLOCHIA SPECIES USING MULTIPLEX PCR

4.1 Introduction

“Krai-Krue” is a name of a crude drug used in many Thai folk medicines. The drug is derived from dried roots of three *Aristolochia* plants, *A. pothieri* (Athikomkulchai and Ruangrungri 2001), *A. pierrei* and *A. tagala* (Sathornviriyapong, Picheansoonthon et al. 2007). It can also be derived from dried roots of *Raphistemma pulchellum* (Apocynaceae) (Vuthithammavech 1997), *Jasminum* spp. (Oleaceae) (Picheansoonthon, Chawalit et al. 2001), and *Gymnopetalum integrifolium* (Cucurbitaceae) (Vuthithammavech 1997). Crude drug “Krai-Krue” is available in the local dispensaries in the forms of powders and dried root slices, which no longer bear the original morphological characters, making them difficult to identify.

Recently, many types of DNA-based molecular technique have been developed for rapid and reliable herbal materials identification tool (Zhao, Hu et al. 2006), and the ITS region is the most frequently used (Li, Cao et al. 2011). Multiplex PCR based on polymorphic sites of nucleotide sequence is a suitable technique for the authentication of herbal medicines by amplification of more than two different loci simultaneously (Lee, Kim et al. 2012).

In this study, a multiplex PCR method using diagnostic primers via interspecific variation analysis of the ITSS2 region was developed for the convenient and rapid identification of the three *Aristolochia* species used as Krai-Krue herbs, *A. pothieri*, *A. pierrei* and *A. tagala*. To the best of our knowledge, this is the first study on the discrimination of the herb using the multiplex PCR technique.

4.2 Materials and methods

4.2.1 Plant materials

Forty-two *Aristolochia* samples as shown in Table 2 were studied. Other Krai-Krue herbs comprising *Raphistemma pulchellum* Wall, *Jasminum sp*, *J. sambac* (L.) Aiton, *J. adenophyllum* Wall. Ex C.B. Clarke and *Gymnopetalum integrifolium* Kurz. collected from various locations were also included in this study (Table 10).

Table 10 Plant samples used as sources of Krai-Krue.

Sample	Place of collections (Thailand, Province)	Voucher number
Raphistemma pulchellum Wall	Bangkok	MUS-5414
Gymnopetalum integrifolium Kurz.	Sakaeo	MUS-5415
	Phetchaburi	MUS-5416
Jasminum sambac (L.) Aiton	Bangkok	MUS-5417
Jasminum adenophyllum Wall. Ex C.B. Clarke	Bangkok	MUS-5418
Jasminum sp	Bangkok	MUS-5419

4.2.2 Genomic DNA extraction

Total genomic DNA was extracted from 100 mg of leaves from each individual plant specimen and was frozen using liquid nitrogen and ground with a mortar and pestle to obtain a fine powder. The isolation of the total DNA from the powder was performed using genomic DNA extraction kit as described previously.

4.2.3 Multiplex PCR of the ITS2

4.2.3.1 ITS2 multiple sequence alignment

The DNA sequences of ITS region from eleven *Aristolochia* plants were aligned using ClustalX Version 2.1 software. According to the multiple sequence alignment, polymorphic sites were detected on the ITS region. The diagnostic forward

primer was designed to be complementary to a region of the PCR product where interspecific variation of interest occurs (Figure 7). The primer pairs that would produce different sizes of PCR products were designed as shown in Table 11. Each primer was designed to anneal to a specific region of each interested *Aristolochia* plants. The two common primers, ITS-Aris-371F and ITS4, were also designed to amplify DNA sequence as an internal amplification control.

Table 11 Species-specific primers used in multiplex PCR in this study.

Primer	Specificity	Sequence (5'⟶3')	Tm (^{o}C)
ITS2-poF	*A. pothieri*	GCC GCG AGG ACC CAA TG	60.0
ITS2-piF	*A. pierrei*	GAC TAC TGG TGG CTC CAC GCA	63.7
ITS2-taF	*A. tagala*	GGC GGG GGC GAG CAG GC	67.2
ITS-Aris-371F	Internal control	AAT TGC AGA ATC CCG CGA AC	57.3
ITS4	Internal control	TCC TCC GCT TAT TGA TAT GC	55.3

(The underlined nucleotide(s) were specifically designed for target sequence(s))

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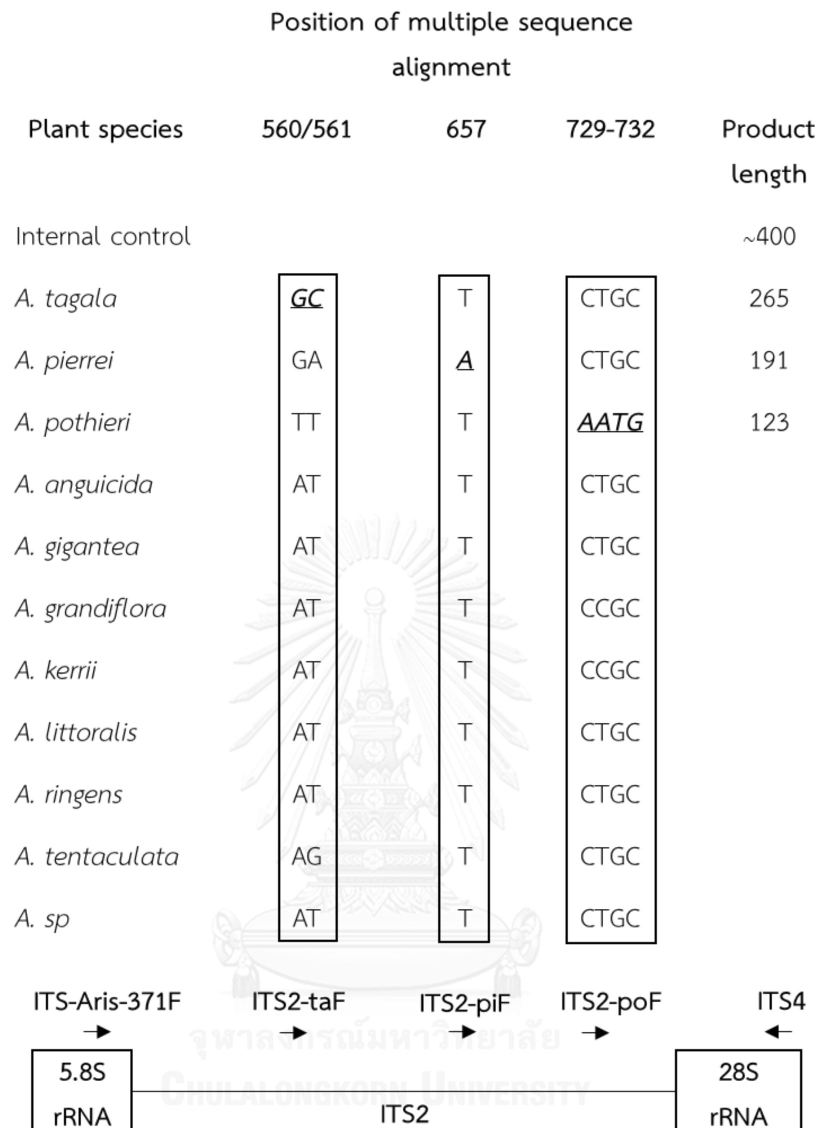

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Figure 7 Positions of diagnostic primers for multiplex PCR for discrimination of *Aristolochia* plants used as Krai-Krue herbs.

4.2.3.2 Multiplex PCR

Nucleotide polymorphisms-based multiplex PCR was performed to authenticate three *Aristolochia* plants using species-specific primers. The multiplex PCR should be first examined the specificity of diagnostic primers by singleplex PCR (Sint, Raso et al. 2012). Each diagnostic primer pair was conducted separately using genomic DNA of each species as template. With the multiplex PCR system, three diagnostic

primers and two common primers were included in the multiplex PCR reaction using genomic DNA of each species as template. The amplification reaction was performed in 25 µL of GoTaq® Flexi DNA Polymerase reaction mixture, consisting of 5X PCR buffer, 25 mM $MgCl_2$, 2.5 mM of each dNTP, 10 mM of each primer, 5U *Taq* polymerase (Promega, USA), and 10–100 ng of total genomic DNA as a template.

Annealing temperatures were determined by gradient PCR with temperatures increasing from 52 to 58°C. The optimal PCR condition obtained was at 96°C for 3 min, followed by 30 cycles of 96°C for 60 s, 55°C for 1 min and 72°C for 30 sec, and final extension at 72°C for 10 min. The amplified products were detected by 1.7% agarose gel electrophoresis in 1X TAE buffer at 80 V for 40 min. Separated PCR products were then visualized by ethidium bromide staining under UV light. Fragment sizes were estimated by comparison with DNA marker. This experiment was repeated in all samples in the same species for three times to verify the stability and reproducibility of banding patterns.

4.3 Results

4.3.1 ITS2 sequences analysis and species-specific primers for multiplex PCR

According to the multiple sequence alignments of ITS regions of eleven *Aristolochia* plants, three interspecific variation sites specific to each of *A. pothieri*, *A. pierrei* and *A. tagala* were detected. These sites were chosen to design three diagnostic primers. The nucleotide at positions 560-561 was GC which specific in *A. tagala*. The nucleotide at positions 657 was T in all *Aristolochia* plants, whereas in *A. pierrei* was A. The positions 729-732 in *A. pothieri* was AATG, but not in the other species. These diagnostic forward primers were designed for specific amplification using the available variation sites on the ITS2 region of eleven *Aristolochia* plants. The forward primers, ITS2-poF, ITS2-piF and ITS2-taF, were used to amplify specific fragments of 123, 191 and 265 bp for *A. pothieri*, *A. pierrei* and *A. tagala*, respectively.

The common forward primer, ITS-Aris-371F, and universal primer, ITS4, were used as internal amplification control to all *Aristolochia* plants with ~ 400 bp fragment (Figure 7).

4.3.2 Multiplex PCR analysis

The specificity of each primer was done using singleplex PCR. DNA template of each species was amplified individually with three pairs of diagnostic primers. The results revealed that only species-specific product was amplified. Each primer pairs generated specific fragments with different size. For example, only specific fragment of 123 bp was generated in *A. pothieri*. Likewise, *A. pierrei* and *A. tagala* generated their specific fragments of 191 and 265 bp of PCR products, respectively. These results indicated that each primer was specific to each species (data not shown).

In multiplex PCR reaction, each specific PCR product was amplified specifically from its target species with the combination of the three diagnostic forward primer and common forward primer. The PCR result was observed by gel electrophoresis. Three individual species-specific fragments were shown with different sizes. The fragments of 123, 191 and 265 bp was amplified by the primer pairs ITS2-poF/ITS4, ITS2-piF/ITS4 and ITS2-taF/ITS4 specifically for *A. pothieri*, *A. pierrei* and *A. tagala*, respectively, while the 400 bp fragment generated from internal control primer pair of ITS-Aris-371F/ITS4 in all target species. The PCR product was determined by visualization under UV light after gel electrophoresis. Therefore, two different size of PCR products were simultaneously amplified in all of individual target species. The result analysis was based on the condition of one fragment used as an internal control and one fragment used as specific fragment of each species (Figure 8). To confirm the reproducibility of this method, the experiment was repeated in all samples in the same species for three times.

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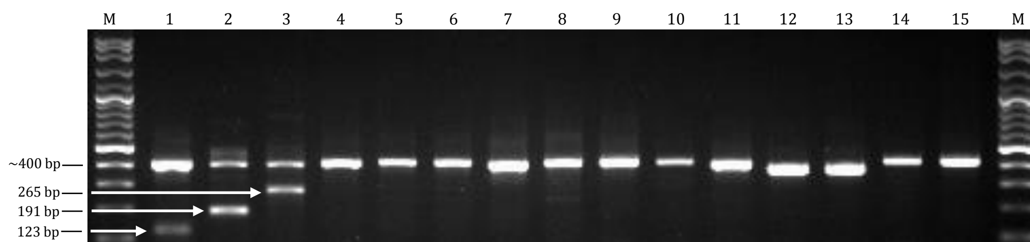

Figure 8 1.7% agarose gel electrophoresis image of PCR products generated with a set of species-specific PCR primers on ITS2 region of Krai-Krue herb. Lane;

1: *Aristolochia pothieri*
2: *A. pierrei*
3: *A. tagala*
4: *A. ringens*
5: *A. kerrii*
6: *A. littoralis*
7: *A. grandiflora*
8: *A. gigantea*
9: *A. tentaculata*
10: *A. anguicida*
11: *Gymnopetalum integrifolium*
12: *Raphistemma pulchellum*
13: *Jasminum* sp
14: *J. sambac*
15: *J. adenophyllum*
M: VC 100-bp plus DNA ladder

4.4 Discussion

Dried roots of three *Aristolochia* plants, *A. pothieri* (Athikomkulchai and Ruangrungri 2001), *A. pierrei* and *A. tagala* (Sathornviriyapong, Picheansoonthon et al. 2007) and other plants, *Raphistemma pulchellum* (Apocynaceae) (Vuthithammavech 1997), *Jasminum* spp. (Oleaceae) (Picheansoonthon, Chawalit et al. 2001), and *Gymnopetalum integrifolium* (Cucurbitaceae) (Vuthithammavech 1997) have been recognized as sources of Krai-Krue. Such misidentification of herbs is particularly common for species that share a similar name or similar features but significantly vary in their medicinal or toxic properties. Therefore, the proper identification of Krai-Krue herbs is needed, especially when the herbs appear in the form of a powder, shredded material, or a formulated mixture. To ensure the safety of using these herbs, species-specific method for the discrimination of *A. pothieri*, *A. pierrei* and *A. tagala* is neccessary.

Morphological characteristics of plants have been widely used for plant classification. Regarding the botanical origins of Krai-Krue, generally, *A. pothieri* has been clearly differentiated by leaf morphology from *A. pierrei* and *A. tagala* (Phuphathanapong 1987). But *A. pierrei* and *A. tagala* are difficult to differentiate by morphology. The discrimination of these *Aristolochia* plants is difficult especially when they are ground into small pieces or powder. To ensure the safety of consumers from Krai-Krue containing herbal products, the development of a simple and reliable method for discrimination of these *Aristolochia* plants is necessary. In this study, based on the obtained nucleotide sequences of four DNA regions (*rbc*L, *mat*K, ITS and *trn*H-*psb*A) from eleven *Aristolochia* species as described in the previous chapter, the ITS2 region was used as a tool for discrimination of the three *Aristolochia* plants because it is a high variable region and has been successfully used in several herbal identifications (Li, Cao et al. 2011). Based on the diagnosed polymorphic sites from sequencing technique as in Figure 7, the three *Aristolochia* Krai-Krue can be differentiated by multiplex PCR.

Multiplex PCR was developed for identification of the three *Aristolochia* plants using a species-specific primer set. The three forward species-specific primers located on ITS2 region, ITS2-poF, ITS2-piF and ITS2-taF, were specifically designed for *A. pothieri*, *A. pierrei* and *A. tagala*, respectively to generate PCR products of 123, 191 and 265 bp in length. The generated amplicon size of each specific fragment should be different when detected by gel electrophoresis (Sint, Raso et al. 2012). The internal control of amplification was included in the experiment design to ensure that the reaction was successful because the PCR product of internal control should always be amplified even though there is no target DNA sequence. The PCR products of ITS region from all samples were amplified with the internal control primers ITS-Aris-371F and ITS4 located on conserved region of 5.8s rDNA and 28s rDNA to generate 400 bp fragments in all samples. The test of specificity of each diagnostic primer before starting

the multiplex PCR was performed by singleplex PCR. The conditions of PCR for both singleplex and multiplex PCR were optimized by gradient PCR. The optimal annealing temperature was at 55 °C for all primers (data not shown). The result indicated that multiplex PCR simultaneously amplified two fragments, one corresponding to target sequence for each species and the other to an endogenous sequence as an internal control for the PCR. Interestingly, only one specific fragment of each species was amplified by its specific primer. This result indicated that the each species-specific primer designed was highly specific to its target sequence. The three *Aristolochia* plants used as sources of Krai-Krue herbs could be discriminated individually between species with their specific primers. The results from this study are consistent with previous studies showing that the multiplex PCR could be applied as an effectively authenticate medicinal plants tool (Lee, Doh et al. 2008, Jigden, Wang et al. 2010).

4.5 Conclusion

The multiplex PCR based on ITS2 region was examined and successfully applied for the discrimination of three *Aristolochia* plants used as Krai-Krue herbs from other sources. The results indicated that this method is a convenient and specific tool for raw material identification. This is the first report of the authentication of three *Aristolochia* species used as Krai-Krue herbs by molecular approach, and this could be adapted for identification of other medicinal plants in a simple, accurate, time-saving and cost-effective method.

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CHAPTER V
APPLICATION OF MULTIPLEX PCR FOR IDENTIFICATION OF KRAI-KRUE

5.1 Introduction

Krai-Krue is a crude drug used as an ingredient in Thai folk medicinal formulas for tonic, muscle relaxant, diuretic, antipyretic, analgesic, anti-rheumatism, immunostimulant, emmenagogue, abortive agent and liver enhancer (Vuthithammavech 1997). It is also one of ingredients in 10 herbal recipes on the Thailand list of Herbal Medicinal Products A.D. 2006, for example, Ya hom Nawakod *(ยาหอมนวโกฐ),* Ya hom Inthajuk (ยาหอมอินทจักร์), Ya Ummaruekawatee (ยาอัมฤควาที), Ya Tatbunjob (ยาธาตุบรรจบ), Ya Wisumpayayai (ยาวิสัมพยาใหญ่), Ya Munthatat (ยามัณฑธาตุ), Ya Kheawhom (ยาเขียวหอม), Ya Treehom (ยาตรีหอม), Ya Prasaganplu (ยาประสะกานพลู), Ya Prasajettapungkee (ยาประสะเจตพังคี) (Health 2006). According to microscopic, morphological and chemical profiling approaches, Krai-Krue can be derived from dried roots of the three *Aristolochia* species, *A. pothieri* Pierre ex Lecomte (Vuthithammavech 1997, Athikomkulchai and Ruangrungri 2001)*, A. pierrei* Lecomte and *A. tagala* Cham. (Sathornviriyapong, Picheansoonthon et al. 2007). In 2013, the National Drug Committee have issued an order that demands the removal of Krai-Krue from all formulas within one years after April 19th, 2013 (Control 2013, Health 2013). However, despite the warnings, Krai-Krue still be bought from local dispensaries.

In this study, a novel multiplex PCR technique was developed based on nucleotide sequence of ITS2 region for the discrimination of *Aristolochia* Krai-Krue herbs and was used to authenticate crude Krai-Krue drugs purchased from various local dispensaries.

5.2 Materials and Methods

5.2.1 Crude drugs named Krai-Krue and Thai traditional formulas containing Krai-Krue

Seven commercial crude drug samples claimed to be Krai-Krue (i.e. C1–C7) and twenty-three formulas claimed to contain Krai-Krue were randomly purchased from various traditional drug stores. The list of samples used in this study was shown in Table 12.

5.2.2 Genomic DNA extraction

Total genomic DNA was extracted from *20-50* mg of dried crude drug samples (as shown in Figure 9) using genomic DNA extraction kit as mentioned in Material and Methods of Chapters III.

5.2.3 Multiplex PCR

The multiplex PCR protocol was performed as mentioned in Material and Methods of Chapter IV. The amplification reaction was performed in 25 µL of reaction mixture GoTaq® Flexi DNA Polymerase, consisting of 5X PCR buffer, 25 mM $MgCl_2$, 2.5 mM of each dNTP, 10 mM of each primer, 5U *Taq* polymerase (Promega, USA), and 3.5 µL of total genomic DNA as a template.

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Table 12 Details of Krai-Krue crude drugs and formulas analyzed in this study.

Sample	Code	Date of manufacture	Purchased location (Thailand, Province)	Proportion of Krai-Krue in formulas
Krai-Krue	C1	27/12/12	Bangkok	-
Krai-Krue	C2	27/12/12	Bangkok	-
Krai-Krue	C3	16/04/13	Nakhon Si Thammarat	-
Krai-Krue	C4	27/07/13	Phetchaburi	-
Krai-Krue	C5	17/09/13	Ayutthaya	-
Krai-Krue	C6	20/08/14	Bangkok	-
Krai-Krue	C7	20/08/14	Bangkok	-
Ya Ummaruekawatee	R151	23/12/11	Sakaeo	0.1000
	R153	23/02/13	Bangkok	0.1000
Ya Kheawhom	R122	09/08/13	Bangkok	0.0526
	R123	06/03/13	Bangkok	0.0526
Ya Tatbunjob	R072	26/07/13	Bangkok	0.0370
	R073	15/01/13	Bangkok	0.0370
	R075	10/04/12	Mahasarakham	0.0370
Ya Hom Nawakod	R052	17/08//13	Bangkok	0.0185
	R053	28/01/13	Bangkok	0.0185
	R054	12/07/12	Prachinburi	0.0185
Ya Wisumpayayai	R112	05/08/13	Bangkok	0.0185
	R113	15/10/12	Bangkok	0.0185
Ya Treehom	R043	22/04/13	Bangkok	0.0156
Ya Prasa Ganplu	R082	24/07/13	Bangkok	0.0154
	R083	01/12/12	Bangkok	0.0154
Ya Prasa Jettapungkee	R093	02/04/12	Bangkok	0.0152
Ya Munthatat	R103	09/04/13	Bangkok	0.0108

Ya Hom Inthajuk	R062	12/06/13	Bangkok	0.0102
	R063	02/07/13	Bangkok	0.0102
	R064	16/07/12	Prachinburi	0.0102
Ya Juntaleela	R012	25/05/13	Bangkok	0.1212
	R013	20/05/13	Bangkok	0.1212
Ya Hom Kaelomwingwean	R143	28/08/13	Bangkok	N/A

([a] Manufacturing date of the products)

5.3 Results

The multiplex PCR was performed under the conditions described above. The experimental DNA admixtures containing the genomic DNA of three *Aristolochia* species were prepared and subjected to multiplex PCR analysis to test the accuracy of this technique. After gel electrophoresis, the combined electrophoresis patterns were resolved, and PCR products observed on agarose gel represented different species in the mixtures (Figure 10 and Table 13). Each fragment exhibited the unique characteristic of the species. The DNA admixture containing *A. pothieri, A. pierrei and A. tagala* presented a combined pattern of four fragments: a 123-bp fragment from *A. pothieri*, a 191-bp fragment from *A. pierrei*, a 265-bp fragment from *A. tagala* and a 400-bp fragment from internal amplification control. The PCR products from C1-C5 were observed by two different sizes at 191-bp and 400-bp. The results indicated that C1-C5 were derived from *A. pierrei*, while the fragments of C6 and C7 at 400-bp in length indicated that they were not of *A. pothieri*, *A. pierrei* or *A. tagala*. They were probably derived from other species of Krai-Krue herbs.

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Table 13 Details of commercially available crude drugs analyzed.

Claimed crude drugs	Code	Purchased location (Thailand, Province)	Purchase date	Detected species
Krai-Krue	C1	Bangkok	2012/08/27	*A. pierrei*
Krai-Krue	C2	Bangkok	2012/08/27	*A. pierrei*
Krai-Krue	C3	Nakhon Si Thammarat	2013/04/16	*A. pierrei*
Krai-Krue	C4	Phetchaburi	2013/07/22	*A. pierrei*
Krai-Krue	C5	Ayutthaya	2013/09/17	*A. pierrei*
Krai-Krue	C6	Bangkok	2014/12/20	N/D
Krai-Krue	C7	Bangkok	2014/12/20	N/D

Figure 9 Samples of crude drugs “Krai-Krue” C1-C7

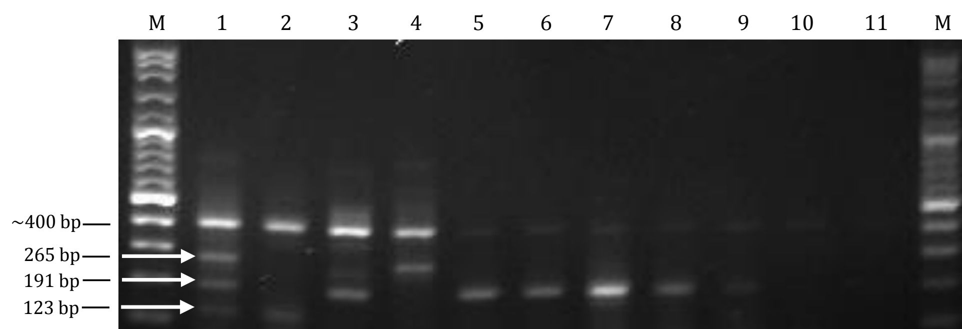

Figure 10 1.7% agarose gel electrophoresis image of species-specific PCR primers on ITS2 region of Krai-Krue herb. Seven commercially available crude drugs (C1 – C7) and DNA markers (M) in bp are indicated. Lane 1: mixed Krai-Krue, 2: *Aristolochia pothieri*, 3: *A. pierrei*, 4: *A. tagala*, 5-10: C1-C7 respectively.

5.4 Discussion

The nephrotoxic herb, many *Aristolochia* species, share a similar crude drug name in Thai as "Krai-Krue". The National Drug Committee has legally issued the announcement to manufacturer to remove Krai-Krue from registered formulas within one year after 19th April 2013. The identification of Krai-Krue herbs is challenging when they are in form of powders or small pieces. Unfortunately, using DNA barcoding of ITS2 sequence for identification of commercial Krai-Krue crude drugs failed (data not shown). The BLAST of C1-C7 sequencing results indicated that they were naturally contaminated by fungal and endophyte (data not shown). It might be affected by storaging for a long time or DNA is highly degraded or contaminated by microorganism, making the poor quality amplification of DNA for sequencing or fingerprinting (Singh, Srivastava et al. 2008, Gautam and Bhadauria 2009, Gautam, Sharma et al. 2011, Li, Zhang et al. 2011). Indeed, DNA barcoding can be used to identify individual species. However, there are the alternative molecular methods as well as multiplex PCR which provides reliable and rapid protocols, and can also be applied for species identification of closely related herb. Moreover, multiplex PCR reactions that can routinely amplify barcode markers will significantly reduce laboratory costs (Heubl 2013). For example,

discrimination of Korean *Artemisia iwayomogi* from other *Artemisia* herbs (Lee, Doh et al. 2008), identification of Mediterranean olive (Consolandi, Palmieri et al. 2008), authentication of *Dendrobium* species used in China (Chiang, Yu et al. 2012), authentication of *Anemarrhena asphodeloides* (Jigden, Wang et al. 2010) and discrimination of Phyllanthus taxa used in China (Lee, Li et al. 2006).

The multiplex PCR technique could be successfully applied to distinguish the three *Aristolochia* plants used as Krai-Krue herbs. The results disclosed that there are still crude drugs derived from *A. pierrei* in local dispensaries in Thailand. Two samples are not derived from *A. pothieri*, *A. pierrei* or *A. tagala*, and could not be identified at species level. Likewise for the formulas purchased from traditional drug stores, multiplex PCR could not be used for detection of Krai-Krue herbs. The possible causes might be the DNA degradation after formulation processes including heat, grinding, mixing and too low amounts of Krai-Krue in the formulas. However, the failure of this process probably causes from the limitation of multiplex PCR such as competition of primers and limited of resources in reaction (Edwards and Gibbs 1994). Nested PCR has been used in cases in which direct PCR proved impossible. However, combination with other identification tools such as chemical fingerprint is needed to confirm that whether they are derived from *Aristolochia* species.

5.5 Conclusion

This multiplex PCR method could serve as a rapid and reliable identification tool of raw material Krai-Krue herbs with specific for *A. pothieri*, *A. pierrei* and *A. tagala*. Although, various identification methods are available, each method has its own pros and cons; and no single method can be sufficient to authenticate herbal materials. To assure the identification results, another identification tool is need such as chemical assessment by HPTLC with standard chemical marker.

CHAPTER VI
CHEMICAL PROFILES OF KRAI-KRUE AND THAI TRADITIONAL FORMULAS

6.1 Introduction

Chemical profiling is a basic approach for herbal material identification used as complement to other methods for better quality control. The major chemical constituents in the whole plant of *Aristolochia* are the human carcinogens, aristolochic acid I (AAI) and aristolochic acid II (AAII) (NTP 2011). Aristolochic acid I (AAI) has been used as a chemical marker for the quality control of herbs and herbal products containing *Aristolochia* species (Blatter and Reich 2004, Li, Au et al. 2012, Phadungrakwittaya, Akarasereenont et al. 2012). Since 2012, High-performance thin layer chromatography (HPTLC) pattern is recommended by European Pharmacopoeia and British Pharmacopoeia for screening of aristolochic acids at levels equal to or greater than 2 ppm (Commission and Britain 2012, Pereira Sena, Ashton-Prolla et al. 2012). In the present study, the HPTLC was performed to detect aristolochic acid I in seven Krai-Krue crude drugs and twenty-three Krai-Krue containing formulas available in market.

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6.2 Materials and Methods

6.2.1 Crude drug “Krai-Krue” and Thai traditional formulas containing Krai-Krue

Krai-Krue samples and Krai-Krue containing Thai traditional formulas were purchased from various local dispensaries in Thailand as shown in Table 14.

Table 14 Details of Krai-Krue crude drugs and samples analyzed in this study.

Sample	Code	Date of manufacture	Purchased location (Thailand, Province)	Proportion of Krai-Krue in formula
Krai-Krue	C1	27/12/12	Bangkok	-
Krai-Krue	C2	27/12/12	Bangkok	-
Krai-Krue	C3	16/04/13	Nakhon Si Thammarat	-
Krai-Krue	C4	27/07/13	Phetchaburi	-
Krai-Krue	C5	17/09/13	Ayutthaya	-
Krai-Krue	C6	20/08/14	Bangkok	-
Krai-Krue	C7	20/08/14	Bangkok	-
Ya Ummaruekawatee	R151	23/12/11	Sakaeo	0.1000
	R153	23/02/13	Bangkok	0.1000
Ya Kheawhom	R122	09/08/13	Bangkok	0.0526
	R123	06/03/13	Bangkok	0.0526
Ya Tatbunjob	R072	26/07/13	Bangkok	0.0370
	R073	15/01/13	Bangkok	0.0370
	R075	10/04/12	Mahasarakham	0.0370
Ya Hom Nawakod	R052	17/08//13	Bangkok	0.0185
	R053	28/01/13	Bangkok	0.0185
	R054	12/07/12	Prachinburi	0.0185
Ya Wisumpayayai	R112	05/08/13	Bangkok	0.0185
	R113	15/10/12	Bangkok	0.0185
Ya Treehom	R043	22/04/13	Bangkok	0.0156
Ya Prasa Ganplu	R082	24/07/13	Bangkok	0.0154
	R083	01/12/12	Bangkok	0.0154
Ya Prasa Jettapungkee	R093	02/04/12	Bangkok	0.0152
Ya Munthatat	R103	09/04/13	Bangkok	0.0108
Ya Hom Inthajuk	R062	12/06/13	Bangkok	0.0102

	R063	02/07/13	Bangkok	0.0102
	R064	16/07/12	Prachinburi	0.0102
Ya Juntaleela	R012	25/05/13	Bangkok	0.1212
	R013	20/05/13	Bangkok	0.1212
Ya Hom Kaelomwingwean	R143	28/08/13	Bangkok	N/A

([a] Manufacturing date of the products, N/A = Not available)

6.2.2 High performance thin layer chromatography (HPTLC)

Detection of aristolochic acid I was determined using a CAMAG Linomat 5 automatic sample spotter (Muttenz, Switzerland) under a flow of nitrogen gas for Krai-Krue crude drugs and manually spotted for Krai-Krue containing formulas. The test solution was prepared by extracting 2.25 g of the powdered herbal drug with 10 ml of anhydrous formic acid, water, methanol (1:9:40 V/V/V), then sonicated at room temperature for 10 min and centrifuged at 14,000 rpm for 5 min. The clear solution was used as the test solution for 1 µL (for C1-C5), 20 µL (for C6-C7 and all formulas). The solution samples were spotted in the bands of width 8 mm with a CAMAG microlitre syringe on a HPTLC Silica gel 60 F_{254} glass plate (20x10 cm). The plate was developed in a CAMAG glass twin-through chamber (20×20 cm) which was presaturated with 25 ml mobile phase of an upper layer of the mixture of anhydrous formic acid, water, ethyl acetate, toluene (1:1:10:20 V/V/V/V) for 30 min at room temperature. Solvent fronts of the mobile phases were allowed to ascend 8 cm above the line of sample application. Subsequently, developed HPTLC plates were air dried and sprayed with a 100 g/L solution of stannous chloride in dilute hydrochloric acid until the plate is slightly wet, and then heated at 100°C for 1 min. The chromatograms were observed under long ultraviolet wavelengths (365 nm). HPTLC plates were air dried and scanned with a CAMAG TLC scanner 3 and analyzed by winCATS software version 1.4.4.

Aristolochic acid I (Sigma-Aldrich, USA) was used as standard at concentration 2 and 5 ppm for 20 µL. All materials and reagents were of analytical grade.

6.3 Results

HPTLC chromatogram of standard aristolochic acid of AAI was developed using the mobile phase and the detection method described above. The result showed that Rf value of AAI was 0.46. The chromatogram of AAI showed greenish-blue zone. The lighted yellow zone, a characteristic fluorescence of AAII, was observed at high concentration of AAI (Figure 11). The linear calibration curves of AAI were within the concentration range of 0.5-20 ppm. A linear calibration equation, $y= 396.28x+823.09$ was obtained with a correlation coefficient of 0.9835 (Figure 12).

HPTLC profiles of extracts of Krai-Krue samples and Thai traditional formulas were examined. Standard AAI at concentration 2 and 5 ppm were used as reference markers. Seven Krai-Krue crude drugs were tested. The result showed that only C1-C5 have the same Rf value as standard AAI. (Figure 13). The AAI contents of crude drug extracts of C1-C5 were 0.135, 0.225, 0.144, 0.141 and 0.159 %w/w, respectively.

The solutions of extracts prepared from twenty-three formulas were used as test samples. The chromatogram showed that thirteen formulas (R151, R123, R072, R073, R053, R054, R093, R112, R113, R043, R062, R063, R064) have a band with a greenish-blue color and an Rf value of AAI. While the other ten formulas (R153, R122, R075, R052, R082, R083, R103, R012, R013, R143) did not show (Figure 14 and Table 15).

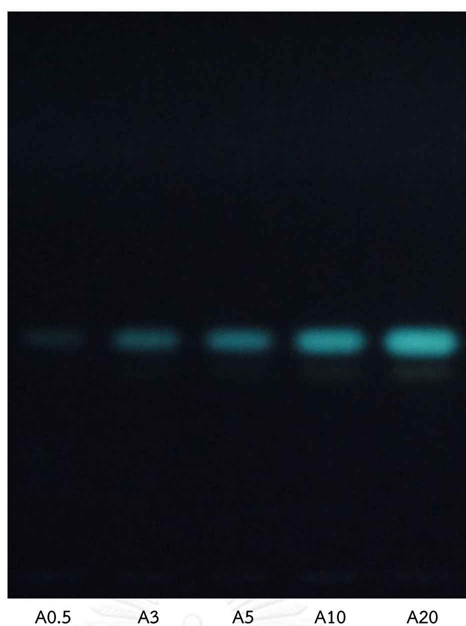

Figure 11 HPTLC profiles of standard aristolochic acid I at concentration 0.5, 3, 5, 10 and 20 ppm respectively (lane 1-5).

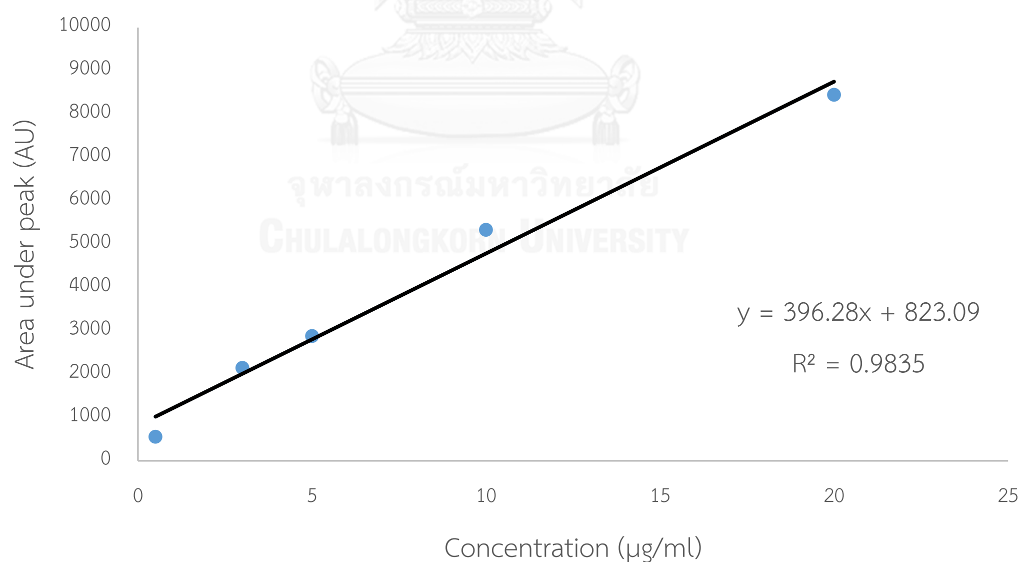

Figure 12 Calibration curve of AAI by TLC-densitometric method.

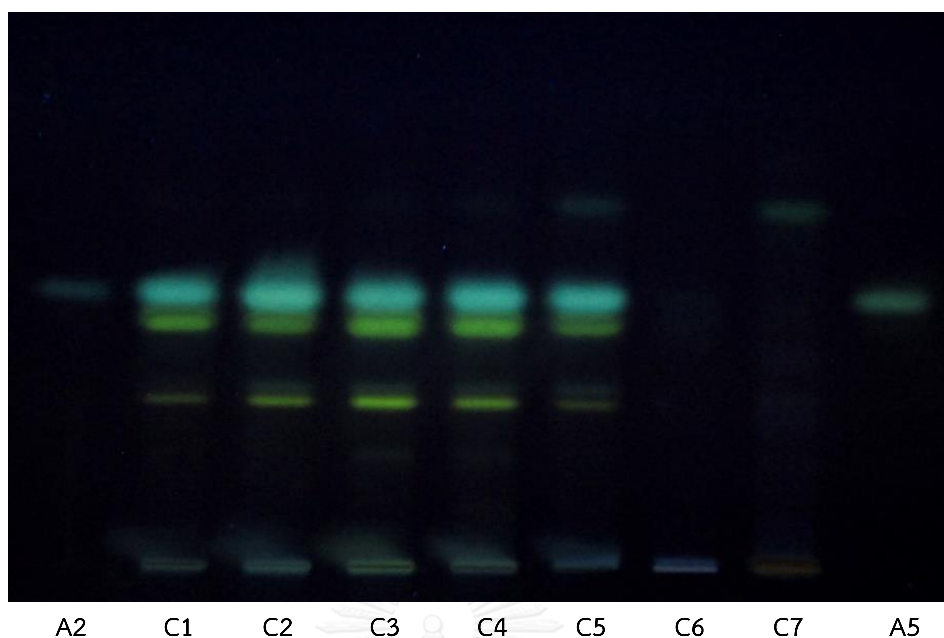

Figure 13 HPTLC profile of Krai-Krue herbs; lane 1 (A2) is standard AAI 2 ppm (20 µl), lane 2-8 are Krai-Krue from traditional herb stores (1 µl for C1-C5 and 20 µl for C6-C7) and lane 9 (A5) is standard AAI 5 ppm (20 µl).

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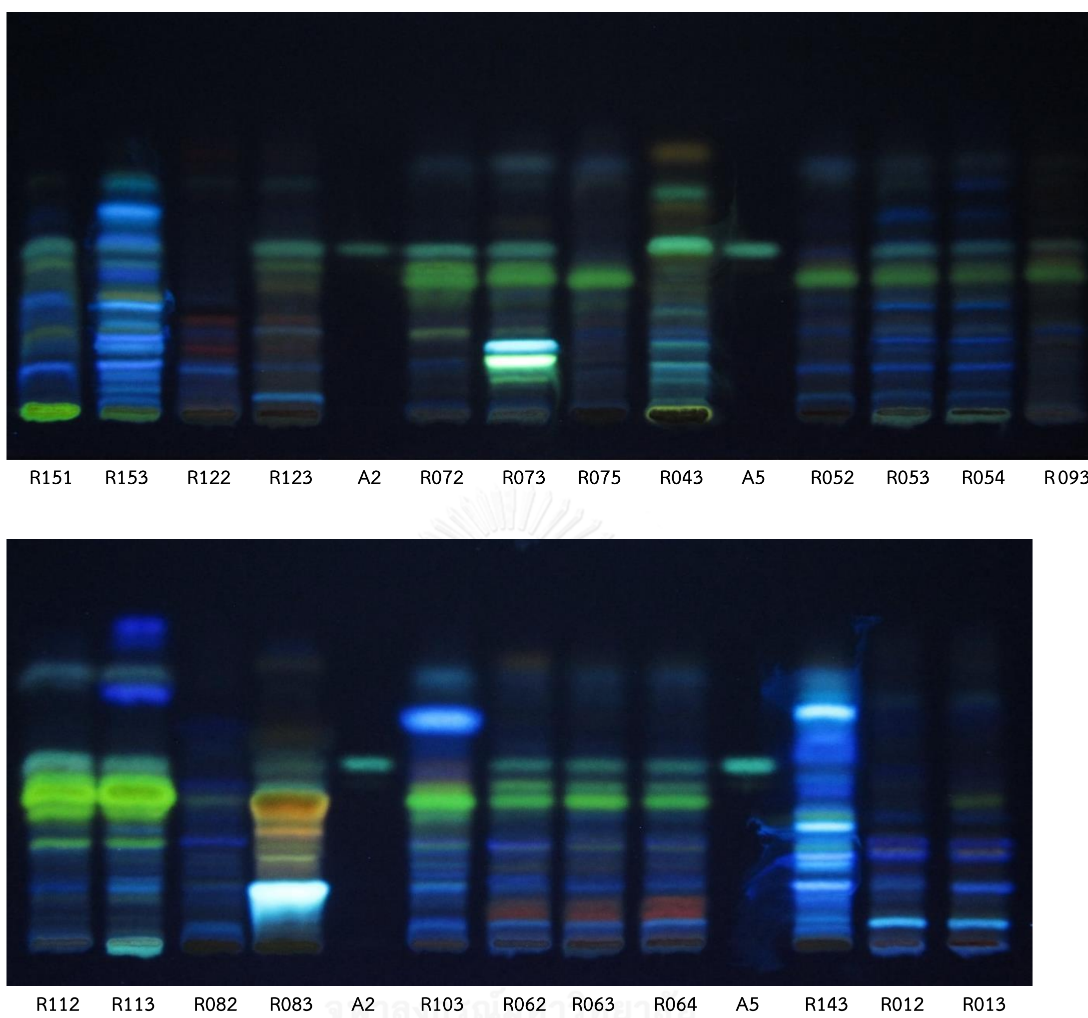

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Figure 14 HPTLC profiles of 23 available formulas containing Krai-Krue.

Table 15 The detection of AAI in Krai-Krue crude drugs and Krai-Krue containing formulas collected from the herb and traditional medicine markets analyzed in this study.

Sample	Code	Date of manufac-ture	Purchased location (Thailand, Province)	Proportion of Krai-Krue in formula	Detection of AAI
Krai-Krue	C1	27/12/12	Bangkok	-	+
Krai-Krue	C2	27/12/12	Bangkok	-	+
Krai-Krue	C3	16/04/13	Nakhon Si Thammarat	-	+
Krai-Krue	C4	27/07/13	Phetchaburi	-	+
Krai-Krue	C5	17/09/13	Ayutthaya	-	+
Krai-Krue	C6	20/08/14	Bangkok	-	-
Krai-Krue	C7	20/08/14	Bangkok	-	-
Ya Ummaruekawatee	R151	23/12/11	Sakaeo	0.1000	+
	R153	23/02/13	Bangkok	0.1000	-
Ya Kheawhom	R122	09/08/13	Bangkok	0.0526	-
	R123	06/03/13	Bangkok	0.0526	+
Ya Tatbunjob	R072	26/07/13	Bangkok	0.0370	+
	R073	15/01/13	Bangkok	0.0370	+
	R075	10/04/12	Mahasara-kham	0.0370	-
Ya Hom Nawakod	R052	17/08//13	Bangkok	0.0185	-
	R053	28/01/13	Bangkok	0.0185	+
	R054	12/07/12	Prachinburi	0.0185	+
Ya Wisumpayayai	R112	05/08/13	Bangkok	0.0185	+
	R113	15/10/12	Bangkok	0.0185	+
Ya Treehom	R043	22/04/13	Bangkok	0.0156	+
Ya Prasa Ganplu	R082	24/07/13	Bangkok	0.0154	-
	R083	01/12/12	Bangkok	0.0154	-
Ya Prasa Jettapungkee	R093	02/04/12	Bangkok	0.0152	+
Ya Munthatat	R103	09/04/13	Bangkok	0.0108	-
Ya Hom Inthajuk	R062	12/06/13	Bangkok	0.0102	+
	R063	02/07/13	Bangkok	0.0102	+

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	R064	16/07/12	Prachinburi	0.0102	+
Ya Juntaleela	R012	25/05/13	Bangkok	0.1212	-
	R013	20/05/13	Bangkok	0.1212	-
Ya Hom Kaelomwingwean	R143	28/08/13	Bangkok	Not available	-

6.4 Discussion

In Thailand, dried root of the three *Aristolochia* species, *A. pothieri* Pierre ex Lecomte (Athikomkulchai and Ruangrungri 2001), *A. pierrei* Lecomte and *A. tagala* Cham. (Sathornviriyapong, Picheansoonthon et al. 2007), have been reported as sources of medicinal crude drugs called “Krai-Krue”.

In order to ensure consumer safety, chemical profile is a basic approach for herbal material identification. As far as chemical substances are concerned, chemical profiling using aristolochic acid I as standard reference were also used to analyze raw material of Krai-Krue by thin-layer chromatography (TLC) (Sathornviriyapong, Picheansoonthon et al. 2007), high-performance thin layer chromatography (HPTLC) (Phadungrakwittaya, Akarasereenont et al. 2012), high-performance liquid chromatography (HPLC) and liquid chromatography/mass spectroscopy (LC/MS) (Tripatara, Onlamul et al. 2012), and ultra-high performance liquid chromatography (UHPLC) (Wattanarangsan 2012).

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From these techniques, the optimum HPTLC system for detection of AAI in Krai-Krue and Ya Homnawakod was conducted with chloroform, methanol and acetic acid (65:20:1 v/v) as mobile phase with Rf value 0.55. The lower limit of detection (LOD) at 8 ng for AA-I and 5 ng for AA-I salt. In addition, For RP-TLC analysis, acetonitrile, methanol and water (3:0.5:1 v/v) were used as mobile phase. AAI appeared at Rf value 0.48 with the lower limits detected for both AA-I and AA-I salt were 15 ng (Phadungrakwittaya, Akarasereenont et al. 2012).

The amount of AA-I determined by UHPLC in *A. tagala* was 0.237 %w/w. Limit of detection (LOD) and limit of quantification (LOQ) were 0.8 and 2.0 μg/mL

(Wattanarangsan 2012). Whereas other study found that the amount of AA-I in *A. tagala* was 0.24 %w/w and *A. tagala* and Ya Homnawakod showed the same profiles of HPLC and LC/MS (Tripatara, Onlamul et al. 2012). However, HPTLC is a method of choice for screening of AAI due to a more convenient, rapid and cheaper procedure.

In this study, HPTLC screening method of aristolochic acid recommended by British Pharmacopoeia and European Pharmacopoeia was performed. The results showed that 1 µl of C1-C5 extracts possibly contain aristolochic acid I and the source of these crude drugs are *Aristolochia* plants. In contrast to 20 µl of C6 and C7, there are no band chromatogram at the same Rf value as aristolochic acid I. This method is suitable for screening of *Aristolochia* containing herbal products and it requires more test to quantify AAI in products.

As the results, the amount of AAI in C1-C5 were 0.135, 0.225, 0.144, 0.141 and 0.159 %w/w, respectively. the average amount of AAI in C1-C5 were 0.1608 %w/w. According to proportion of Krai-Krue in formulas ranged from 0.0102-0.1212, the amount of AAI in formulas ranged from 0.00164-0.01949 %w/w. The results agreed with previous study that chromatogram of AAI was shown at the same Rf value but the UV absorbance of that was not detected. It might be caused by the amount of AAI is too low for HPTLC analysis or there are some substances from other plants in recipes that interfered the UV absorbance of AAI *(*Phadungrakwittaya, Akarasereenont et al*. 2012)*. Therefore more sensitivity methods such as liquid chromatography (LC) or liquid chromatography coupled with mass spectrometry are recommended for confirmation of AAI (Commission and Britain 2012).

All formulas used as samples in this study were randomly purchased for both of before and during the regulation of removing Krai-Krue from herbal formula was announced. The ratios of Krai-Krue to all ingredients of 22 formulas were calculated as shown in Table 14except for one formula (R143), the detail of ingredients could not be found. The chromatograms of 20 µl showed that AAI were detected in

only 13 formula including R151, R123, R072, R073, R053, R054, R093, R112, R113, R043, R062, R063 and R064 which were manufactured before and during the regulation. The other ten formulas (R153, R122, R075, R052, R082, R083, R103, R012, R013 and R143), AAI did not contain AAI Figure 14 HPTLC profiles of 23 available formulas containing Krai-Krue. (Figure 14 and Table 14) which may due to the source of Krai-Krue (not *Aristolochia* sp.), small amounts of Krai-Krue in the formulas and also the production processes which affected the stability of the finished products. Because of the complexity of different ingredients in the recipes and the unassignable target peak, spectra could not be detected by CAMAG TLC Scanner 3. In addition, the different chemical patterns were found for the same formula indicating that the standardization and quality control of registered herbal products in Thailand are needed.

6.5 Conclusion

The HPTLC method was found to be a simple and rapid method for AAI of suspected *Aristolochia* containing crude drugs and formulas. These data should be useful for further regulation and legislation. Moreover, this technique should be included as a primary step for the standardization and quality control of plant raw materials and herbal formulations available in the market.

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CHAPTER VII
DISCUSSION AND CONCLUSIONS

Recently, natural products have become a popular supplement for maintaining good health. The misidentification of material derived from toxic herbs such as *Aristolochia* herbs have been reported as one of the global concerns (Debelle, Vanherweghem et al. 2008). To assure the correct authentication of natural materials, many identification tools have been developed (Heubl 2013). Currently, the DNA barcodes of many herbal *Aristolochia* plants have been studied. Chemical profiling has been often combined with DNA fingerprinting to investigate the botanical sources of suspected herbal materials (But, Shaw et al. 2007, Li, Au et al. 2012, Chan 2014, Li, Au et al. 2014, Liu, Chuang et al. 2015).

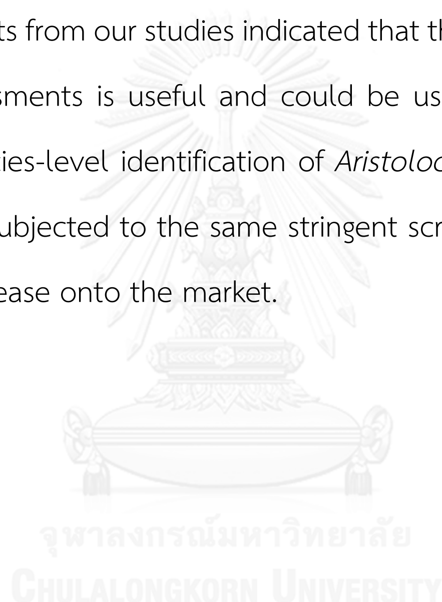
The present studies provide genetic and phytochemical tools for identification of *Aristolochia* plants. They were applied for identification of the crude drug called "Krai-Krue" which was derived from different plants including dried roots of *A. pothieri*, *A. pierrei*, *A. tagala*, *Raphistemma pulchellum*, *Jasminum* spp and *Gymnopetalum integrifolium*. Genetic assessment by DNA barcoding technique of four standardized DNA regions (*rbc*L, *mat*K, ITS and *trn*H-*psb*A) were conducted on eleven *Aristolochia* plants, *A. anguicida*, *A. gigantea*, *A. grandiflora*, *A. kerrii*, *A. littoralis*, *A. pierrei*, *A. pothieri*, *A. ringens*, *A. tagala*, *A. tentaculata* and *A.* sp. The nucleotide variations of these species were found in the order of ITS (28.96%) > *trn*H-*psb*A (13.35%) > *mat*K (11.22%) > *rbc*L (3.29%). Although intraspecific variations of samples were detected, the information could still be served as an effective approach to discriminate different or confusing plant species of the same genus. The obtained nucleotide sequences are also useful as the centralized nucleotide database for global uses. The combined data of many DNA regions could be used for further study such as development of molecular markers, phylogenetic analysis and forensic science.

The systematic of *Aristolochia* has been based on morphological characters such as perianth tubes, leaves, number of styles and anthers on gynostemium and fruit and chromosome number (González 1999, Murata, Ohi et al. 2001, Ohi-Toma, Sugawara et al. 2006). In this study, the phylogenetic tree constructed from nucleotide sequences of complete *mat*K gene of *Aristolochia* were analyzed. The phylogenetic analysis supported the morphological criteria, habitat and chromosome number. This result agrees well with the previously published *mat*K phylogenetic tree (Murata, Ohi et al. 2001, Ohi-Toma, Sugawara et al. 2006) and *trn*L-*trn*F phylogenetic tree (Neinhuis, Wanke et al. 2004). In addition, this is the first study of *A. pothieri*.

The high level of ITS2 sequence variations in eleven *Aristolochia* plants, was used for detection of Krai-Krue sources. Unfortunately, direct DNA amplification and sequencing process by universal primer of this region failed to discriminate crude drugs bought from local dispensaries. The results indicated that crude drugs were contaminated with the microorganism during post-harvesting processes and storage conditions (data unpublished). However, the sequences were analyzed for the development of other molecular markers. Multiplex PCR based on the ITS2 region were then employed. The method successfully differentiated the three *Aristolochia* Krai-Krue from the other botanical origins by different sizes of PCR products on agarose gel electrophoresis. In addition, HPTLC using AAI as standard reference was helpful in aiding the multiplex PCR to identify the botanical sources of Krai-Krue herbs. The results indicated that crude drugs “Krai-Krue” from various local dispensaries were derived from *A. pierrei* and other species. These studies confirmed that the combined identification tools, multiplex PCR and HPTLC, are suitable, convenient and specific technique for the discrimination of Krai-Krue herbs used in Thailand. The combined techniques can be modified for the identification of other several pharmaceutical herbs or individual species in herbal drug formulations.

Nevertheless, this PCR based method was not suitable for Krai-Krue containing formulas because there are very low amount of Krai-Krue in those formulas. Therefore, the twenty-three Krai-Krue containing formulas manufactured before and during the Krai-Krue removal regulations were assessed by HPTLC. According to the result, 13 formulas probably contained AAI. This method could be used as AAI-screening test by the herbal industries and regulatory authorities. As recommendations, the more sensitivity of molecular markers is needed in this case for example real-time PCR analysis for further study.

The results from our studies indicated that the combination of genetic and phytochemical assessments is useful and could be used as suitable tools for both genus level and species-level identification of *Aristolochia* plants. Moreover, natural products should be subjected to the same stringent scrutiny and controls as modern drugs before their release onto the market.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มูลนิธิฟื้นฟูส่งเสริมการแพทย์ไทยเดิมอายุรเวทวิทยาลัย(ชีวกโกมารภัจจ์) (2535). ตำราการแพทย์ไทยเดิม (แพทยศาสตร์สงเคราะห์) ฉบับอนุรักษ์. กรุงเทพ, โรงพิมพ์สามเจริญพานิช.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

APPENDIX

APPENDIX A

Morphology of plant samples used in this study

จุฬาลงกรณ์มหาวิทยาลัย
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Figure A1 *Aristolochia anguicida* Jacq. The plant (A); leaf (B); flower (C); fruit (D).

Figure A2 *Aristolochia gigantea* Mart. et Zucc. The plant (A); stem (B); flower (C); stipule (D), fruit (E); fruit (F); seeds (G).

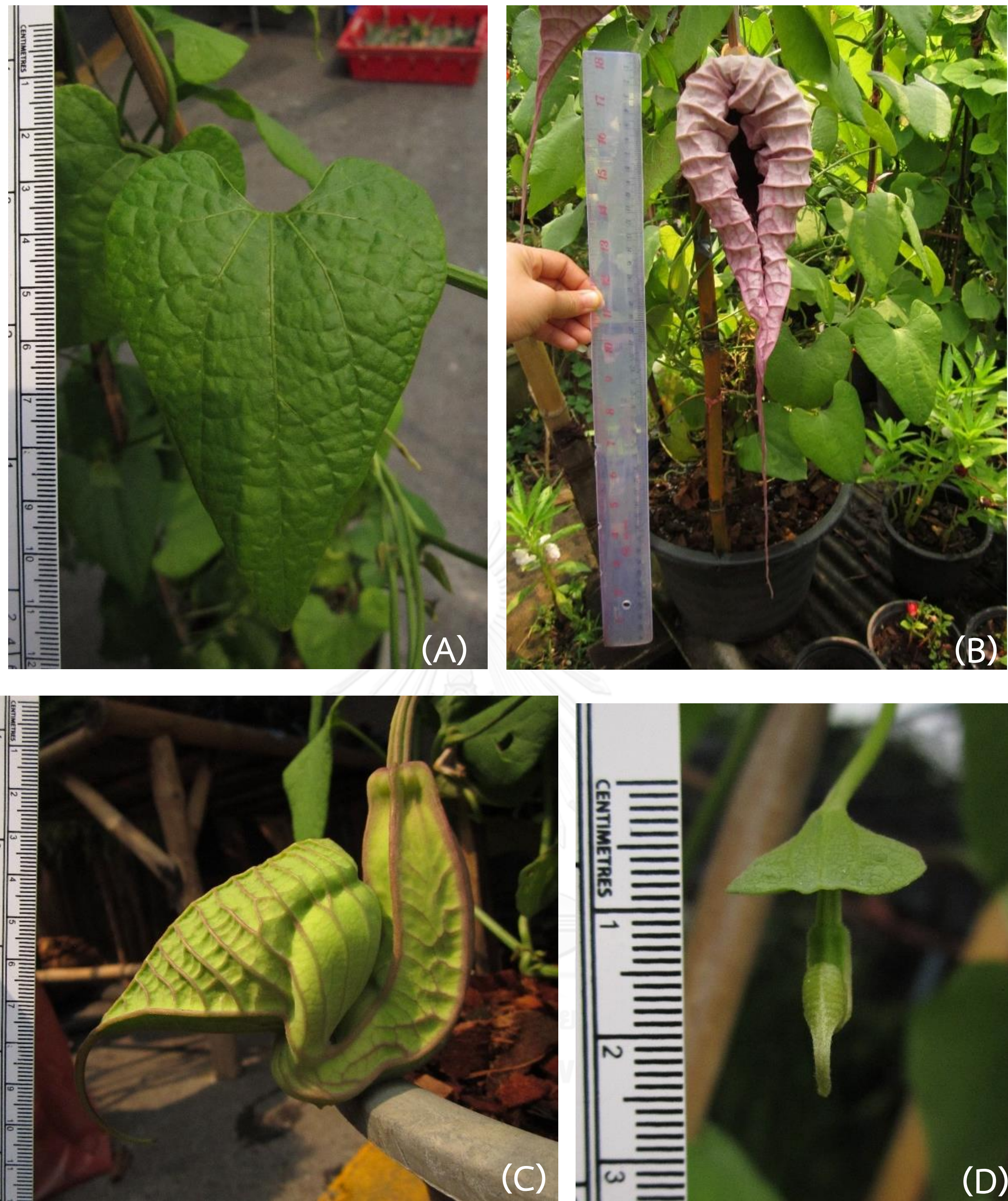

Figure A3 *Aristolochia grandiflora* Sw. Leaf (A); flower (B); flower (C); flower (D).

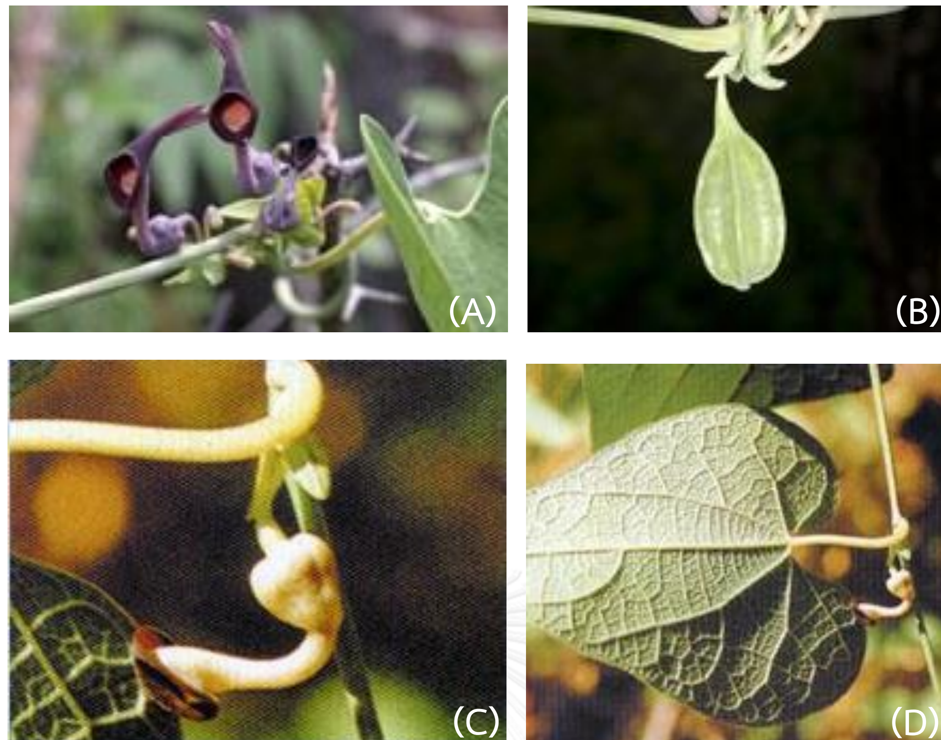

Figure A4 *Aristolochia kerrii* Craib.

Flowers (A); fruit (B) [cited 2015 March 12] Available from:

http://web3.dnp.go.th/botany/detail.aspx?wordsnamesci=Aristolochia0kerrii0Craib

Flower (C); leaf (D) [cited 2015 March 12] Available from:

http://www.rspg.or.th/plants_data/rare_plants/scien_name_a24.htm

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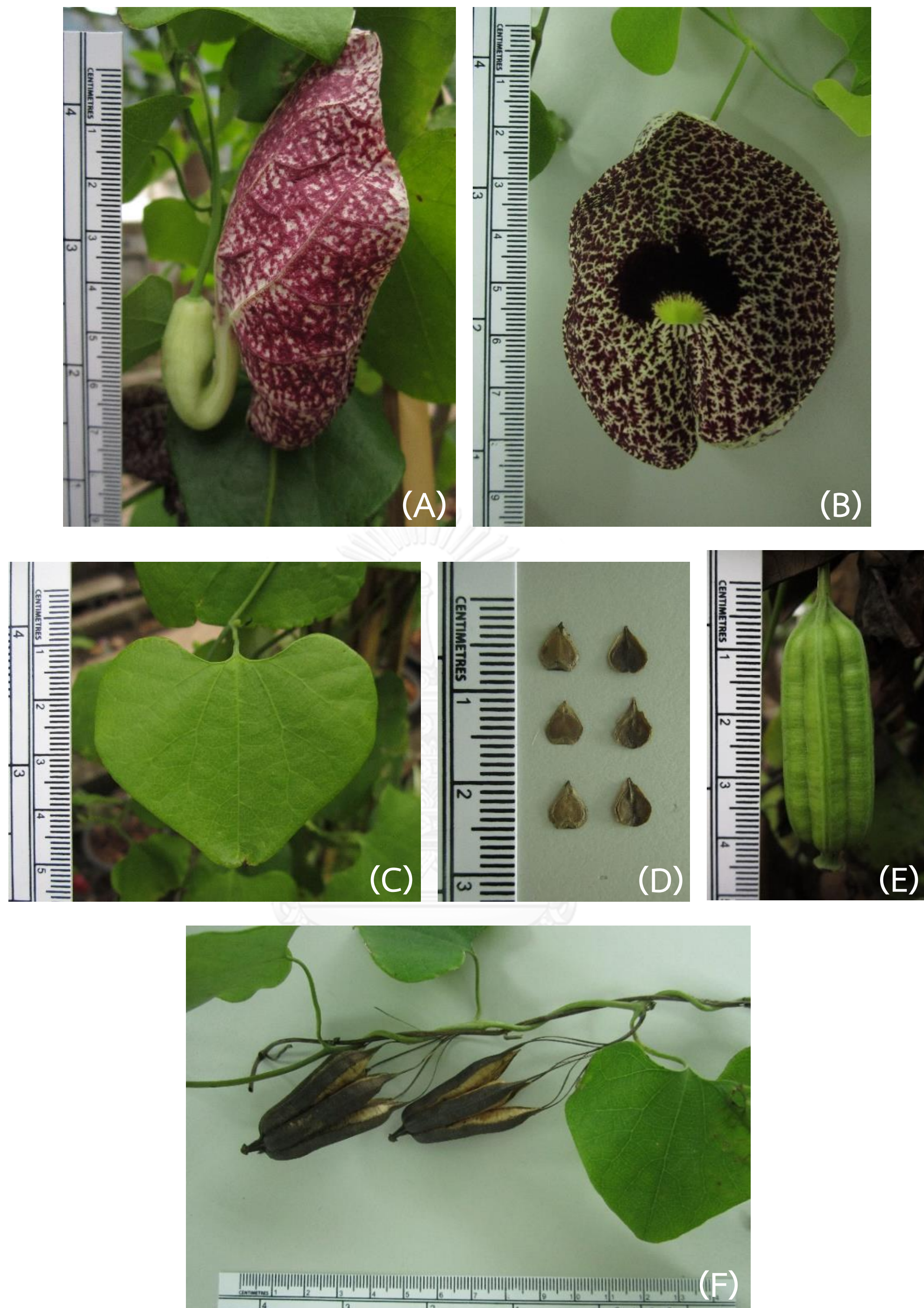

Figure A5 *Aristolochia littoralis* D. Parodi. Flower (A); flower (B); leaf (C); seeds (D); fruit (E); dry fruit (F).

Figure A6 *Aristolochia ringens* Vahl. Leaf and stipule (A); flower and leaf (B); seeds (C); dry fruit (D).

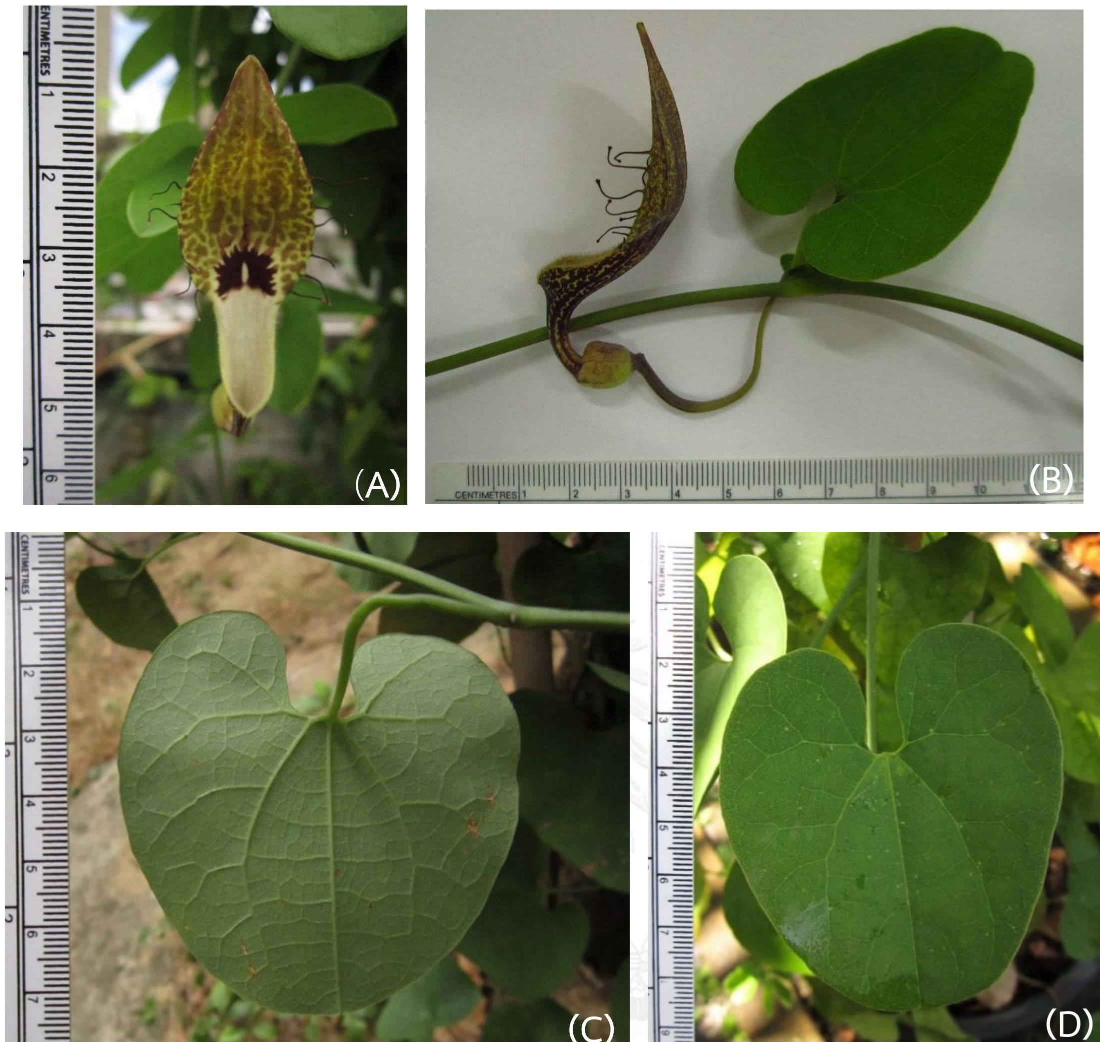

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Figure A7 *Aristolochia tentaculata* Schmidt in Fedde. Flower (A); flower and leaf (B); leaf (C); leaf (D).

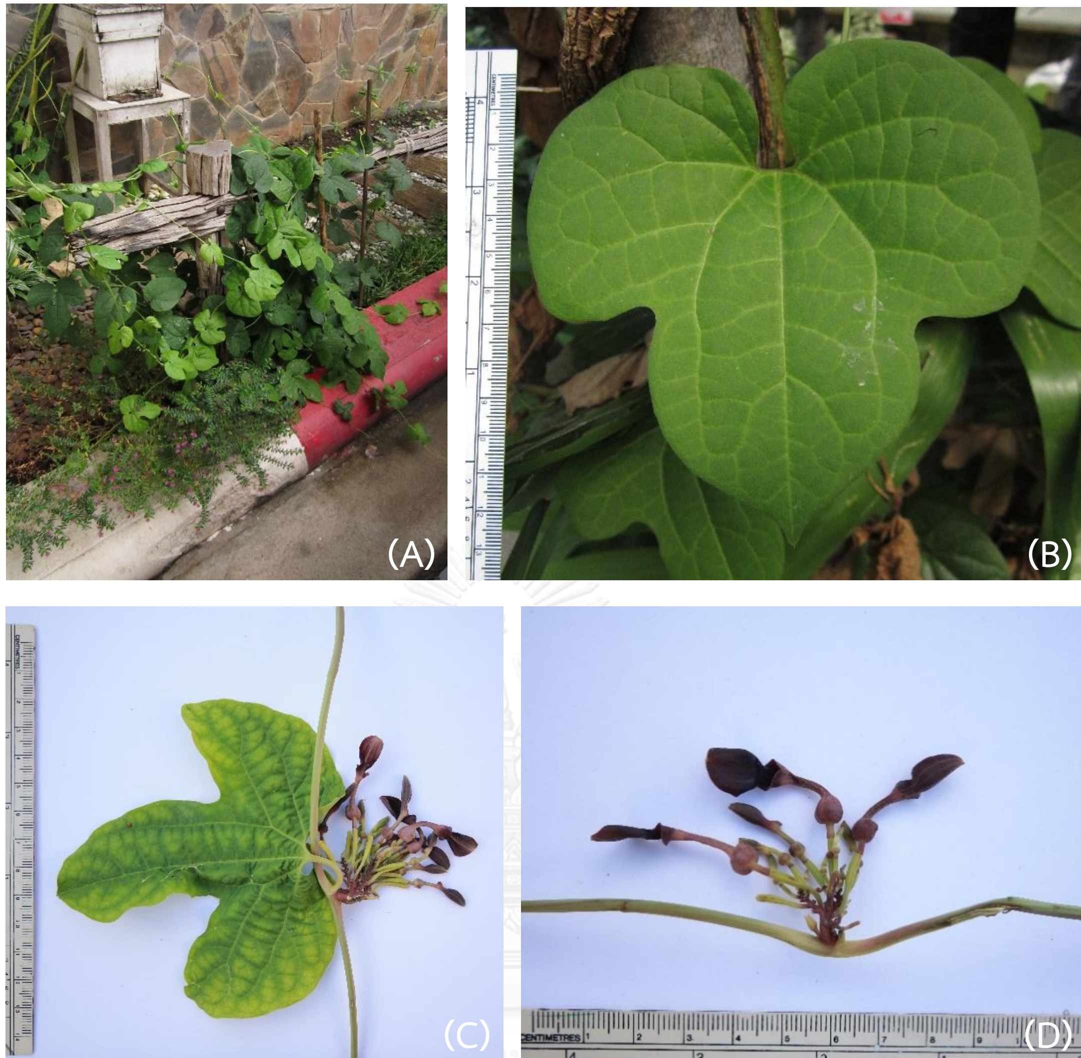

Figure A8 *Aristolochia pothieri* Pierre ex Lecomte. The plant (A); leaf (B); flowers and leaf (C); flowers (D).

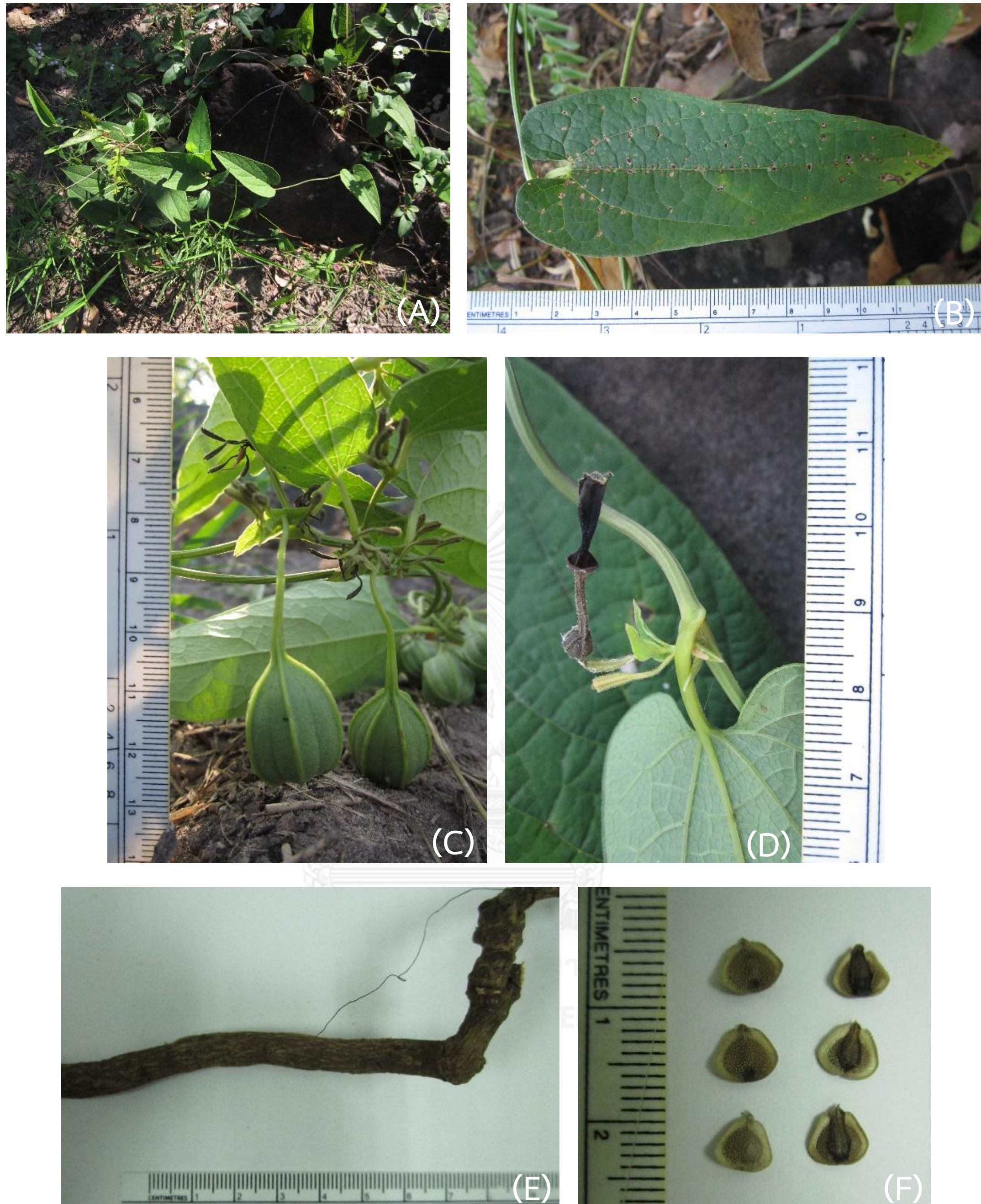

Figure A9 *Aristolochia pierrei* Lecomte. The plant (A); leaf (B); flowers and fruits (C); flower (D); stem and root (E); seeds (F).

Figure A10 *Aristolochia tagala* Cham. The plant (A); flowers and leaves (B); flowers (C); seeds (D); seeds (E); dry fruit (F).

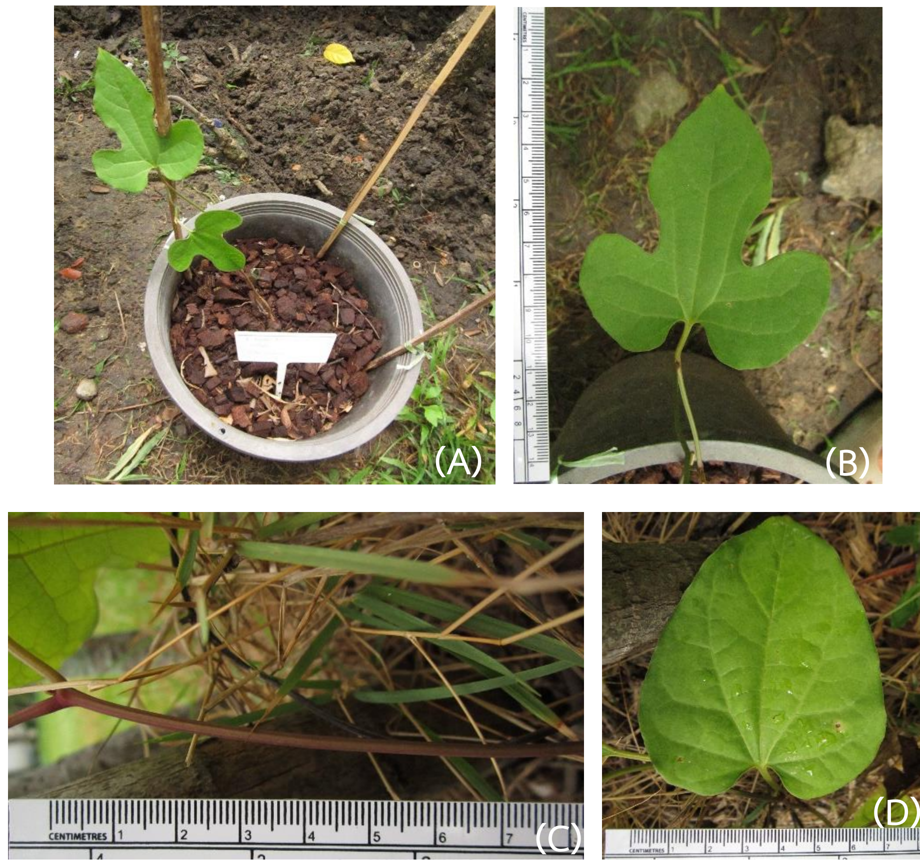

Figure A11 *A.* sp. The plant (A); leaves (B); stem (C); leaf (D).

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Figure A12 *Raphistemma pulchellum* (Roxb.) Wall Craib.

The plant (A), flowers (B), flowers (C) [cited 2015 March 12] Available from:

http://www.rspg.or.th/plants_data/herbs/herbs_29.htm

Flowers (D) [cited 2015 March 12] Available from:

http://www.qsbg.org/database/botanic_book%20full%20option/search_detail.asp?botanic_id=2047

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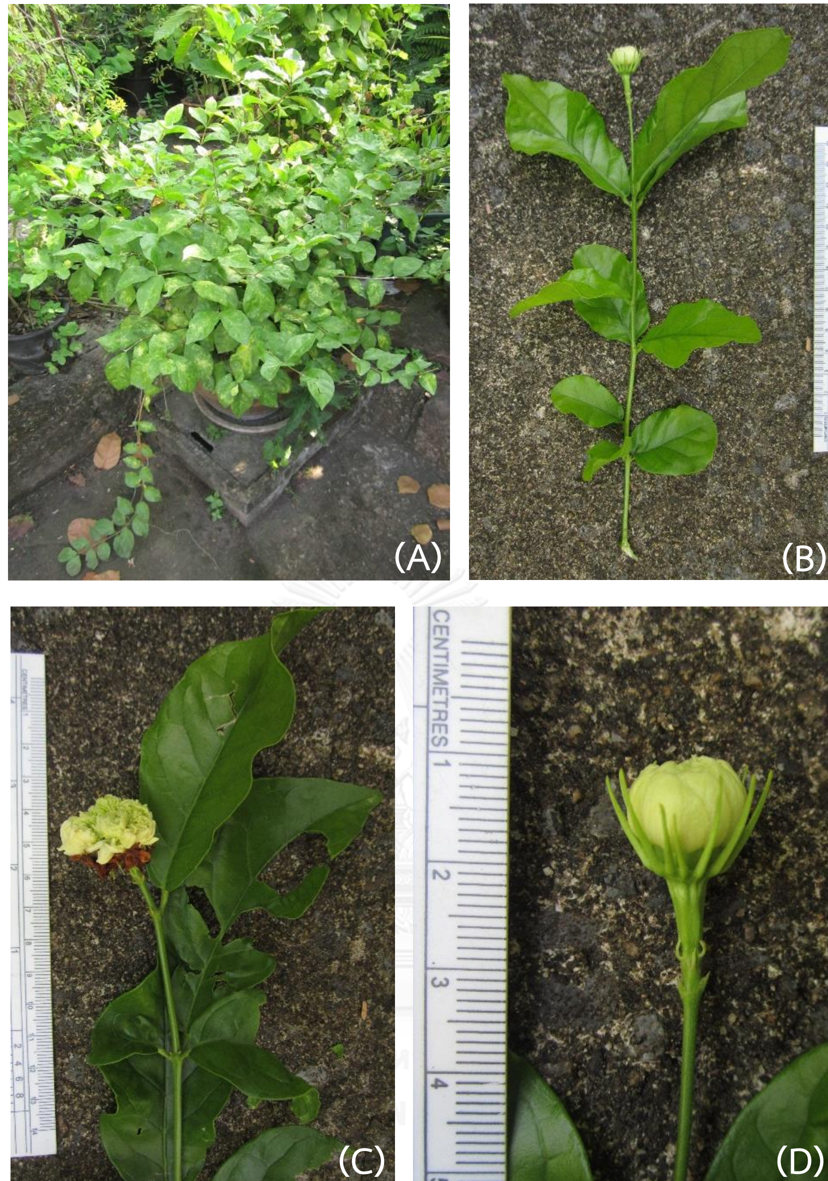

Figure A13 *Jasminum sambac* (L.) Aiton. The plant (A); flower and leaves (B); flower and leaves (C); flower (D).

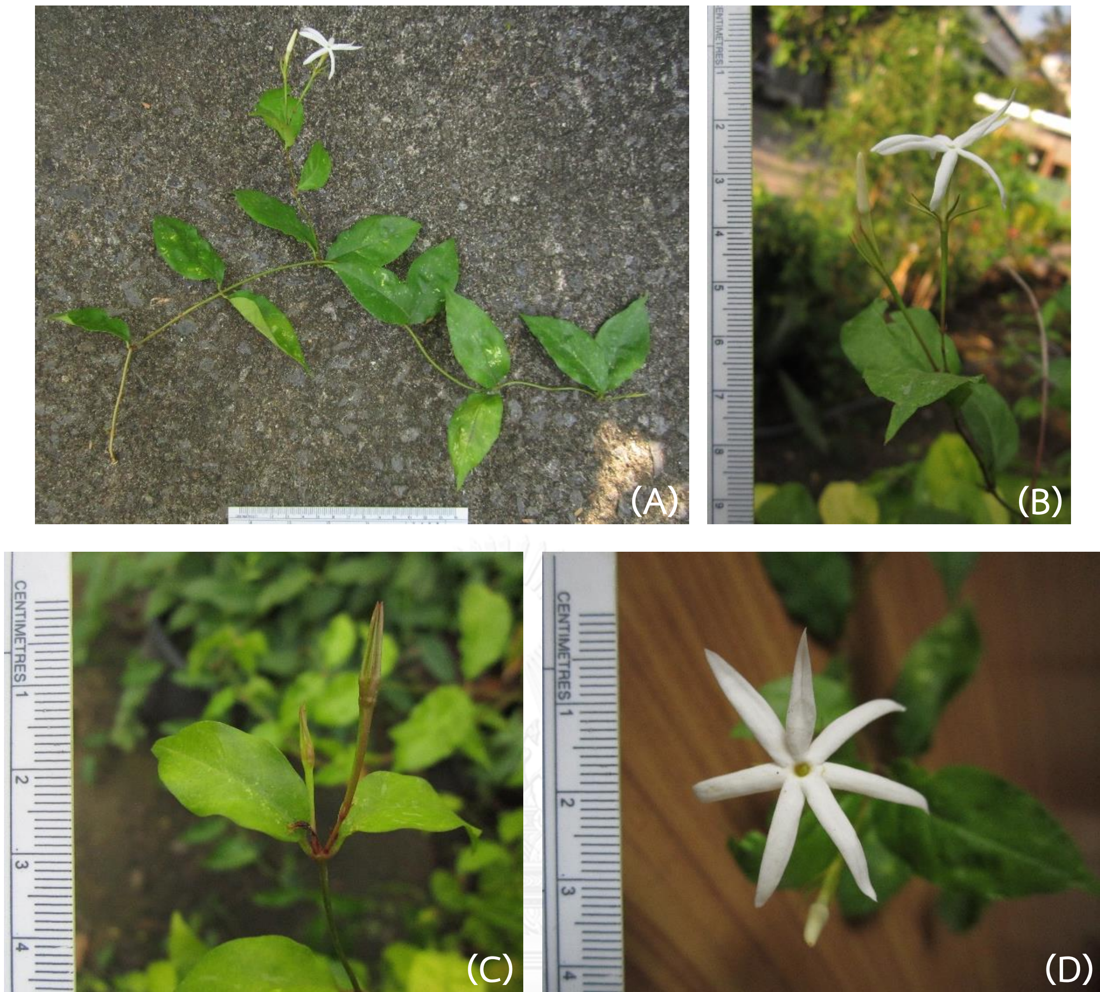

Figure A14 *Jasminum adenophyllum* Wall. Ex C.B. Clarke. Flowers and leaves (A); flowers and leaves (B); flowers (C); flower (D).

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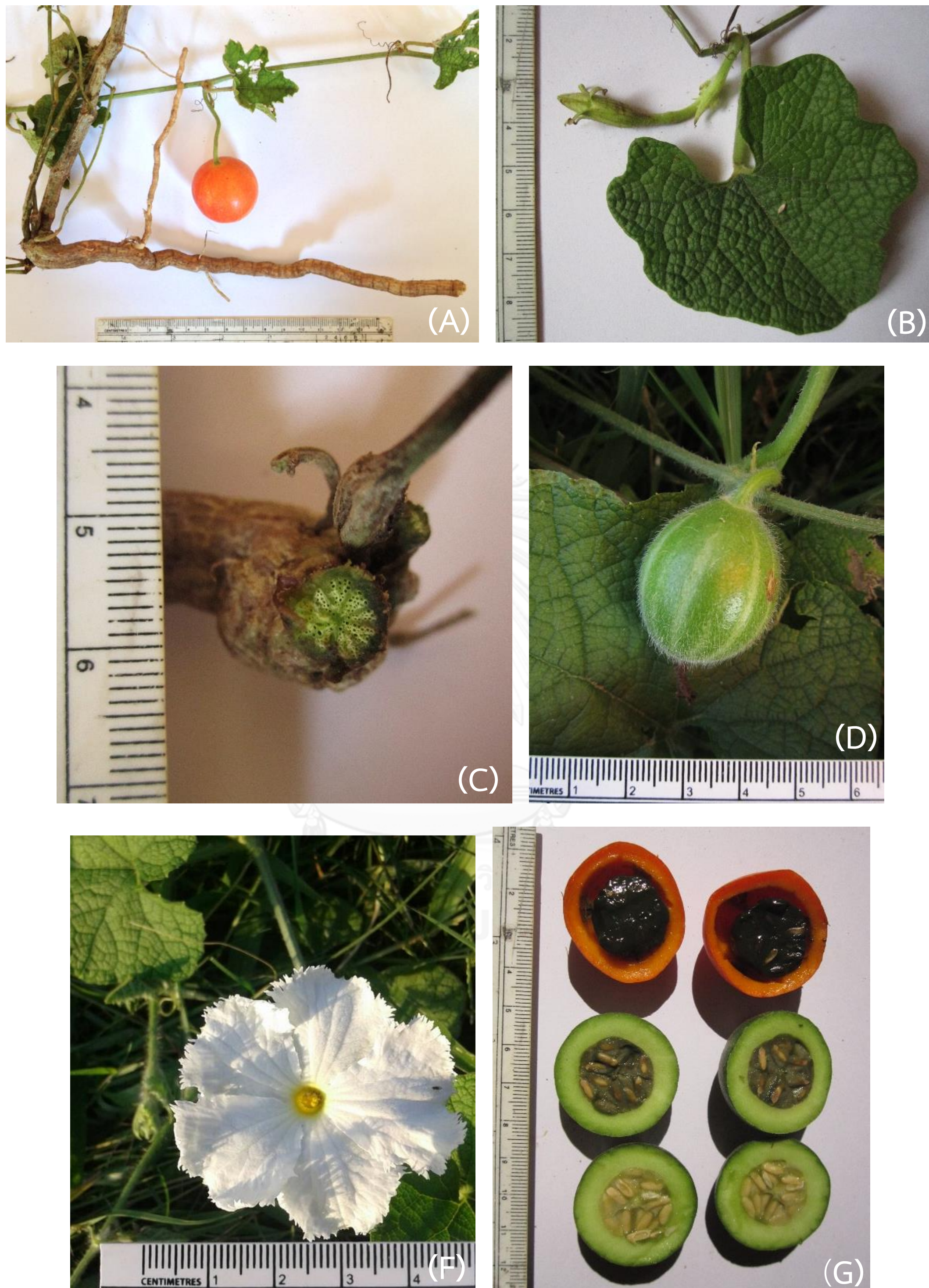

Figure A15 *Gymnopetalum integrifolium* Kurz. Fruit, stem and root (A); flowers and leaf (B); stem (C); fruit (D); flower (E); fruits (G)

APPENDIX B

Multiple sequence alignments of the eleven *Aristolochia* plants

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Figure B16 Sequence alignment of full length *rbc*L genes of eleven *Aristolochia* plants.

The numbers on the top line represent the base numbers in sequence alignment. The altered bases indicate the sequence differences. '.' represents the base being identical to the first sequence.

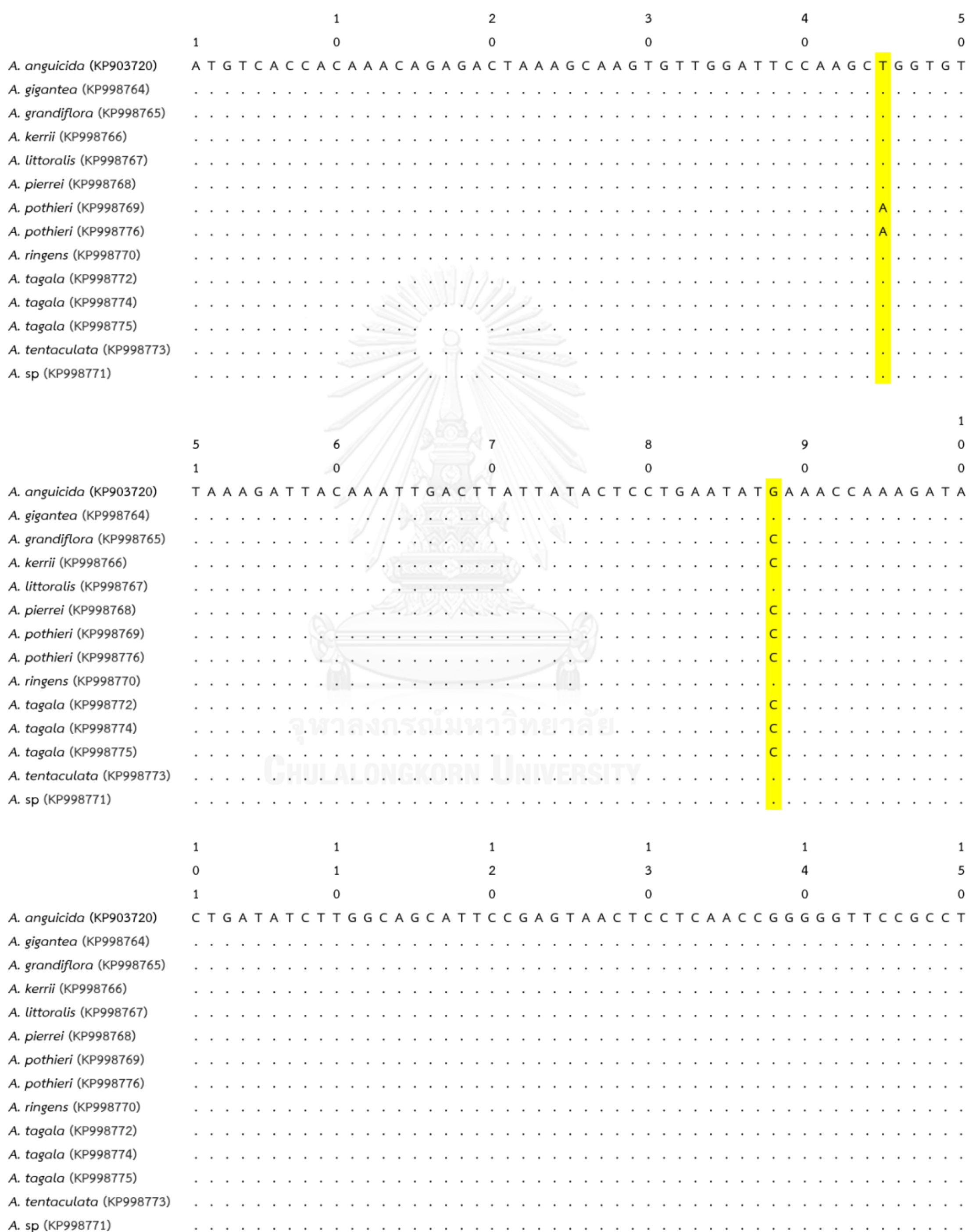

Figure B1 Sequence alignment of full length *rbc*L genes of eleven *Aristolochia* plants. (continued)

Species	151–200
A. anguicida (KP903720)	GAGGAAGCAGGGGCTGCAGTAGCTGCCGAATCTTCTACTGGTACATGGAC
A. gigantea (KP998764)	....
A. grandiflora (KP998765)	......................................A...........
A. kerrii (KP998766)	....
A. littoralis (KP998767)	......................................C...........
A. pierrei (KP998768)	....
A. pothieri (KP998769)	...........T......................................
A. pothieri (KP998776)	...........T......................................
A. ringens (KP998770)	....
A. tagala (KP998772)	....
A. tagala (KP998774)	....
A. tagala (KP998775)	....
A. tentaculata (KP998773)	...................................G..............
A. sp (KP998771)	....

Species	201–250
A. anguicida (KP903720)	AACTGTGTGGACTGATGGACTTACTAGCCTTGATCGTTACAAAGGACGAT
A. gigantea (KP998764)	....
A. grandiflora (KP998765)	....
A. kerrii (KP998766)	....
A. littoralis (KP998767)	....
A. pierrei (KP998768)	....
A. pothieri (KP998769)	......A...
A. pothieri (KP998776)	......A...
A. ringens (KP998770)	....
A. tagala (KP998772)	....
A. tagala (KP998774)	....
A. tagala (KP998775)	....
A. tentaculata (KP998773)	....
A. sp (KP998771)	....

Species	251–300
A. anguicida (KP903720)	GCTACCACATCGAGCCCGTTGCTGGGGAGGAAAATCAATATATTGCTTAT
A. gigantea (KP998764)	....
A. grandiflora (KP998765)	.....G..
A. kerrii (KP998766)	....................C............G.....T..........
A. littoralis (KP998767)	....
A. pierrei (KP998768)	....................C............G.....T..........
A. pothieri (KP998769)	....................C............G.....T..........
A. pothieri (KP998776)	....................C............G.....T..........
A. ringens (KP998770)	....
A. tagala (KP998772)	....................C............G.....T..........
A. tagala (KP998774)	....................C............G.....T..........
A. tagala (KP998775)	....................C............G.....T..........
A. tentaculata (KP998773)	....
A. sp (KP998771)	....

Figure B1 Sequence alignment of full length *rbc*L genes of eleven *Aristolochia* plants. (continued)

Species	301–350
A. anguicida (KP903720)	GTAGCTTACCCTTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTT
A. gigantea (KP998764)	....
A. grandiflora (KP998765)	....
A. kerrii (KP998766)	....
A. littoralis (KP998767)	....
A. pierrei (KP998768)	....
A. pothieri (KP998769)	....
A. pothieri (KP998776)	....
A. ringens (KP998770)	....
A. tagala (KP998772)	....
A. tagala (KP998774)	....
A. tagala (KP998775)	....
A. tentaculata (KP998773)	....
A. sp (KP998771)	....

Species	351–400
A. anguicida (KP903720)	TACTTCCATTGTAGGTAATGTATTTGGGTTCAAAGCCTTACGAGCTTTAC
A. gigantea (KP998764)	....
A. grandiflora (KP998765)	....
A. kerrii (KP998766)	.....................G............................
A. littoralis (KP998767)	....
A. pierrei (KP998768)	.....................G............................
A. pothieri (KP998769)	.....................G............................
A. pothieri (KP998776)	.....................G............................
A. ringens (KP998770)	....
A. tagala (KP998772)	.....................G............................
A. tagala (KP998774)	.....................G............................
A. tagala (KP998775)	.....................G............................
A. tentaculata (KP998773)	....
A. sp (KP998771)	....

Species	401–450
A. anguicida (KP903720)	GTCTGGAGGATCTACGAATTCCTCCCGCTTATATCAAAACTTTCCAAGGT
A. gigantea (KP998764)	....
A. grandiflora (KP998765)	.......................A.........................G
A. kerrii (KP998766)	.......................A..T......................C
A. littoralis (KP998767)	....
A. pierrei (KP998768)	............................G....................C
A. pothieri (KP998769)	.......................A.........................C
A. pothieri (KP998776)	.......................A.........................C
A. ringens (KP998770)	....
A. tagala (KP998772)	.......................A....G....................C
A. tagala (KP998774)	.......................A....G....................C
A. tagala (KP998775)	.......................A....G....................C
A. tentaculata (KP998773)	.......................A.........................C
A. sp (KP998771)	....

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Figure B1 Sequence alignment of full length *rbc*L genes of eleven *Aristolochia* plants. (continued)

```
                               451       460       470       480       490       500
A. anguicida (KP903720)        CCTCCCCATGGTATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGTCG
A. gigantea (KP998764)         ..................................................
A. grandiflora (KP998765)      ..................................................
A. kerrii (KP998766)           ........C.........................................
A. littoralis (KP998767)       ..................................................
A. pierrei (KP998768)          ........C.........................................
A. pothieri (KP998769)         ........C.........................................
A. pothieri (KP998776)         ........C.........................................
A. ringens (KP998770)          ..................................................
A. tagala (KP998772)           ........C.........................................
A. tagala (KP998774)           ........C.........................................
A. tagala (KP998775)           ........C.........................................
A. tentaculata (KP998773)      ........C.........................................
A. sp (KP998771)               ..................................................

                               501       510       520       530       540       550
A. anguicida (KP903720)        TCCTCTATTGGGATGTACTATTAAACCAAAATTGGGGTTATCGGCGAAGA
A. gigantea (KP998764)         ..................................................
A. grandiflora (KP998765)      ..................................................
A. kerrii (KP998766)           ..................................................
A. littoralis (KP998767)       ..................................................
A. pierrei (KP998768)          ..................................................
A. pothieri (KP998769)         ..................................................
A. pothieri (KP998776)         ..................................................
A. ringens (KP998770)          ...........................T......................
A. tagala (KP998772)           ..................................................
A. tagala (KP998774)           ..................................................
A. tagala (KP998775)           ..................................................
A. tentaculata (KP998773)      ..................................................
A. sp (KP998771)               ..................................................

                               551       560       570       580       590       600
A. anguicida (KP903720)        ACTACGGTAGAGCGGTTTATGAATGTCTCCGCGGTGGACTTGATTTTACC
A. gigantea (KP998764)         ..................................................
A. grandiflora (KP998765)      ..................................................
A. kerrii (KP998766)           ..................................................
A. littoralis (KP998767)       ..................................................
A. pierrei (KP998768)          ..................................................
A. pothieri (KP998769)         ..................................................
A. pothieri (KP998776)         ..................................................
A. ringens (KP998770)          ..................................................
A. tagala (KP998772)           ..................................................
A. tagala (KP998774)           ..................................................
A. tagala (KP998775)           ..................................................
A. tentaculata (KP998773)      ..................................................
A. sp (KP998771)               ..................................................
```

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Figure B1 Sequence alignment of full length *rbc*L genes of eleven *Aristolochia* plants. (continued)

```
                            6                 6                   6                   6                   6                   6
                            0                 1                   2                   3                   4                   5
                            1                 0                   0                   0                   0                   0
A. anguicida (KP903720)     A A G G A C G A T G A G A A C G T G A A C T C C C A A C C A T T T A T G C G T T G G A G A G A C C G
A. gigantea (KP998764)      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. grandiflora (KP998765)   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. kerrii (KP998766)        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. littoralis (KP998767)    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. pierrei (KP998768)       . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. pothieri (KP998769)      . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . .
A. pothieri (KP998776)      . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . .
A. ringens (KP998770)       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. tagala (KP998772)        . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. tagala (KP998774)        . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. tagala (KP998775)        . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. tentaculata (KP998773)   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. sp (KP998771)            . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

                            6                 6                   6                   6                   6                   7
                            5                 6                   7                   8                   9                   0
                            1                 0                   0                   0                   0                   0
A. anguicida (KP903720)     T T T C T T A T T T T G T G C C G A A G C A C T T T A T A A A G C A C A G G C C G A A A C A G G T G
A. gigantea (KP998764)      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . . . . . . .
A. grandiflora (KP998765)   . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. kerrii (KP998766)        . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . T . . . . . . . . . . . . . . . . . .
A. littoralis (KP998767)    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. pierrei (KP998768)       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. pothieri (KP998769)      . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. pothieri (KP998776)      . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. ringens (KP998770)       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. tagala (KP998772)        . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. tagala (KP998774)        . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. tagala (KP998775)        . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. tentaculata (KP998773)   . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. sp (KP998771)            . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

                            7                 7                   7                   7                   7                   7
                            0                 1                   2                   3                   4                   5
                            1                 0                   0                   0                   0                   0
A. anguicida (KP903720)     A A A T C A A A G G A C A T T A C T T A A A T G C A A C T G C G G G T A C A T G T G A A G A A A T G
A. gigantea (KP998764)      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. grandiflora (KP998765)   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. kerrii (KP998766)        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. littoralis (KP998767)    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. pierrei (KP998768)       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. pothieri (KP998769)      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. pothieri (KP998776)      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. ringens (KP998770)       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. tagala (KP998772)        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. tagala (KP998774)        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. tagala (KP998775)        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. tentaculata (KP998773)   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. sp (KP998771)            . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
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Figure B1 Sequence alignment of full length *rbc*L genes of eleven *Aristolochia* plants. (continued)

Species	751 – 760 – 770 – 780 – 790 – 800
A. anguicida (KP903720)	ATGAAAAGGGCGATATTTGCCAGAGAATTGGGAGTTCCTATCGTTATGCA
A. gigantea (KP998764)	....
A. grandiflora (KP998765)	...........C......................C...............
A. kerrii (KP998766)	...........C......................................
A. littoralis (KP998767)	....
A. pierrei (KP998768)	...........C........T.............................
A. pothieri (KP998769)	...........C....G...T.............................
A. pothieri (KP998776)	...........C....G...T.............................
A. ringens (KP998770)	....
A. tagala (KP998772)	...........C........T.............................
A. tagala (KP998774)	...........C........T.............................
A. tagala (KP998775)	...........C........T.............................
A. tentaculata (KP998773)	...........T....G.................................
A. sp (KP998771)	....

Species	801 – 810 – 820 – 830 – 840 – 850
A. anguicida (KP903720)	TGACTACTTAACGGGAGGATTCACTGCAAATACTAGCTTGGCTCATTATT
A. gigantea (KP998764)	....
A. grandiflora (KP998765)	....
A. kerrii (KP998766)	....
A. littoralis (KP998767)	....
A. pierrei (KP998768)	....
A. pothieri (KP998769)	....
A. pothieri (KP998776)	....
A. ringens (KP998770)	....
A. tagala (KP998772)	....
A. tagala (KP998774)	....
A. tagala (KP998775)	....
A. tentaculata (KP998773)	....
A. sp (KP998771)	....

Species	851 – 860 – 870 – 880 – 890 – 900
A. anguicida (KP903720)	GCCGAGACAATGGCCTACTTCTTCACATCCATCGCGCAATGCATGCAGTT
A. gigantea (KP998764)	....
A. grandiflora (KP998765)	...............................C..................
A. kerrii (KP998766)	.............A....................................
A. littoralis (KP998767)	....
A. pierrei (KP998768)	....
A. pothieri (KP998769)	...............................C..................
A. pothieri (KP998776)	...............................C..................
A. ringens (KP998770)	....
A. tagala (KP998772)	....
A. tagala (KP998774)	....
A. tagala (KP998775)	....
A. tentaculata (KP998773)	....
A. sp (KP998771)	....

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Figure B1 Sequence alignment of full length *rbc*L genes of eleven *Aristolochia* plants. (continued)

```
Position                    901, 910, 920, 930, 940, 950
A. anguicida (KP903720)     ATTGATAGACAGAAGAATCATGGTATGCACTTCCGAGTACTTGCAAAAGC
A. gigantea (KP998764)      ..................................................
A. grandiflora (KP998765)   ..................................................
A. kerrii (KP998766)        ...........A......................................
A. littoralis (KP998767)    ..................................................
A. pierrei (KP998768)       ..................................................
A. pothieri (KP998769)      ..................................................
A. pothieri (KP998776)      ..................................................
A. ringens (KP998770)       ..................................................
A. tagala (KP998772)        ..................................................
A. tagala (KP998774)        ..................................................
A. tagala (KP998775)        ..................................................
A. tentaculata (KP998773)   ..................................................
A. sp (KP998771)            ..................................................

Position                    951, 960, 970, 980, 990, 1000
A. anguicida (KP903720)     ATTACGTTTGTCCGGTGGAGATCATATTCACGCCGGTACCGTAGTAGGTA
A. gigantea (KP998764)      ..................................................
A. grandiflora (KP998765)   ..................................................
A. kerrii (KP998766)        ..................................................
A. littoralis (KP998767)    ..................................................
A. pierrei (KP998768)       .......A..........................................
A. pothieri (KP998769)      .......A..........................................
A. pothieri (KP998776)      .......A..........................................
A. ringens (KP998770)       ..................................................
A. tagala (KP998772)        .......A..........................................
A. tagala (KP998774)        .......A..........................................
A. tagala (KP998775)        .......A..........................................
A. tentaculata (KP998773)   ..................................................
A. sp (KP998771)            ..................................................

Position                    1001, 1010, 1020, 1030, 1040, 1050
A. anguicida (KP903720)     AACTGGAAGGGGAACGGGAAATCACTTTGGGTTTTGTTGATTTACTACGT
A. gigantea (KP998764)      ..................................................
A. grandiflora (KP998765)   ..................................................
A. kerrii (KP998766)        ..................................................
A. littoralis (KP998767)    ..................................................
A. pierrei (KP998768)       ..........T.......................................
A. pothieri (KP998769)      ..........T.......................................
A. pothieri (KP998776)      ..........T.......................................
A. ringens (KP998770)       ..................................................
A. tagala (KP998772)        ..........T.......................................
A. tagala (KP998774)        ..........T.......................................
A. tagala (KP998775)        ..........T.......................................
A. tentaculata (KP998773)   ..................................................
A. sp (KP998771)            ..................................................
```

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Figure B1 Sequence alignment of full length *rbc*L genes of eleven *Aristolochia* plants. (continued)

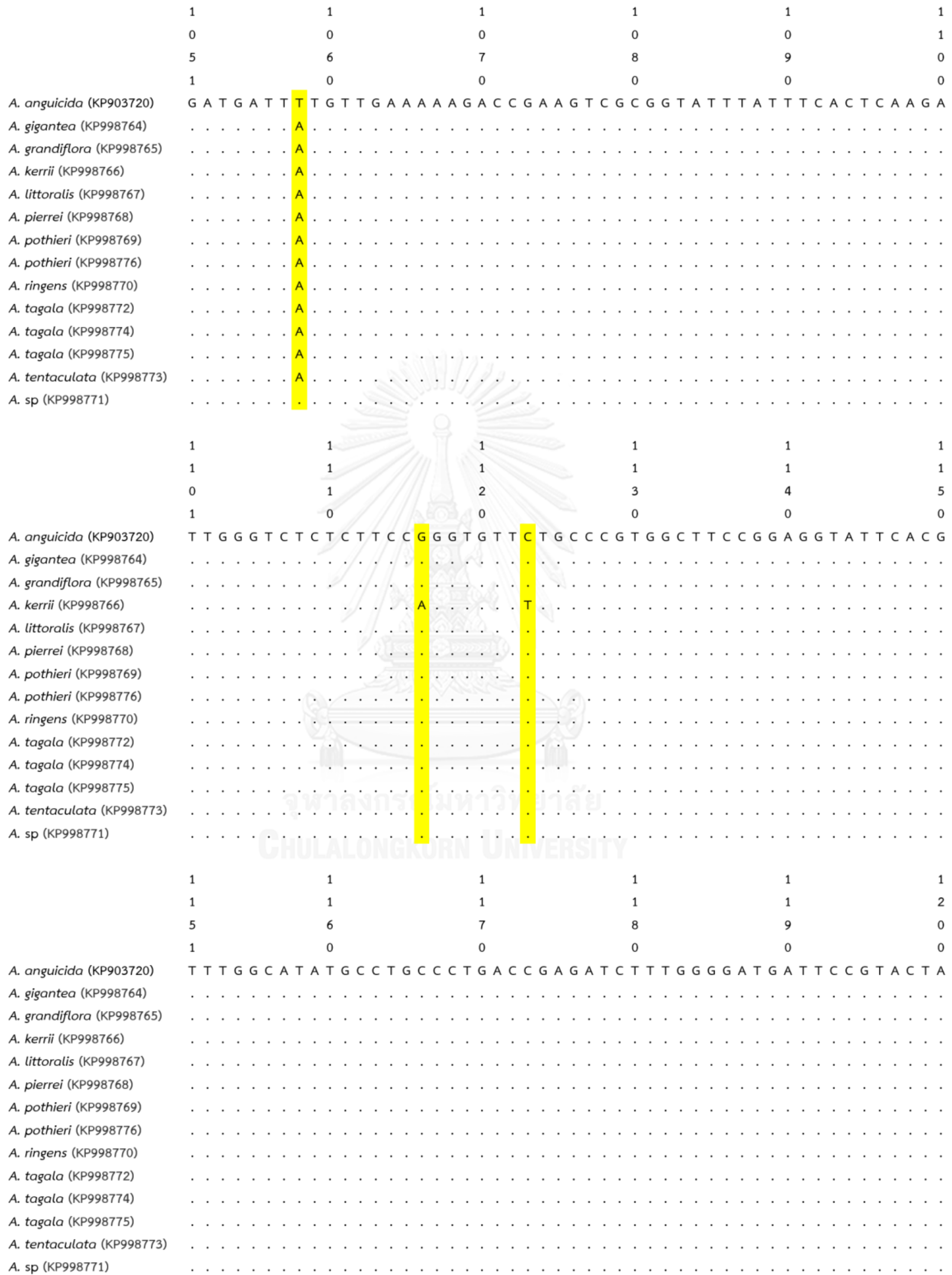

Figure B1 Sequence alignment of full length *rbc*L genes of eleven *Aristolochia* plants. (continued)

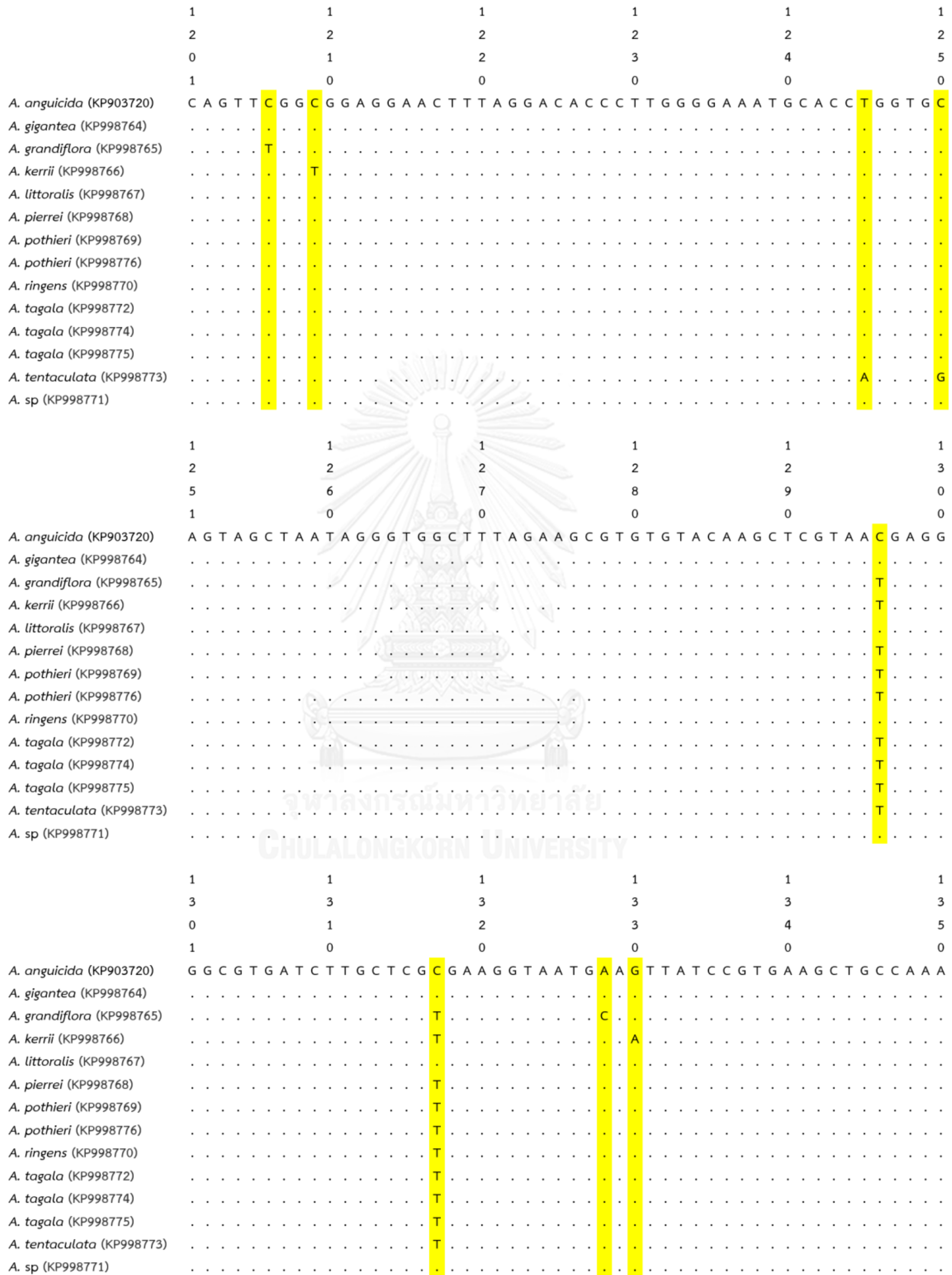

Figure B1 Sequence alignment of full length *rbc*L genes of eleven *Aristolochia* plants. (continued)

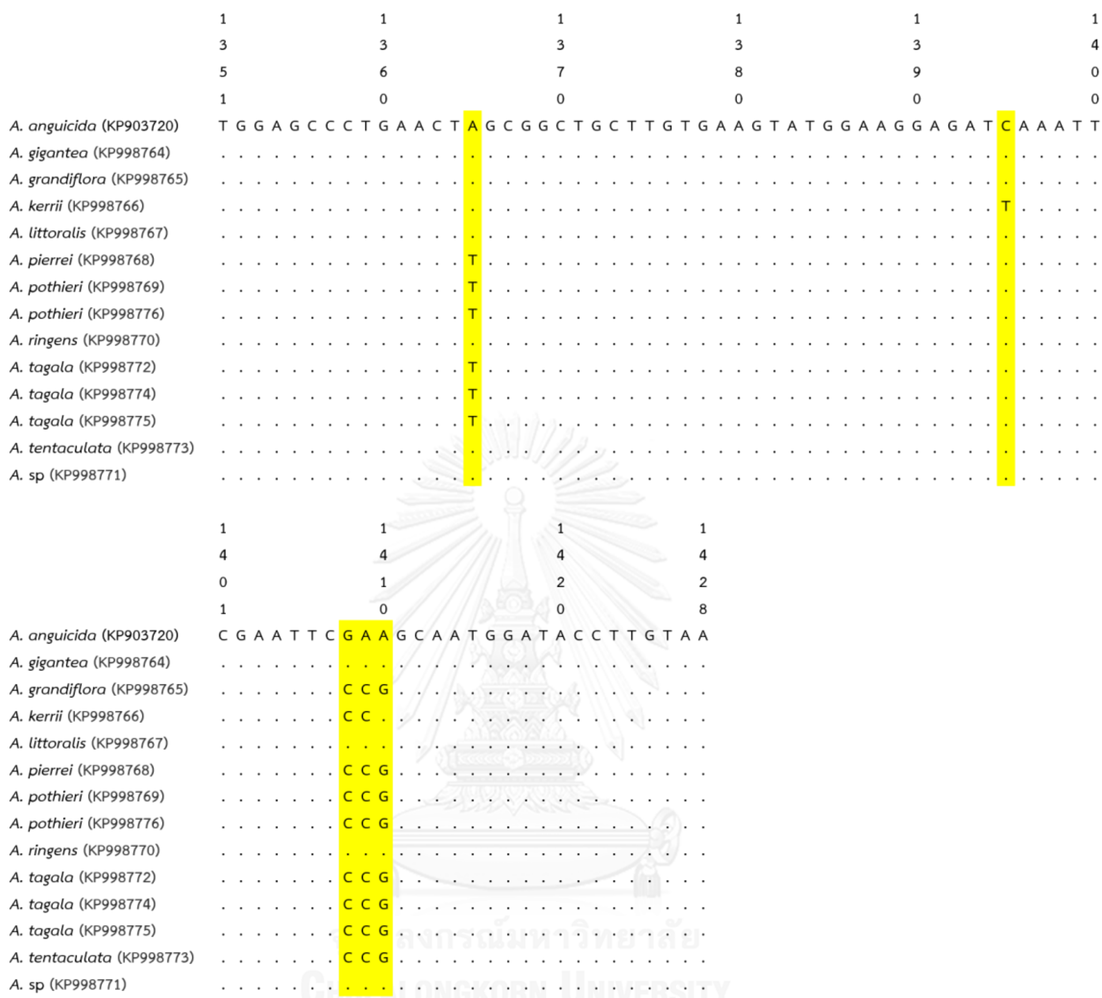

Figure B17 Sequence alignment of full length *mat*K genes of eleven *Aristolochia* plants.

The numbers on the top line represent the base numbers in sequence alignment. The altered bases indicate the sequence differences. '.' represents the base being identical to the first sequence. '–' represents gap.

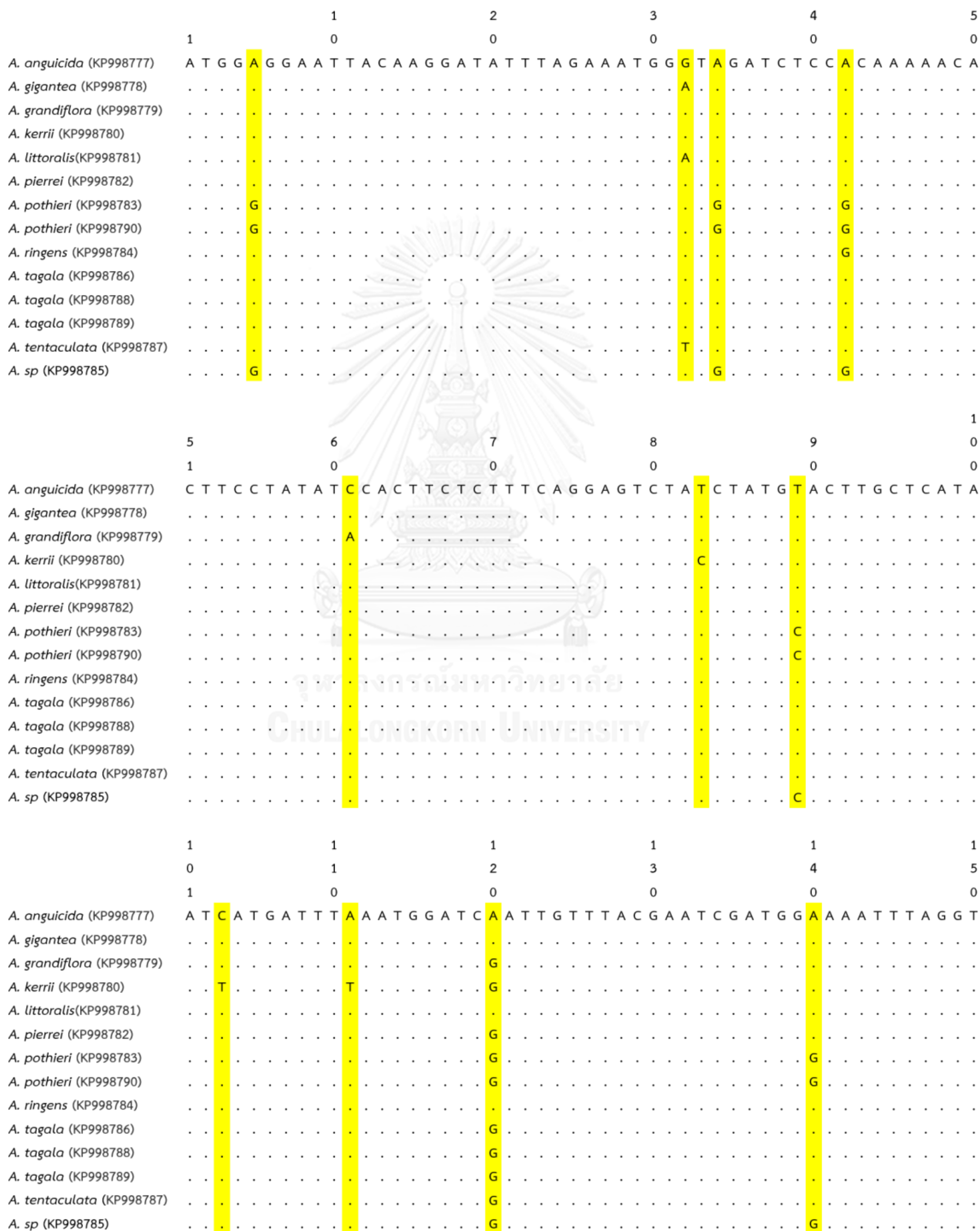

Figure B2 Sequence alignment of full length *mat*K genes of eleven *Aristolochia* plants. (continued)

```
                              151      160       170       180       190       200
A. anguicida (KP998777)       TATGACAAAAGATTCAGTTCACTAATTGTGAAACGTTTAATTATTCGAAT
A. gigantea (KP998778)        ..................................................
A. grandiflora (KP998779)     ..........A...A...................................
A. kerrii (KP998780)          ..........A............T..........................
A. littoralis(KP998781)       ..................................................
A. pierrei (KP998782)         ..........A.......................................
A. pothieri (KP998783)        ..........A...A....T..............................
A. pothieri (KP998790)        ..........A...A....T..............................
A. ringens (KP998784)         .....................A............................
A. tagala (KP998786)          ..........A.......................................
A. tagala (KP998788)          ..........A.......................................
A. tagala (KP998789)          ..........A.......................................
A. tentaculata (KP998787)     ..................................................
A. sp (KP998785)              ..........A...A....T..............................

                              201      210       220       230       240       250
A. anguicida (KP998777)       GCATCAACAGAACCATTTTTTTATTTTGGTTACCAATTTT------CACC
A. gigantea (KP998778)        ...................A....................------A...
A. grandiflora (KP998779)     .............T............C.......T.....------....
A. kerrii (KP998780)          ..............C....A......C.......T.....------....
A. littoralis(KP998781)       ...................A....................------....
A. pierrei (KP998782)         ..............T..------...C.............------....
A. pothieri (KP998783)        ..............T..------...C.............CACAAA...A
A. pothieri (KP998790)        ..............T..------...C.............CACAAA...A
A. ringens (KP998784)         ..........................C.............------....
A. tagala (KP998786)          ..............T..------...C.............------....
A. tagala (KP998788)          ..............T..------...C.............------....
A. tagala (KP998789)          ..............T..------...C.............------....
A. tentaculata (KP998787)     ..........................C.............------....
A. sp (KP998785)              ..............------..T...C.............CACAAA...A

                              251      260       270       280       290       300
A. anguicida (KP998777)       AAAATAGATTCGTTAGGAAAAGCAATAATTTGGATTATCAAACCATATCA
A. gigantea (KP998778)        .....G............................................
A. grandiflora (KP998779)     ..............G......CA..A......T...C.............
A. kerrii (KP998780)          .....G.........AA.........T....T....C.............
A. littoralis(KP998781)       ..................................................
A. pierrei (KP998782)         ......................A..A...G......C.............
A. pothieri (KP998783)        ....GG................A..A...G......C.....T.......
A. pothieri (KP998790)        ....GG................A..A...G......C.....T.......
A. ringens (KP998784)         ....................................C.............
A. tagala (KP998786)          ......................A..A...G......C.............
A. tagala (KP998788)          ......................A..A...G......C.............
A. tagala (KP998789)          ......................A..A...G......C.............
A. tentaculata (KP998787)     ....................................C.............
A. sp (KP998785)              ....GG................A..A...G......C.....T.......
```

Figure B2 Sequence alignment of full length *mat*K genes of eleven *Aristolochia* plants. (continued)

	301–350
A. anguicida (KP998777)	GAGGCTTTTGCAGTCATTATGGAAATTCCACTCTCCCTGCAATTAGTATC
A. gigantea (KP998778)	....
A. grandiflora (KP998779)	....
A. kerrii (KP998780)	....
A. littoralis(KP998781)	....
A. pierrei (KP998782)	....
A. pothieri (KP998783)	..............T......................C............
A. pothieri (KP998790)	..............T......................C............
A. ringens (KP998784)	....
A. tagala (KP998786)	....
A. tagala (KP998788)	....
A. tagala (KP998789)	....
A. tentaculata (KP998787)	....................................AG............
A. sp (KP998785)	..............T......................C............

	351–400
A. anguicida (KP998777)	TTGCCTGGAAGAAAAAGAAATAGCCAAATCTCATAATTTACGATCTATTC
A. gigantea (KP998778)	........................A.........................
A. grandiflora (KP998779)	.....A..................A.........................
A. kerrii (KP998780)	.............C..........A.........................
A. littoralis(KP998781)	........................A.........................
A. pierrei (KP998782)	........................A....A....................
A. pothieri (KP998783)	........................A....A...A................
A. pothieri (KP998790)	........................A....A...A................
A. ringens (KP998784)	........................A.........................
A. tagala (KP998786)	........................A....A....................
A. tagala (KP998788)	........................A....A....................
A. tagala (KP998789)	........................A....A....................
A. tentaculata (KP998787)	........................A.........................
A. sp (KP998785)	........................A....A...A................

	401–450
A. anguicida (KP998777)	ATTCAATATTTCCCTTTTTCGAGGACAAATTATCACATTTAAATCATGTG
A. gigantea (KP998778)	....
A. grandiflora (KP998779)	..........C.......................................
A. kerrii (KP998780)	...................T..............................
A. littoralis(KP998781)	....
A. pierrei (KP998782)	...................T..............................
A. pothieri (KP998783)	...................T..............................
A. pothieri (KP998790)	...................T..............................
A. ringens (KP998784)	....
A. tagala (KP998786)	...................T..............................
A. tagala (KP998788)	...................T..............................
A. tagala (KP998789)	...................T..............................
A. tentaculata (KP998787)	....
A. sp (KP998785)	...................T..............................

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Figure B2 Sequence alignment of full length *mat*K genes of eleven *Aristolochia* plants. (continued)

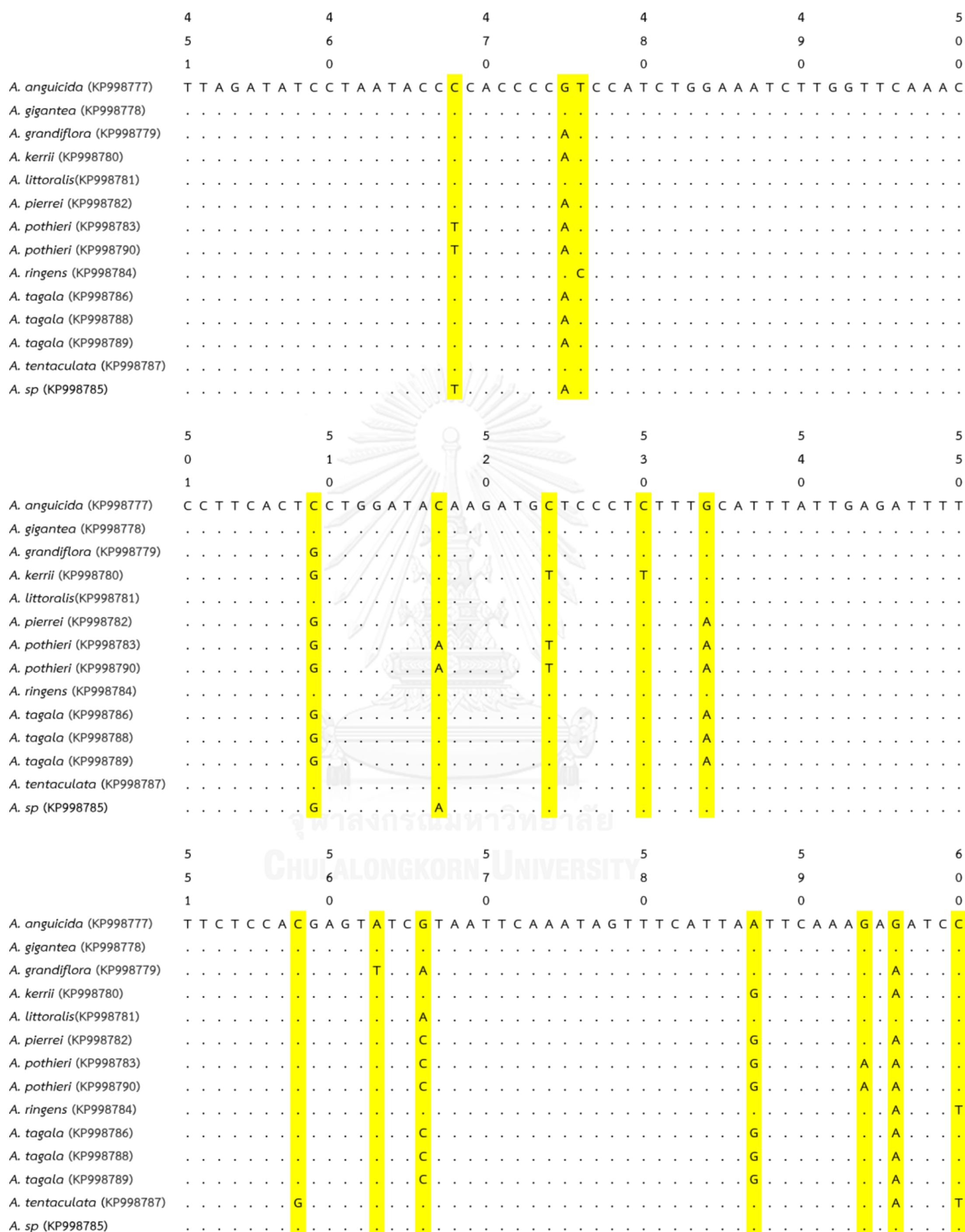

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Figure B2 Sequence alignment of full length *mat*K genes of eleven *Aristolochia* plants. (continued)

```
                              601       610       620       630       640       650
A. anguicida (KP998777)       TTTTTTTTTTTCTC---------------AAAAGAGAATCAAAGAAAAGA
A. gigantea (KP998778)        ..............AAAAGAGAATCAAAG.....................
A. grandiflora (KP998779)     A.....A....A..------------------------------......
A. kerrii (KP998780)          ...........T..---------------------------TCA...GA.
A. littoralis(KP998781)       ..............---------------.....................
A. pierrei (KP998782)         A...C.........------------------------------......
A. pothieri (KP998783)        A...C.........------------------------------.....G
A. pothieri (KP998790)        A...C.........------------------------------.....G
A. ringens (KP998784)         A.............---------------.....................
A. tagala (KP998786)          A...C.........------------------------------......
A. tagala (KP998788)          A...C.........------------------------------......
A. tagala (KP998789)          A...C.........------------------------------......
A. tentaculata (KP998787)     A.............------------------------------......
A. sp (KP998785)              ..............---------------.....................

                              651       660       670       680       690       700
A. anguicida (KP998777)       GAATCAAAGATTATTCTTGTTCCTATATAATTCTCATGTATATGAATGCG
A. gigantea (KP998778)        ..................................................
A. grandiflora (KP998779)     .................................G................
A. kerrii (KP998780)          ............C........T............................
A. littoralis(KP998781)       .................................................A
A. pierrei (KP998782)         ..................................................
A. pothieri (KP998783)        ..................................................
A. pothieri (KP998790)        ..................................................
A. ringens (KP998784)         ..................................................
A. tagala (KP998786)          ..................................................
A. tagala (KP998788)          ..................................................
A. tagala (KP998789)          ..................................................
A. tentaculata (KP998787)     ...............A..................................
A. sp (KP998785)              ..................................................

                              701       710       720       730       740       750
A. anguicida (KP998777)       AATCCATATTCGTTTTTCTCGGTAAACAAGCCTCTCGTTTACGATCAACA
A. gigantea (KP998778)        ..................................................
A. grandiflora (KP998779)     ..........GT......................................
A. kerrii (KP998780)          ..................................................
A. littoralis(KP998781)       ..........................................A.......
A. pierrei (KP998782)         .....C........................T...................
A. pothieri (KP998783)        ..........T...................T...................
A. pothieri (KP998790)        ..........T...................T...................
A. ringens (KP998784)         ..................................................
A. tagala (KP998786)          ..............................T...................
A. tagala (KP998788)          ..............................T...................
A. tagala (KP998789)          ..............................T...................
A. tentaculata (KP998787)     ....................................A.............
A. sp (KP998785)              ..................................................
```

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Figure B2 Sequence alignment of full length *mat*K genes of eleven *Aristolochia* plants. (continued)

```
                          7                 7                   7                   7                   7                   8
                          5                 6                   7                   8                   9                   0
                          1                 0                   0                   0                   0                   0
A. anguicida (KP998777)   T C C C C T G G A G C C T T T C T T G A G C G A A C A T A T T T C T A T G G A A A A A T G G A G C A
A. gigantea (KP998778)    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. grandiflora (KP998779) . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . A . . . .
A. kerrii (KP998780)      . . . . . . . . . . . . C . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . .
A. littoralis(KP998781)   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. pierrei (KP998782)     . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. pothieri (KP998783)    . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. pothieri (KP998790)    . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. ringens (KP998784)     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. tagala (KP998786)      . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. tagala (KP998788)      . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. tagala (KP998789)      . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. tentaculata (KP998787) . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. sp (KP998785)          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

                          8                 8                   8                   8                   8                   8
                          0                 1                   2                   3                   4                   5
                          1                 0                   0                   0                   0                   0
A. anguicida (KP998777)   T T T T T T A G T A G T G C T T T G T A A T C A T T T T C A G A A G A C C T T G T G G T T A C T T A
A. gigantea (KP998778)    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . .
A. grandiflora (KP998779) . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . . . . . . .
A. kerrii (KP998780)      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . . . . . . .
A. littoralis(KP998781)   . . . . . . . . . . . G . . . . . . . . . . . . . . . . . . A . . A . . . . . . G . . . . . . . . .
A. pierrei (KP998782)     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . . . . . . .
A. pothieri (KP998783)    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . . . . . . .
A. pothieri (KP998790)    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . . . . . . .
A. ringens (KP998784)     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. tagala (KP998786)      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . . . . . . .
A. tagala (KP998788)      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . . . . . . .
A. tagala (KP998789)      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . . . . . . .
A. tentaculata (KP998787) . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. sp (KP998785)          . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . A . . . . . . G . . . . . . . . .

                          8                 8                   8                   8                   8                   9
                          5                 6                   7                   8                   9                   0
                          1                 0                   0                   0                   0                   0
A. anguicida (KP998777)   A G G A T C C T T T T G C A C A T T A T G T C A G A T A C C G A G G A A A A T C A A T T C T G G C T
A. gigantea (KP998778)    . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . .
A. grandiflora (KP998779) . . . . . . . . . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. kerrii (KP998780)      . . . . . . . . . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. littoralis(KP998781)   . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . .
A. pierrei (KP998782)     . . . . . . . . . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. pothieri (KP998783)    . . . . . . . . . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . T . .
A. pothieri (KP998790)    . . . . . . . . . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . T . .
A. ringens (KP998784)     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. tagala (KP998786)      . . . . . . . . . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. tagala (KP998788)      . . . . . . . . . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. tagala (KP998789)      . . . . . . . . . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. tentaculata (KP998787) . . . . . . . . . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. sp (KP998785)          . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . .
```

Figure B2 Sequence alignment of full length *mat*K genes of eleven *Aristolochia* plants. (continued)

```
                           9        9         9         9         9         9
                           0        1         2         3         4         5
                           1        0         0         0         0         0
A. anguicida (KP998777)    TCAAAGGGGACTCATTTTATGATGAAGAAATGGAAATATCATCTTGTCAA
A. gigantea (KP998778)     ..........................A.......................
A. grandiflora (KP998779)  ........T......C.......................T..........
A. kerrii (KP998780)       ............T..C.......................T..........
A. littoralis(KP998781)    ..........................A.......................
A. pierrei (KP998782)      ...............C.......................T..........
A. pothieri (KP998783)     ........A......C.......................T..........
A. pothieri (KP998790)     ........A......C.......................T..........
A. ringens (KP998784)      ..................................................
A. tagala (KP998786)       ...............C.......................T..........
A. tagala (KP998788)       ...............C.......................T..........
A. tagala (KP998789)       .....A.........C.......................T..........
A. tentaculata (KP998787)  ..................................................
A. sp (KP998785)           ..........................A.......................

                                                                            1
                           9        9         9         9         9         0
                           5        6         7         8         9         0
                           1        0         0         0         0         0
A. anguicida (KP998777)    TTTATGGCAGTGTCATTTTCACTTGTGGTCTCAACCGGGCAGAATCCATA
A. gigantea (KP998778)     .........................G........................
A. grandiflora (KP998779)  .............T....................................
A. kerrii (KP998780)       ........................C.........................
A. littoralis(KP998781)    .........................G...............A........
A. pierrei (KP998782)      .......................................T..........
A. pothieri (KP998783)     ......................................AT..........
A. pothieri (KP998790)     ......................................AT..........
A. ringens (KP998784)      ..................................................
A. tagala (KP998786)       .......................................T..........
A. tagala (KP998788)       .......................................T..........
A. tagala (KP998789)       .......................................T..........
A. tentaculata (KP998787)  ..................................................
A. sp (KP998785)           .........................G........................

                           1        1         1         1         1         1
                           0        0         0         0         0         0
                           0        1         2         3         4         5
                           1        0         0         0         0         0
A. anguicida (KP998777)    TAAACCGATTATGCAATCATTCCTTCTATTTTATGGGCTATCTCTCAAGT
A. gigantea (KP998778)     ......A...........................................
A. grandiflora (KP998779)  ......A.....A.....................................
A. kerrii (KP998780)       ...........GA....A...T............................
A. littoralis(KP998781)    ..................................................
A. pierrei (KP998782)      ............A.....................................
A. pothieri (KP998783)     ......A.....A.....................................
A. pothieri (KP998790)     ......A.....A.....................................
A. ringens (KP998784)      ............A.....................................
A. tagala (KP998786)       ............A.....................................
A. tagala (KP998788)       ............A.....................................
A. tagala (KP998789)       ............A.....................................
A. tentaculata (KP998787)  ............A..............T......................
A. sp (KP998785)           ..................................................
```

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Figure B2 Sequence alignment of full length *mat*K genes of eleven *Aristolochia* plants. (continued)

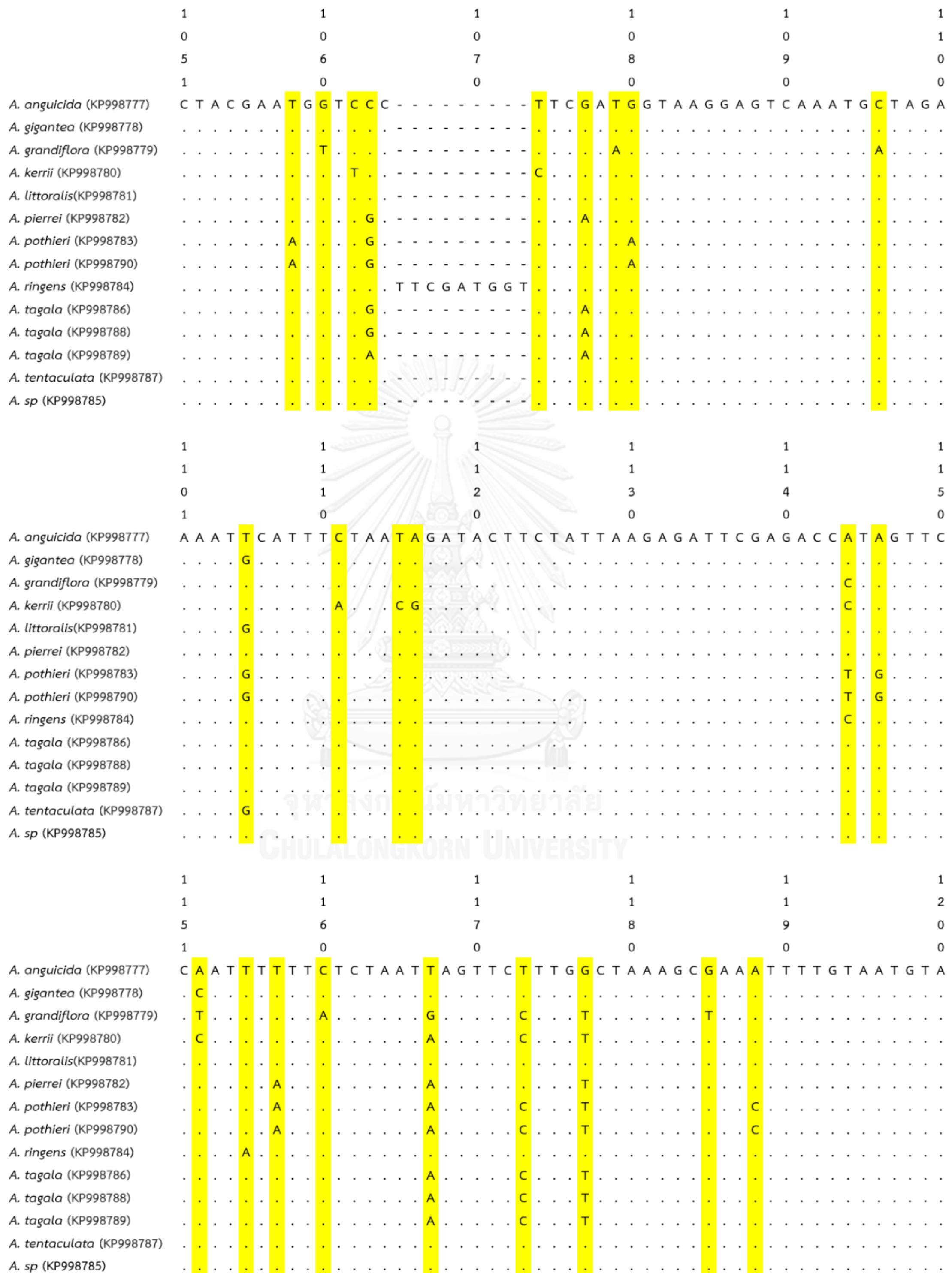

Figure B2 Sequence alignment of full length *mat*K genes of eleven *Aristolochia* plants. (continued)

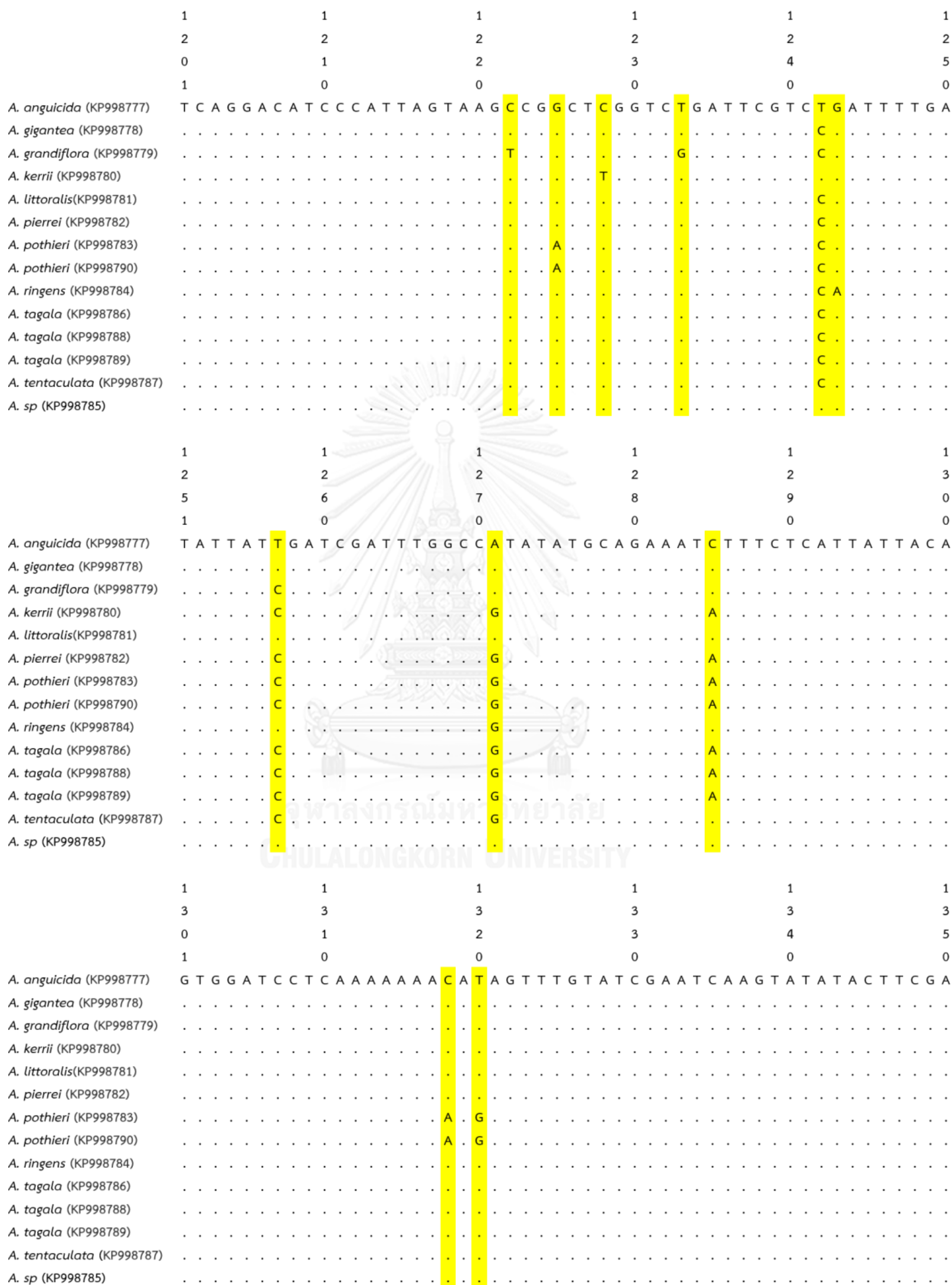

Figure B2 Sequence alignment of full length *mat*K genes of eleven *Aristolochia* plants. (continued)

```
                            1        1         1         1         1         1
                            3        3         3         3         3         4
                            5        6         7         8         9         0
                            1        0         0         0         0         0
A. anguicida (KP998777)     CTTTCATGTGCTAGAACTTTGGCTCGTAAACACAAAAGTACGGTACGCAC
A. gigantea (KP998778)      ..................................................
A. grandiflora (KP998779)   ..................................................
A. kerrii (KP998780)        ..................................................
A. littoralis(KP998781)     ..................................................
A. pierrei (KP998782)       ..................................................
A. pothieri (KP998783)      ..................................................
A. pothieri (KP998790)      ..................................................
A. ringens (KP998784)       ..................................................
A. tagala (KP998786)        ..................................................
A. tagala (KP998788)        ..................................................
A. tagala (KP998789)        ..................................................
A. tentaculata (KP998787)   ..................................................
A. sp (KP998785)            ..................................................

                            1        1         1         1         1         1
                            4        4         4         4         4         4
                            0        1         2         3         4         5
                            1        0         0         0         0         0
A. anguicida (KP998777)     CTTTTTGAAAAAATTAGGTTCGGAATTCTTAGAGGAATTCCTTATGGAGG
A. gigantea (KP998778)      ..................................................
A. grandiflora (KP998779)   T.....T.....................................C.....
A. kerrii (KP998780)        T...........................................C...A.
A. littoralis(KP998781)     ............T.....................................
A. pierrei (KP998782)       T.......................................T...C...A.
A. pothieri (KP998783)      T..........................A............T...C...A.
A. pothieri (KP998790)      T..........................A............T...C...A.
A. ringens (KP998784)       ............................................C.....
A. tagala (KP998786)        T.......................................T...C...A.
A. tagala (KP998788)        T.......................................T...C...A.
A. tagala (KP998789)        T.......................................T...C...A.
A. tentaculata (KP998787)   ............................................C.....
A. sp (KP998785)            ..................................................

                            1        1         1         1         1         1
                            4        4         4         4         4         5
                            5        6         7         8         9         0
                            1        0         0         0         0         0
A. anguicida (KP998777)     AAGAACAAGTTCTTTCTTTGATCTTCCCAAGAACCTCTTCTATTTCACAT
A. gigantea (KP998778)      ..................................................
A. grandiflora (KP998779)   ...........A..................................G...
A. kerrii (KP998780)        ..............................................G...
A. littoralis(KP998781)     ..................................................
A. pierrei (KP998782)       ..............................................G...
A. pothieri (KP998783)      ........................................A.....G...
A. pothieri (KP998790)      ........................................A.....G...
A. ringens (KP998784)       ...........T..................................G...
A. tagala (KP998786)        ..............................................G...
A. tagala (KP998788)        ..............................................G...
A. tagala (KP998789)        ..............................................G...
A. tentaculata (KP998787)   .....................................C........G...
A. sp (KP998785)            ..................................................
```

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Figure B2 Sequence alignment of full length *mat*K genes of eleven *Aristolochia* plants. (continued)

```
                            1501      1510      1520      1530      1540      1550
A. anguicida (KP998777)     AAGTTACATAAGGAATGTATTTGGTATTTGGATATTATCCGTATCACCGA
A. gigantea (KP998778)      ..................................................
A. grandiflora (KP998779)   .G..................................T..........T..
A. kerrii (KP998780)        .G.............................................T..
A. littoralis(KP998781)     ..................................................
A. pierrei (KP998782)       .G...........................................G.T..
A. pothieri (KP998783)      .G..............T.C............................T..
A. pothieri (KP998790)      .G..............T.C............................T..
A. ringens (KP998784)       .G.............................................T..
A. tagala (KP998786)        .G...........................................G.T..
A. tagala (KP998788)        .G...........................................G.T..
A. tagala (KP998789)        .G...........................................G.T..
A. tentaculata (KP998787)   .G.............................................T..
A. sp (KP998785)            ..................................................

                            1551      1560     1569
A. anguicida (KP998777)     CCTGGCTAATCATGAATGA
A. gigantea (KP998778)      .T.................
A. grandiflora (KP998779)   ......C...T.CTC....
A. kerrii (KP998780)        .............TC....
A. littoralis(KP998781)     ...................
A. pierrei (KP998782)       .........C...TC....
A. pothieri (KP998783)      ......C......TC....
A. pothieri (KP998790)      ......C......TC....
A. ringens (KP998784)       ......C......TC....
A. tagala (KP998786)        .............TC....
A. tagala (KP998788)        .............TC....
A. tagala (KP998789)        .............TC....
A. tentaculata (KP998787)   ......C............
A. sp (KP998785)            ...................
```

Figure B18 Sequence alignment of ITS regions of eleven *Aristolochia* plants.

The numbers on the top line represent the base numbers in sequence alignment. The altered bases indicate the sequence differences. '.' represents the base being identical to the first sequence. '–' represents gap.

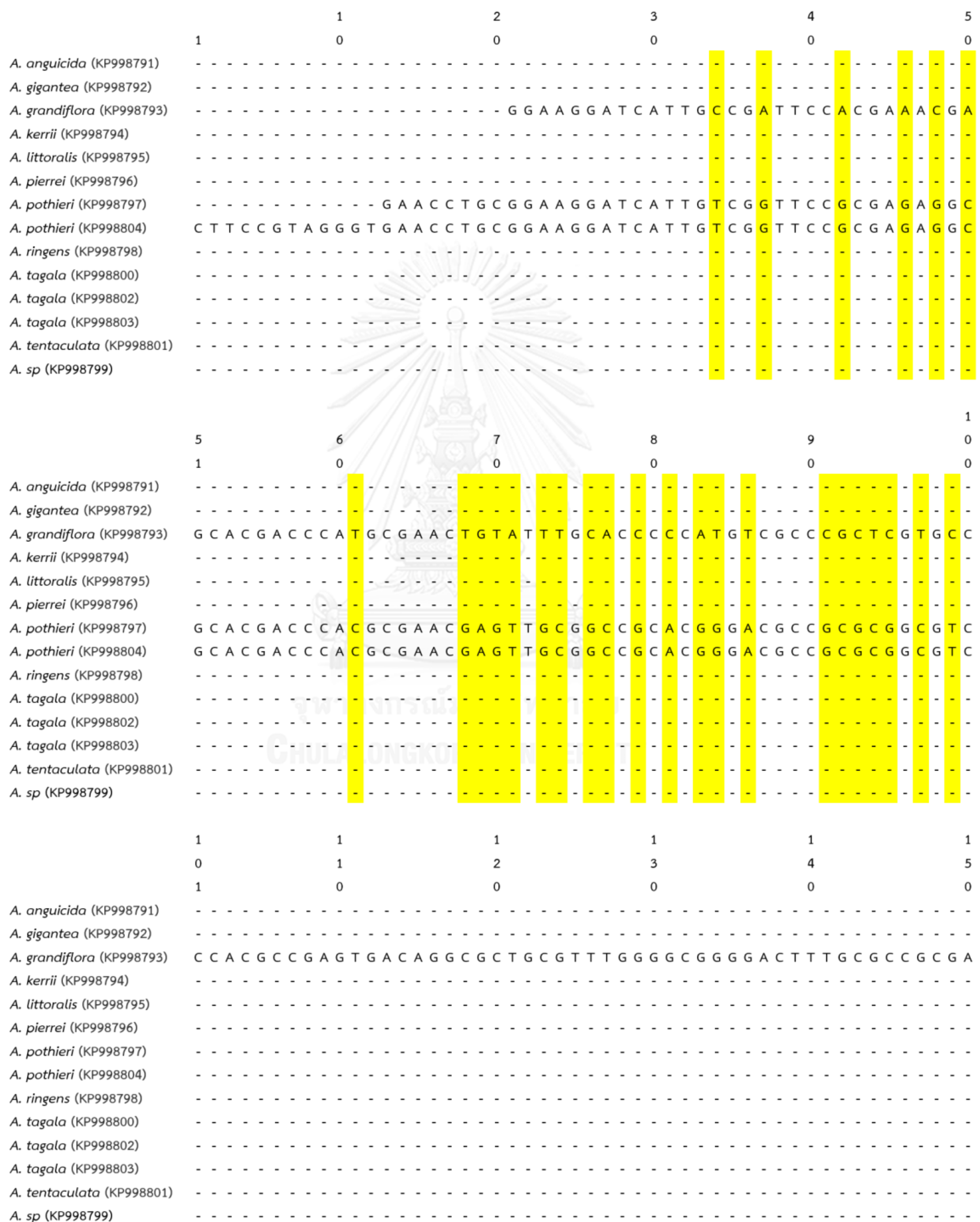

Figure B3 Sequence alignment of ITS regions of eleven *Aristolochia* plants. (continued)

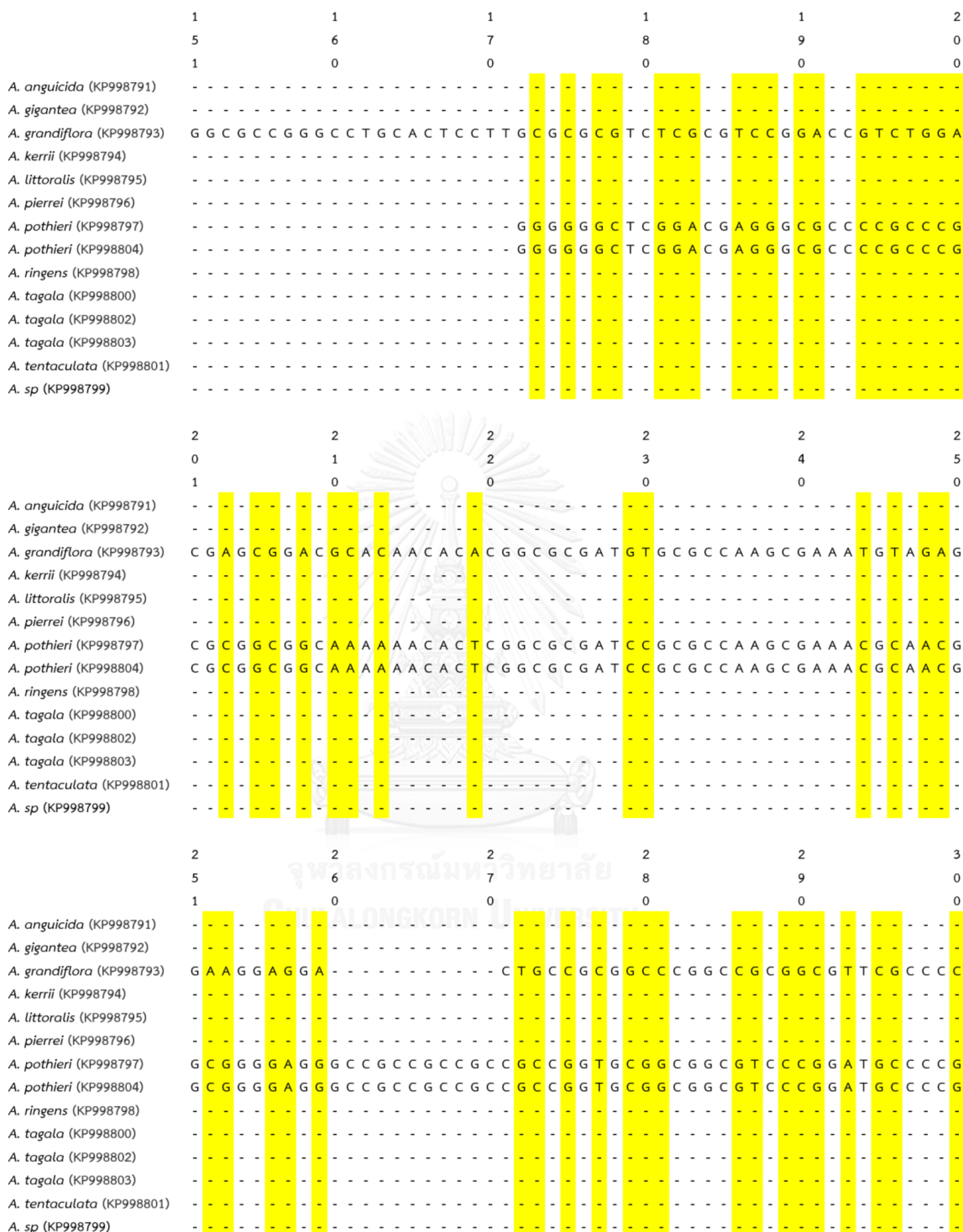

Figure B3 Sequence alignment of ITS regions of eleven *Aristolochia* plants. (continued)

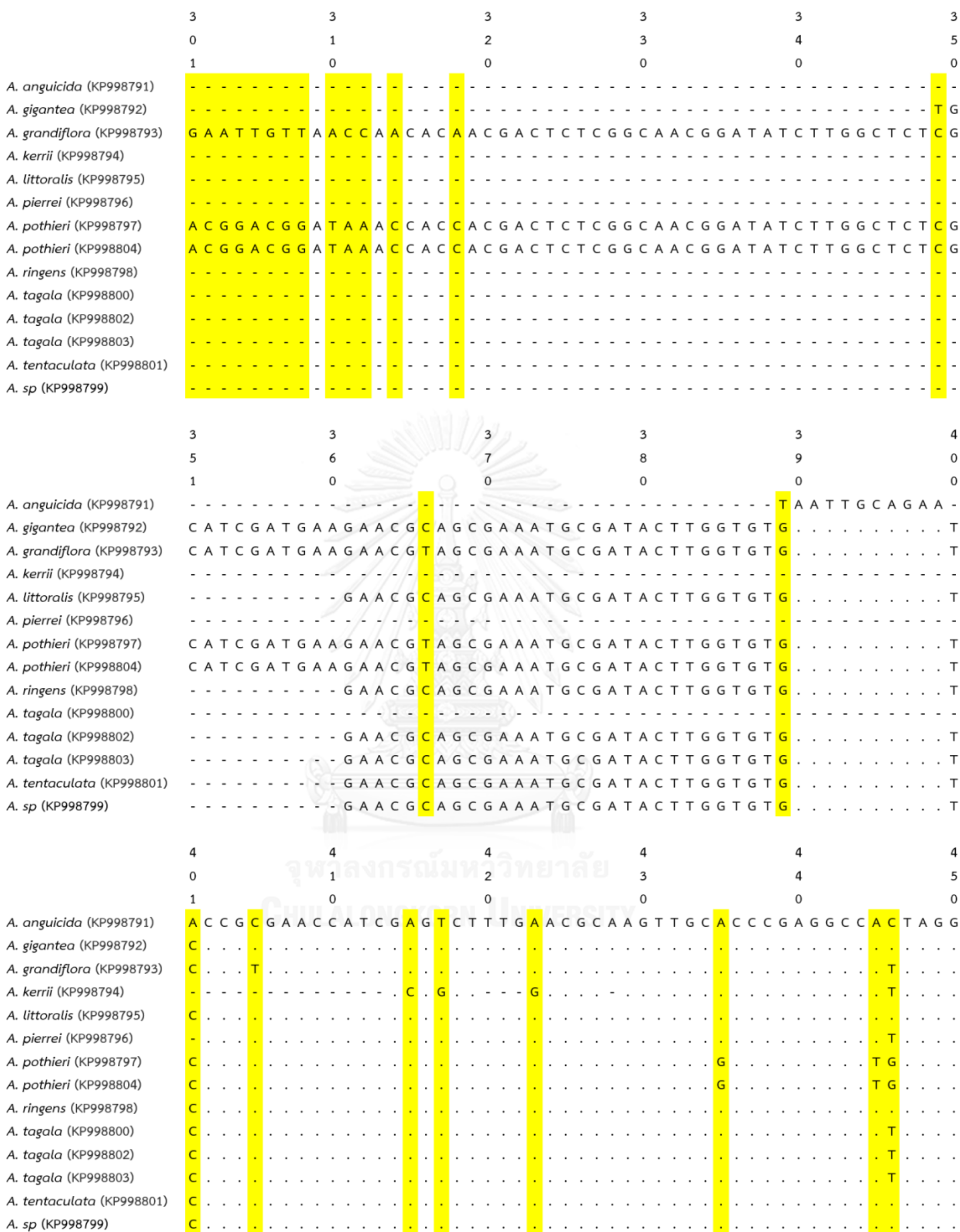

Figure B3 Sequence alignment of ITS regions of eleven *Aristolochia* plants. (continued)

```
                            451                                              500
A. anguicida (KP998791)     TCGAGGGTACACCTGCCTGGGCGTCACGCCCGAC--GTGTTCT-CCCCCC
A. gigantea (KP998792)      ..................................--...C...-......
A. grandiflora (KP998793)   ..................................--.......---....
A. kerrii (KP998794)        C......C..........................--.CC....-......
A. littoralis (KP998795)    ..................................--...C...C......
A. pierrei (KP998796)       C.A...............................--.C.....-.T....
A. pothieri (KP998797)      C......C..........................--.C.....C....A.
A. pothieri (KP998804)      C......C..........................--.C.....C....A.
A. ringens (KP998798)       ..................................--.C.C...-......
A. tagala (KP998800)        C.A...............................--.C.....-......
A. tagala (KP998802)        C.A...............................--.C.....-......
A. tagala (KP998803)        C.A...............................--.C.....-......
A. tentaculata (KP998801)   C.................................GT.......C.....A
A. sp (KP998799)            ..................................--.......-......

                            501                                              550
A. anguicida (KP998791)     ACCCCGCGCGCCCCCGCCCCGCCGCGGGCGGCGCCGCGCGTCG-ACGGG-
A. gigantea (KP998792)      G..........T........AT............G........-.....-
A. grandiflora (KP998793)   C..T.....-------------A............AC.C.......C---G.TC.-
A. kerrii (KP998794)        G........----------------------TCG.........CC....-
A. littoralis (KP998795)    G..........T........A............GG........-.....-
A. pierrei (KP998796)       G........----------------------A..G........-T....-
A. pothieri (KP998797)      G..T.C-------------------------.TT....G.GGATG....C
A. pothieri (KP998804)      G..T.C-------------------------.TT....G.GGATG....C
A. ringens (KP998798)       G........C.........AA........C....G........-.....-
A. tagala (KP998800)        G........----------------------T........G..-G....-
A. tagala (KP998802)        G........----------------------T........G..-G....-
A. tagala (KP998803)        G........----------------------T........G..-G....-
A. tentaculata (KP998801)   C...T....C.T......G.AG.....TC.T..G........-T....-
A. sp (KP998799)            ...........................................-.....-

                            551                                              600
A. anguicida (KP998791)     GGCGAGCAGATTGGCCGTCCCTGCCCCCC----GGGGCGAGGTTGGCTGA
A. gigantea (KP998792)      ..............T.A............----.................
A. grandiflora (KP998793)   .............................--CG..........C....T.
A. kerrii (KP998794)        .........-...................CTCC..........C...CT.
A. littoralis (KP998795)    ..............T..............----.................
A. pierrei (KP998796)       .........GA..........C.......CCGG..T.......C...CT.
A. pothieri (KP998797)      ..G......T...........C......G-CGA..........C...CC.
A. pothieri (KP998804)      ..G......T...........C......G-CGA..........C...CC.
A. ringens (KP998798)       ..............T..C...........----.................
A. tagala (KP998800)        .........GC............G.....-CGG..T.......C....T.
A. tagala (KP998802)        .........GC..................-CGG..T.......C....T.
A. tagala (KP998803)        .........GC..................-CGG..T.......C....T.
A. tentaculata (KP998801)   ..........G...T..............----................-
A. sp (KP998799)            .............................----.................
```

Figure B3 Sequence alignment of ITS regions of eleven *Aristolochia* plants. (continued)

```
                           6                 6                   6                   6                   6                   6
                           0                 1                   2                   3                   4                   5
                           1                 0                   0                   0                   0                   0
A. anguicida (KP998791)    A A A A - C G C - T G T G C - C C C G C - - G G G C A C T T C G G C A C G A C G A G T G G T G G C T
A. gigantea (KP998792)     . . . . A T . . - . T . . . - . . . A . - - . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. grandiflora (KP998793)  . . T . T . A - - - - - . . - . . . . . - - . . . . T . - A . . . . G . . . . T . . . . . . . . . .
A. kerrii (KP998794)       . . . G G A A T C G . A . A G . . . . . G C . . . . T . - G . . . . G . . . . C G T . . . . . . . .
A. littoralis (KP998795)   . . . . A T . . - . T . . . - . . . A . - - . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. pierrei (KP998796)      . . . G G . A A - - - - . . - . . . A . - - . . . . T . - G . . . . G . . . . T . C . . . . . . . .
A. pothieri (KP998797)     . . . G C . . G - - - - . . - . . . C . G G . . . . T . - G . . . . G . . . . T G C . . . . . . . .
A. pothieri (KP998804)     . . . G C . . G - - - - . . - . . . C . G G . . . . T . - G . . . . G . . . . T G C . . . . . . . .
A. ringens (KP998798)      . . . . - . . . - . T . . . - . . . A . - - . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. tagala (KP998800)       . . . G G . A A - - - - . . - . . . A . - - . . . . T . - G . . . . G . . . . T . C . . . . . . . .
A. tagala (KP998802)       . . . G G . A A - - - - . . - . . . A . - - . . . . T . - G . . . . G . . . . T . C . . . . . . . .
A. tagala (KP998803)       . . . G G . A A - - - - . . - . . . A . - - . . . . T . - G . . . . G . . . . T . C . . . . . . . .
A. tentaculata (KP998801)  . . . T G . . . T . T . . . - . . . A . - - . . . . G . . G . . . . . . . . . . . . . . . . . . . .
A. sp (KP998799)           . . . . - . . . - . . . . . - . . . . . - - . . . . . . . . . . . . . . . . . . . . . . . . . . . .
```

```
                           6                 6                   6                   6                   6                   7
                           5                 6                   7                   8                   9                   0
                           1                 0                   0                   0                   0                   0
A. anguicida (KP998791)    C C G C C C T - C C C C G G C C C T - - G C G T G G C T C G A A G T C G T G T C C G A G G - - C C C
A. gigantea (KP998792)     . . . . . . . - . . . T . . . . . . - - . . . . . . . . . . . . . . . . . . . . . . . . . - - . . .
A. grandiflora (KP998793)  . G . . . . . - . . . . . . . . T . T - . T T A . . . . . . . . . . . . . . . . . . T . . C C . . .
A. kerrii (KP998794)       . G A A G . . C . . . . . . . . . C C A . . C G . . . C . . . . . . . . C . C . . . C . T C C . . .
A. littoralis (KP998795)   . . . . . . . - . . . . . . . . . . - - . . . . . . . . . . . . . . . . . . . . . . . . . - - . . .
A. pierrei (KP998796)      . . A . G . A C . . . . . . . . T . G - . . C G . . . C . . . . . . . . C . C A . . C . A A C . . .
A. pothieri (KP998797)     . G A T G . . C - - - - - - - - - - - - . . C G . . G G . . . . . . . . C . A T . . C . A A - . . .
A. pothieri (KP998804)     . G A T G . . C - - - - - - - - - - - - . . C G . . G G . . . . . . . . C . A T . . C . A A - . . .
A. ringens (KP998798)      . . . . . . . - . . . . . . . . . . - - . . . . . . . . . . . . . . . . . . . . . . . . . - - . . .
A. tagala (KP998800)       . . A . G . . C . . . . . . . . T . G - . . C G . . . C . . . . . . . . C . C . . . C . A A C . . .
A. tagala (KP998802)       . . A . G . . C . . . . . . . . T . G - . . C G . . . C . . . . . . . . C . C . . . C . A A C . . .
A. tagala (KP998803)       . . A . G . . C . . . . . . . . T . G - . . C G . . . C . . . . . . . . C . C . . . C . A A C . . .
A. tentaculata (KP998801)  . . . . . . . - . . . . . . . . . . - - . . . . . . . . . . . . . . . . . . . . . . . . . - - . . .
A. sp (KP998799)           . . . . . . . - . . . . . . . . . . - - . . . . . . . . . . . . . . . . . . . . . . . . . - - . . .
```

```
                           7                 7                   7                   7                   7                   7
                           0                 1                   2                   3                   4                   5
                           1                 0                   0                   0                   0                   0
A. anguicida (KP998791)    G G G C - T G T G A A G - - - - - - - - A A G G A C C C C T G C C G G C A G T C G C C T C - - - C G
A. gigantea (KP998792)     . . . . - . C C . . . . - - - - - - - - . . . C . . . . . . . . . . . . . . C . . . . . . C G - . .
A. grandiflora (KP998793)  C . T T - - - - . . G . G C T A C - - - G . . C . . . . . C . . . . . . T . C T . . . . . C G G T .
A. kerrii (KP998794)       C C . . T C . G . . . . G C C C C G A G G . . . . . . . . C . . . . . . C . C T T . G C . C T C . .
A. littoralis (KP998795)   . . . . - . C C . . . . - - - - - - - - . . . . . . . . . . . . . . . . . . C . . . . . . C G C . .
A. pierrei (KP998796)      C C . . - . . G . . . . T C T G C - - - G . . . . . . . . . . . . . . . T . C . . . G C . T C - . T
A. pothieri (KP998797)     C . . . - C . G . . . . G C C G C - - - G . . . . . . . A A T G . . . . - . C . C . G C . C - - . .
A. pothieri (KP998804)     C . . . - C . G . . . . G C C G C - - - G . . . . . . . A A T G . . . . - . C . C . G C . C - - . .
A. ringens (KP998798)      A . . . - . C C . C . . - - - - - - - - . . . . . . . . . . . . . . . . . . . . . . . . . - - - . .
A. tagala (KP998800)       C C . . - . . G A . . . G C T G C - - - G . . . . . . . . . . . . . . . T . C . . . G C . T C - . .
A. tagala (KP998802)       C C . . - . . G A . . . G C C G C - - - G . . . . . . . . . . . . . . . C . C . . . G C . T C - . .
A. tagala (KP998803)       C C . . - . . G A . . . G C T G C - - - G . . . . . . . . . . . . . . . T . C . . . G C . T C - . .
A. tentaculata (KP998801)  . . . . A . T . . . G A - - - - - - - - G . . . . . . . . . . . . . . . . . . . . . . . . - - - . .
A. sp (KP998799)           . . . . - . . . . . . . - - - - - - - - . . . . . . . . . . . . . . . . . . . . . . . . . - - - . .
```

Figure B3 Sequence alignment of ITS regions of eleven *Aristolochia* plants. (continued)

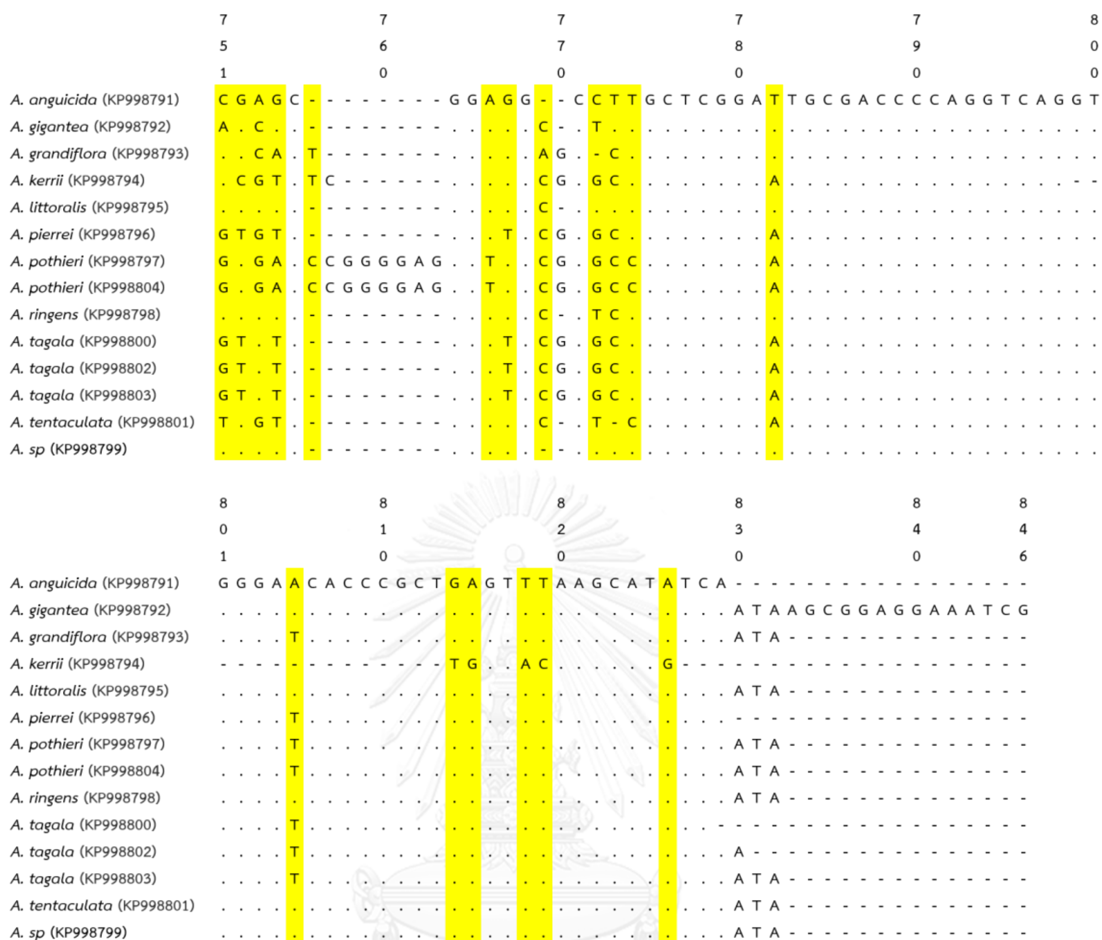

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Figure B19 Sequence alignment of *trn*H-*psb*A regions of eleven *Aristolochia* plants.

The numbers on the top line represent the base numbers in sequence alignment. The altered bases indicate the sequence differences. '.' represents the base being identical to the first sequence. '–' represents gap.

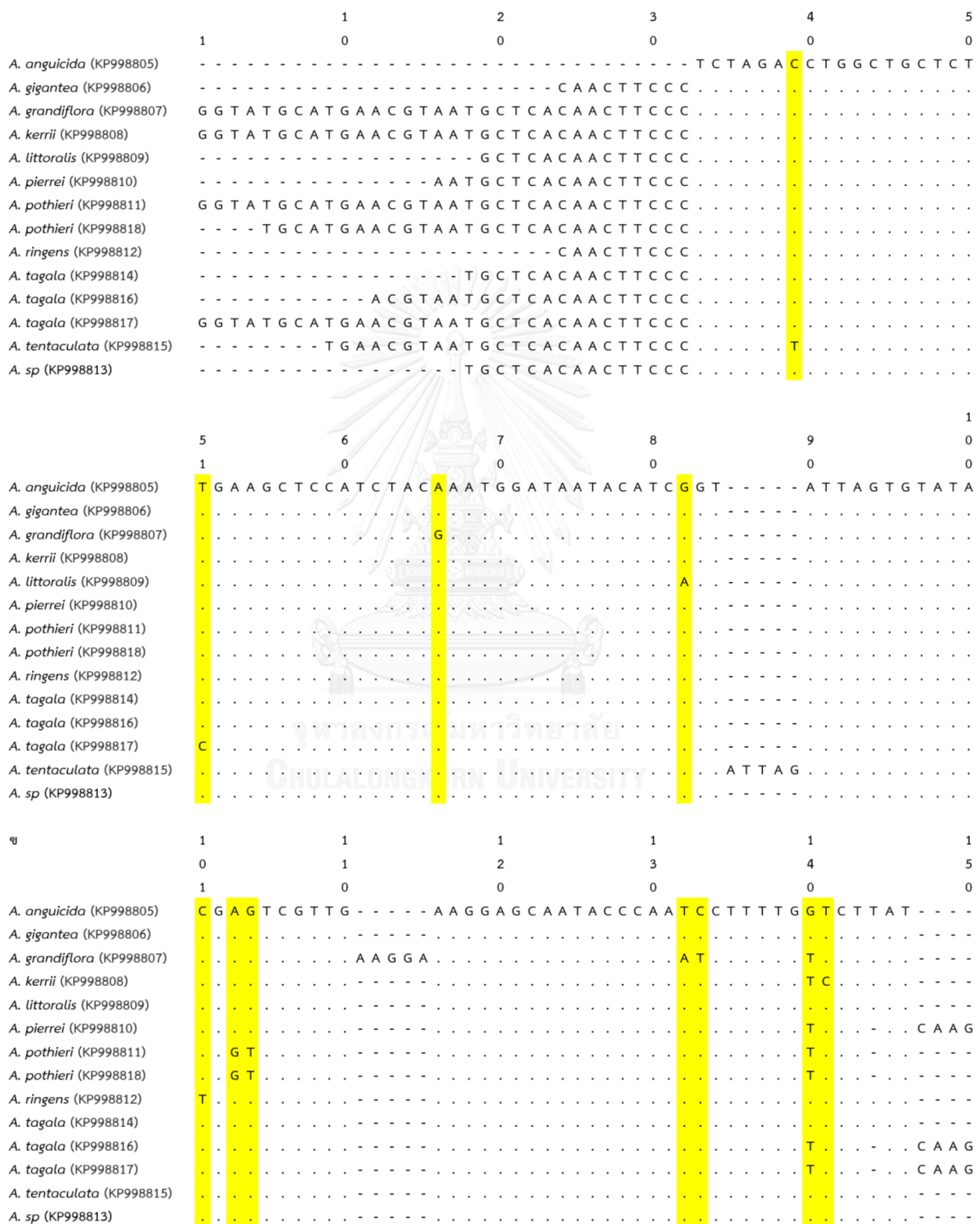

Figure B4 Sequence alignment of *trn*H-*psb*A regions of eleven *Aristolochia* plants. (continued)

```
                              1                 1                   1                   1                   1                   2
                              5                 6                   7                   8                   9                   0
                              1                 0                   0                   0                   0                   0
A. anguicida (KP998805)       - C A A G A G G T G G G T A T T G T T C C G C T C A A T T C A C T A T T A A T T C A A T A G A G T T
A. gigantea (KP998806)        - . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . .
A. grandiflora (KP998807)     - T . . A . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . C . . . . . . C . G T . . . .
A. kerrii (KP998808)          - . . . . . . . . . . . . . . . . . . . . . T . T . . . . . . . . . . C . . . . . . C . . T . . . .
A. littoralis (KP998809)      - . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . .
A. pierrei (KP998810)         A . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . C . . . . . . .
A. pothieri (KP998811)        - . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . C . . . . T . .
A. pothieri (KP998818)        - . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . C . . . . T . .
A. ringens (KP998812)         - . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . C . . . G . . .
A. tagala (KP998814)          - . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A. tagala (KP998816)          A . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . C . . . . . . .
A. tagala (KP998817)          A . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . C . . . . . . .
A. tentaculata (KP998815)     - . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . C . . . . . . .
A. sp (KP998813)              - . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

                              2                 2                   2                   2                   2                   2
                              0                 1                   2                   3                   4                   5
                              1                 0                   0                   0                   0                   0
A. anguicida (KP998805)       T T A C T T C T A T T G C A T A - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - A A T A
A. gigantea (KP998806)        . . . . . C A . . . . . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - . . . .
A. grandiflora (KP998807)     . . . . . C A . . . C A . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - . . . .
A. kerrii (KP998808)          . . . . . C A . . . C . . . A . A - - - - - - - - - - - - - - - - - - - - - - - - - - - A A . . . .
A. littoralis (KP998809)      . . . . . C A . . . . . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - . . . .
A. pierrei (KP998810)         . . . . . C A . . . C . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - . . . .
A. pothieri (KP998811)        . . . . . C A . . . C . T . . . A T C C A C A A T A C A A T A C A A T A T A A T A T G C A T . . . .
A. pothieri (KP998818)        . . . . . C A . . . C . T . . . A T C C A C A A T A C A A T A C A A T A T A A T A T G C A T . . . .
A. ringens (KP998812)         . . . . . C A . . . . . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - . . . .
A. tagala (KP998814)          . . . . . . . . . . . . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - . . . .
A. tagala (KP998816)          . . . . . C A . . . C . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - . . . .
A. tagala (KP998817)          . . . . . C A . . . C . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - . . . .
A. tentaculata (KP998815)     . . . . . C . . . . . . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - . . . .
A. sp (KP998813)              . . . . . . . . . . . . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - . . . .

                              2                 2                   2                   2                   2                   3
                              5                 6                   7                   8                   9                   0
                              1                 0                   0                   0                   0                   0
A. anguicida (KP998805)       G G C A T T T - - - - - - - - - - - - - - - - - - - - - - - - - - T - - - - - - - - - - - T T C T T
A. gigantea (KP998806)        . . . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - . A T C T A - - - - - - . A T . .
A. grandiflora (KP998807)     T . . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - . A T - - - - - - - - - . . T . A
A. kerrii (KP998808)          . . . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - A C T C C - - - - - - - . . T . .
A. littoralis (KP998809)      . . . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - . A T C T A - - - - - - . A T . .
A. pierrei (KP998810)         T . . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - . A T C T A T T T - - - . . T . .
A. pothieri (KP998811)        T . . . . . . T A T A T T T A T A T A T A T A T A T A T A T A T A . A T A T A T T T G T A . . T G .
A. pothieri (KP998818)        T . . . . . . T A T A T T T A T A T A T A T A T A T A T A T A T A . A T A T A T T T G T A . . T G .
A. ringens (KP998812)         . . . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - . A T C T A - - - - - - . A T . .
A. tagala (KP998814)          . . . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - . - - - - - - - - - - - . . . . .
A. tagala (KP998816)          T . . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - . A T C T A T T T T T T . . T . .
A. tagala (KP998817)          T . . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - . A T C T A T T T T T T . . T . .
A. tentaculata (KP998815)     . . . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - . A T C T A - - - - - - . A T . .
A. sp (KP998813)              . . . . . . . - - - - - - - - - - - - - - - - - - - - - - - - - - . - - - - - - - - - - - . . . . .
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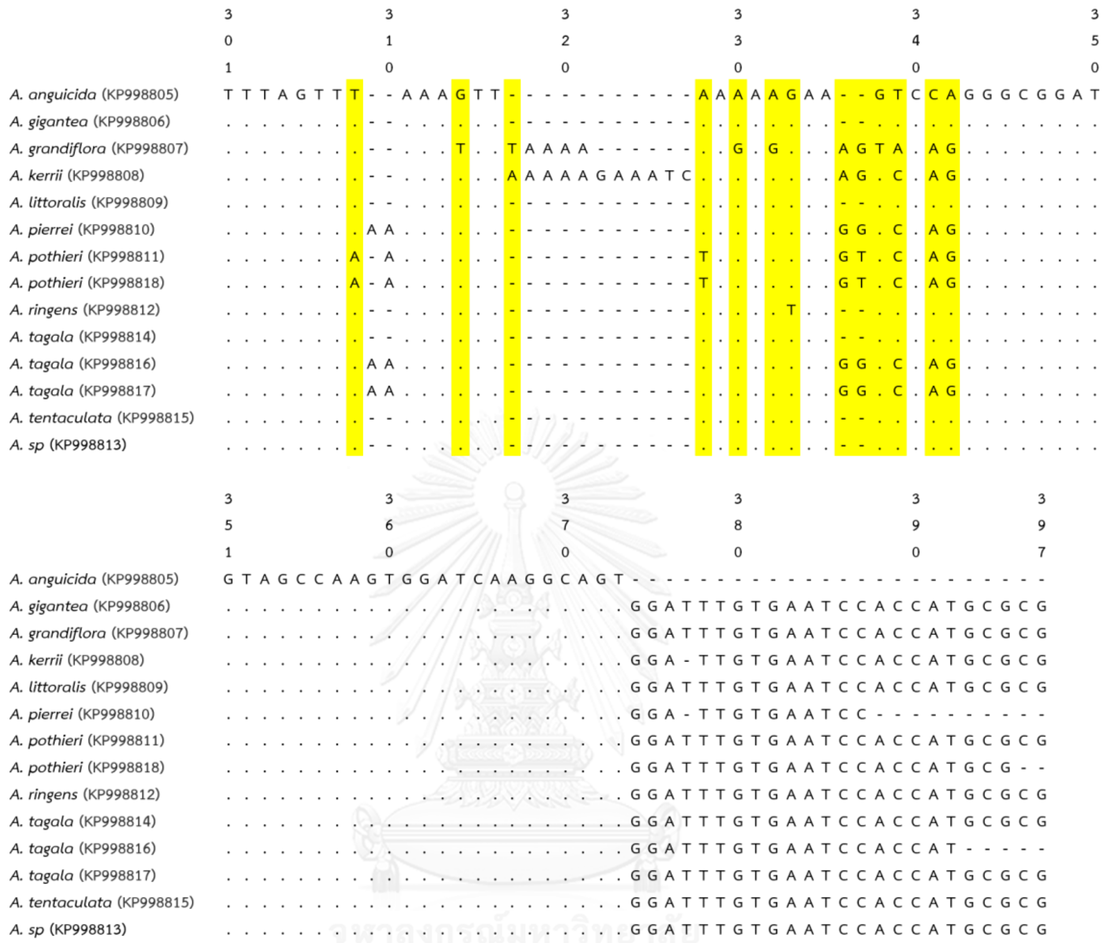
Figure B4 Sequence alignment of *trn*H-*psb*A regions of eleven *Aristolochia* plants. (continued)



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VITA

Miss Piroonrat Dechbumroong was born on October 26, 1988 in Phetchaburi, Thailand. She received her Bachelor's Degree of Science in Pharmacy in 2012 from the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

Poster presentation

1. Dechbumroong P., Amnuoypol S., Sukrong S.. 2015. Sequencing analysis of the matK DNA of Aristolochia plants used as "Krai-Krue" herb. The 5th National and International Graduate Study Conference 2015; "Creative Education: Intellectual Capital toward ASEAN", July, 16-17, 2015, Bangkok, Thailand.

Research

1. Senior project "Isolation of alpha-glucosidase inhibitors from Mamecylon plebejum Kurz. var. ellipsoideum Craib leaf located at Samae-san Island, Chonburi". Undersupervisor of Associate Professor Dr. Surattana Amnouypol.

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