MICROSCOPIC, MOLECULAR AND SCOPOLAMINE CONTENT EVALUATIONS OF *DATURA METEL* L. VAR. *METEL* AND *DATURA METEL* L. VAR. *FASTUOSA*

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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สมชาย อิสระวาณิชย์ : การประเมินลักษณะทางจุลทรรศน์ อณูโมเลกุล และ ปริมาณสารสโคโปลามีนของลำโพงขาว และ ลำโพงกาสลัก (MICROSCOPIC, MOLECULAR AND SCOPOLAMINE CONTENT EVALUATIONS OF *DATURA METEL* L. VAR. *METEL* AND *DATURA METEL* L. VAR. *FASTUOSA*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : อ. ดร. กาญจนา รังษีหิรัญรัตน์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ. ดร. นิจศิริ เรืองรังษี, 180 หน้า.

ลำโพงขาว และ ลำโพงกาสลักเป็นพืชสมุนไพรในจีนัส *Datura* ที่พบได้ในเอเชียตะวันออกเฉียงใต้รวมทั้งประเทศไทย การแพทย์แผนโบราณมีการใช้ลำโพงเป็นยาขยายม่านตา และใช้ต้านระบบประสาทพาราซิมพาเธติคมานาน ฤทธิ์ทางเภสัชวิทยาของสารอัลคาลอยด์โทรเปนในต้นลำโพงที่พบในส่วนต่างๆ ของพืชทั้งสอง ได้แก่ ไฮออสซีน (สโคโปลามีน) และ ไฮออสไซยามีน หรือ อะโทรปีน เนื่องจากข้อมูลเกี่ยวกับพืชทั้งสองชนิดมีค่อนข้างจำกัด ดังนั้นวัตถุประสงค์ของการศึกษาวิจัยนี้เพื่อประเมินคุณลักษณะของลำโพงขาว และ ลำโพงกาสลัก โดยอาศัยเทคนิคทางจุลทรรศน์ และอณูโมเลกุล รวมทั้งปริมาณสารสโคโปลามีนในพืชทั้งสอง. ผลการประเมินทางมหทรรศน์พบว่าพืชทั้งสองมีสัณฐานวิทยาที่แตกต่างกัน แต่มีคุณลักษณะทางจุลทรรศน์ของภาคตัดขวางลำต้นและเส้นกลางใบที่คล้ายคลึงกัน ค่าคงที่ของใบ (ค่าจำนวนปากใบ ค่าดัชนีปากใบ และค่าอัตราส่วนเซลล์รั้ว) ซึ่งเป็นคุณสมบัติที่สำคัญในการจำแนกชนิดของพืช พบว่ามีความแตกต่างของค่าคงที่ดังกล่าว การเพิ่มปริมาณสารพันธุกรรมในบริเวณ ITS โดยวิธี PCR ได้ผลผลิต PCR ขนาดประมาณ 670 คู่เบส ซึ่งมีความใกล้เคียง 99-100 เปอร์เซ็นต์ โดยพบความแตกต่างของนิวคลีโอไทด์โพลีมอร์ฟิซึม 2 ตำแหน่งในบริเวณ 5.8S และ 4 ตำแหน่งในบริเวณ ITS2 ตำแหน่ง 512 และ 614 ในบริเวณ ITS2 สามารถใช้จำแนกพืชทั้งสองได้ ส่วนการเพิ่มปริมาณสารพันธุกรรมในบริเวณยีน *rbc*L และ *atp*B ซึ่งมีขนาด 1.5 กิโลเบส พบว่านิวคลีโอไทด์มีความใกล้เคียง 95-100 และ 94-99 เปอร์เซ็นต์ ตามลำดับ ปริมาณสารสโคโปลามีนในพืชลำโพงทั้งสอง จากการเปรียบเทียบผลระหว่างวิธี TLC image และ HPLC พบว่ามีความสัมพันธ์ของปริมาณสารจากการวิเคราะห์ในทิศทางเดียวกันโดยพบปริมาณสารสโคโปลามีนมากในส่วนดอกในลำโพงขาว และพบมากในส่วนผลของลำโพงกาสลัก. จากข้อมูลดังกล่าว คุณลักษณะทางมหทรรศน์ ค่าคงที่ของใบ และเทคนิคทางอณูโมเลกุลสามารถใช้จำแนกลำโพงทั้งสองที่มีความใกล้เคียงกัน และพืชทั้งสองดังกล่าวมีศักยภาพในการใช้เป็นแหล่งผลิตสารสโคโปลามีน

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SOMCHAI ISSARAVANICH : MICROSCOPIC, MOLECULAR AND SCOPOLAMINE CONTENT EVALUATIONS OF *DATURA METEL* L. VAR. *METEL* AND *DATURA METEL* L. VAR. *FASTUOSA*.
THESIS ADVISOR : KANCHANA RUNGSIHIRUNRAT, Ph.D., THESIS CO-ADVISOR: ASSOC PROF NIJSIRI RUANGRUNGSI, Ph.D., 180 pp.

Datura metel L. var. *metel* and *Datura metel* L. var. *fastuosa* (Thai name Lamphong- Khaao and Lam-Phong-Ka-Sa-Lak, respectively) are indigenous herb in genus *Datura*, that can be found in Southeast Asia, including Thailand. They have a long history of usage in folkloric medicine as parasympatholytic and mydriatic agents. Their pharmacological actions are due to tropane alkaloids, namely hyoscine (scopolamine) and hyoscyamine/atropine, presented in all parts of the plant. Due to the limited data in these medicinal plants, the purpose of this study is to evaluate the characteristics of *D. metel* L. var. *metel* and *D. metel* L. var. *fastuosa* by microscropic and molecular technique, including their scopolamine contents. According to the results, macroscopic and microscopic analysis of these plants revealed the different morphology and almost similar of stem and midrib cross section. The constant values of leaves (stomatal number, stomata index and palisade ratio), which are the important property for species identification showed different constant numbers. The PCR amplification of ITS region generated the PCR product approximately 670 bp in size, which indicated 99-100% homology. There are two polymorphisms within the 5.8S region, four polymorphisms within the ITS2. The two positions of single nucleotide polymorphism (SNP) were shown at positions 512 and 614 of ITS2 region could be classified these closely related plants.The PCR amplification of *rbc*L, and *atp*B region generated the PCR product approximately 1.5 kp in size, which indicated 95-100% and 94-99 % homology, respectively. In this study, the scopolamine contents of two varieties of *D. metel* L. were compared by TLC image and HPLC method. The comparison of two methods showed good correlation. The results of this study showed the most of scopolamine content in flower part of *D. metel* L. var. *metel*, while *D. metel* L.var. *fastuosa* showed the most of scopolamine content in fruit part, respectively. According to these evidences, the combinations of macroscopic, constant values of leaves and molecular method are able to authenticate these closely related plants and both of them have a potency to be a source of scopolamine production**.**

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

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Co-advisor's Signature.....

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LIST OF ABRIVIATIONS

^{o} C	=	degree Celsius
µg	=	microgram
µg/µl	=	microgram per microliter
µg/spot	=	microgram per spot
µl	=	microliter
µm	=	micrometer
µM	=	micromolarity
ACN	=	acetonitrile
adh	=	alcohol dehydrogenase
AFLP	=	Amplified Fragment Length Polymorphism
AOAC	=	Association of Official Agricultural Chemists
AP-PCR	=	Arbitrarily Primed PCR
AR	=	analytical reagent grade
ArgD	=	arginine decarboxylase
ARMS	=	amplification refractory mutation system
ATP	=	adenosine triphosphate
*atp*B	=	ATP synthase beta subunit
BKF	=	Forrest Herbarium Thailand
bp	=	base pair
CE	=	Capillary Electrophoresis
$CHCl_3$	=	chloroform
cm	=	centimeter
cpDNA	=	chloroplast DNA
CTAB	=	cetyltrimethylammonium (cetrimonium) bromide
DAF	=	DNA Amplification Fingerprinting
DALP	=	Direct Amplification of Length Polymorphism
DAMD	=	Direct Amplification of Minisatellite-region DNA
dATP	=	deoxyadenosine triphosphate
dCTP	=	deoxycytidine triphosphate
ddNTPs	=	dideoxy nucleoside triphosphates

dGTP	=	deoxyguanosine triphosphate
DNA	=	deoxyribonucleic acid
dNTPs	=	deoxyribonucleotide triphosphate
dsDNA	=	double stranded DNA
dTTP	=	deoxythymidine triphosphate
EDTA	=	ethylenediaminetetraacetic acid
ETS	=	external transcribed region
g	=	gram
*gap*A	=	glyceraldehydes-3-phosphate dehydrogenase
GC	=	Gas Chromatography
GC-MS	=	Gas Chromatography-Mass Spectrometry
H6H	=	hyoscyamine 6β-hydroxylase
HCl	=	hydrochloric acid
HPLC	=	High Performance Liquid Chromatography
HPTLC	=	High Performance Thin Layer Chromatography
ICH	=	International Conference on Harmonisation
IGS	=	intergenic spacer
ISSR	=	Inter-Simple Sequence Repeat
ITIS	=	Integrated Taxonomic Information System
ITS	=	intergenic transcribed spacer
KCl	=	potassium chloride
KH_2PO_4	=	potassium dihydrogen orthophosphate
kp	=	kilobase
LC-MS	=	Liquid Chromatography-Mass Spectrometry
LOD	=	limit of detection
LOQ	=	limit of quantification
m	=	meter
M	=	molarity
*mat*K	=	maturase K
mg	=	milligram
mg/g	=	milligram per gram
mg/ml	=	milligram per milliliter

$MgCl_2$	=	magnesium chloride
min	=	minute
ml	=	milliliter
ml/min	=	milliliter per minute
mm	=	millimeter
mM	=	milimolarity
mm^2	=	square millimeter
mtDNA	=	mitochondrial DNA
N	=	normality
Na_2SO_4	=	sodium sulphate
NCBI	=	National Center for Biotechnology Information
nDNA	=	nuclear DNA
NH_4OH	=	ammonium hydroxide
nm	=	nanometer
NTS	=	non-transcribed spacer
ODS	=	octa decyl silane
OrnDC	=	ornithine decarboxylase
PCR	=	Polymerase Chain Reaction
PCR-RFLP	=	Polymerase Chain Reaction-Restriction Fragment Length Polymorphism
PDA	=	photodiode array detector
pgi	=	phosphoglucose isomerase
PMT	=	putrescine N-methyltransferase
PTFE	=	polytetrafluoroethylene
r^2	=	correlation coefficient
RAPD	=	Random Amplified Polymorphic DNA
*rbc*L	=	ribulose 1, 5 bisphosphate carboxylase/oxygenase
rDNA	=	ribosomal DNA
R*f*	=	retention factor
RNA	=	ribonucleic acid
rpm	=	round per minute
RSD	=	relative standard deviation

Rt	=	retention time
SCAR	=	Sequence Characterized Amplified Polymorphic
SD	=	standard deviation
sec	=	second
SNP	=	Single Nucleotide Polymorphism
SPSS	=	statistical package for the social sciences
SSCP	=	Single Strand Conformation Polymorphism
SSR	=	Single Sequence Repeat
Taq	=	*Taq* DNA Polymerase
TBE buffer	=	tris-boric EDTA buffer
TE buffer	=	tris-EDTA buffer
TIF	=	tagged image file
TLC	=	Thin Layer Chromatography
Tm	=	temperature for annealing
TMC	=	traditional Chinese medicine
TPAs	=	tropane alkaloids
TR-I	=	tropinone reductase I
TR-II	=	tropinone reductase II
UV	=	ultraviolet
V	=	volt
v	=	volume
v/v	=	volume by volume
w/w	=	weight by weight
WHO	=	World Health Organization volume
$\overline{X}$	=	mean

CHAPTER I
INTRODUCTION

1 Background and Significance of the Study

Lamphong (Thorn Apples) is a general name for *Datura metel* L. in Thailand. It is an annual herb or perennial undershrub belonging to the family Solanaceae or Nightshade family and has a long history usage in Thai traditional medicine. The Wat Pho texts mention *Datura* as a remedy for many ailments. Powder from the dried seeds of the *Datura* plant is used in small doses to treat fever and as a cerebral tonic. The flower is dried and smoked by asthmatics as a bronchodilator and also curbs nausea. Decoction of the root is also used to treat asthma, as well as bronchitis and cough. Decoction of the leaves is used traditionally to treat mucous or blood in the stool and the juice of the fruit is administered in drops to treat infections of the ear (Punyarajun and Tipduangta, 1981; Salguero, 2003).

All part of this plant contains chiefly tropane alkaloids**,** hyoscine (also known as scopolamine), hyoscyamine, datumetine and atropine. According to these tropane alkaloids, scopolamine is the main constituents and used worldwide in medicine as mydriatic, anticholinergic and parasympatholytic agents that act on the parasympathetic nervous system (Alexander et al., 2008). Because of its widespread occurrence throughout Thailand, it may be considered as an attractive source for the production of medicinally useful tropane alkaloids, particularly scopolamine (Dechatiwongse et al., 1993). These are several methods for analysis of tropane alkaloids including thin layer chromatography (TLC), gas chromatography (GC), high performance liquid chromatography (HPLC), and capillary electrophoresis (CE). Each method has their advantage and disadvantage aspect. Thus development of rapid and simple analytical methods is interested (Tantivatana et al., 1978; 7. Benslimani et al., 2011; Kursinszki et al., 2005; Cherkaoui et al., 1997).

There are two varieties of *D. metel* L., the white variety called *Datura metel* L. var. *metel* (or Lamphong Khaao) and the purple variety called *Datura metel* L. var. *fastuosa* (or Lamphong Kaa-sa-lak) (Smitinand, 1980). They are widely distributed in

South America, tropical area especially Asia and also in Thailand (Avery et al., 1959). Although these two varieties of *D. metel* L. commonly have highly variable morphological characteristics, identification based on morphology is not always conclusive in process material. The use of DNA technology is considered because of the uniqueness of genetic information within the species. The identification using molecular marker has been widely application in medicinal plant variation. It may be necessary to employ more than one DNA region to attain species or variety level discrimination. Combination of two or more candidate DNA regions to yield variety level unique identification is now needed (Houghton and Mukherjee, 2009; สุชาดา, 2553).

In order to clarify whether there are any different between these two varieties of *Datura metel* L. collected in Thailand, microscopic evaluation of transverse section and leaf measurements, molecular evaluation of three candidate DNA markers (ITS, *rbc*L and *atp*B) and scopolamine content evaluation by TLC image method and HPLC method were evaluated and compared.

2. Research Question

2.1 Do the differences of leaf measurement (stomatal number, stomatal index and palisade ratio) between two varieties *D. metel* L. can be distinguished by microscopic evaluation method?

2.2 Do the differences of Internal Transcribe Spacer (ITS), *rbc*L and *atp*B sequences of the two varieties plant can be discriminated by molecular evaluation?

2.3 Do the differences of scopolamine content of the two varieties *D. metel* L. can be distinguished by phytochemical evaluation?

3. Objectives of the Study

3.1 To study of leaf constant value of stomatal number, stomatal index and palisade ratio between *D. metel* L. var. *metel* and *D. metel* L. var. *fastuosa*

3.2 To study the ITS, *rbc*L and *atp*B sequence between *D. metel* L. var. *metel* and *D. metel* L. var. *fastuosa*

3.3 To study the scopolamine content by thin layer chromatography (TLC) image and high performance liquid chromatography (HPLC) method between *D. metel* L. var. *metel* and *D. metel* L. var. *fastuosa*

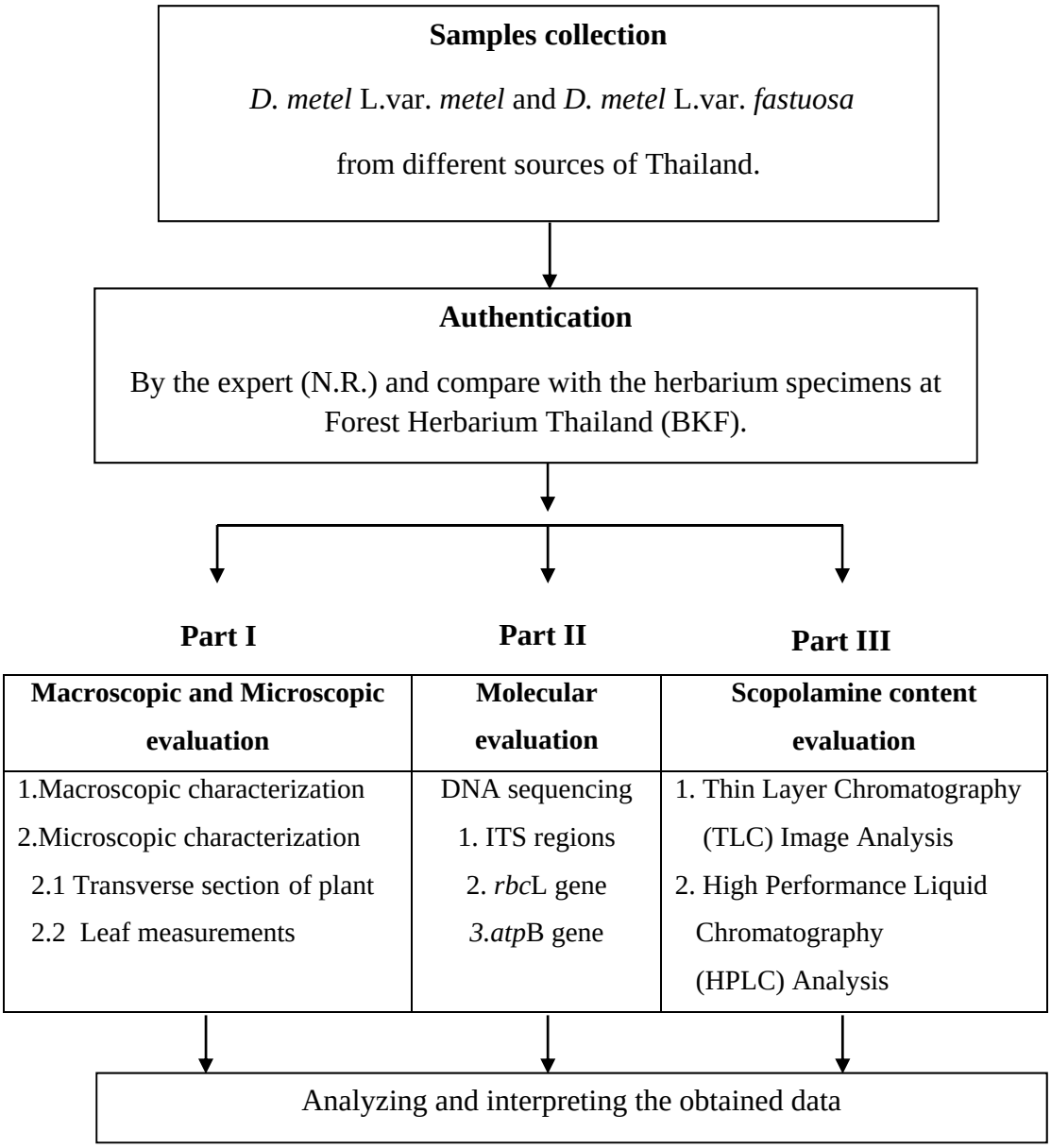
4. Contributions of the study

4.1 Evaluate of leaf measurements value which are composed of stomatal number, stomatal index and palisade ratio can be used for discriminating and comparing the differences between *D. metel* L. var. *metel* and *D. metel* L. var. *fastuosa*.

4.2 Sequence variation of ITS, *rbc*L and *atp*B can be developed for further study such as PCR-RFLP, DNA fingerprinting, DNA barcode and submitted to GenBank for public nucleotide data searching.

4.3 The scopolamine content of the two varieties of *D. metel* L. using thin layer chromatography (TLC) image and high performance liquid chromatography (HPLC) analysis can be used as phytochemical fingerpringting.

5. Conceptual framework



CHAPTER II

REVIEW OF RELATED LITERATURE

1. Botanical aspect of *Datura metel* L.

The genus *Datura* belongs to the tribe Datureae and the family Solanaceae. This family is also extremely important as a source of drugs in medicine, pharmacology but many are poisonous when used in excess. The generic name of *Datura* was first used by Linnaeus (1737). This genus comprises of 10-12 species occurring the tropical and warm temperature regions of the world (Avery et al., 1959). Of these, several are found in warmer part of the globe and several others are occasionally cultivated for their great trumpet-like odorous flowers but some are widespread weeds. *Datura metel* L. is the most common garden *Datura*, also run wild and naturalized. The plant is found in open waste land throughout Thailand. It occurs in all parts of Thailand even in the waste land and roadside (Ratana Teeyapant, 1987).

In 1753, Linnaeus wrote his first description about *Datura metel* L and validly published.in his first edition of Species Plantarum. It is based on the Hortus Cliffortianus name. He stated that it came from Asia and Africa and has spread to all other parts of the world (Avery et al., 1959).

According to Safford (1921) the member of genus *Datura* were divided into four sections; I. *Stramonium* II. *Dutra* III. *Ceratocaulis* and IV. *Brugmansia* (Safford, 1921).

Section I. *Stramonium* (Tournefort) Bernhardi.

The species belonging to this section were

D. stramonium L.

- *D. stramonium* L. var. *tatula*
- *D. stramonium* L. var. *inermis*
- *D. stramonium* L. var. *laevis*
- *D. stramonium* L. var. *bertolonii*

D. ferox L. (synonym : *D. Stramonium ferox* Boccone.)

D. quercifolia H.B.K.

Section II. *Dutra* Bernhardi

Six species belong to this section were

D. pruinosa Greenm.

D. leichhardtii Muell.

D. meteloides DC. in Dunal.

D. metel L.

- *D. metel* L. var. *fastuosa*

- *D. metel* L. var. *metel* (synonym : *D. alba* Numph. Ex. Nees.)

D. discolor Bernh.

D. innoxia Mill.

Section III. *Ceratocsulis* (Spach.) Bernhardi.

There is only the single species in this section

D. Ceratocaulis Ort.

Section IV. *Brugmansia* (Persoon) Bernhardi.

The species forming the section *Brugmansia* were first placed in a distinct genus by Persoon (1805). The taxonomy of the *Brugmansia* is rather complicated. They are widely cultivated as ornamentals. Its range of distribution is widely spread in tropical and subtropical regions. They produce large, white or colored trumpet-shaped flowers.

In 1983, Hammer, Romeike, and Titel worked out a dichotomous key. This key also enables the plant enthusiast to classify the different species with relative certainty. The properties used for classification are explained briefly below:

Table 1. The dichotomous key of classifying the different of *Datura* species.

If this statement is true		Then, go to
1	Plants are like trees, flowers pendulous or nodding	*Brugmansia*
1’	Plants are wood-like, partly woody flowers are upright	2
2	Fruits hang downwards	3
2’	Fruits are upright sec. *Datura*	8
3	Fruits are bald, when ripe fall apart irregularly sec. *Ceratocaulis*	*D.ceratocaula*
3’	Fruits are spiny or have conical humps sec. *Datura*	4
4	Fruits open regularly, four flaps	*D.discolor*
4’	Fruits, when ripe, fall apart irregularly; rarely fall as a whole	5
5	Fruits have conical humps	*D.metel*
	Flowers are white or yellow	a
	Flowers are violet to red (at least partly)	c
	a Flowers are simple, white	var. *metel*
	a’ Flowers are double	b
	b Flowers are white	var. *muricata*
	b’ Flowers are yellow	var. *chlorantha*

	c Flowers are single violet	var. *rubra*
	violet	f. *rubra*
	red	f. *sanguinea*
	c' Flowers are double	d
	d Flowers are single color, violet or red	var. *obscura*
	violet	f. *obscura*
	red	f. *atropurpurea*
	d' Flowers (outer) are violet or red,	
	(inner) white	var. *fastuosa*
	(outer) violet	f. *fastuosa*
	(outer) red	f. *malabarica*
5'	Fruits usually have sharp piercing spines	6
6	Flowers are relatively small, up to 3 in (7 cm) long, usually few opening	*D. leichhardtii*
	Plants are usually taller than 18 in (0.5m), leaves and shoots are lightly furry	ssp. *leichhardtii*
	Plants are not usually taller than 18 in (0.5m), leaves and shoots are lightly furry	ssp. *pruinosa*
6'	Flowers are relatively large, more than 4 in (10 cm) long	7
7	Interacuminal peak is extremely short, flower edge is evenly rounded, flowers in upper section are usually violet or pale violet, seeds are yellowish	*D. wrightii*
7'	Interacuminal peak is longer, flowers edge is wavy, seeds are medium brown	*D. inoxia*
8	Fruits is either bald or covered with spines, all of which are almost the same length	*D. stramonium*
	a Plants are green, flowers are white	b
	a' Plants are anthocyanin colored, flower are violet	c
	b Fruits is spiny (sometimes bald and spiny fruit on one plant)	var. *stramonium*
	Fruits is all spiny	f. *stramonium*
	Bald and spiny fruit on one plant	f. *labilis*
	b' Fruit without spines	var. *inermis*
	c Fruit spin	var. *tatula*
	Anthocyanin coloring is less noticeable	f. *tatula*
	Anthocyanin coloring is very noticeable	f. *bernhardii*
	c' Fruit does not have spines	var. *godronii*
8'	Spines are very strong, longer in upper part of the fruit	9
9	Upper spines are almost as long as the fruit capsule, leaves are toothed irregularly, undulated	*D. ferox*
9'	Upper spines are about a third of the length of the fruit, leaves deeply undulated, lobed	*D. quercifolia*

Classification Key
sec. = section, section, var. = varietas,variety, spp. = sub-species, f. = forma,form
(Source: Preissel, U., and Preissel, H. G. 2002).

Taxonomy of the genus *Datura*

The taxonomic hierarchy of the genus *Datura* can be classified as follow: (Integrated Taxonomic Information System, ITIS; The NCBI taxonomy database; [Online] Available from http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=30513 [2012, July 26].

Kingdom Plantae
Subkingdom Viridaeplantae
Infrakingdom Streptophyta
Division Tracheophyta
Subdivision Spermatophytina
Infradivision Angiospermae
Class Magnoliopsida
Superorder Asteranae
Order Solanales
Family Solanaceae
Tribe Datureae
Genus *Datura* L.

***Datura metel* L.**

D. metel L. have two varieties, the white variety called *Datura metel* L. var. *metel* or *Datura alba* Nees and the purple variety called *Datura metel* L. var. *fastuosa* (Bernh.) Danert are known as "white" and "black" *Datura* respectively. The former of two varieties can be distinguished from each other by the number of corolla of a flower, main vein, and the color of the stem and flower (Safford, 1921; Haegi, 1976).

D. metel L. is the only one species of genus *Datura* naturally found in Thailand. *Datura metel* L. var. *metel* ,Thai name is called "Lamphong Khaao" while, *Datura metel* L. var. *fastuosa* (Bernh.) Danert, Thai name is called "Lumphong Kaasalak".

D. metel L. var. *metel* (synonym: *Datura alba* Nees)

Vernacular names: Ma-Khuea-Ba or Mad Egg-plant (Northern, Northeastern), Mang-To-Lo (Chinese-Bangkok), La-Ang-Ka (Suai-Surin), Lam-Phong-Khao (Central), Liak (Khmer-Surin), Thorn Apple (Smitinand, 1980).

D. metel L. var. *fastuosa* (Bernh) Danert (synonym: *Datura fastuosa* L.)
Vernacular names: Ka-Salak (Lampang), Lam-Phong-Ka-Lak (Chumphon, Surat Thani), Lam-Phong-Ka-Sa-Lak (Central, Sukhothai), Ma-Khuea-Ba-Dok-Dam (Lampang) (Smitinand, 1980).

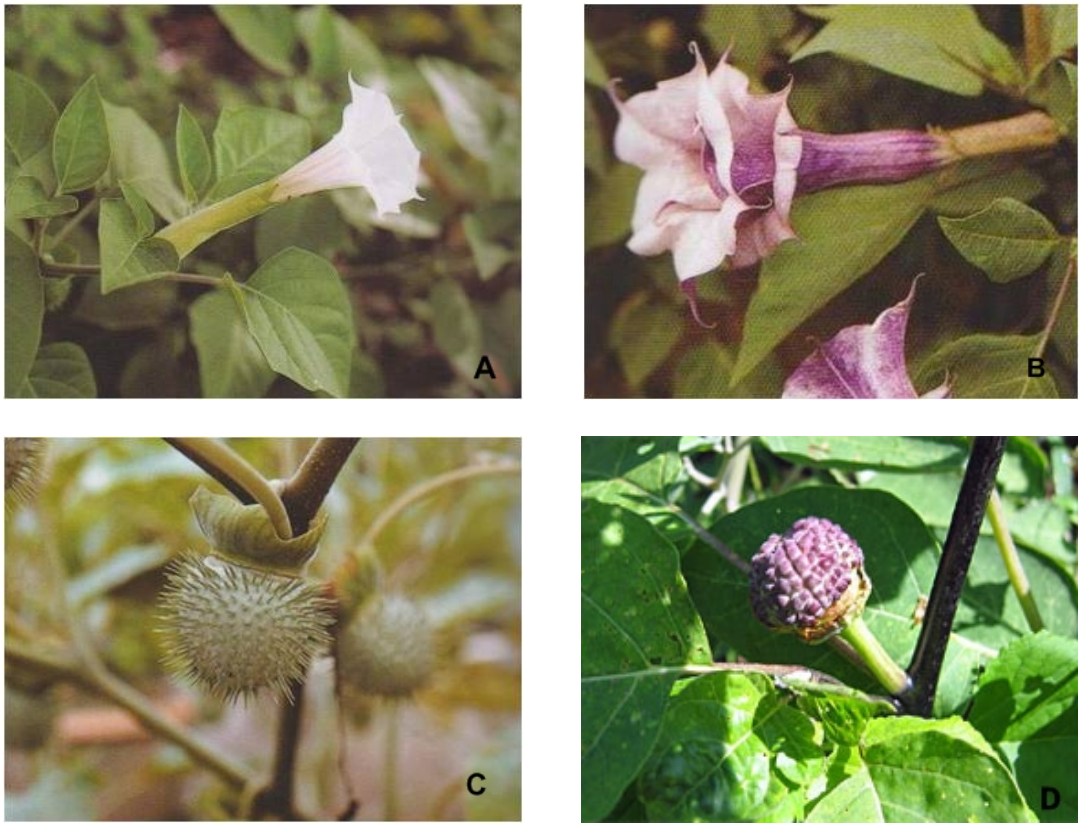


Figure 1. Comparison the visible parts between *D. metel* L. var. *metel* (A: flower, C: fruit) and *D. metel* L. var. *fastuosa* (B: flower, D: fruit)

Plant description

" *Datura metel* L. is an annual herb. It is an erect, branched, short shrub and reaches its mature size of 1.5-3 ft (0.5-1m) tall after a few months. The plants are slightly furry and the shoots are usually dark violet in color. The oval to broad oval, undulate or coarsely toothed leaves often have the same coloring. The flower, which are immensely varied, are most conspicuous and most interesting characteristic **(Figure 1)**. During the day they emit a pleasant scent. According to variety and shape they can be single or double, five to nine peaks and can be colored pure white, cream, yellow, purple or purple outside and white inside. Its large, funnel-shaped flower come in a wealth of colors and

shapes. Danert (1954) differentiated into 11 different groups. Corolla trumplet-shaped, simple, double, or triple by the irregular petaloid outgrowth of the stamens and inner corolla surface; about 14 to 15 cm long. Calyx regular, 5 to 7 cm long, evenly five-lobed, less than half as long as the corolla. Style 11 to 13 cm long. Capsule globose, inclined, 4 to 6 cm in diameter, covered with very short spines or tubercles. Leaves ovate, nearly entire or with a few teeth. Stems green in forms with white or yellow flowers and purple in those with purple flowers. Leaf scars conspicuous on the stems. Stem erect, about 0.3 meter in some varieties, reaching to 1.5 meters in others. Stem and leaves glabrous. The very short spines or tubercles on the capsules and the glabrous condition of the stem and leaves are the main characters that distinguish this species from the other large species of the Dutra section. *D. metel* L. has conspicuous, heart-shape cuts in place of the interacuminal peaks. These make decorative dividers on the large flowers that are 6-8 in (14-20 cm) in size. The upright, ovate- to round-shaped fruit capsule develops after successful pollination. Its surface is lightly furry and it covered with numerous conical humps and a few spines. After the ripening period, the capsule falls apart irregularly and releases between 200-300 seeds. They are colored dark to brownish yellow and have a conspicuous elaiosome. The mass of a thousand seeds weighs nearly 15-20 g" (Preissel, 2002).

Distribution

The type locality of *D. metel* L. is Asia. The range of distribution includes tropical and subtropical Asia, Africa, and America. The plant is often cultivated throughout warm regions of the world (Avery, 1959).

Chemical constituents studies from *D. metel* L.

The major isolated compounds of *D. metel* L. were tropane alkaloids such as scopolamine (hyoscine), hyoscyamine, and atopine. The alkaloids found in this entire plant are present in **Table 2**.

Table 2. Alkaloids found in *D. metel* L.

Plant part	Alkaloids
Stem	Scopolamine, Hyoscyamine, Meteloidine, Atropine
Seed	Hyoscyamine, Scopolamine, Isoquinoline alkaloid, 7-hydroxy-3,6-Ditigloyloxytropane, Atropine
Flower	Anisodamine,Aatropine, Isoquinoline alkaloid, Hyoscyamine, Tropine
Leave	Scopolamine, Atropine, Datumitine, Isoquinoline alkaloid, Hyoscyamine
Root	Hyoscyamine, Littorine, Dopamine, Scopolamine, Tropine, Pseudotropine, Tigloidine, Cuskhygrine, 3,6-Ditigloyloxytropane

Beside tropane alkaloids, steroids and flavonoids were also isolated from the arial parts of *D. metel* L. such as: Datumetelin, *Datura*metelin A-F, *Datura*metelin G-Ac, Daturiline, Daturilinol, Physalindicanol A, Quercetine, Stigmastero, β-Sitosterol, Withametelin, WithametelinB, Withametelin B-Ac, Withastramonolide, and 12-Deoxywithastramonolide (Sirichan Pattanapongsirikul, 2002).

In many other species of *Datura* (e,g. *D. ferox; D. metel; D. meteoides*), scopolamine is the principle alkalid of the leaves at all time and these species are used to isolate scopolamine (Muhtadi, 1990). In Thailand, Scopolamine is the one of main constituents which found in *D. metel* L. (Tantivatana et al., 1978; Dechatiwongse et al., 1993).

Traditional Use

The *D. metel* L. plant has been well known for its use in traditional Chinese and Indian systems of medicine for centuries as a narcotic, anodyne and antispasmodic (Rajesh-Sharma, 2002). In South and Central America where the greatest concentration of solanaceous plants exists, a wide range of species is used, although *Datura* species and *Solanum* species are particularly important (Ayensu, 1981). *D.metel* L. is different importance in different societies as can be shown on **Table 3**.

Table 3. Traditional used of *D. metel* L.

Country/Continent	Species	Uses
South/Central America	*Datura metel* L.	Anaesthetic, wounds, and bruises, arthritis, ulcers, prolapse haemorrhoids, neuralgia, fever, asthma, flu, headache and tumours
Africa	*Datura fastuosa* L.	Abortifacient
China	*Datura metel* L.	Cough, asthma, analgesic
India	*Datura metel* L.	Headache, asthma, leprosy, sores, epilepsy, convulsion, veneral disease, mumps.

(Source: James, 1991)

Medicinal and Pharmacological activities

All parts of *Datura* plants contain dangerous levels of tropane alkaloids (highly poisonous) and may be fatal if ingested by humans or other animals, including livestock and pets. *D. metel* L. may be toxic if ingested in a tiny quantity, symptomatically expressed as flushed skin, headaches, hallucinations, and possibly convulsions or even a coma. The principal toxic elements are tropane alkaloids. Accidentally (or intentionally) ingesting even a single leaf could lead to severe side effects (Quisumbing, 1951; Alexander et al., 2008; Phua et al., 2008).

All parts of *D. metel* L. (leaves, flowers, seeds and roots) have a long history of usage in folkloric medicine. These parts possess narcotic, anodyne, antispasmodic properties and are useful in neuralgia and antispasmodic (Hahn, 2003).

Datura possesses properties analogous to those of Belladonna which due to their similar active constituents. Juice of *Datura*' s leaves and causes dilution of pupil. Leaves and flowers are cut into small pieces and smoked to relieve the attack of bronchial asthma. Many usages are also claimed in folkloric medicine (Sezik et al., 1992).

Datura acts chiefly by virtue of its tropane alkaloids presented, hyoscine and hyoscyamine/atropine. Both are of considerable pharmaceutical interests because of their parasympatholytic, anticholinergic, antiemetic and sedative actions are antispasmodic and antisecretory which make uses of *Datura* and / or its constituents in many pharmaceutical

preparations. Atropine sulfate is antidote for poisoning caused by cholinesterase inhibitors such as organophosphate insecticides (Bliss, 2001).

Hyoscine, like hyoscyamine, is a potent mydriatic. Its action resembles hyoscyamine in peripheral actions but differs greatly in its central effects. Hyoscine is a central depressant with sedative and tranquilizing properties (Shutt and Bowes, 1979). Hyoscine or scopolamine has been used with morphine in acute mania and delirium, including delirium tremens. It has been tried for the relief of withdrawal symptoms in various treatments of morphine dependence (Bowler et al., 1944). It has also been used in the symptomatic treatment of idiopathic and post-encephalitic parkinsonism (Reynolds, 1982).

D. metel L. is popular all over the world for its medicinal uses. It is known for its use in fever with catarrh, cerebral complications, diarrhea, skin diseases, antiseptic, animal bites, anti helmenthic and in herpetic diseases and also has healing potential on burn wounds (Phiya et al., 2002). It is also known for its antibacterial activity against burn pathogens (Gnanamani et al., 2003), antifungal activity against phytopathogens (Dabur et al., 2004) and herbicidal activity against *Phalaris minor* Retz., one of the most problematic weeds of wheat (Javaid et al., 2008).

2. Method in plant authentication

The major methods employed in the authentication of herbal materials are macroscopic and microscopic examination, and chromatography. These methods are rapid and inexpensive. The chemical analysis is one of the best methods for the detection of active ingredients or contaminants that can be used for plant identification. Nowadays, the authentication of plant using bio-molecular methods is widely useful for assortment of medicinal plants (Zhao et al., 2006).

2.1 Macroscopic evaluation

Macroscopic evaluation method is an assessment of plant material, either with the naked eye or with a hand lens or stereo-microscope. It typically includes gross morphological characteristic or organoleptic sensation is used to determine the color, odor, taste, form, size, shape, *etc*. of plant material, so the similar species of plant can share similar morphological characteristics and appropriate training is needed to acquire the macroscopic identification skills.

2.2 Microscopic evaluation

Microscopic evaluation method of medicinal plant is based on the observation of the cellular structure, and their content of plant material by use of a microscope. It reveals plant histological characteristics (Trease and Evans, 2009). A number of leaf measurements are used to distinguish between some closely related species not easily characterized by general microscopy.

Determination of stomatal number and stomatal index

Stomata are openings (the stomata pores or apertures) epidermis bounded by two specialized epidermal cells, the guard cells, which by changes in shape bring about the opening and closure of the aperture. It is convenient to apply the term stoma to the entire unit, the pore and the two guard cells. The structure of the epidermis and stomata are of first importance in the microscopical identification of leaves. The stomata may be surrounded by cells resembling the other epidermal cells that differ in shape and sometimes also in content from the ordinary epidermal cells. These distinct cells are called subsidiary cell of the stoma. The subsidiary cells may or may not be closely relate onto genetically to the guard cells (Eames and MacDaniels, 1974).

The stomatal number and the stomatal index are the very specific criteria for identification and characterization of leafy crude drugs. In the mature leaves, four significant types of stomata are distinguished by their form and the arrangement of the surrounding cells, especially the subsidiary cells (WHO, 1998).

- The anomocytic or ranunculaceous (irregular-celled) type; the stoma is surrounded by a varying number of cells, generally not different from those of the epidermis (**Figure 2a**).
- The anisocytic or cruciferous (unequal-celled) type; the stomata is usually surrounded by three or four subsidiary cells, one of which markedly small than the other (**Figure 2b**).
- The diacytic or caryophyllaceous (cross-celled) type; the stomata is accompanied by two subsidiary cells, the common wall of which is at right angle to the stoma (**Figure 2c**).
- The paracytic or rubiaceous (parallel-celled) type; the stomata has two subsidiary cells, of which the long axis of the stomata (**Figure 2d**).

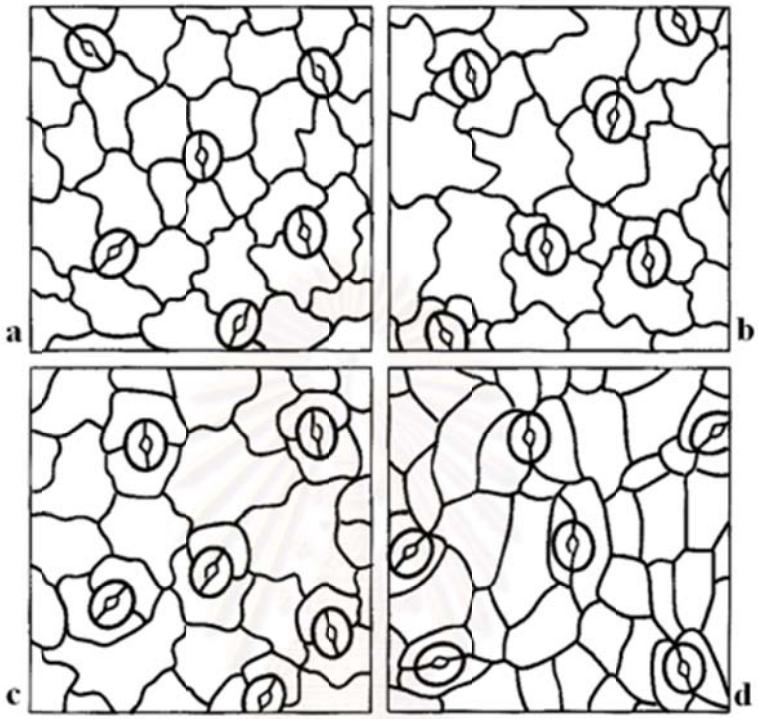


Figure 2. Surface view of epidermis illustrates four patterns of stomata type. a: anomocytic; b: anisocytic; c: diacytic; d: paracytic (WHO, 1998)

- **Stomatal number**

Stoma is another type of epidermal structure processing great diagnostic value. A stoma consists of two similar cells, the guard cells, placed with their long axis parallel and having a small cellular space, the porous between them. By variations of the turgidity of the guard cells, the size of porous is altered. In surface view the guard cell often appear crescent shaped, their concave faces being adjacent to one another. During the formation of stomata, the cell cuts off from the mother cell often acquire a shape and size differing from those of the other epidermal cells and are therefore termed the subsidiary cells. The two guard cells and the porous counted as 1 cell stoma. The average number of stomata per square millimeter of epidermis is termed the stomata number. In recording results the range as well as the average value should be recorded or each surface of the leaf and the ratio of values for the two surfaces. The actual number of stomata per square millimeter is variable for the same plant, this being especially noticeable if records are made for different years. In certain cases this

ratio may be of diagnostic importance (Wallis, 1960; Eames and MacDaniels, 1974; Trease and Evans, 2009).

Stomatal number is the number of stomata per unit area of leaves. It was designed by Timmerman, (1927) (Youngken, 1948).

$$\text{Stomatal number} = \frac{\text{Number of stomata}}{\text{Area of epidermal cell (mm}^2\text{)}}$$

- **Stomatal index**

The significance of the number of stomata per unit area of leaf was investigated by Timmerman in 1927. Salisbury showed that a high correlation coefficient exists between the number of stomata and the number of epidermal cell per unit area of leaf surface of a given species. Stomatal index is the percentage proportion of the ultimate divisions of the epidermis of a leaf which have been converted into stomata (Youngken, 1948).

In other words, stomatal index is defined as the percentage of stomata from the total number of epidermal cells, which can be explained as:

$$\text{Stomatal index} = \frac{S}{E+S} \times 100$$

Where; S = the number of stomata per unit area.

E = the number of ordinary epidermal cells in the same unit area.

Stomatal number varies considerably with the age of the leaf, stomatal index is highly constant for a given species and many be determined on either or powdered samples (Trease and Evans, 2009).

- **Palisade ratio**

Palisade cells are a type of photosynthetic cells of the mesophyll of leaf occurring mostly just beneath the upper epidermal surface layer (**Figure 3**) (Wallis, 1960). The cells are elongated and more cylindrical and arranged in one or more rather regular, relatively compact layer near the ventral, or upper side of the leaf with

the long axis of the cells perpendicular to the leaf surface (Eames and MacDaniels, 1974).

The term “palisade ratio” was introduced by two British pharmacognosists, T.E. Wallis and T. Dewar, in 1933. It represents a figure obtained by counting the total number of palisade cells beneath four upper epidermal cells and dividing the number by four (Youngken, 1948). The average number of palisade cells beneath each upper epidermal cell is termed the palisade ratio. Fine powders can be used for the determination (Trease and Evans, 2009). This value remains constant within a range a range for a given plant species and is of diagnostic value in differentiating the species. This value does not alter based on geographical variation and differs from species to species and that is why it is a very useful diagnostic feature for characterization and identification of different plant species (Mukherjee, 2007).

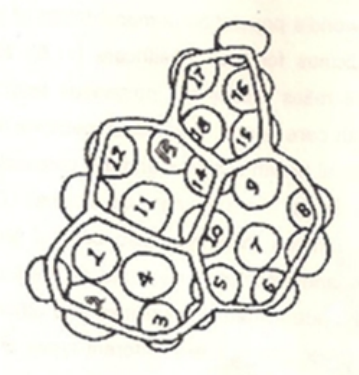


Figure 3. Four upper contiguous epidermal cells with underlying palisade cells in surface view

2.3 Molecular evaluation

The molecular marker is used as a marker for analysis of genetic diversity and relatedness between or within different populations, species, and individuals (Weising et al., 2005). Benefiting from molecular cloning and polymerase chain reaction (PCR) techniques, DNA technique has now become a popular mean for identification and authentication of plant and animal species. Not only for generating the diversity of plants, it can be also applied for detecting of the adulteration in herbal drugs by the advantages of plants DNA identification. DNA technology provides a useful and independent tool to complement chemical analyses for the authentication and quality assurance of medicinal materials. DNA-based markers are less affected by age, physiological condition of

samples and environmental factors. They are not tissue-specific and thus can be detected at any phase of organism development. Only a small amount of sample is sufficient for analysis and the physical form of the sample does not restrict detection (Shaw et al., 2002).

Prior to investigate the plants by molecular method, the genomic DNA is needed to isolate from the plants' cells. Accordingly, an enormous number of plant DNA isolation protocols (and modifications of existing procedures) have been published. The majority of methods aim at isolating total cellular DNA, which is a suitable substrate for almost all PCR-based marker methods. However, there are also numerous protocols that are specifically designed for the isolation of nuclear DNA (nDNA), chloroplast DNA (cpDNA), and mitochondrial DNA (mtDNA), respectively. Plant DNA isolation methods differ in many respects, including the disruption of tissues and cells, the composition of extraction and lysis buffers, and in the way that DNA is purified from other cell ingredients (such as protein, RNA, membranes, polysaccharides, and polyphenols) (Weising et al., 2005). Besides the commercial instant DNA extraction kit, isolation DNA by standard CTAB procedure is considered to be a widely isolation method for DNA preparation, that is sufficiently pure for PCR analyses in many plant species (Sahoo, 2003). There are several regions in the DNA from various origins, which were used for studying the divergence or identity of plants.

Plant genome is all the genetic material in the plant cells, which contain the nuclear genome and organelle genome. Organelle genome can be divided into two parts: the chloroplast genome or chloroplast DNA, and mitochondria genome or mitochondria DNA. Plant genome covers the genic DNA, which act as proteins synthesis for the cell, and the non-genic DNA, which is mainly found in the genome and it's not clear what its role (สุชาดา สุขหร่อง, 2553). The obtained genomic DNA is then used as a DNA template for amplification. There are several regions in the plant genome can be used for studying the divergence or identity of plants, such as;

Nuclear genome

Nuclear genome (nDNA) is a linear DNA packed closely on the chromosome. It is the largest components in the nucleus. Nuclear genome is composed of information

inherited equally from both parents (heterozygous) and mostly used in taxonomic studies (Chase et al., 2005).

Ribosomal DNA (rDNA)

ribosomal DNA has several properties that make it useful for studying genetic variability and divergence within and between species: tandemly repeated genes, secary structure of transcribed regions, differential rates of evolution between spacers and coding regions, and concerted evolution. rDNA consists of a tandem repeat of a unit segment, an operon, composed of non-transcribed spacer (NTS), external transcribed region (ETS), 18S, ITS1, 5.8S, ITS2, and 28S tracts (Richard et al., 2008).

- **Internal Transcribed Spacers (ITS)**

Internal transcribed spacers (ITS) are sequences located in angiosperm 18S-26S ribosomal DNA genes, including ITS1 (between 18S and 5.8S rRNA coding regions), 5.8S, and ITS2 (between 5.8S and 26S rRNA coding regions) of the nuclear ribosomal DNA (**Figure 4**). The ITS region is highly repeated in the plant nuclear genome which is present in the form of up to many thousands of copies arranged in tandem repeats (Souframanien et al., 2003).

The studies of restriction site variation in the ribosomal DNA (rDNA) in populations of animals and plants by Gerbi (1985) indicated that the spacer regions are high variable while the coding regions are conserved (Gerbi, 1985). In angiosperm systematics, sequences from the rapidly evolving ITS region have had similar impact on the understanding of interspecific and intergeneric relationships, while also yielding insights on speciation and biogeography. Moreover, the divergent ITS sequences have been widely used for plant phylogenetics (Alejandro et al., 2005).

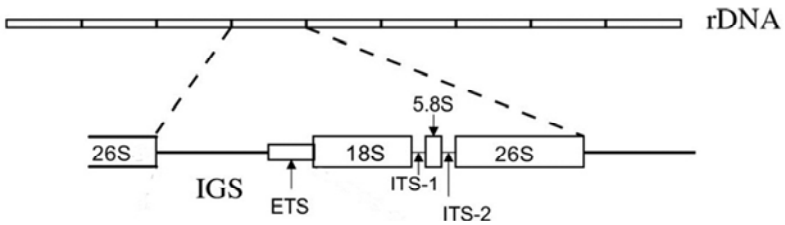


Figure 4. The ITS region which separated by intergenic spacer (IGS)

The ITS region is now the most widely sequenced DNA region. It has typically been most useful for molecular systematics at the species level and even within species. Because of its higher degree of variation than other genic regions of rDNA, variation among individual rDNA repeats can sometimes be observed within both the ITS and IGS regions (White et al., 1990).

Table 4. The universal primers have been designed for ITS amplification

primer name	Direction	sequence (5'->3')	Tm (°C)
ITS1	Forward	TCCGTAGGTGAACCTGCGG	65
ITS2	Reverse	GCTGCGTTCTTCATCGATGC	62
ITS3	Forward	GCATCGATGAAGAACGCAGC	62
ITS4	Reverse	TCCTCCGCTTATTGATATGC	58
ITS5	Forward	GGAAGTAAAAGTCGTAACAAGG	63

(Source: White et al., 1990)

Other regions in nuclear genome that are used in evolution analysis of plants but are not generally used in DNA fingerprint in herbal drug such as phy gene (phytochrome), *gap*A gene (glyceraldehydes-3-phosphate dehydrogenase), adh gene (alcohol dehydrogenase) and pgi gene (phosphoglucose isomerase) (สุชาดา สุขหร่อง, 2553).

Chloroplast genome

The chloroplast genomes (cpDNA) of several plants have been completely sequenced, leading to the identification of many of the genes contained in the organelle DNAs. These chloroplast genes encode both RNAs and proteins involved in gene expression, as well as a variety of proteins that function in photosynthesis. The genomes of chloroplasts consist of circular DNA molecules present in multiple copies per organelle, ranging from 120 to 170 kb, and there is a relatively high degree of conservation in size, structure, gene content, and linear order of the genes in land plants (Downie and Palmer, 1992). The regions of chloroplast genome that commonly used in DNA fingerprint of herbal drug such as;

- **The *atp*B gene**

The *atp*B gene is located in the large single-copy region of the chloroplast genome contiguous with *atp*E gene and downstream from the *rbc*L gene, from which it is separated by an approximately 900 bp intergenic spacer region (**Figure 5**). The *atp*B gene encodes the β subunit of ATP synthase (other subunits are encoded in either the chloroplast or the nuclear genomes). ATP synthase has a highly conserved structure that couples proton translocation across membranes with the synthesis of ATP (Zurawski et al., 1982; Gatenby et al., 1989). Many features of the *atp*B gene suggest that it may be valuable for comparative sequence studies at higher taxonomic levels. It is short enough (1497 bp) for ease of sequencing but long enough to be potentially phylogenetically information, given broadly comparable rates of evolution to *rbc*L (Wolfe, 1991; Hoot et al., 1995).

- **The *rbc*L gene**

The *rbc*L gene (a single copy gene is approximately 1430 base pairs in length) is free from length mutations except at the far 3' end, and has a fairly conservative rate of evolution. The function of the *rbc*L gene is to code for the large subunit of ribulose 1, 5 bisphosphate carboxylase/oxygenase (RUBISCO or RuBPCase). The sequence data of the *rbc*L gene are widely used in the reconstruction of phylogenies throughout the seed plants. It's a gene involved in catalyzing the primary chemical reaction by which inorganic carbon enters the biosphere which is first major step of carbon fixation. This gene has slow substitution rate and extensive database of sequences make *rbc*L sequence data well suited for phylogenetic studies at a variety of higher taxonomic levels, from interfamilial to subclass (Donoghue et al., 1993; Frascaria et al., 1993).

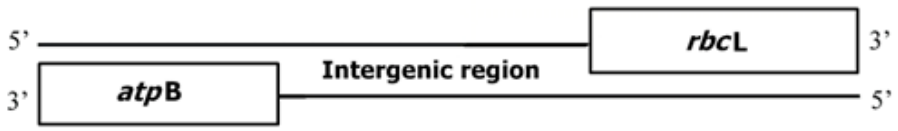


Figure 5. Structure of *rbc*L gene and *atp*B gene including the DNA (intergenic region) flanking between *atp*B and *rbc*L gene

- **The *mat*K gene and *trn*K gene**

The *mat*K gene (Maturase K, approximately 1550 base pairs), is located within the intron of the chloroplast gene *trn*K (**Figure 6**). This gene can encode to enzyme maturase which presumably helps fold the intron RNA into the catalytically-active structure (Hilu and Liang, 1997; Muller et al., 2006). In plant molecular systematics and evolution, the *mat*K gene is emerging as another valuable gene to study because of its reasonable size, high substitution rate, evenly distributed codon position variation, low transition and transversion ratio, and the easiness of amplification due to its two flanking coding *trn*K gene. The *mat*K gene has fast evolution that, it's not possible to be used as the universal primer (Johnson et al., 1996).

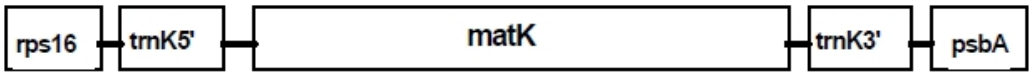


Figure 6. Structure of *mat*K gene which flanking between *trn*K gene

Other chloroplast genomes are also used for investigating the herbal plants such as; gene *ndh*F, the region in the area of gene *trn*T, *trn*L, *trn*F, and intergenic region of *trn*H-*psb*A gene (สุชาดา สุขหร่อง, 2553).

Mitochondrial genome

Mitochondrial genome (mtDNA) in plants is normally depicted as a circular molecule and located in mitochondria which is involves in converting the chemical energy from food into a form that cells can use, adenosine triphosphate (ATP). The plant mitochondrial genome is very large and highly variable in size between species, moreover, substitute rate of the nucleotide in plants mitochondrial genome is slower than those of animals approximately 40-100 times and slower than those of nuclear genome and chloroplast genome around 12 and 3-4 times, respectively. Thus this mitochondrial genome for comparative sequencing is rather limited or rarely used in authentication of herbal plants (Palmer, 1992).

To amplify the desire region, polymerase chain reaction (PCR) is commonly used.

The polymerase Chain Reaction

The polymerase chain reaction (PCR) is an *in vitro* technique, which was invented by Kary B. Mullis in 1983. The PCR technique allows the amplification of a specific deoxyribonucleic acid (DNA) region that lies between two regions of know DNA sequence by the simultaneous primer extension of complementary strands of DNA. Million copies of the target DNA sequence can be synthesized from a low amount of starting DNA template within a few hours. The PCR reaction component consist of genomic DNA used as DNA template for copies, a pair of primers for amplified in target sequences, dNTPs (dATP, dCTP, dGTP and dTTP), buffer and *Taq* DNA polymerase. The amplification reaction consists of three steps; (1) denaturation of dsDNA at high temperature, (2) annealing to allow primers to form hybrid molecules at the optimal temperature, and (3) extension of the anneal primers by heat-stable *Taq* DNA polymerase (**Figure 7**). The cycle is repeated for 20-40 times. Finally, the amplification products are examined by electrophoresis (Mullis and Faloona, 1987). The essential step in each cycle is thermal denaturation of double stranded target molecules, primer annealing to both DNA strands and enzymatic synthesis of DNA (Vosberg, 1985). Primers are short, single stranded DNA molecules between 20 – 30 nucleotides in length, which are complementary to the ends of a defined sequence of DNA template. A DNA polymerase in the presence of deoxynucleoside triphosphate (dNTPs) extends the bond primers on single-stranded denatured DNA template under suiTable reaction conditions (Erlich, 1989; Newton and Graham, 1994). This results in the synthesis of new DNA strands complementary to the template strands. These strands exist at this stage as double stranded DNA, annealing of primers by cooling the mixture and primer extension by DNA polymerase at a temperature suiTable for enzyme reaction. Each repetition of strand synthesized becomes a template for any further cycle of amplification and so the amplified target DNA sequence is selectively amplified cycle after cycle (Figure 7). This technique is capable of synthesis over a million copies of a specific target DNA sequence in a few hours. The products of a successful first round of amplification are heterogeneously sized DNA molecules, whose lengths may exceed the distance between the binding sites of the two primers. In the sec round, these molecules generate DNAs of defined length that will accumulate in an exponential fashion in later rounds of amplification and will form the dominant products of the reaction. Although longer

molecules continue to be produced from the original template DNAs in every round, they accumulate only at a linear rate and therefore do not contribute significantly to the final mass of target sequence (Sambrook et al., 1989). This results in the exponential accumulation of the specific target fragment at approximately 2^n, where n is the number of cycles.

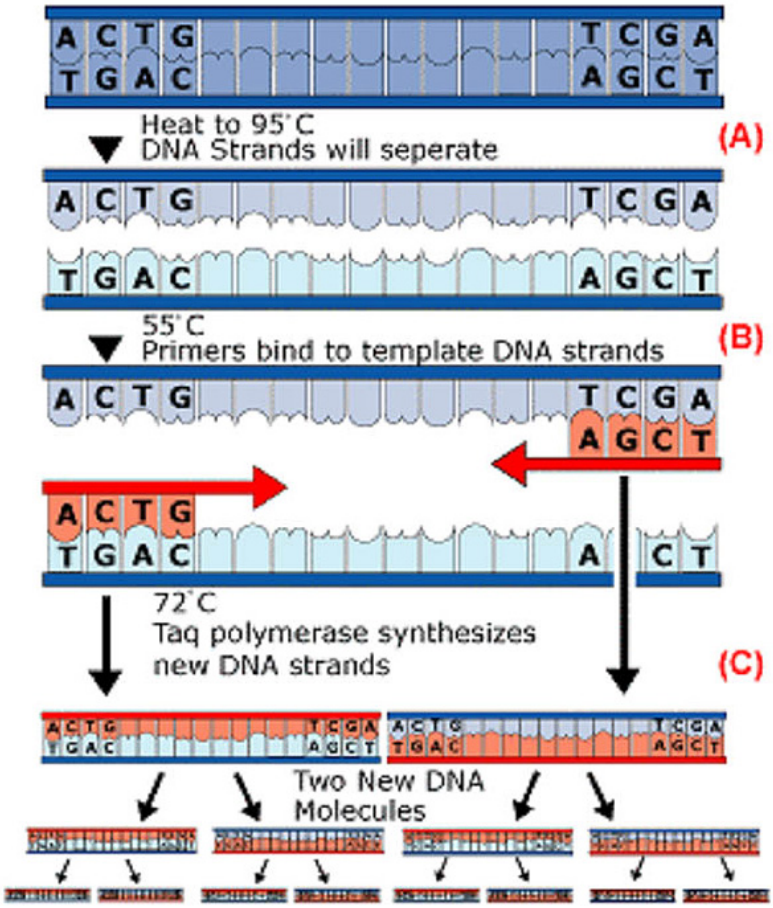


Figure 7. Illustration of the polymerase chain reaction (PCR). Step 1: Solution is heated to 95°C to denature the two strands of the target DNA (A). Step 2: Solution is cooled to ~55°C to allow the primers to anneal (bind) to the ends of the DNA strands (B). Step 3: Solution is reheated to ~72°C to allow *Taq* polymerase to synthesize complementary copies of each strand (C).

Image from http://oceanexplorer.noaa.gov/explorations/04etta/background/dna/media/dna_1.html [30/04/2012].

Various types of DNA-based molecular techniques are utilized to evaluate DNA polymorphism. The DNA-based molecular methods can be divided into three major techniques namely hybridization-based method, PCR-based method, and DNA sequencing-based method.

Hybridization-based method

Hybridization-based method or non PCR-based method including Restriction Fragment Length Polymorphism (RFLP), DNA is digested and hybridized by restriction enzymes that reveal a pattern difference between DNA fragment sizes and labeled probes in individual organisms, respectively. On an agarose gel, RFLP can be visualized using radiolabeled complementary DNA sequence. Polymorphism is analyzed after hybridization by observing present or absent bands (Joshi et al., 2004). In present, the popularity of using RFLP fingerprint technique was diminished due to the complicated procedures, and the safety in working with radioactive materials. In addition, there are other ways to access and easier, including PCR based method.

PCR-based method

PCR-based method are the amplification of DNA fragments or loci *in vitro* with the oligonucleotide primers and the thermosTable DNA polymerase enzymes (Shaw et al., 2002). For examples, random amplified polymorphic DNA (RAPD), arbitrarily primed PCR (AP-PCR), DNA amplification fingerprinting (DAF), amplified fragment length polymorphism (AFLP), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), single strand conformation polymorphism (SSCP), sequence characterized amplified polymorphic (SCAR), amplification refractory mutation system (ARMS), single sequence repeat (SSR) analysis, direct amplification of length polymorphism (DALP), inter-simple sequence repeat (IISR) and direct amplification of minisatellite-region DNA (DAMD) (Yip et al., 2007). For example, Mace et al. (1999) used AFLP technique to evaluate and assess species relationships within the tribe Datureae (Mace et al., 1999).

DNA sequencing-based method

Polymorphism at the DNA level can be studied by several methods but the direct strategy is determination of nucleotide sequences of a defined region. For this technique, the primer is specifically designed based on a defined region of gene sequences. Variation due to transitions, transversion, insertion or deletion can be assessed directly and information on a defined locus can be obtained (Joshi et al., 2004). The nucleotide sequencing is one of the most techniques to utilize the phylogenetic history. DNA sequence data are the power of informative tool for molecular systematics and comparative analysis of DNA sequences is becoming increasingly importance in plant systematics and evolution (Zhang et al., 2007). From previous studied of DNA sequencing-based method, Carles M., et al. (2005) designed a silicon-based DNA microarray for the authentication of toxic traditional Chinese medicine (TMC). Genomic DNA was extracted from fresh leaves of *D.metel* L. The spacer region of the 5S-RNA gene was amplified by PCR and subsequently sequenced. Oligonucleotide probes were spotted on to silicon-based chip. DNA corresponding to the 5S-rRNA gene of the toxic TCM plants was amplified by asymmetric PCR and hybridized to the microarrays. *D.metel* L. was discriminated based on the difference on the hybridization patterns (Carles et al., 2005).

The nuclear (nDNA) and chloroplast genome (cpDNA) are commonly able to investigate in the molecular systematics and taxonomy of plants. The nDNA is more complexity and repetitive properties. On the other hand, the cpDNA is well suiTable for evolutionary and phylogenetics studies above the species level because cpDNA; 1) is a relative abundant component of total DNA, 2) contains primarily single copy gene, 3) has a conservative rate of nucleotide substation. The most common genes in nuclear ribosomal gene consists of a transcribed region that comprises an external transcribed spacer (ETS), followed by 18s rDNA, an internal transcribed spacer (ITS-1), the 5.8s rDNA, a second internal transcribed spacer (ITS-2), and finally the 26s rDNA. Each repeat is separated from the next repeat by an intergenic spacer (IGS) (Soltis et al., 1998).

In 1970's, two DNA sequencing techniques for longer DNA molecules were invented. These were the Sanger (chain termination) method and the Maxam-Gilbert (chemical cleavage) method. The Maxam-Gilbert method is based on nucleotide-

specfic cleavage by chemicals and is best used to sequence oligonucleotides (short nucleotide polymers, usually smaller than 50 base-pairs in length) (Maxam and Gilbert, 1977). The Sanger method is more commonly used because it has been proven technically easier to apply, and, with the advent of PCR and automation of the technique, is easily applied to long strands of DNA including some entire genes. This technique is based on chain termination by dideoxy nucleotides during PCR elongation reactions (Sanger et al., 1977).

In the Sanger method, the DNA strand to be analyzed is used as a template and DNA polymerase is used in a PCR reaction to generate complimentary strands using primers. Four different PCR reaction mixtures are prepared, each containing a certain percentage of dideoxy nucleoside triphosphate (ddNTPs) analogs to one of the four nucleotides (dATP, dCTP, dGTP or dTTP). Synthesis of the new DNA strand continues until one of these analogs is incorporated, at which time the strand is prematurely truncated. Each PCR reaction will end up containing a mixture of different lengths of DNA strands, all ending with the nucleotide that was dideoxy labeled for that reaction. Gel electrophoresis is then used to separate the strands of the four reactions, in four separate lanes, and determine the sequence of the original template based on what lengths of strands end with what nucleotide.

In the automated Sanger reaction, primers are used that are labeled with four different colored fluorescent tags (**Figure 8**). PCR reactions, in the presence of the different dideoxy nucleotides, are performed as described above. However, next, the four reaction mixtures are then combined and applied to a single lane of a gel. The color of each fragment is detected using a laser beam and the information is collected by a computer which generates chromatograms showing peaks for each color from which the template DNA sequence can be determined (Weising et al., 2005).

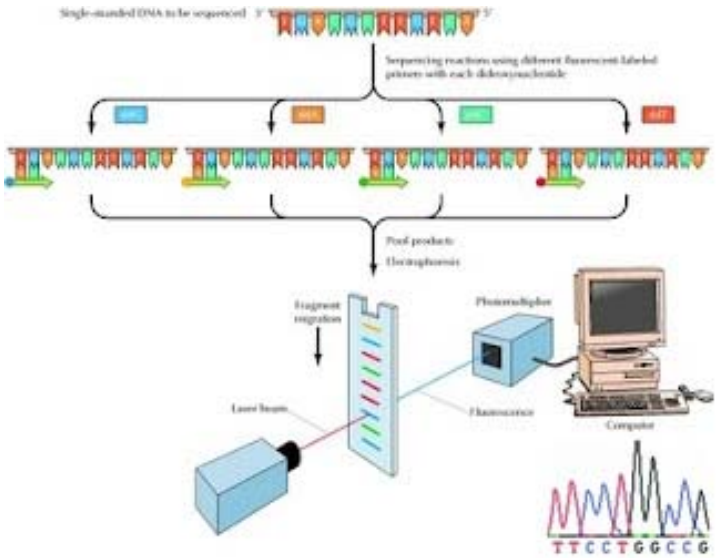


Figure 8. Automated DNA sequencing

Image from http://dnasequencing-humandnasequence.blogspot.com/2011_04_01_archive. html [30/04/2012]

2.4 Scopolamine content evaluation

Tropane alkaloids

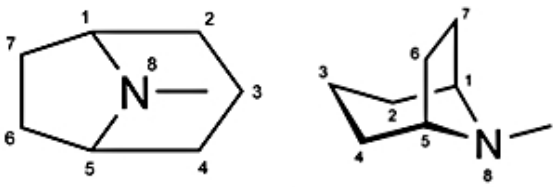
Tropane alkaloids mainly occur in Solanaceae, Erythroxylaceae, and Convolvulaceae plant families. They were found in the following plant genera: e.g., *Atropa. Datura, Hyoscyamus*, *Brugmansia, Duboisia, Mandragora, Solanum*, *Scopolia*, *Withania, Anisodus* from Solanaceae family, *Erythroxylum* from Erythroxylaceae, and *Convolvulus* and *Calystegia* from Convolvulaceae (Griffin and Lin, 2000). The alkaloids are localized both in underground (roots) and aerial parts (especially leaves and seeds) of the plants (Bruneton, 1999). The principle alkaloids of this group are (-) hyoscyamine, atropine [(±)-hyoscyamine], and scopolamine (also known as hyoscine) (**Figure 9**). Atropine is an antidote in cases of poisoning caused by cholinesterase inhibitors such as physostigmine and organophosphate insecticides. Scopolamine has a depressant activity on the central nervous system and is used to treat motion sickness. It is also employed for preanaesthetic sedative and for obstetric amnesia in conjunction with analgesics, and to calm delirium (Brown and Taylor, 2001).

Tropane alkaloids are also found in *D. metel* L. which is widely distributed in Thailand. Scopolamine, hyoscyamine and its racemic form, atropine, are the

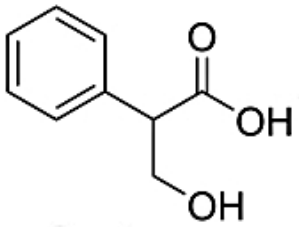
important tropane alkaloids present in the plant. They are of high therapeutic value and are frequently used as sedatives, antispasmodics and mydriatics (Palazon et al., 2008).

Tropane is a dicyclic compound formed by the condensation of a pyrrolidine precursor (ornithine) with three acetate-derived carbon atoms. Both pyrrolidine and piperidine ring systems can be discerned in the molecule. The 3-hydroxy derivatives or tropane is known as tropine. Its esterification with (-) tropic acid yields hyoscyamine, which may be racemized to form atropine (Mukherjee, 2007).

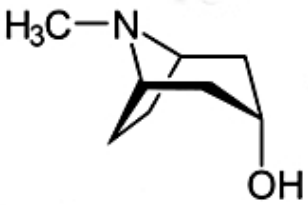
The main tropane alkaloid of *D. metel* L. is hyoscine (scopolamine), while *l*-hyoscyamine is also presented in trace amount. Hyoscine is an ester of 6,7- epoxy- 3-hydroxytropane or scopoline and *l*-tropic acid . *l*-Hyoscyamine is an ester of 3-hydroxytropane or tropine and *l*-tropic acid. It naturally exits in plants but during extraction and purification process it usually isomerizes to d *l*-form which is known as atropine [Siddigui et al., 1986].



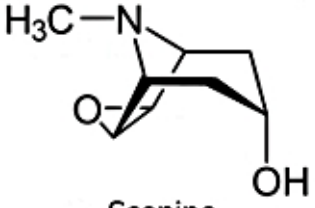
Tropane



Tropic acid



Tropanol



Scopine

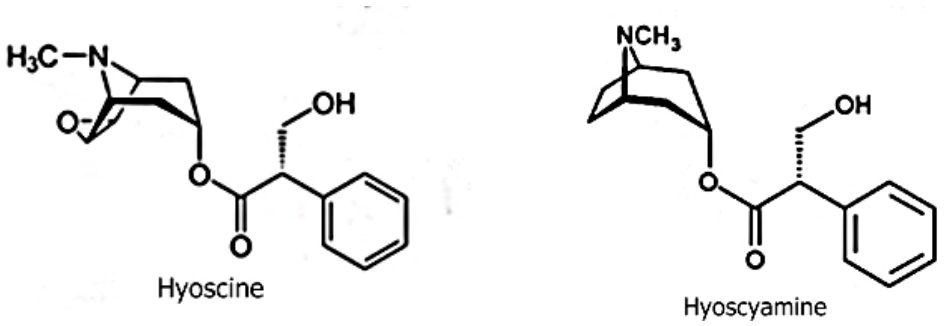


Figure 9. Structural formular of tropane, tropic acid, tropanol, scopine, hyoscine (scopolamine) and hyoscyamine
Image from http://en.wikipedia.org/wiki/ [20/07/2012]

Biosynthesis of tropane alkaloids

The characteristic of tropane alkaloids (TPAs) is ester of hydroxytropane (the alkaline part) and various acids (the acidic part). Most investigations of their biosynthesis have been performed extensively on various species of *Datura* but all the evidences have shown the similar pathways operate in other tropane alkaloids producing plants (Trease and Evans, 2009).

Tropane alkaloids occur mainly in the Solanaceae and include the anti-cholinergic drugs atropine, hyoscyamine, and scopolamine, and the narcotic tropical anesthetic cocaine. Although nicotine is not a member of the tropane class, the N-methyl-1^1-pyrrolinium cation involved in TPA biosynthesis is also an intermediate in the nicotine pathway. N-Methyl-1^1-pyrrolinium cation formation begins with the decarboxylation of ornithine and/or arginine by ornithine decarboxylase (OrnDC) and arginine decarboxylase (ArgD), respectively. These enzymes are involved in the formation of putrescine either directly by OrnDC, or via agmatine and N-carbamoylputrescine in the case of ArgD; thus, the early steps of TPA and nicotine biosynthesis are also common to polyamine metabolism. The first committed step in TPA and nicotine biosynthesis is catalyzed by a SAM-dependent putrescine N-methyltransferase (PMT). Subsequently, N-methylputrescine is oxidatively deaminated by a diamine oxidase to 4-aminobutanol, which undergoes spontaneous cyclization to form the reactive N-methyl-11-pyrrolinium cation. The N-methyl-1^1-pyrrolinium cation is thought to condense with acetoacetic acid to yield hygrine as a precursor of the tropane ring, or with nicotinic acid to form nicotine,

although the enzymology of these steps is not known. Tropinone is located at a branch point in the TPA pathway and is the first intermediate with a tropane ring. Two related dehydrogenases, tropinone reductase I (TR-I) and tropinone reductase II (TR-II), reduce the 3-keto group of tropinone to the 3α- and 3β- groups of the stereospecific alkamines tropine and 9-tropine, respectively. Hyoscyamine is produced by the condensation of tropine and the phenylalanine-derived intermediate tropic acid (**Figure 10**) (Facchini, 2001; Facchini, 2006).

Hyoscyamine can be converted to its epoxide scopolamine by Hyoscyamine 6β-hydroxylase (H6H) of the tropane ring followed by intramolecular epoxide formation via removal of the 7β-hydrogen (**Figure 11**) (Robbers et al., 1996; Trease and Evans, 2009; บุญชู ศรีตุลารักษ์, 2553).

In 2010, Pramod KK, et al. have founded that the H6H protein and its transcript were found only in roots but not in the aerial parts via. stems and leaves. The immunolocalization studies performed on leaf, stem, root as well as hairy root tissues showed that H6H was present only in the pericycle cells of young lateral and hairy roots. These studies suggest that the conversion of hyoscyamine to scopolamine takes place in the root pericycle cells, and the alkaloid biosynthesized in the roots gets translocated to the aerial parts in *D. metel* L. (Pramod et al., 2010).

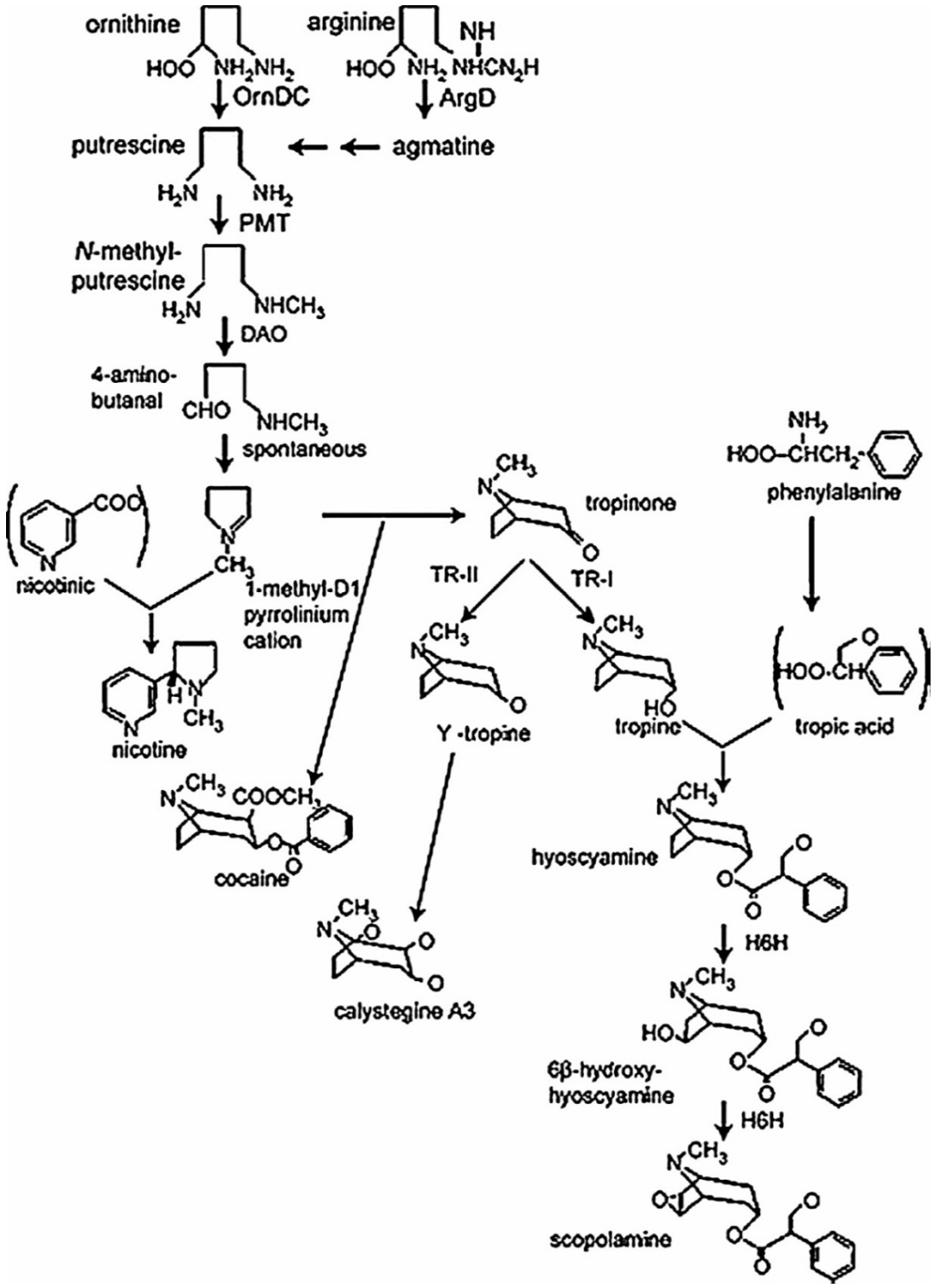


Figure 10. Biosynthesis of scopolamine from ornithine

Image from http://www.pnas.org/content/101/17/6786/F1.large.jpg [20/07/2012]

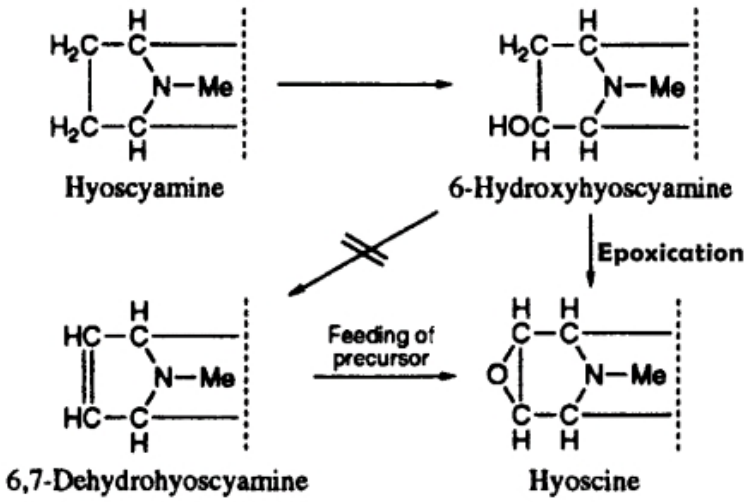


Figure 11. Route for the formation of hyoscine from hyoscyamine (partial formulae) (Trease and Evans, 2009)

Determination of tropane alkaloids

1. **Extraction of tropane alkaloids**

Extraction methods vary with the scale and purpose of the operation, and with the raw material. Tropane alkaloids are isolated from the powder sample of Solanaceous plants by one of this procedure (Trease and Evans, 2009):

Process A: The powdered material is moistened with water and mixed with lime or alkali which combines with acids, tannins and other phenolic substances and sets free the alkaloids (if they exist in the plant as salts). Extraction is then carried out with organic solvents such as ether, chloroform or petroleum spirit. The concentrated organic liquid is then shaken with aqueous acid and allowed to separate. Alkaloid salts are now in the aqueous liquid, while many impurities behind in the organic liquid.

Process B: The powdered material is extracted with water or aqueous alcohol containing dilute acid. Pigments and other unwanted materials are removed by shaking with chloroform or other organic solvents. The free alkaloids are then precipitated by the addition of excess sodium bicarbonate or ammonia and separated by filtration or by extraction with organic solvents.

The extraction methods, process A (the sample is extracted with alkali in organic solvent first, then followed by acid-base shaking) has been prominent. Various literatures

follow this procedure for extraction of tropane alkaloids.

2. Methods of tropane alkaloids analysis

Tropane alkaloids are currently analyzed by several methods including thin layer chromatography (TLC) (Mroczek, 2008), high performance thin layer chromatography (HPTLC) (Sharma et al., 2009), gas chromatography (GC) (Drager, 2002), gas chromatography mass spectrometry (GC-MS) (Elisabetta et al., 2001), high performance liquid chromatography (HPLC) (Ceyhan et al., 2001), liquid chromatography mass spectrometry (LC-MS) (Steenkamp et al., 2004), and capillary electrophoresis (CE) (Cataldi and Bianco, 2008).

Thin layer chromatography (TLC) or planar chromatography is a type of liquid chromatography in which the stationary phase is in the form of a layer on a glass, an aluminum, or plastic support. It is still frequently used for tropane alkaloids as a common method of choice for herbal analysis. The classical capillary-action TLC is an inexpensive and easy technique, that require little instrumentation, which is used for separation of simple mixtures and for qualitative identification or semi-quantitative, visual analysis of samples (Liang et al., 2004).

The advantages of TLC are due to its simplicity, a small quantity of solvents used, analyzing samples with minimum sample preparation, and the possibility of separating many samples and standards simultaneously on a single plate, leading to high throughput, low cost analyses and also the ability to construct calibration curves from standards chromatographed under the same conditions as the samples (Sherma, 2005). Previous studied of TLC solvent systems and detection methods had been reported for their identification of scopolamine and also other tropane alkaloids (Muhtadi and Hassan, 1990; Mroczek, 2008; Wagner and Bladt, 2009).

Until recently, the use of TLC-image analysis has been applied for content determination of several compounds. With a combination of single computer technology and image analysis software for evaluation of TLC chromatogram, the quantitative TLC method based on image analysis is more convenient and less expensive than other chromatographic methods. Commercial and free web-based image software for TLC-image analysis are available in which performances are based on sensitivity of spot

detection, background compensation algorithms, intensity resolution, precision and accuracy of image analysis (Hung et al., 2001; Amber, 2007; Johnsson et al., 2007).

The simultaneous quantification of hyoscyamine and scopolamine in different plant parts such as, leaves, roots and seeds of wild morphotypes of *D. metel* L. by the high performance thin layer chromatography (HPTLC) technique was performed in 2009 by Sharma et al. The advantage of HPTLC is a rapid, reproducible, accurate, selective, and high sample throughput, which results from the small amount of sample preparation required and the simultaneous quantification of several samples (Sharma et al., 2009).

Gas chromatography (GC) and gas chromatography mass spectroscopy (GC-MS) are well known for the analysis volatile chemical components. The advantages of GC or GC-MS clearly lie in its high sensitivity for the detection of almost all volatile compounds. The high selectivity of capillary columns enables separation of many tropane alkaloids simultaneously within comparatively short times (Miraldi et al., 2001). However, the most serious disadvantage of the method is that it is not suiTable for analyzing polar samples and non-volatile compounds (Liang et al., 2004).

High performance liquid chromatography (HPLC) is one type of liquid chromatography (LC) that is a physical separation technique conducted in the liquid phase. It is a high throughput technique for determining small amount of impurities of herbal materials with the advantages of high sensitivity and high reliability. Components of analyses are separated by distributing between the mobile phase (a flowing liquid) and a stationary phase (sorbents packed inside column). HPLC is very popular technique for analysis of herbal extract because it is easy to use and it not limited by the volatility or stability of the samples (Dong, 2006). Furthermore, HPLC can successfully use for the separation and quantitative determination of closely related tropane alkaloids. Thus rapid, simple, robust, reproducible and sensitive analytical methods are needed to enable the analysis of samples in a short analytical time (Banyai et al., 2006). Liquid chromatography mass spectroscopy (LC-MS) for tropane alkaloid analysis is a promising approach which will be increasingly used in the future, as improved interfaces and volatile but highly selective solvent systems become increasingly available. The metabolites and catabolites of tropane alkaloids, which no UV absorption can be measured by LC-MS (Drager, 2002; Aehle and Drager, 2010; Jakabova et al., 2012).

For capillary electrophoresis (CE), appears well suited to tropane alkaloid analysis because these compounds are natural cations, if the appropriate acidic buffer pH is chosen. Migration of the analyses in the usual cationic mode (sample introduction at the anode and detection and outlet at the cathode) is caused by the charged nitrogen atom. The sample volume in CE is very low, a few nanoliters, and therefore very sensitive is required (Suntornsuk, 2002). Detection in CE is usually achieved by a DAD system, a drawback for CE separation of tropane alkaloids due to their low UV light absorption (Bogusz and Erkens, 1994). While the tropic acid esters and others esters with aromatic carboxylic acids may be measured, the free amino alcohols like tropine and pseudo-tropine and other metabolites like hygrine and cuscohygrine are not detected at all (Aehle and Drager, 2010).

CHAPTER III
MATERIALS AND METHODS

Part I. Macroscopic and microscopic evaluation

1.1 . Macroscopic evaluation

Plant sample

D. metel L. var. *metel* was collected from The Somdej Phra Thepraratanarajsuda Medicinal Plants Garden, Petroleum Authority of Thailand, Rayong Province (LPK081001) and *D. metel* L.var. *fastuosa* was collected from Chatuchak Plant Market, Bangkok Province (KSL071001) in July-August 2010, then, were authenticated by Assoc. Prof. Dr. Nijsiri Ruangrungsi, (N.R.), Department of Pharmacognocy, Faculty of Pharmaceutical Sciences, Chulalongkorn University and compared with the herbarium specimens at Forrest Herbarium Thailand (BKF). Vouchers were deposited at the College of Public Health Sciences, Chulalongkorn University.

Apparatus

- 0.2 mm Line width black micro pigment pen (Sakura Corp., Japan)
- Drawing board
- Drawing paper 100 gram (Master art, Thailand)
- HB pencil and eraser (Pentel, Thailand)

Procedure

A complete branch of each plant was subjected to thoroughly observed and compared for the differences. The drawing outline of the two plant samples was illustrated in the proportion size related to the original and approved by the expert (N.R.).

1.2 . Microscopic evaluation

Plant sample

Mature leaves of *D. metel* L.var. *metel* were collected from three locations;

1. The Somdej Phra Thepraratanarajsuda Medicinal Plants Garden, Petroleum Authority of Thailand, Rayong Province (LPK081001, August, 2010)

2. Sirirukkachat garden, Faculty of Pharmacy, Mahidol University, Salaya, Nakhonpathom Province (LPK091002, September, 2010)
3. Bang Ra Jan District, Singburi Province (LPK011103, January, 2011)

Mature leaves of *D. metel* L.var. *fastuosa* were collected from three locations;

1. Chatuchak Plant Market, Bangkaen District, Bangkok Province (KSL071001, July, 2010).
2. Bang Nam Priao District, Chachoengsao Province (KSL041104, April, 2011)
3. Muang District, Chonburi Province (KSL051105, May, 2011)

The samples were authenticated separately for each location by the expert (N.R.).

Chemical and Reagent

- 70 Degree ethyl alcohol
- Distilled water
- Sodium hypochlorite (Haiter Bleach, Kao industrial, Thailand)

Apparatus

- 0.2 mm Line width black micro pigment pen (Sakura Corp., Japan)
- Beaker 250 ml. (Pyrex, Germany)
- Compound microscope (Zeiss model Axioskop, Germany)
- Digital Camera (Power Shot A640, Canon Inc., Japan)
- Drawing board
- Drawing paper 100 g (Master Art, Thailand)
- Forceps
- Glass slide and cover glass
- HB pencil and eraser (Pentel, Thailand)
- Hot plate Model HP-A191 (Thermolyne, USA)
- Razors (Gillette blade, USA)

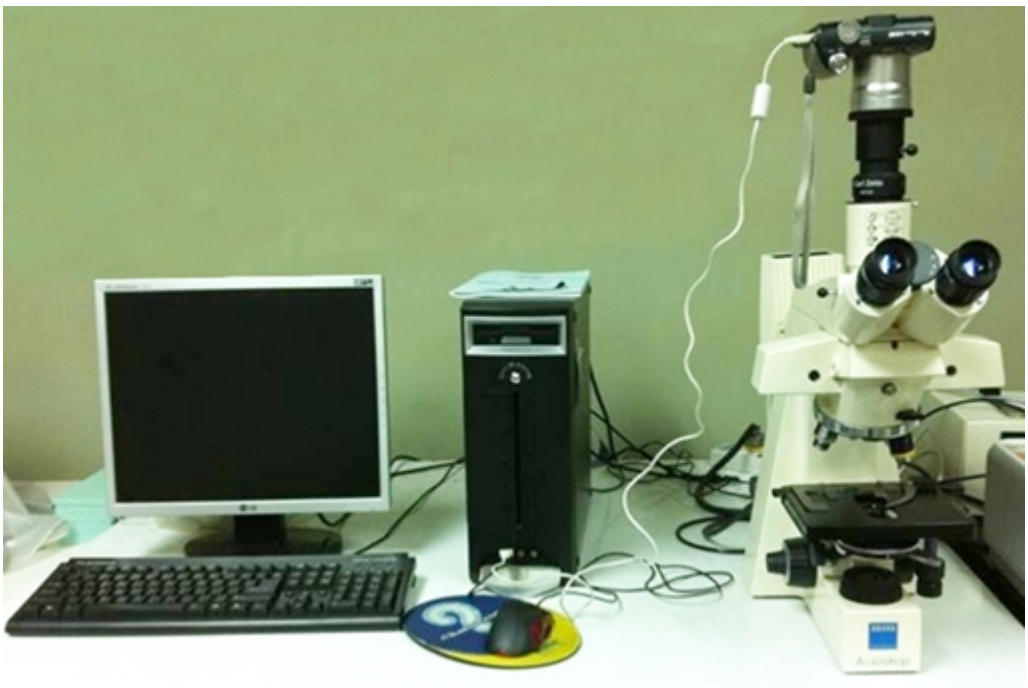


Figure 12. Zeiss compound microscope model Axioskop attached with digital camera

Procedure

Stomatal number and stomatal index

This protocol was adapted from the method of Geisler (Geisler et al., 2000).

1. Paint a thick patch of clear nail polish on the both side of fresh leaf surface being studied. Make a patch at least one square centimeter.
2. Allow the nail polish to dry completely.
3. Tape a piece of clear cellophane tape to the dried nail polish patch. (The tape must be clear, do not use any other opaque tape)
4. Gently peel the nail polish patch from the leaf by pulling on a corner of the tape and peeling the fingernail polish off the leaf. This is the leaf impression will be examined.
5. Tape the peeled impression to a very clean microscope slide, use scissors to trim away any excess tape, and label the slide as appropriate for the specimen being examined.
6. Examine the leaf impression under a light compound microscope (**Figure 12**). Search for areas where there are numerous stomata, and where there are no dirt, thumb prints, damaged areas, and large leaf veins.

7. A 20 X magnification of objective lens of compound microscope, with an attached digital camera was used and recorded the images. The images were scaled for the area of 0.5 mm^2 using program AxioVision version 4.1 prior counting the stomata and epidermal cells (**Figure 13-14**).
8. The number of stomata and epidermal cells was multiplied by 4 in order to give total number of stomata and epidermal cells in the area of 1 mm^2. The area of the sample was to be changed and recorded not less than 30 images from several fractions of leaves from one location.

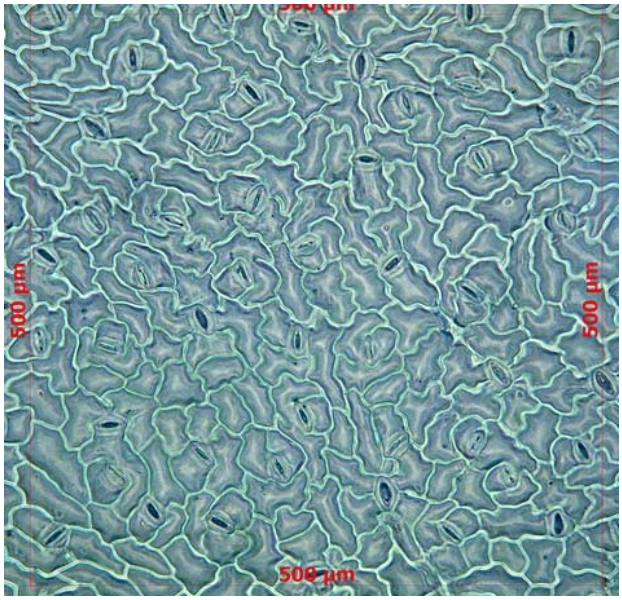


Figure 13. The upper epidermal layer of *D.metel* L. leaf in the area of 0.5 mm^2 (20X magnification)

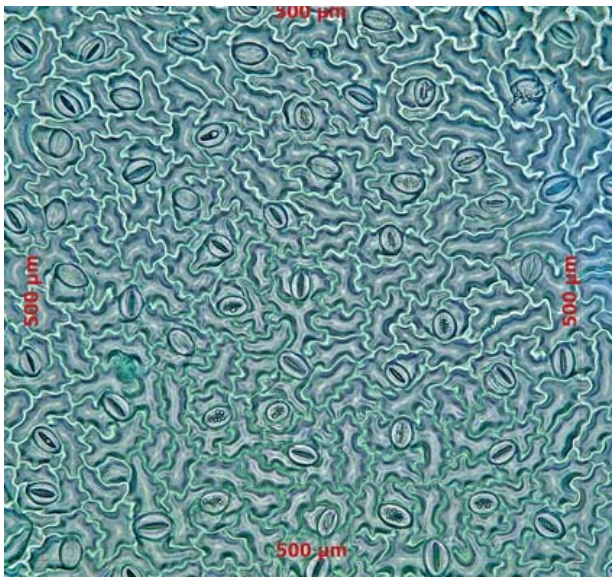


Figure 14. The lower epidermal layer of *D.metel* L. leaf in the area of 0.5 mm^2 (20X magnification)

Palisade ratio

The procedure was also modified from the method described in Pulok K. Mukherjee (Mukherjee, 2007).

1. Gently put the fractions of leaf, which were cut off from the middle of the leaf into the mixture of sodium hypochlorite: water (1:1), which was warmed on hot plate (the leaf had been soaked in 70 degree alcohol for at least 2-3 weeks prior before used).
2. Let the fractions of leaf to boil in sodium hypochlorite solution until the samples were transparent. Then, the samples were rinsed with distilled water until the samples were cleaned and mounted the samples on slide and covered with cover glass.
3. The pieces of sample were kept separately on a glass slide with its upper epidermal layer kept uppermost side. A 40X magnification of objective lens of compound microscope, with an attached digital camera was used and recorded the images.
4. The image of 4 clear continuous epidermal cells consist of the round, closely packed palisade cells was observed (**Figure 15**) and recorded by using digital camera.

5. The area of the sample was to be changed and recorded not less than 30 images. The palisade cells inside the boundary and those that are 50% or more inside the outer boundary of 4 epidermal cells were taken into account. The number of total palisade cells was divided by 4, which gave the average number of palisade cell under each epidermal cell.

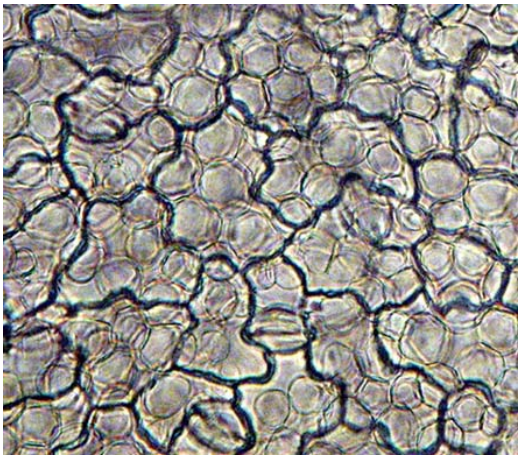


Figure 15. The round, closely packed palisade cells in the boundary of four clear continuous epidermal cells of *D.metel* L. leaf (40X magnification)

Stem and midrib cross section

Mature stem and midrib from mature leaf of *D.metel* L. var. *metel* and *D.metel* L. var. *fastuosa* were thinly cross sectioned with a razor blade by hand then, separately placed a complete piece on the glass slide and covered with a cover glass. The stem and midrib cross section were observed under microscope with magnification of 10X to 40X to evaluate the fine details and recorded the images (**Figure 16** and **Figure 17**). The images were illustrated to evaluate the differences.

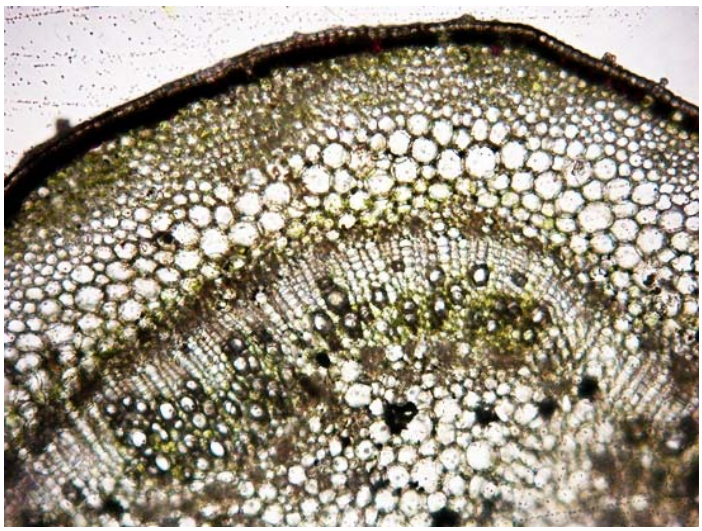


Figure 16. *D.metel* L. stem cross section (10X magnification)

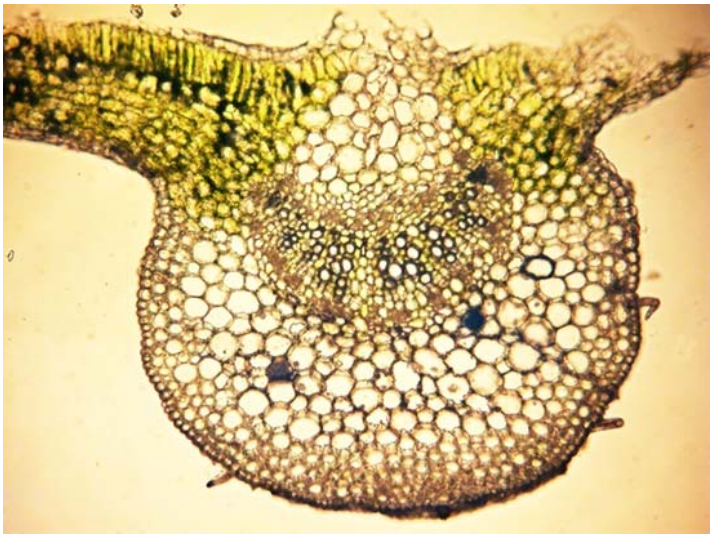


Figure 17. *D.metel* L. midrib cross section (10X magnification)

Statistic analysis

The data of stomatal number, stomatal index, and palisade ratio were calculated as mean and standard deviation, and statistically analyzed using independent sample t-test by SPSS version 17.0 for windows program for analyzing of significant difference between two varieties *D.metel* L.

Part ll. Molecular evaluation

Plant sample

Table 5. Fresh aerial part authentic samples

Sample no.	Plant	Habitat (Province)	Collecting date (Month, Year)	Voucher ID
1	*D. metel* L.var. *fastuosa*	Chatuchak Plant Market, Bangkaen District, Bangkok Province	July, 2010	KSL071001
2	*D. metel* L.var. *fastuosa*	Nakhonchaisri District, Nakhonpathom Province	July, 2010	KSL071002
3	*D. metel* L. (hybrid)	Chatuchak Plant Market, Bangkaen District, Bangkok Province	July, 2010	LHB071001
4	*D. metel* L. (hybrid)	The Somdej Phra Thep Rattana Rajsuda Medicinal Plants Garden, Petroleum Authority of Thailand, Rayong Province	August, 2010	LHB081002
5	*D. metel* L.var. *metel*	The Somdej Phra Thep Rattana Rajsuda Medicinal Plants Garden, Petroleum Authority of Thailand, Rayong Province	August, 2010	LPK081001
6	*D. metel* L.var. *metel*	Sirirukkachat garden, Faculty of Pharmacy, Mahidol University, Salaya, Nakhonpathom Province	September, 2010	LPK091002
7	*D. metel* L. (hybrid)	Kanchanabhishek Institute of Medical and Public Health Technology, Chai Noi District, Nonthaburi Province	September, 2010	LHB091003
8	*D. metel* L. (hybrid)	Krok Phra District, Nakornsawan Province	October, 2010	LHB101004
9	*D. metel* L (hybrid)	Sirirukkachat Garden, Faculty of Pharmacy, Mahidol University, Salaya Campus, Nakhonpathom Province	October, 2010	LHB101005
10	*D. arborea* L. *	Pong Nam Ron District, Chanthaburi Province	November, 2010	TNF111001
11	*D. metel* L.var. *metel*	Bang Ra Jan District, Singburi Province	January, 2011	LPK011103
12	*D. metel* L. (hybrid)	Bang Bor District, Samutprakarn Province	February, 2011	LHB021106
13	*D. metel* L.var. *fastuosa*	Royal Agricultural Research Center, Muang District, Chiangmai Province	March, 2011	KSL031103
14	*D. metel* L. (hybrid)	Royal Agricultural Research Center, Muang District, Chiangmai Province	March, 2011	LHB031107

Sample no.	Plant	Habitat (Province)	Collecting date (Month, Year)	Voucher ID
15	*D. metel* L.var. *fastuosa*	Bang Nam Priao District, Chachoengsao Province	April, 2011	KSL041104
16	*D. metel* L.var. *fastuosa*	Muang District, Chonburi Province	May, 2011	KSL051105
17	*D. metel* L. (hybrid)	Herbal Botanical Garden of Khung Ta Phao Temple, Mueang District, Uttaradit Province	June, 2011	LHB061108
18	*D. metel* L. (hybrid)	Hang Dong District, Chiangmai Province	July, 2011	LHB071109
19	*D. metel* L. (hybrid)	Chon Dan District, Petchaboon Province	August, 2011	LHB081110
20	*D. metel* L. (hybrid)	The Queen Sirikit Botanic Garden, Mae Rim District, Chiangmai Province	August, 2011	LHB081111
21	*D. metel* L. (hybrid)	Muang District, Pathumthani Province	September, 2011	LHB091112

***** assign for out group

DNA extraction

Chemical and Reagent

- 2-Mercaptoethanol (AR grade, BDH Chemical, England)
- 3 M Sodium acetate (pH 5)
- 70 Degree ethyl alcohol
- Absolute ethanol (Merck, Germany)
- CTAB (Cetyl trimethylammonium bromide) buffer
- Liquid nitrogen
- Phenol: chloroform: isopropanol (25: 24: 1)
- Saturated phenol (AR grade, BDH Chemical, England)
- TE (Tris-EDTA) buffer

Apparatus

- 1.5 ml Microcentrifuge tubes
- -20°c Freezer (Sharp, Japan)
- Micropipette (Biohit, Finland)
- Centrifugation machine (Sigma, Germany)
- Mortar and pestle
- Shaking water bath (GFL model 1086, Germany)
- Spatula
- Vortex mixer model K-550-GE (Scientific Equipment, USA)

Plant genomic DNA was individually extracted from the fresh young leaves using a modified CTAB technique (Doyle and Doyle, 1987).

1. Freeze the fresh young leaves rapidly in liquid nitrogen and grind to a power with mortar and pestle. Transfer into 1.5 ml microcentifuge tube.

2. Add 500 µl of CTAB buffer into microcentifuge tube then, incubate and shaking in water bath at 65 °C for 1 hour.

3. Centrifuge the microcentifuge tube at 10,000 round per min (rpm) for 10 min and transfer supernatant into a new clean 1.5 ml microcentifuge tube.

4. Add 500 µl of saturated phenol to get rid of other phenolic compounds and

proteins then, vortex 1 min and centrifuge the microcentifuge tube at 10,000 rpm, 10 min.

5. Transfer the aqueous phase (upper layer) to a new 1.5 ml microcentifuge tube and added 500 µl of phenol: chloroform: isopropanol (25: 24: 1) to get rid of the excessive phenol and proteins from the DNA then, mixed well by vortex mixer. Centrifuged the samples 10,000 rpm for 10 min, then transfered the aqueous phase to a fresh microcentifuge tube.

6. Add 1:10 volume of 3 M sodium acetate (pH 5) and invert tube 2-3 times. Add 2 volume of cold absolute ethanol to precipitate DNA, invert tube 2-3 times and keep at -20°C for 1 hour.

7. Centrifuge the microcentifuge tube at 10,000 rpm for 10 min then, discard the supernatant. DNA pellet was washed with 1 ml of cold 70% ethanol and centrifuged 10,000 rpm for 10 min. Discarded the supernatant and dried DNA pellet at room temperature and dissolve DNA in 50 -100 µl of TE buffer was added to the DNA pellet and left to dissolve homogeneously, and then store at 4^{o}C refrigerator.

8. The extracted DNA was stored at -20^{o}C for further use.

DNA qualification and DNA amplification by Polymerase Chain Reaction (PCR)

Chemical and Reagent

- 100 bp, and 1 kb DNA ladder marker (Fermentas, USA)
- 1X Loading dye
- 1X TBE buffer
- 10 mM dNTPs
- 10 µM Forward primer (ITS5) (Fermentas, USA)
- 10 µM Reverse primer (ITS4) (Fermentas, USA)
- 10 µM *rbc*L_F Forward primer (*rbc*L_F) (Fermentas, USA)
- 10 µM *rbc*L_R Reverse primer (*rbc*L_R) (Fermentas, USA)
- 10 µM *atp*B_F Forward primer (*atp*B_F) (Fermentas, USA)
- 10 µM *atp*B_R Reverse primer (*atp*B_R) (Fermentas, USA)
- 10X PCR Buffer (Fermentas, USA)

- 5 Unit/µl *Taq* DNA polymerase (Recombinant) (Fermentas, USA)
- 25 mM $MgCl_2$ (Fermentas, USA)
- Agarose gel (Merck, Geramany)
- Distilled deionized water
- DNA template (Extracted DNA solution)
- Ethidium bromide
- PCR purification kit (QIAGEN, USA)

Apparatus

- 100 µl Microcentrifuge tubes (Axygen, USA)
- Centrifugation machine (Sigma, Germany)
- Electrophoresis chamber and power supply (Biorad, model 200/ 2.0 power)
- Micropipette 1.00-10.00 µl, 100-1,000 µl (Biohit, Finland) and tips
- PCR tubes (Axygen, USA)
- Thermal cycler (GeneAmp PCR system 9700, Applied Biosystems, USA)
- UV visualize gel documentation machine (Auto Chemi System, USA)

DNA qualification

The quality of extracted DNA was estimated by comparing the band intensity of the extracted DNA with 1 kb DNA ladder marker (Promega, USA) in agarose gel. A 1.5 % (w/v) agarose/TBE gel staind with ethidium bromide. Five microliter of extracted DNA solution was mixed with 1X loading dye and loaded onto the gel, which is placed in an electrophoresis chamber filled with 1X TBE buffer. Electrophoresis was carried out at 100 V for 30 min or until the dye migrated to a sufficient distance. The gel was photographed under UV light using UV transilluminator gel documentation machine (Auto Chemi System, USA).

DNA amplification

A pair of ITS, *rbc*L and *atp*B primer were used to amplify the ITS, *rbc*L, and *atp*B regions for DNA analysis. All the primer sequences were shown in **Table 6**. The PCR amplifications were conducted in a GeneAmp PCR system 9700 (Applied

Biosystems, USA).

Table 6. Primers used for PCR amplification and sequencing

Primer	Direction	Primer sequences (5' – 3')	No. of bases	Tm (°c)
ITS4 [1]	Forward	5' TCCTCCGCTTATTGATATGC 3'	20	55.0
ITS5 [1]	Reverse	5' GGAAGTAAAAGTCGTAACAAGG 3'	22	56.0
*rbc*L_F [2]	Forward	5' TGTCACCACAAACAGAAACTAAAGCAAGT 3'	29	62.4
*rbc*L_R [2]	Reverse	5' CTTTTAGTAAAGATTGGGCCGAG 3'	23	58.9
*atp*B_F [3]	Forward	5' TCAGTACACAAAGATTTAAGGTCAT 3'	25	56.2
*atp*B_R [3]	Reverse	5' TATGAGAATCAATCCTACTACTTCT 3'	25	56.9

[1] Primers designed by White et al. (1990)

[2] Primers designed by Razafimandimbison and Bremer (2001)

[3] Primers designed by Hoot et al. (1995)

The ITS region

An approximately 750 bp fragment of the ITS region was amplified using universal primers, ITS4 and ITS5. The position of these primers is shown in **Figure 18**. PCR for the ITS was performed in a 20 µl reaction volume mixture containing 1 µl of genomic DNA, 0.1 mM of dNTPs, 1X PCR buffer (100mM Tris-HCl (pH 8.0), 500 mM KCl, 0.8% (v/v) Nonidet P40), 2.5 mM of $MgCl_2$, 0.5 Unit/µl of *Taq* DNA polymerase (Fermentas), 0.1 µM of each primer, and sterile deionized water, with the following thermocycling conditions: an initial denaturation at 95°Cfor 5 min, followed by 30 cycles of denaturation at 95° C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec and ended with a final extension at 72° C for 5 min.

Figure 18. Diagram of the ITS region showing the position of primers and the predicted 750 bp PCR product

The *rbc*L gene

An approximately 1.5 kb fragment of *rbc*L gene was amplified using two synthetic primers, *rbc*L_F and *rbc*L_R. The position of these primers is shown in **Figure 19**. PCR amplification was performed in a 20 µl reaction volume containing 1 µl of genomic DNA, 0.1 mM of dNTPs, 1X PCR buffer (100 mM Tris-HCl (pH 8.0), 500 mM KCl, 0.8% (v/v) Nonidet P40), 2.5 mM of $MgCl_2$, 0.5 Unit/µl of *Taq* DNA polymerase, 0.1 µM of each primer, and sterile deionized water, with the following thermocycling conditions: an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 60 sec, annealing at 58°C for 60 sec and extension at 72°C for 60 sec and ended with a final extension at 72°C for 5 min.

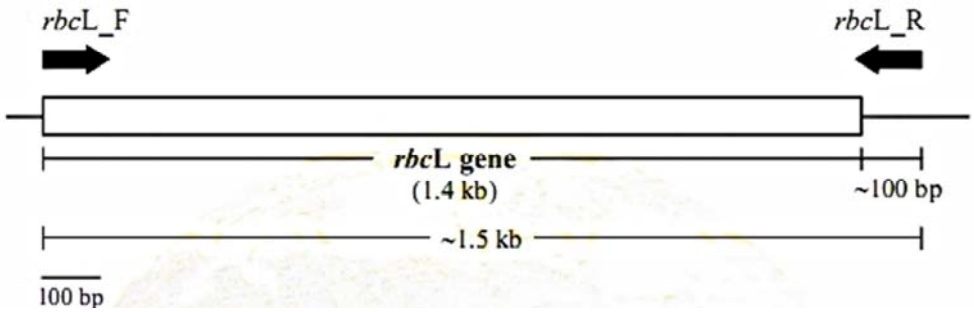


Figure 19. Diagram of the *rbc*L region showing the position of *rbc*L_F (5'end of gene) and *rbc*L_R primers (100 bp downstream of the termination codon), and the predicted 1.5 kb PCR product

The *atp*B gene

An approximately 1.5 kb fragment of *atp*B was amplified by two universal primers, which are *atp*B_F and *atp*B_R The position of these primers is shown in **Figure 20**. PCR amplification was performed in a 20 µl reaction volume containing 3 µl of genomic DNA, 0.1 mM of dNTPs, 1X PCR buffer (100 mM Tris-HCl (pH 8.0), 500 mM KCl, 0.8% (v/v) Nonidet P40), 2.5 mM of $MgCl_2$, 0.5 unit/µl of *Taq* DNA polymerase, 0.1 µM of each primer, and sterile deionized water, with the following thermocycling conditions: an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 60 sec, annealing at 56°C for 60 sec and extension at 72°C for 60 sec and ended with a final extension at 72°C for 5 min.

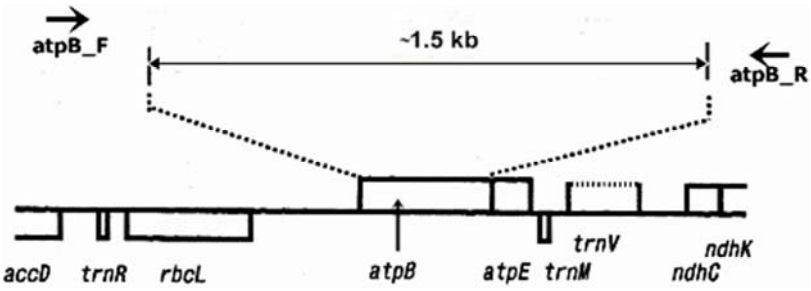


Figure 20. Diagram of the *atp*B region showing the predicted 1.5 kb PCR product

Detection of PCR product

The PCR products were separated by agarose gel electrophoresis. The 1.5 % (w/v) agarose/TBE gel was prepared (by weighting 1.5 g of agarose in 100 ml of 1X TBE buffer and then melt until completely). Then, add 2 µl of 10 mg/ml ethidium bromide were added to the warm gel, and gently mixed before pouring into a gel tray. After gel solidification, the gel was transferred into the electrophoresis chamber filled with 1X TBE buffer. PCR products were mixed with loading dye, which is used for loading PCR products into gel wells and tracked migration of the DNA fragments during electrophoresis. 1 kb DNA ladder was loaded along with the DNA samples for size comparison. A voltage of 100 V was applied to run the gel for 30 min or until the dye migrated to a sufficient distance. After that, the gel was examined under UV light and photographed using UV visualize gel documentation machine (Auto Chemi System, USA).

Purification of PCR product

The PCR products were purified from primers, nucleotides, polymerases, and salts using QIAquick PCR Purification Kit (QIAGEN, USA) prior DNA sequencing. According to the protocol (QIAGEN, 2002), all centrifugation steps were carried out at 13,000 rpm. Five volumes of PB buffer was added to 1 volume of PCR sample and mixed. The sample was applied to the QIAquick Spin Column sitting on the 2 ml collection tube provided, to allow DNA binding to the column. After centrifuged for 60 sec, the flow-through was discarded and the column was placed back on the same collection tube. Then, 0.75 ml of PE buffer was added into the column for washing and centrifuged for 60 sec. The flow-through was discarded and the column was placed back into the same tube. The column was centrifuged for another 60 sec to remove residual of ethanol in PE buffer. The column was placed on a clean 1.5 ml microcentrifuge tube. After that, 50 µl of buffer EB (10 mM Tris-Cl, pH 8.5) was added to the center of the QIAquick membrane and the column was centrifuged for 60 sec. The purified DNA was stored at -20°C for DNA sequencing (ABI system).

DNA sequencing analysis

ClustalW2 - multiple sequence alignment program (Online; available from http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used to multiple aligned the sequences of the three regions; ITS, *rbc*L, and *atp*B.

Part III. Scopolamine content evaluation

Material

- 20x20 cm Aluminum sheets silica gel plate , G60 F254 (Merck, Germany)
- Filter paper no.4, 125 mm (Whatman, England)
- pH paper (Merck, Germany)
- Syringe filter PTFE type, 13 mm, 0.45µm with luer lock (Fortune Scientific, Thailand)

Chemical and Reagent

- 25 % Ammonium hydroxide (AR grade, Merck, Germany)
- Acetonitrile (HPLC grade, RCI-Asia Labscan, Thailand)
- Chloroform (HPLC grade, JT Baker Chemical, USA)
- Diethylamine (AR grade, RCI-Asia Labscan, Thailand)
- Dragendorff's reagent
- Ethyl acetate (AR grade, BDH Chemical, England)
- Methanol (HPLC grade, RCI-Asia Labscan, Thailand)
- Orthophosphoric acid (AR grade, BDH Chemical, England)
- Potassium dihydrogen orthophosphate (AR grade, Merck, Germany)
- (-)-Scopolamine hydrochloride (Sigma-Aldrich, Singapore)
- Sodium sulphate (AR grade, Merck, Germany)
- Toluene (AR grade, BDH Chemical, England)

Equipments and instruments

- Analytical balance 4 digits (Adventurer™ Ohaus Crop., USA)
- Digital scanner HP Deskjet F2280 (Hewlett-Packard, Thailand)
- Graduated cylinders 25 ml, 100 ml, and 500 ml.
- High performance liquid chromatography (HPLC) Model LC-20A series with LC solution workstation software (Shimadzu, Japan)
- Image J software (http://rsbweb.nih.gov/ij/)
- Micropipette 10-100 µl, 100-1000 µl and tips (Biohit, Finland)
- pH meter model UB-10 (Denver Instrument, USA)
- Rotary vacuum evaporator R-200 (Buchi, Switzerland)
- Soxhlet apparatus
- TLC chamber (Camag, Switzerland)

- Ultrapure water system NW series (Heal Force Bio-Meditech Holdings, China)
- Ultra sonic chamber (Analytical Lab Science, Thailand)
- Vacuum pump with pressure regulator, model DOA-P504-BN (GAST Manufacturing, Inc., USA)
- Round bottle flask 500 ml.
- Volumetric flasks 250 ml, and 500 ml.
- Volumetric pipettes 1.00 ml, 5.00 ml, and 10.00 ml.
- Vortex mixer, model K-550-GE (Scientific Industries, Inc., USA)

Determination of scopolamine content

Plant sample

Whole plants of *D. metel* L.var. *metel* were collected from three locations;

1. The Somdej Phra Thepraratanarajsuda Medicinal Plants Garden, Petroleum Authority of Thailand, Rayong Province (LPK081001, August, 2010)
2. Sirirukkachat garden, Faculty of Pharmacy, Mahidol University, Salaya, Nakhonpathom Province (LPK091002, September, 2010)
3. Bang Ra Jan District, Singburi Province (LPK011103, January, 2011)

Whole plants of *D. metel* L.var. *fastuosa* were collected from three locations;

1. Chatuchak Plant Market, Bangkaen District, Bangkok Province (KSL071001, July, 2010).
2. Bang Nam Priao District, Chachoengsao Province (KSL041104, April, 2011)
3. Muang District, Chonburi Province (KSL051105, May, 2011)

Sample preparation

The dry samples (leaves, flowers and fruits) were ground into a coarse powder with a blender and through 40 meshed sieve for further extraction and analysis.

Sample extraction

By following the modified method of Gontier (Gontier et al., 1994), the approximately 1 to 6 g of dried powder of each sample was weighed accurately and subjected to the extraction of tropane alkaloids. This sample was extracted for 4-5 hours in a soxhlet apparatus with 300 ml of methanol- chloroform- 25 % ammonium hydroxide (50-50-1.5) until it was exhausted. After filtration, the residue was washed twice with 15 ml of chloroform. The pooled filtrate was evaporated under reduced pressure in a rotary evaporator till dryness. The dry extract was washed in 20 ml of 0.1 N hydrochloric acid three times, and extracted twice with 15 ml of chloroform to eliminate impurities. The acid phase was adjusted to pH 10 with 5 ml, 25% ammonium hydroxide, scopolamine was exhaustively extracted three time with 35 ml of chloroform. After addition of anhydrous sodium sulphate (Na_2SO_4), the extract was filtrated and residue was washed with 10 to 20 ml of chloroform. These combined extracted solvent fractions were evaporated under reduced pressure till dryness (**Figure 21**).

For sample solution, each extract was dissolved in methanol to a concentration of 100 mg/ml of leaves, 50 mg/ml of flowers, and 40 mg/ml of fruits, respectively. These solutions need a brief sonication (10 min) at room temperature to enhance complete dissolution and keep in refrigerator for further analysis with thin layer chromatography (TLC) image and high performance liquid chromatography (HPLC) method, respectively.

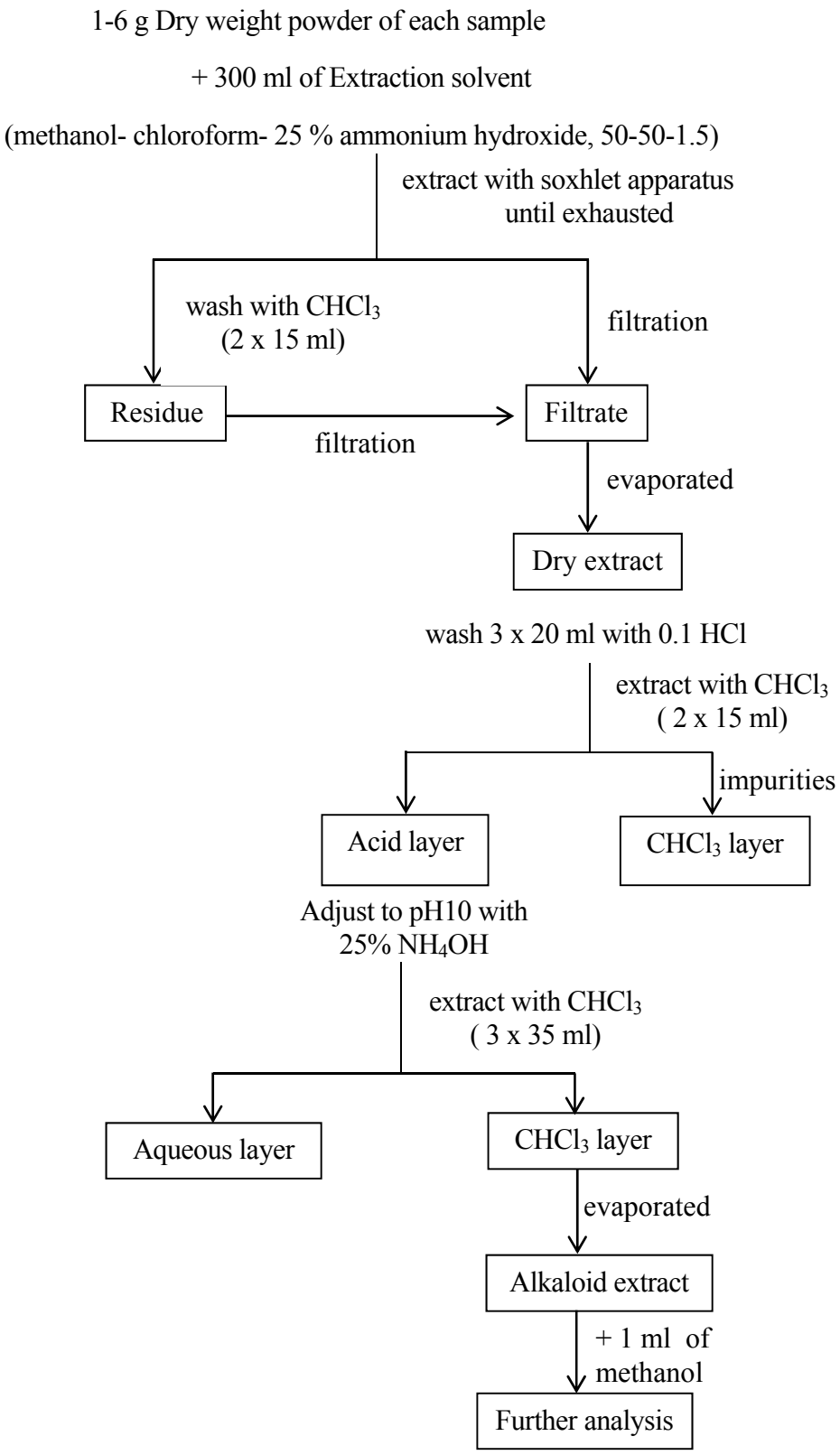


Figure 21. Schematic of alkaloids extraction from *D.metel* L.

Thin layer chromatography (TLC) image analysis

The TLC method was modified from Wagner & Sabine, and Sotanaphun (Wagner and Bladt, 2009; Sotanaphun et al., 2009). Ten microliters of each standard solutions (50 - 500 µg/ml) and alkaloids extracted solutions (leaf, flower, and fruit) were spotted as 10 mm bands in length onto the 20x20 cm aluminum sheets silica gel plate (G60 F254, Merck) 0.25 mm thickness and developed in the solvent system, toluene: ethyl acetate: diethylamine (70: 20: 10 v/v) for at least 1 hour and the developing distance was 18 cm. The distance between each spot was 0.5 cm. The scopolamine spots were detected with dragendorff's reagent.

The TLC chromatogram was scanned by a digital scanner (Hewlett Packard Deskjet F2280) and saved as a tagged image file (TIF) format at a resolution of 600 dpi. Quantification of each band was carried out by an image analysis using image J for windows version 1.45s (http://rsbweb.nih.gov/ij/). The colour image was converted to grayscale by photoshop software. The peak area corresponding to scopolamine was analysed by wand tool (available in the process toolbar) for peak area's measurement.

Method validation for TLC image analysis

In this study, the methods were evaluated for standard calibration curve and linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ).

Standard calibration curve, linearity, and detection range

For determination of standard calibration curve, a stock standard solution of scopolamine was prepared by dissolving 50 mg of standard scopolamine hydrochloride in 10 ml methanol. The working standard solutions of scopolamine were prepared by diluting the stock standard solution to obtain the concentration ranges of 0.5 to 5.0 mg/ml. Ten microliter of each standard solution was spotted onto the TLC plate to obtain the final concentration of 5 to 50 µg/spot, respectively. Each concentration was spotted seven times on the TLC plate. The chromatogram was developed and the image was scanned and analysed as described above. The peak

areas were plotted against the corresponding standard concentrations. The standard calibration curve was obtained from the average of peak areas of each standard concentration by using Microsoft excel program. The scopolamine content was calculated from the standard calibration curve. The sample with scopolamine content over than 5.0 mg/ml was diluted and reanalysed. The content of scopolamine was expressed as milligram per gram (mg/g) of dried weight.

Accuracy

The accuracy of the method was determined by using the standard addition method. (AOAC). Three different concentration (0.50, 1.50, and 2.50 µg/µl) of standard solution were added to the crude extract solution, whereas known amounts of scopolamine. The percentage recovery was calculated by the following equation:

$$\% \text{ Recovery} = \frac{(Cs - C) \times 100}{Ca}$$

Where Cs = the amount of scopolamine that found after adding standard solution

C = the amount of scopolamine that found before adding standard solution

Ca = the amount of reference standard actually added to the sample

Precision

Precision of the method was determined by analyzes the measurement of area under peak of six different concentrations (5-50 µg/spot) of standard solutions in triplicates on the same day (repeatability) and on five different days (intermediate precision) (ICH). The relative standard deviation (RSD) was calculated by the following formula:

$$\% \text{ RSD} = \frac{SD \times 100}{\overline{X}}$$

Where SD = standard deviation

$\overline{X}$ = mean

Limit of detection and limit of quantification (LOD and LOQ)

The LOD and LOQ of this method were determined based on the standard deviation of the response and the slope (ICH). The slope was estimated from the calibration curve of the analytic and the estimate of the standard deviation was carried out from the residual standard deviation of a regression line. The LOD and LOQ were calculated by the following formula:

$$LOD = 3.3\delta / S$$

$$LOQ = 10\ \delta / S$$

Where δ = the standard deviation of y-intercepts of regression lines

S = the slope of the calibration curve

High performance liquid chromatography (HPLC) analysis

The scopolamine content was determined by high performance liquid chromatography (HPLC) by following the modified method of Hoseini (Hoseini et al., 2011) (**Figure 22**). The methanolic solutions of all tested samples were analyzed by HPLC model LC-20A™ series using a ODS-3, C18 column, Inertsil®, sized 5 µm, 250 x 4.6 mm equipped with LC-20 AD binary pump, SPD-M 20 A: UV-PDA detector, automatic vacuum degasser, autosampler, column thermostat compartment, and a 20 µl injection loop. A ODS-3, C18 guard column, Inertsil®, sized 5 µm, 10 x 4 mm was coupled to the analytical column. The samples were analyzed using a buffer containing 50 mM potassium dihydrogen orthophosphate (adjusted to pH 3.0 by orthophosphoric acid prior before used) : acetonitrile (80: 20 v/v). The mobile phase was pumped at a constant flow rate of 1.0 ml/min and the column temperature was maintained at 40ºC. Injection volume of standard and sample solutions were 10 µl. Detector was set at a maximum absorption wavelength 215 nm for monitoring chromatographic profile. The absorbance spectra for every chromatographic run were acquired from 190 to 800 nm.

Table 7. HPLC conditions for determination of scopolamine content

HPLC Parameters	Conditions
Instrument	high performance liquid chromatography (HPLC) Model LC-20A™ series with LC solution workstation software (Shimadzu, Japan)
Analytical column	ODS-3 C_{18} column, Inertsil®, sized 5 µm, 250 x 4.6 mm id.
Guard column	ODS-3, C18 guard column, Inertsil®) sized 5 µm, 10 x 4 mm id.
Mobile phase	50 mM KH_2PO_4 (pH 3.0) : ACN (80 :20 v/v)
Mobile phase flow rate	1.0 ml/min
Column temperature	40 ° C
Injection volume	10 µl
Detector	UV-PDA
Peak width	5 sec
Minimum area	5,000 count

Prior to HPLC column injection, all sample solutions were filtered through a 0.45 µm PTFE Syringe filter. The filtrates were stored in vials until analysis.

Figure 22. High performance liquid chromatography (HPLC) Model LC-20A series with LC solution workstation software (Shimadzu, Japan)

Method validation for HPLC analysis

In this study, the methods were evaluated for standard calibration curve and linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ).

Standard calibration curve, linearity, and detection range

For determination of standard calibration curve, a stock standard solution of scopolamine was prepared by dissolving 50 mg of standard scopolamine hydrochloride in 10 ml methanol (5 mg/ml). The working standard solutions of scopolamine were prepared by diluting the stock standard solution to obtain the concentration ranges of 0.05 to 0.50 mg/ml. The standard solutions of scopolamine were prepared at 6 concentration levels (50 -500 µg/ml) and analysed by HPLC under conditions were described in **Table 7**. Ten microliters containing 50 to 500 µg/ml of standards and samples were injected to HPLC instrument (triplicate injections) respectively. The peak areas were plotted against the corresponding standard concentration to obtain the standard calibration curve by using LC solution

workstation software (Shimadzu, Japan). The standard calibration curve was obtained from the average of peak areas of each standard concentration. The scopolamine content was calculated from the standard calibration curve. The sample with scopolamine content over than 0.50 mg/ml was diluted and reanalysed. The content of scopolamine was expressed as milligram per gram of dried weight.

Accuracy

The accuracy of the method was determined by using the standard addition method. (AOAC). Three different concentration (50, 150, and 250 µg/µl) of standard solution were added to the crude extract solution, whereas known amounts of scopolamine. The percentage recovery was calculated by the following equation:

$$\% \text{ Recovery} = \frac{(Cs - C) \times 100}{Ca}$$

Where Cs = the amount of scopolamine that found after adding standard solution

C = the amount of scopolamine that found before adding standard solution

Ca = the amount of reference standard actually added to the sample

Precision

Precision of the method was determined by analyzes the measurement of area under peak of three different concentrations (100.0, 300.0, and 500.0 µg/ml) of standard solutions in triplicates on the same day (repeatability) and on five different days (intermediate precision) (ICH). The relative standard deviation (RSD) was calculated by the following formula:

$$\% \text{ RSD} = \frac{SD \times 100}{\overline{X}}$$

Where SD = standard deviation

$\overline{X}$ = mean

Limit of detection and limit of quantification (LOD and LOQ)

The LOD and LOQ of this method were determined based on the standard deviation of the response and the slope (ICH). The slope was estimated from the calibration curve of the analytic and the estimate of the standard deviation was carried out from the residual standard deviation of a regression line. The LOD and LOQ were calculated by the following formula:

$$LOD = 3.3\ \sigma/S$$

$$LOQ = 10\ \sigma/S$$

Where σ = the standard deviation of y-intercepts of regression lines

S = the slope of the calibration curve

Statistic analysis

For TLC image method**,** the data will be calculated as grand mean and standard deviation (grand mean ± pooled SD). For determination of scopolamine content, the area under peak will be analyzed using Image J software.

For HPLC method, the data will be calculated as grand mean and standard deviation (grand mean ± pooled SD). For determination of scopolamine content, the area under peak will be analyzed using LC solution workstation software

The scopolamine content was statistically analyzed using paired *t*-test by SPSS version 17.0 for windows program for analyzing of significant difference between two methods.

CHAPTER IV
RESULTS

Part I. Macroscopic and microscopic evaluation of *D.metel* L. var. *metel* and *D.metel* L. var. *fastuosa*

1.1 Macroscopic evaluation

The observation of the areal part such as leaf, flower, stem and fruit of two variety of *D.metel* L. were compared (**Table 8**), and the drawing outline was done as shown in **Figure 23-24.**

D.metel L. var. *metel* is an annual herbaceous plant, green stem colour, erect, 1-1.5 m high. The alternate leaves have petioles 3-7 cm long. Leaves are ovate or broadly ovate, acute or acuminate apex, equal or symmetrical at the base, margins are repand-dentate or angulate with 3-4 coarse teeth. Sizes of leaves are approximately 6 to 15 cm long by 5-11 cm wide. The large tubular flowers are axillary and usually solitary. They are erect or nodding, have a five-toothed, calyx is 4-6 cm long, white or white cream colour, corolla is 8-15 cm long, and often single. The stem and branch are green colour. The fruit is in the form of a spiny and green colour capsule, borne on a short thick peduncle. Seeds are flat, yellowish-brown color, kidney-shaped, about 5 mm long, and have a small fleshy aril, which nearly fill the interior capsule.

D.metel L. var. *fastuosa* is a shrub-like herb with large flower, 1-1.5 m high. The alternate leaves have petioles 3-7 cm long. Leaves are ovate or broadly ovate, acute or acuminate apex, unequal or asymmetrical at the base and often cordate or heart-shaped with sinuate to irregularly toothed edges. Sizes of leaves are approximately 7 to 16 cm long by 4-10 cm wide. Flower is always erectly standing, occur in duplicate or triplicate. Calyx is inflated towards the middle of flower, persistent and reflexes in fruit. Corolla is about double or triple as long as the calyx, white or tinged with green. The stem and branch are purple or dark purple colour. The fruit is in the form of a glabrous or short spines and purple colour capsule, borne on a short thick peduncle. Seeds are flat, yellowish-brown colour, kidney-shaped, about 5 mm long, and have a small fleshy aril, which nearly fill the interior capsule.

Table 8. The comparison of macroscopic character of *D. metel* L.

Part of plant	***D.metel* L. var. *metel***	***D.metel* L. var. *fastuosa***
Flower	Single corolla, large flower with white or white cream colour	Double or triple corolla, large flower with white inside, and violet outside
Stem and branch	Stem and branch are green colour	Stem and branch are purple or dark violet colour
Lamina	Leaves are ovate or broadly ovate, acute or acuminate apex, margins are repand-dentate or angulate with 3-4 coarse teeth, and equal or symmetrical at the base	Leaves are ovate or broadly ovate, acute or acuminate apex, margins are cordate or heart-shaped with sinuate to irregularly toothed edges, and unequal or asymmetrical at the base
Fruit or capsule	Spiny, green colour capsule	Glabrous or short spines, purple colour capsule

Figure 23. Whole plant of *D. metel* L. var. *metel* with flower **(**a), fruiting branch (b), and seed (c)

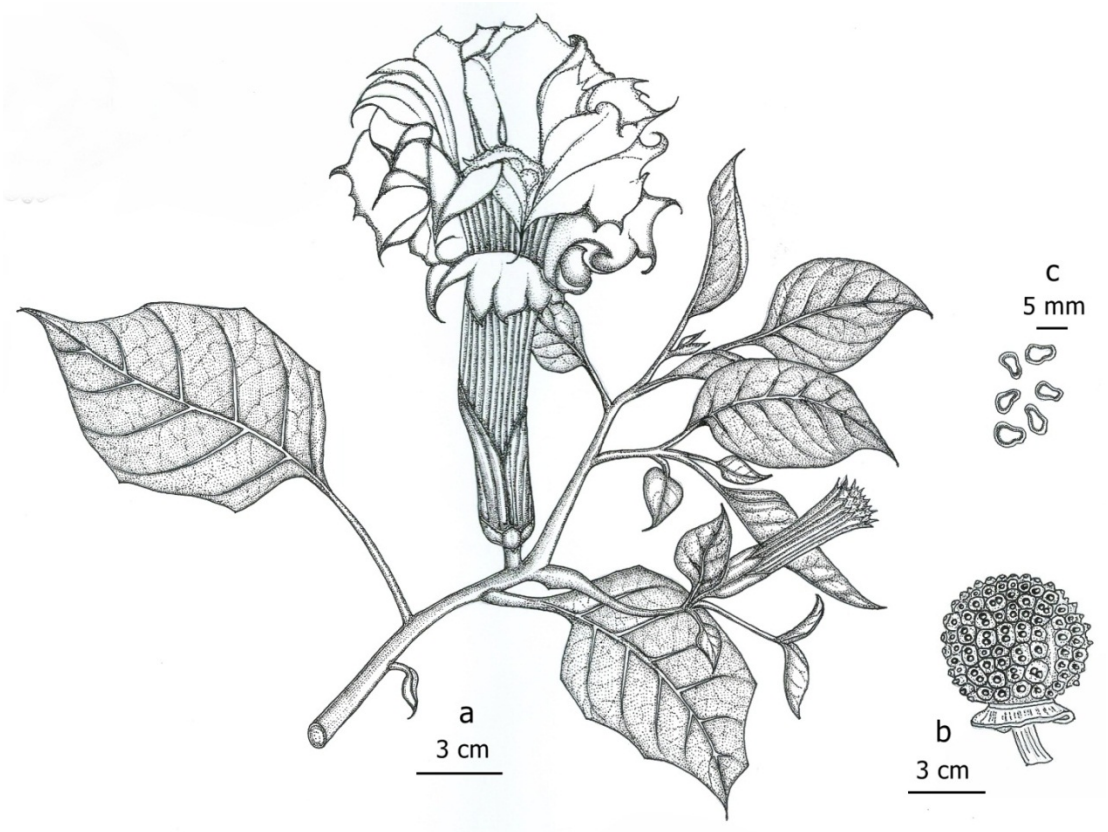


Figure 24. Whole plant of *D. metel* L. var. *fastuosa* with flower **(**a), fruiting branch (b), and seed (c)

1.2 Microscopic evaluation

Microscopic characters of two variety of *D.metel* L. were examined in both upper and lower epidermis, and transverse section of midrib and stem. The stomata of two varieties of *D.metel* L. could be found on both side of the leaves and classified as the anisocytic stomata type**.** A number of stomata of lower (abaxial) epidermis were more presented than upper (adaxial) epidermis **(Figure 25-26)**.

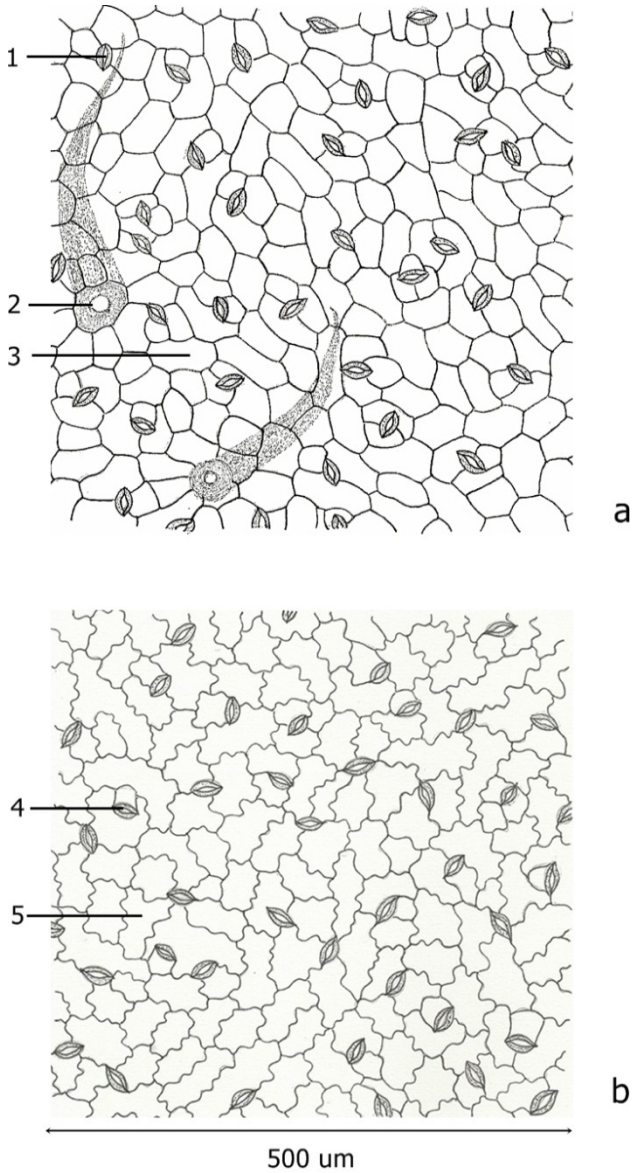


Figure 25. Epidermis of the leaf of *D. metel* L. var. *metel* with 20x magnification

a: Upper epidermis, 1. Anisocytic type stoma, 2. Multicellular uniseriated trichome, 3. Epidermal cell

b: Lower epidermis, 4. Anisocytic type stoma, 5. Epidermal cell

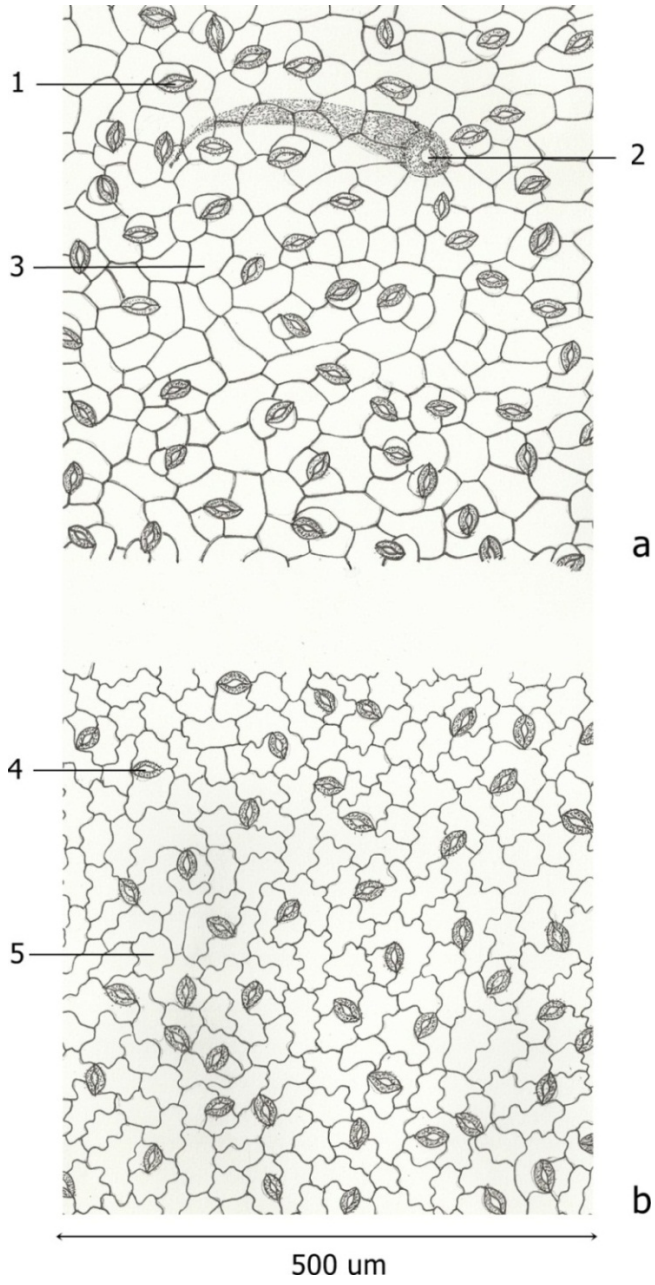


Figure 26. Epidermis of the leaf of *D. metel* L. var. *fastuosa* with 20x magnification

a: Upper epidermis, 1. Anisocytic type stoma, 2. Multicellular uniseriated trichome, 3. Epidermal cell

b: Lower epidermis, 4. Anisocytic type stoma, 5. Epidermal cell

1.2.1 Stomatal number, stomatal index, and Palisade ratio

The constant number of leaf measurements values which consists of stomatal number, stomatal index and palisade ratio were analyzed by microscopic assessment as described in chapter III. The mean and standard deviation of stomatal number (upper and lower epidermis), stomatal index (upper and lower epidermis) in the area of 1 mm^2 and palisade ratio of *D. metel* L. var. *metel* and *D. metel* L. var. *fastuosa* were shown in **Table 9-10**. The independent samples *t*-test of stomatal number (upper and lower epidermis) **(Table 11-12)**, stomatal index (upper and lower epidermis) **(Table 13-14)**, and palisade ratio of two varieties of *D. metel* L. **(Table 15)** were shown. There were significant differences between the two varieties of *D.metel* L. (the significance is less than .05) **(Table 11-15)**.

Table 9. The average leaf measurement values of *D. metel* L. var. *metel* from three locations. (mean ± SD, n = 90)

D. metel* L. var. *metel				
stomatal number		**stomatal index**		**palisade ratio**
upper epidermis	lower epidermis	upper epidermis	lower epidermis	
85.58 ± 18.90	204.53 ± 23.40	13.59 ± 1.00	19.08 ± 0.96	5.11 ± 0.54

Table 10. The average leaf measurement values of *D. metel* L. var. *fastuosa* from three locations. (mean ± SD, n = 90)

D. metel* L. var. *fastuosa				
stomatal number		**stomatal index**		**palisade ratio**
upper epidermis	lower epidermis	upper epidermis	lower epidermis	
190.96 ± 29.03	235.89 ± 31.81	19.29 ± 0.98	20.82 ± 1.16	6.34 ± 0.68

Table 11. The independent samples test of upper stomatal number of *D. metel* L. var. *metel* and *D. metel* L. var. *fastuosa*

Group Statistics

	group	N	Mean	Std. Deviation	Std. Error Mean
Upper stomatal number	1	90	85.58	18.899	1.992
	2	90	190.96	29.030	3.060

1 = *D. metel* L. var. *metel*

2 = *D. metel* L. var. *fastuosa*

Independent Samples Test

Upper stomatal number	Levene's Test for Equality of Variances		t-test for Equality of Means						
								95% Confidence Interval of the Difference	
	F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Equal variances assumed	12.797	.000	-28.860	178	.000	-105.378	3.651	-112.583	-98.172
Equal variances not assumed			-28.860	152.954	.000	-105.378	3.651	-112.591	-98.164

Table 12. The independent samples test of lower stomatal number of *D. metel* L. var. *metel* and *D. metel* L. var. *fastuosa*

Group Statistics

	group	N	Mean	Std. Deviation	Std. Error Mean
Lower stomatal number	1	90	204.53	23.395	2.466
	2	90	235.89	31.809	3.353

1= D. metel L. var. *metel*

2= *D. metel* L. var. *fastuosa*

Lower stomatal number	Levene's Test for Equality of Variances		t-test for Equality of Means						
								95% Confidence Interval of the Difference	
	F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Equal variances assumed	4.243	.041	-7.533	178	.000	-31.356	4.162	-39.569	-23.142
Equal variances not assumed			-7.533	163.489	.000	-31.356	4.162	-39.574	-23.137

Table 13. The independent samples test of upper stomatal index of *D. metel* L. var. *metel* and *D. metel* L. var. *fastuosa*

Group Statistics

	group	N	Mean	Std. Deviation	Std. Error Mean
Upper stomata index	1	90	13.5891	.99703	.10510
	2	90	19.2896	.98475	.10380

1 = *D. metel* L. var. *metel*

2 = *D. metel* L. var. *fastuosa*

Independent Samples Test

Upper stomatal index	Levene's Test for Equality of Variances		t-test for Equality of Means						
								95% Confidence Interval of the Difference	
	F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Equal variances assumed	.122	.728	-38.591	178	.000	-5.70044	.14772	-5. 99194	-5.40894
Equal variances not assumed			-38.591	177.973	.000	-5.70044	.14772	-5.99194	-5.40894

Table 14. The independent samples test of lower stomatal index of *D. metel* L. var. *metel* and *D .metel* L. var. *fastuosa*

Group Statistics

	group	N	Mean	Std. Deviation	Std. Error Mean
Lower stomatal index	1	90	19.0821	.96245	.10145
	2	90	20.8182	1.16014	.12229

1 = *D. metel* L. var. *metel*

2 = *D. metel* L. var. *fastuosa*

Independent Samples Test

Lower stomatal index	Levene's Test for Equality of Variances		t-test for Equality of Means						
								95% Confidence Interval of the Difference	
	F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Equal variances assumed	3.784	.053	-10.926	178	.000	-1.73611	.15889	-2.04967	-1.42255
Equal variances not assumed			-10.926	172.130	.000	-1.73611	.15889	-2.04974	-1.42248

Table 15. The independent samples test of palisade ratio of *D. metel* L. var. *metel* and *D. metel* L. var. *fastuosa*

Group Statistics

	group	N	Mean	Std. Deviation	Std. Error Mean
Palisade ratio	1	90	5.1088	.54255	.05719
	2	90	6.3370	.67592	.07125

1 = *D. metel* L. var. *metel*

2 = *D. metel* L. var. *fastuosa*

Independent Samples Test

Palisade ratio	Levene's Test for Equality of Variances		t-test for Equality of Means						
								95% Confidence Interval of the Difference	
	F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Equal variances assumed	3.267	.072	-13.443	178	.000	-1.22822	.09136	-1.40851	-1.04793
Equal variances not assumed			-13.443	170.042	.000	-1.22822	.09136	-1.40857	-1.04787

1.2.2 Stem and midrib cross section

The stem cross section of *D. metel* L. var. *metel*, and *D. metel* L. var. *fastuosa* showed a single striated layered epidermis cells. The epidermal cells were tabular shaped and covered externally by a fairly thick cuticle, which having a few multicellular uniseriated and glandular trichomes. The chromoplast layer, that contained anthocyanin pigment was found only in *D. metel* L. var. *fastuosa*. The chlorenchyma was located next to the collenchyma. The xylem vessels were align gather in group located next to chlorenchyma. The vascular fibers align gather in group and were interposed horizontally above parenchyma of pith **(Figure 27-28)**.

The midrib cross section of *D. metel* L. var. *metel*, and *D. metel* L. var. *fastuosa* showed multicellular uniseriated trichomes located on the epidermis cells, which covered externally by a fairly thick cuticle . Both of collenchyma located next to the upper and lower epidermis, while parenchyma located next to the collenchyma. The palisade mesophyll consists of a single layer of cells, which lie above spongy mesophyll. The central of midrib, situated vascular tissue is surrounded by parenchyma cells. The xylem vessels and vascular fibers, which align gather in group were sparsely in vascular tissue. Anisocytic stoma type was presented on the both upper and lower epidermis **(Figure 29 - 30)**.

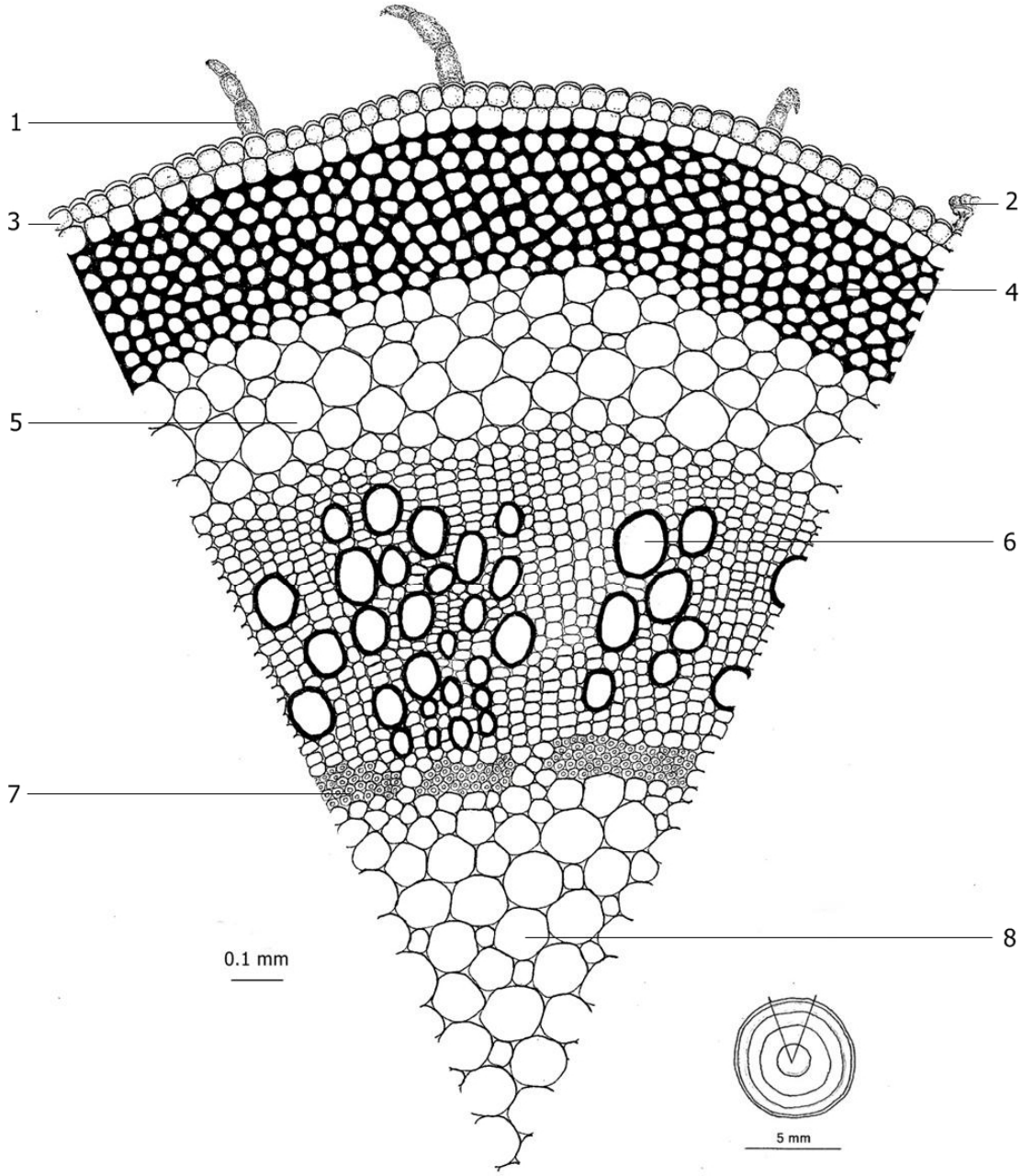


Figure 27. Stem cross section of *Datura metel* L. var. *metel*

1. Multicellular uniseriated trichome
2. Glandular trichome
3. Epidermis
4. Collenchyma
5. Chlorenchyma
6. Xylem vessel
7. Vascular fiber
8. Parenchyma of pith

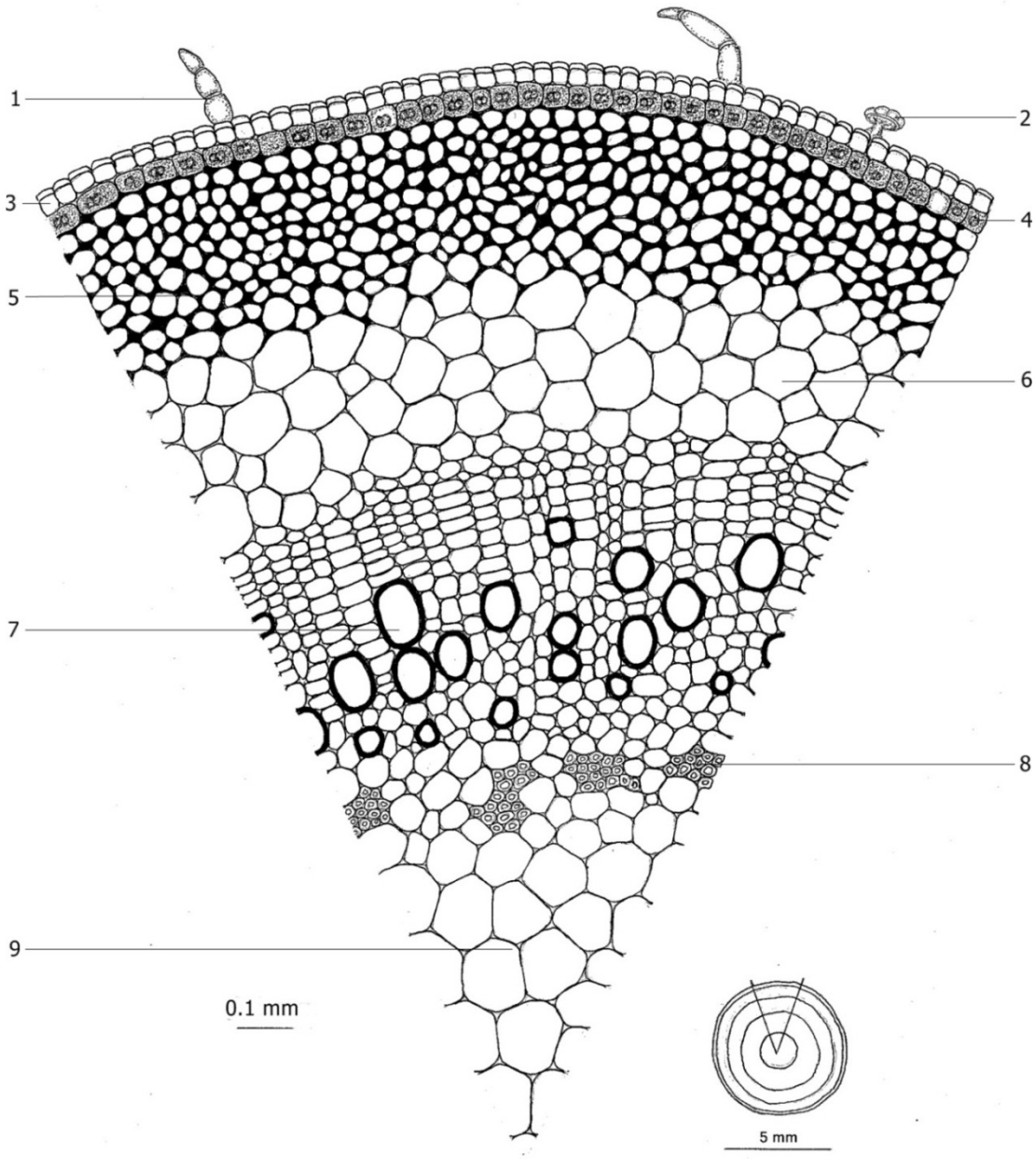


Figure 28. Stem cross section of *Datura metel* L. var. *fastuosa*

1. Multicellular uniseriated trichome 2. Glandular trichome
3. Epidermis 4. Chromoplast containing anthocyanin pigment
5. Collenchyma 6. Chlorenchyma
7. Xylem vessel 8. Vascular fiber
9. Parenchyma of pith

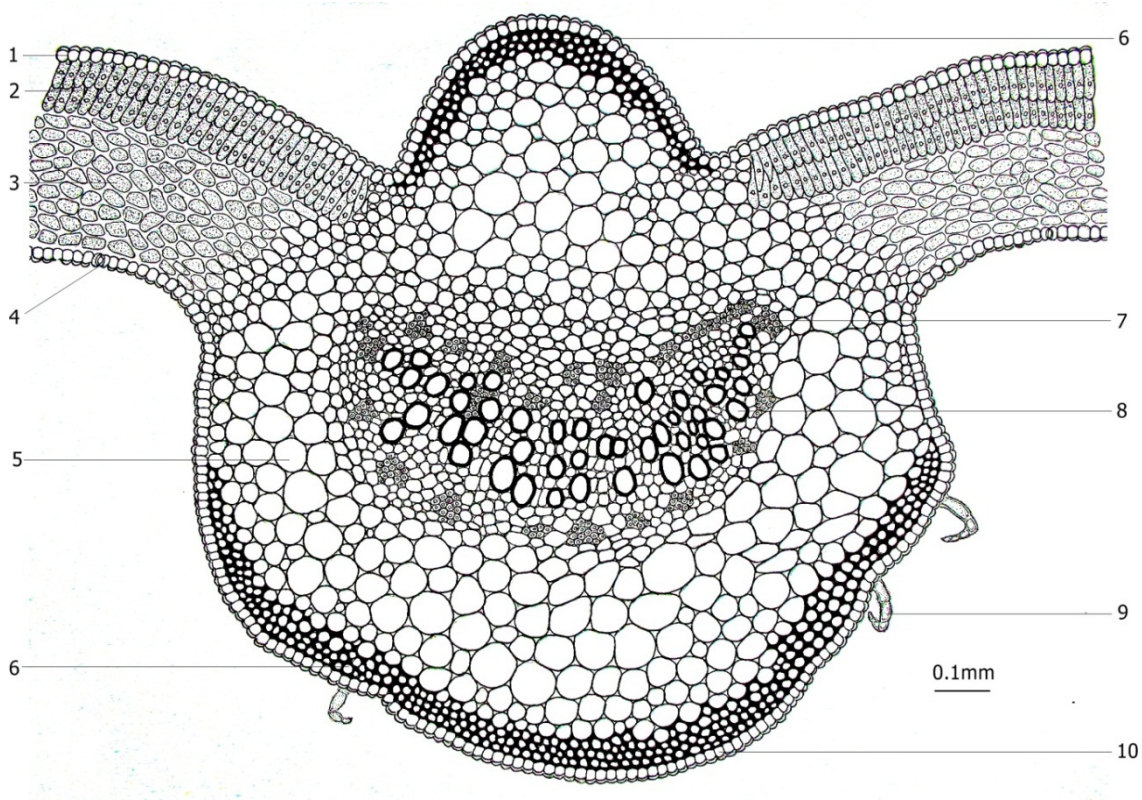


Figure 29. Midrib cross section of *Datura metel* L. var. *metel*

1. Upper epidermis
2. Palisade mesophyll
3. Spongy mesophyll
4. Stomata
5. Parenchyma
6. Collenchyma
7. Vascular fiber
8. Xylem vessel
9. Multicellular uniseriated trichome
10. Lower epidermis

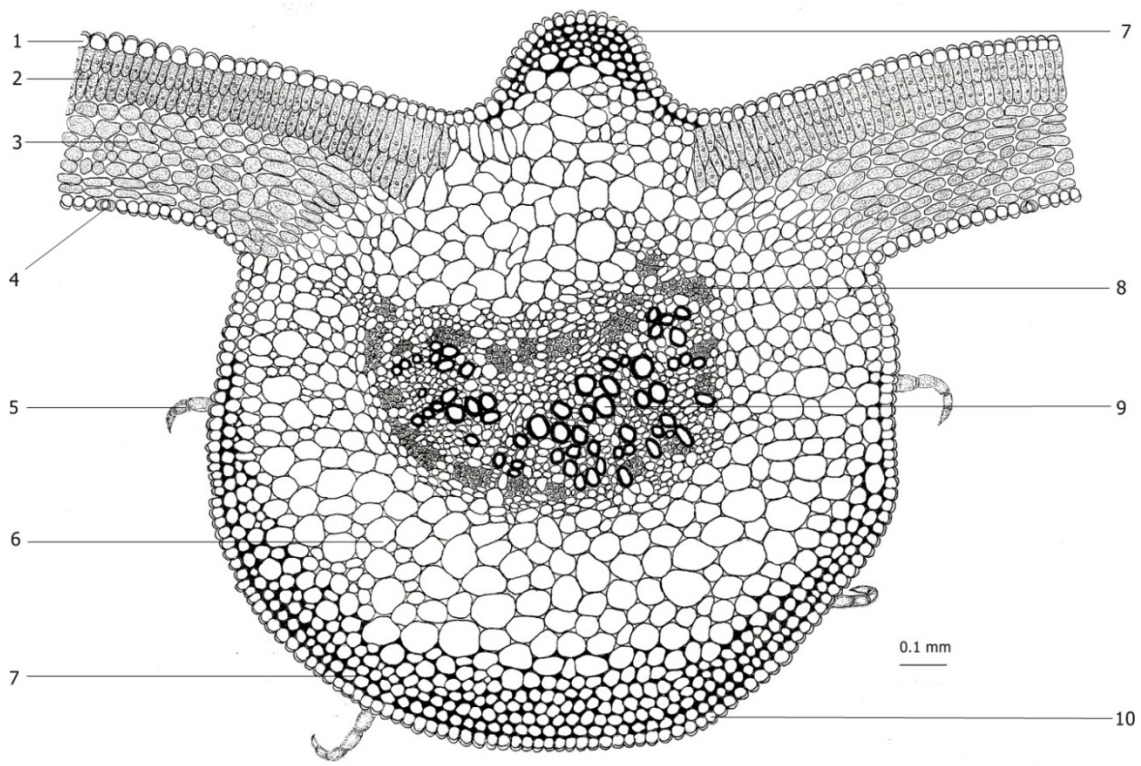


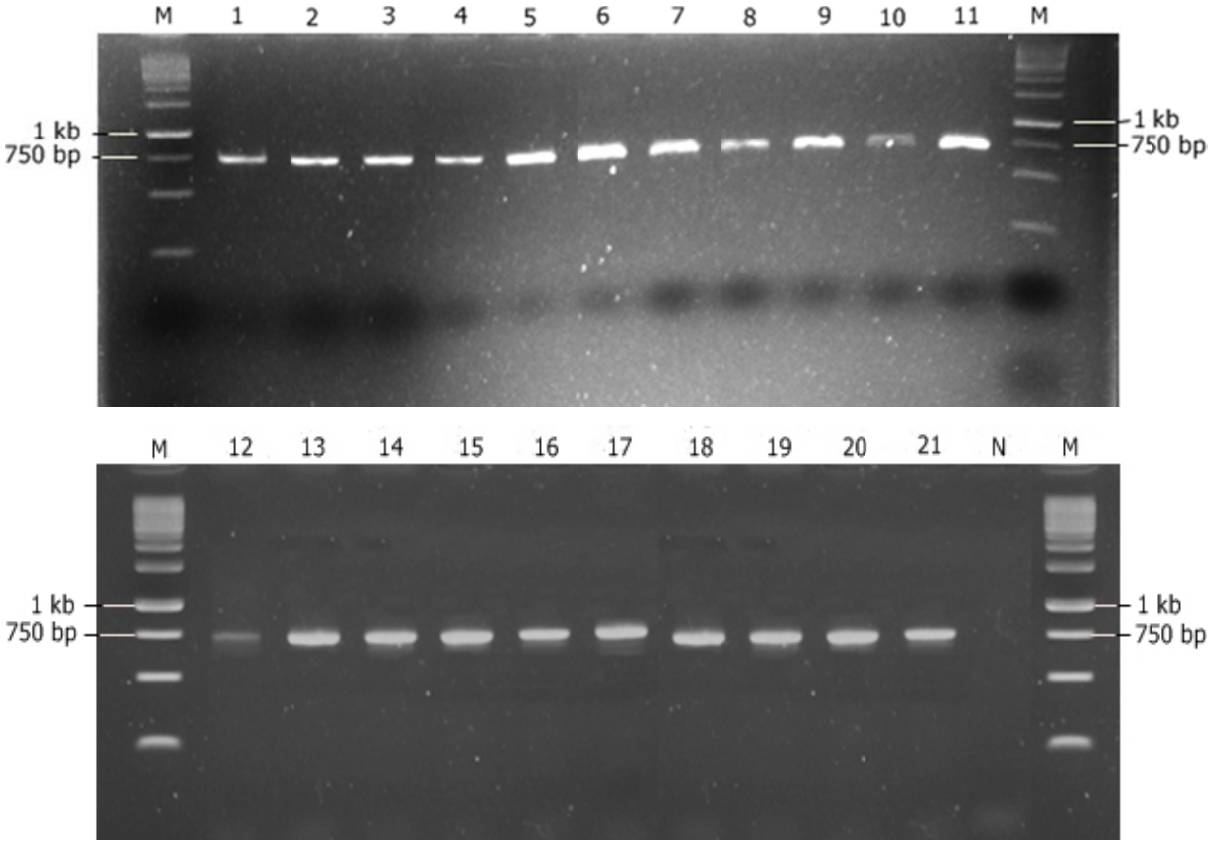
Figure 30. Midrib cross section of *Datura metel* L. var. *fastuosa*

1. Upper epidermis
2. Palisade mesophyll
3. Spongy mesophyll
4. Stomata
5. Multicellular uniseriated trichome
6. Parenchyma
7. Collenchyma
8. Vascular fiber
9. Xylem vessel
10. Lower epidermis

Part ll. Molecular evaluation of *D.metel* L. var. *metel* and *D.metel* L. var. *fastuosa*

2.1 ITS amplification

A pair of universal PCR primers (ITS5 and ITS4) designed from highly conserved regions flanking the Internal transcribe spacer (ITS) region were used for PCR amplification. The PCR products were subjected to electrophoresis using1.5% agarose gel, then stained with ethidium bromide and visualized under UV transilluminator. An approximately 700 bp in size of PCR products comparing to 1 kb DNA ladder (Fermentas, USA) were obtained as shown in **Figure 31**.

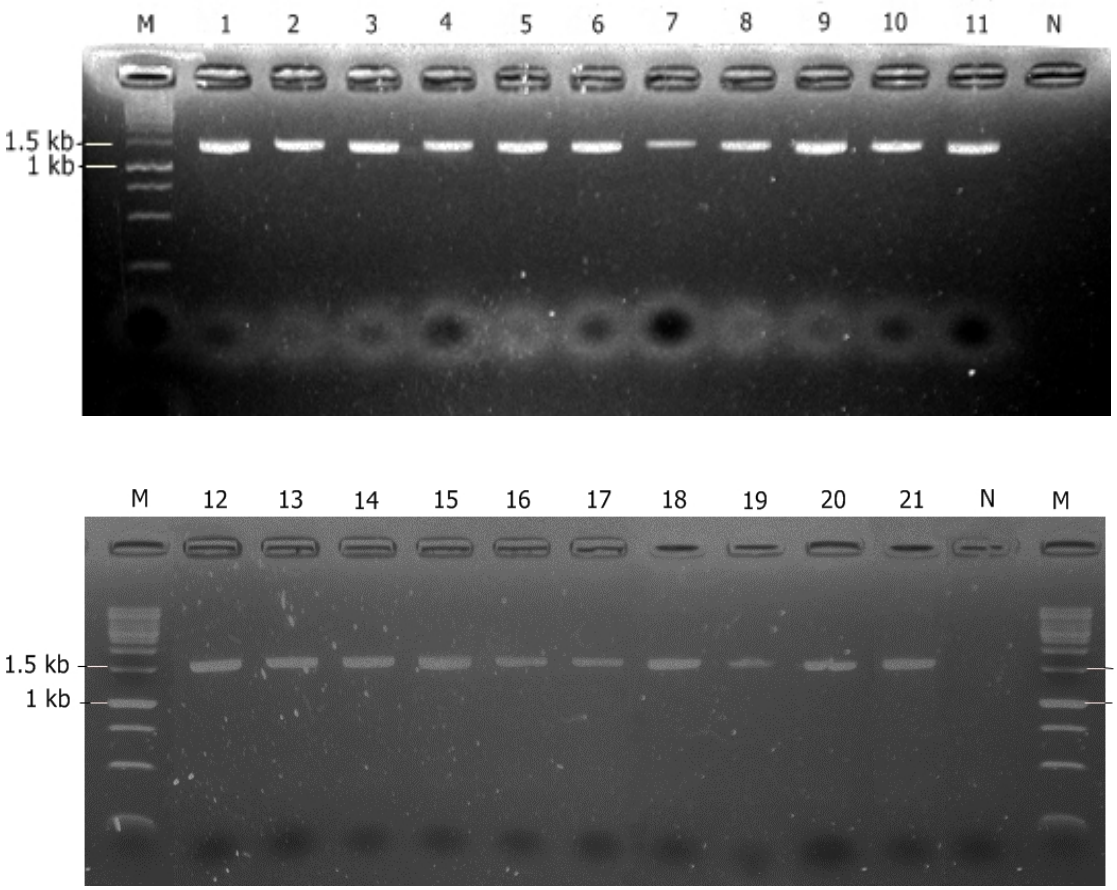


Lane M:	1kb DNA Ladder	Lane 8:	KSL051105	Lane 16:	LHB061108
Lane 1:	LPK081001	Lane 9:	LHB071001	Lane 17:	LHB071109
Lane 2:	LPK091002	Lane 10:	LHB081002	Lane 18:	LHB081110
Lane 3:	LPK011103	Lane 11:	LHB091003	Lane 19:	LHB081111
Lane 4:	KSL071001	Lane 12:	LHB101004	Lane 20:	LHB091112
Lane 5:	KSL071002	Lane 13:	LHB101005	Lane 21:	TNF111001
Lane 6:	KSL031103	Lane 14:	LHB021106	Lane N:	negative control
Lane 7:	KSL041104	Lane 15:	LHB031107		

Figure 31. The ITS amplification products in 1.5% agarose gel electrophoresis

2.2 *rbc*L amplification

A pair of universal PCR primers (*rbc*L-F and *rbc*L-R) were used for PCR amplification. The PCR products were subjected to electrophoresis using1.5% agarose gel, then stained with ethidium bromide and visualized under UV transilluminator. An approximately 1.5kp in size of PCR products comparing to 1 kb DNA ladder (Fermentas, USA) were obtained as shown in **Figure 32**.

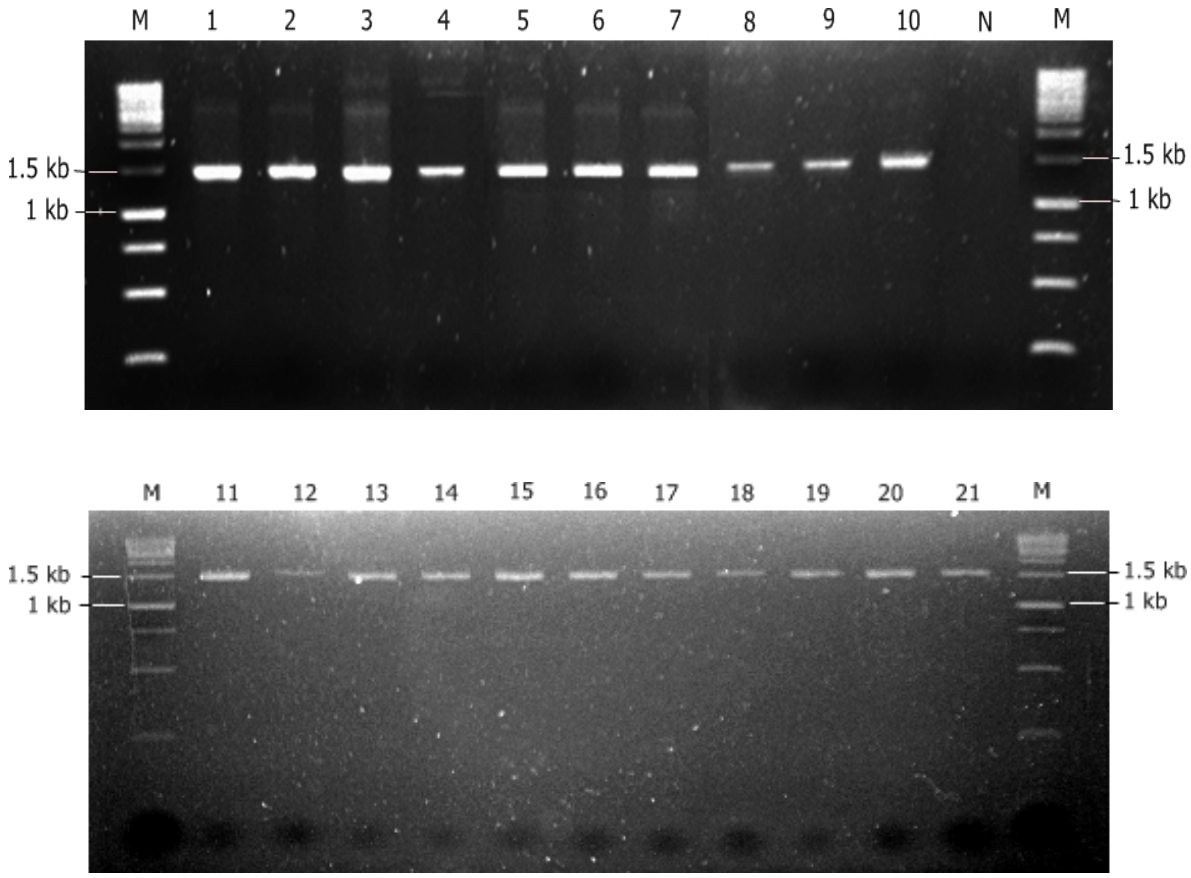


Lane M:	1kb DNA Ladder	Lane 8:	KSL051105	Lane 16:	LHB061108
Lane 1:	LPK081001	Lane 9:	LHB071001	Lane 17:	LHB071109
Lane 2:	LPK091002	Lane 10:	LHB081002	Lane 18:	LHB081110
Lane 3:	LPK011103	Lane 11:	LHB091003	Lane 19:	LHB081111
Lane 4:	KSL071001	Lane 12:	LHB101004	Lane 20:	LHB091112
Lane 5:	KSL071002	Lane 13:	LHB101005	Lane 21:	TNF111001
Lane 6:	KSL031103	Lane 14:	LHB021106	Lane N:	negative control
Lane 7:	KSL041104	Lane 15:	LHB031107		

Figure 32. The *rbc*L amplification products in 1.5% agarose gel electrophoresis

2.3 *atp*B amplification

A pair of universal PCR primers (*atp*B-F and *atp*B-R) were used for PCR amplification. The PCR products were subjected to electrophoresis using1.5% agarose gel, then stained with ethidium bromide and visualized under UV transilluminator. An approximately 1.5kp in size of PCR products comparing to 1 kb DNA ladder (Fermentas, USA) were obtained as shown in **Figure 33.**



Lane M:	1kb DNA Ladder	Lane 8:	KSL051105	Lane 16:	LHB061108
Lane 1:	LPK081001	Lane 9:	LHB071001	Lane 17:	LHB071109
Lane 2:	LPK091002	Lane 10:	LHB081002	Lane 18:	LHB081110
Lane 3:	LPK011103	Lane 11:	LHB091003	Lane 19:	LHB081111
Lane 4:	KSL071001	Lane 12:	LHB101004	Lane 20:	LHB091112
Lane 5:	KSL071002	Lane 13:	LHB101005	Lane 21:	TNF111001
Lane 6:	KSL031103	Lane 14:	LHB021106	Lane N:	negative control
Lane 7:	KSL041104	Lane 15:	LHB031107		

Figure 33. The *atp*B amplification products in 1.5% agarose gel electrophoresis

The total length of the nucleotide fragments of all *D.metel* L. using ITS4 and ITS5 as a universal primers were 670 bp. Sequence comparison showed degree of sequence homology with 99-100% similarity, with 63% GC content. The multiple sequence alignment of ITS region of *D.metel* L. var. *metel*, *D.metel* L. var. *fastuosa*, and hybrid *D.metel* L. were showed in **Appendix B**. The length of ITS1 region is 234 bp from position 1 to 234, 5.8S region is 165 bp from position 235 to 399, and ITS2 region is 271 bp from position 400 to 670. There are two polymorphisms within the 5.8S region, four polymorphisms within the ITS2 as showed in **Table 16.** The phylogenetic relationship of ITS region sequences was generated and showed in **Figure 34**.

Table 16. The alignments of ITS sequences of *D.metel* L. var. *metel*, *D.metel* L. var. *fastuosa*, and hybrid *D.metel* L.

Samples	**Nucleotide Position**					
	5.8S		**ITS2**			
	335	**350**	**499**	**506**	**512**	**614**
D.metel* L. var. *fastuosa						
KSL071001	G	A	T	G	A	G
KSL071002	G	A	A	A	A	G
KSL031103	G	A	T	G	A	G
KSL041104	G	A	T	G	A	G
KSL051105	G	A	T	G	A	G
D.metel* L. var. *metel						
LPK081001	G	A	T	G	C	A
LPK091002	G	A	T	G	C	A
LPK011103	G	A	T	G	C	A
hybrid *D.metel* L.						
LHB071001	G	A	T	G	A	G
LHB081002	C	T	T	G	C	A
LHB091003	G	A	T	G	C	A
LHB101004	G	A	T	G	C	A
LHB101005	G	A	T	G	C	A
LHB021106	G	A	T	G	C	A
LHB031107	G	A	T	G	A	G
LHB061108	G	A	T	G	C	A
LHB071109	G	A	T	G	A	G
LHB081110	G	A	T	G	C	A
LHB081111	G	A	T	G	C	A
LHB091112	G	A	T	G	C	A

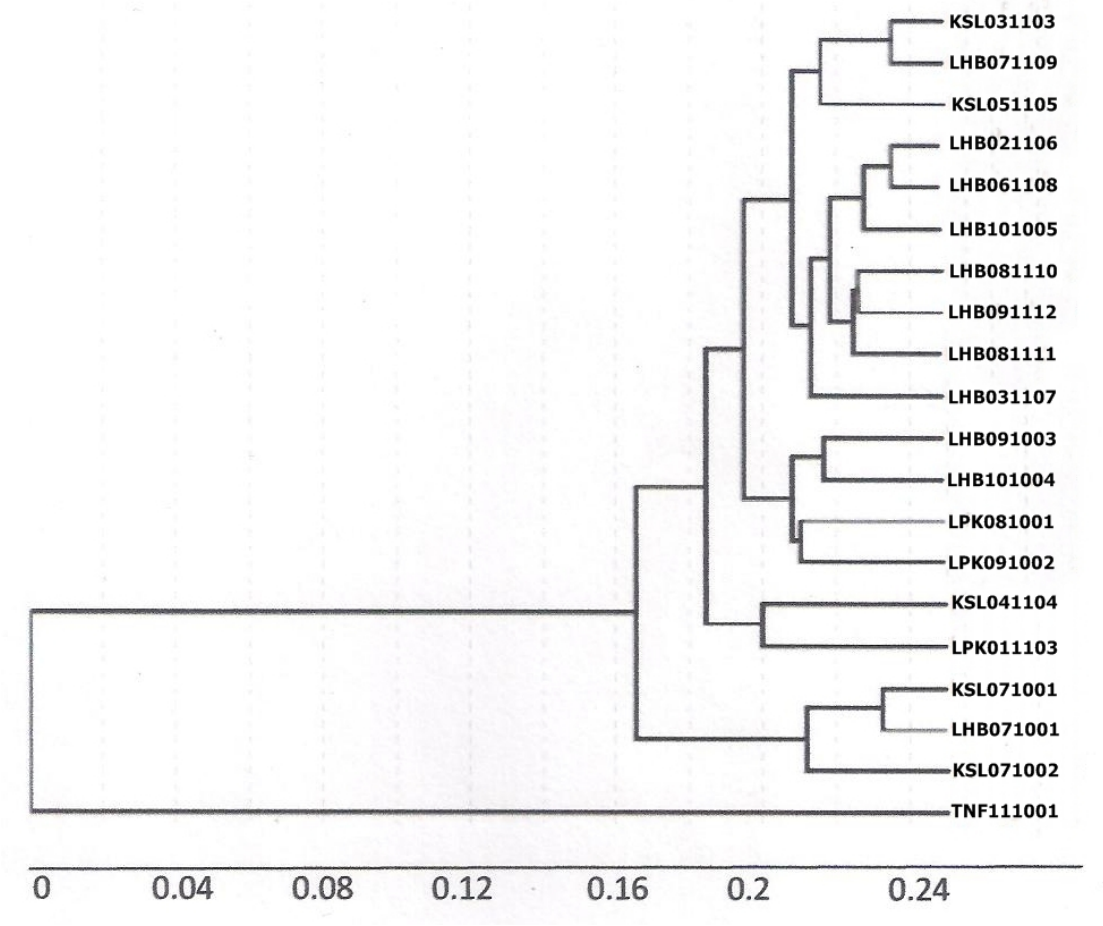


Figure 34. Phylogenetic relationship of nucleotide sequences of ITS region of *D.metel* L. var. *metel*, *D.metel* L. var. *fastuosa*, and hybrid *D.metel* L., (TNF111001 *D. arborea* L. was used as out group)

Both of *atp*B *and rbc*L of PCR products of *D.metel* L. var. *metel*, *D.metel* L. var. *fastuosa*, and hybrid *D.metel* L. were sequenced. The *rbc*L sequences were 1,486-1,523 bp in length, with GC content 43%. The Sequence comparison of all *D.metel* L. showed 95-100 % similarity, while *atp*B sequences were 1,184-1,464 bp in length, with GC content 43%. The Sequence comparison of all *D.metel* L. showed 94-99 % similarity. The multiple sequence alignments of *atp*B, *and rbc*L of *D.metel* L. var. *metel*, *D.metel* L. var. *fastuosa*, and hybrid *D.metel* L. were showed in **Appendix B**.

Part lll. Scopolamine evaluation of *D.metel* L. var. *metel* and *D.metel* L. var. *fastuosa*

3.1 Preparation of crude extract for scopolamine determination

The approximately 1-6 g dried powder of each sample (leaf, flower, and fruit) of *D. metel* L. var. *metel* and *D. metel* L.var. *fastuosa* from six locations were extracted by sample extraction method, that was mentioned in chapter III (**Figure 21**). All samples were extracted by soxhlet apparatus. The yields of crude extracts ranged from 0.76 to 11.03 % dry weight. The yield of crude extract of each sample was shown in **Table 17.**

Table 17. Yield of crude extract of *D. metel* L. var. *metel* and *D. metel* L.var. *fastuosa* from six locations (% w/w of dry weight)

Sample	Location	Part used	Weight of dried powder (gram)	Crude extract (% dry weight)
D. metel L. var. *metel*	Rayong (LPK081001)	Leaf	4.8890	7.63
		Flower	3.0660	6.89
		Fruit	5.1922	9.85
	Singburi (LPK011103)	Leaf	4.0378	0.76
		Flower	4.7602	4.48
		Fruit	5.0084	4.15
	Nakhonpathom (LPK091002)	Leaf	4.8163	1.97
		Flower	1.2832	6.74
		Fruit	5.1220	11.03
D. metel L.var. *fastuosa*	Bangkok (KSL071001)	Leaf	4.3354	2.18
		Flower	4.8188	5.10
		Fruit	5.0186	5.96
	Chonburi (KSL051105)	Leaf	2.7476	10.15
		Flower	3.8450	6.22
		Fruit	4.8964	9.51
	Chachoengsao (KSL041104)	Leaf	6.3974	4.63
		Flower	5.1344	6.72
		Fruit	5.6851	5.94

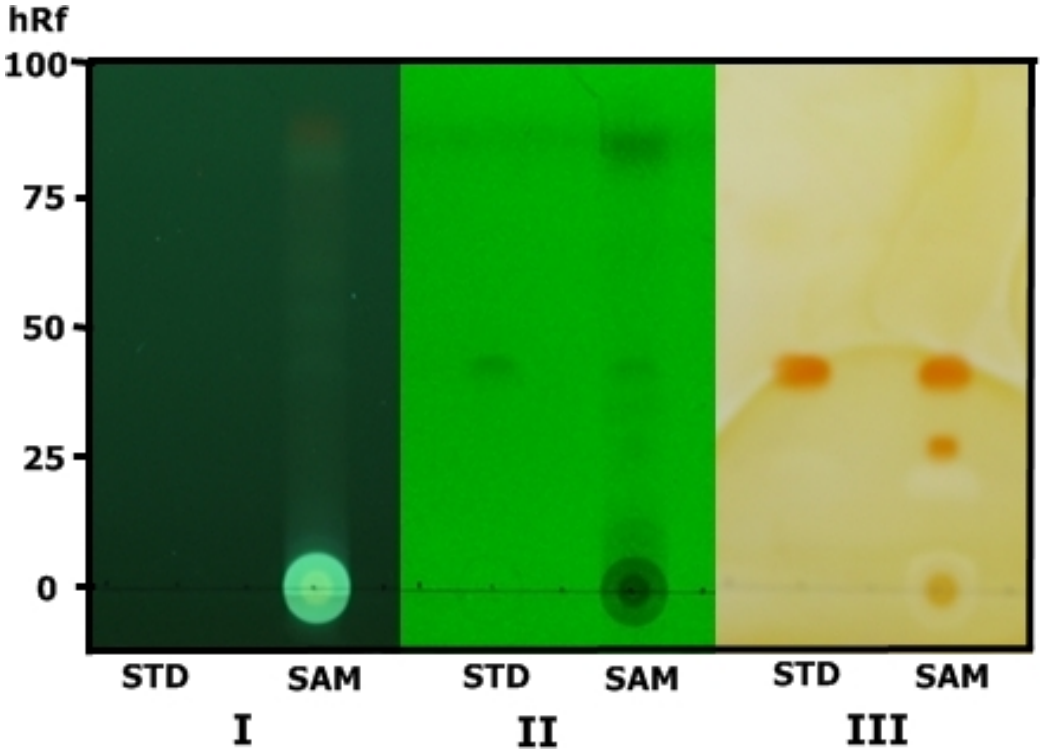


Figure 35. TLC fingerprint of alkaloid extracted of *D.metel* L. var. *metel*

Detection I = detection under UV light 365 nm
II = detection under UV light 254 nm
III = detection with dragendorff's reagent
STD = standard scopolamine hydrochloride
SAM = alkaloid extracted of *D.metel* L. var. *metel*

Developing solvent : Toluene-ethyl acetate-diethylamine = 7-2-1

R*f* values = $\frac{\text{the distance from baseline travelled by the substance}}{\text{the distance of solvent front from the baseline}} = 0.37$

3.2 Determination of scopolamine content by TLC image method

The scopolamine contents of each sample (leaf, flower and fruit) of *D. metel* L. var. *metel* and *D. metel* L.var. *fastuosa* from six different locations were evaluated by TLC Image analysis using Image J software (**Figure 41**). The yields of scopolamine from each sample of six locations were shown in **Table 18**.

Table 18. Scopolamine contents of each sample of *D. metel* L. var. *metel* and *D. metel* L.var. *fastuosa* from six different locations by TLC image method

Sample	Location	Part used	Scopolamine content of dried sample (mg/g dry weight)			
			No.1	No.2	No.3	Mean ± SD
D. metel L. var. *metel*	Rayong (LPK081001)	Leaf	0.196	0.206	0.201	0.201± 0.005
		Flower	3.181	2.924	3.153	3.086± 0.141
		Fruit	0.951	0.903	0.791	0.882± 0.082
	Singburi (LPK011103)	Leaf	0.279	0.295	0.298	0.291± 0.010
		Flower	1.659	1.658	1.675	1.664± 0.009
		Fruit	1.811	1.834	1.817	1.820± 0.012
	Nakhonpathm (LPK091002)	Leaf	1.360	1.415	1.270	1.348± 0.074
		Flower	1.652	1.678	1.425	1.585± 0.139
		Fruit	0.986	1.061	1.125	1.057± 0.070
D. metel L.var. *fastuosa*	Bangkok (KSL071001)	Leaf	0.106	0.095	0.099	0.100± 0.005
		Flower	1.835	1.918	2.328	2.027± 0.264
		Fruit	8.570	8.744	8.000	8.439± 0.389
	Chonburi (KSL051105)	Leaf	1.253	1.116	1.372	1.247± 0.128
		Flower	1.368	1.647	1.597	1.538± 0.149
		Fruit	0.331	0.322	0.347	0.333± 0.013
	Chachoengsao (KSL041104)	Leaf	0.229	0.206	0.211	0.215± 0.012
		Flower	3.373	3.375	3.045	3.264± 0.190
		Fruit	3.416	3.306	3.568	3.430± 0.132

Method validation

Linearity and detection range

The peak areas of standard scopolamine (5.0 - 50.0 µg/spot) were shown in **Table 19**. Six concentrations of scopolamine were plotted against the response (peak area in pixel²) for polynomial calibration curve. The correlation coefficient (r^2) of the curve was 0.9994 and polynomial equation was $y = -1029.5x^2 + 16405x - 5426.1$ (**Figure 36**).

Table 19. The polynomial data of scopolamine by TLC image analysis (n=7)

Concentration (µg/spot)	No. (n)	Peak area (pixel)²	Average	SD	% RSD
5.0	1	2695.8			
	2	5426.3			
	3	7665.9			
	4	2505.8			
	5	6881.0			
	6	6750.8			
	7	3431.6	3063.7	2154.999	70.430
10.0	1	9239.5			
	2	13279.6			
	3	13853.4			
	4	10187.2			
	5	15251.0			
	6	22015.8			
	7	8965.5	9102.5	193.711	2.128
20.0	1	24390.1			
	2	28572.6			
	3	25370.0			
	4	23530.5			
	5	27774.8			
	6	36354.4			
	7	22701.1	23545.6	1194.268	5.072
30.0	1	34321.2			
	2	35320.7			
	3	31617.2			
	4	30773.1			
	5	43357.8			
	6	42459.6			
	7	34616.9	34469.1	209.147	0.607
40.0	1	44765.3			
	2	40962.4			
	3	36635.4			
	4	43810.5			
	5	53560.8			
	6	48219.5			
	7	43073.7	43919.5	1196.082	2.723

	1	51850.9			
	2	47593.8			
	3	42141.6			
50.0	4	58568.8			
	5	68034.7			
	6	64269.6			
	7	49618.7	50734.8	1578.419	3.111

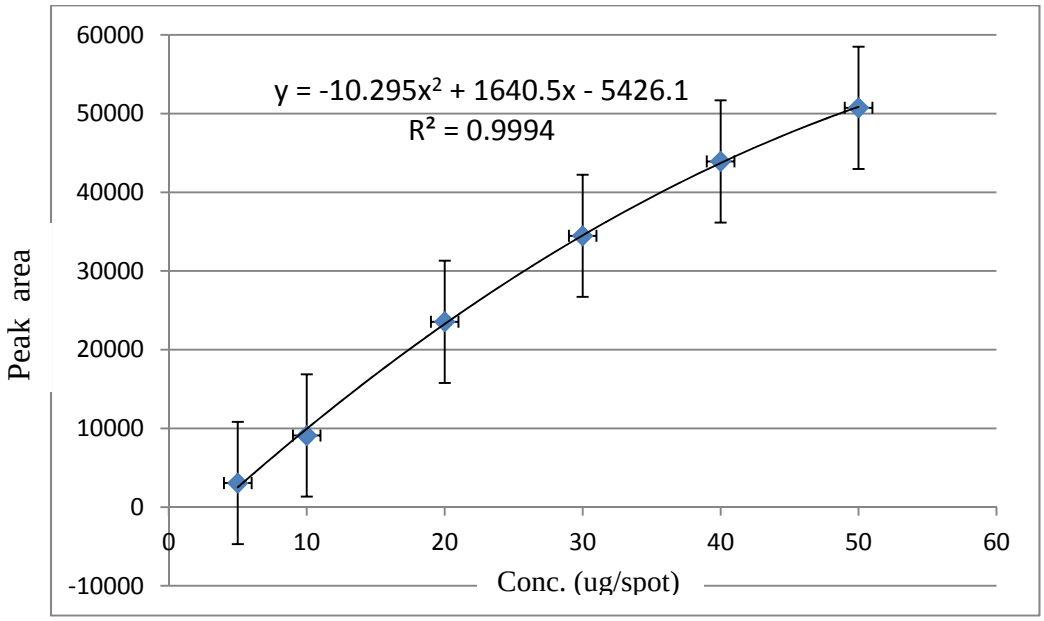


Figure 36. The calibration curve of scopolamine by TLC image analysis

Accuracy

The recovery of scopolamine content from the sample extracted was performed on samples spiked with three different concentration of scopolamine standard (0.50, 1.50, and 2.50 µg/µl). The accuracy of scopolamine content was determined and the average of % recovery was found to be 104.32 ± 8.87 (**Table. 20**)

Table 20. The recovery of scopolamine by TLC image analysis (n=3)

Amount of scopolamine added (µg/spot)	**Amount of scopolamine detected (µg/spot)**	**Recovery (%)**
0.0	5.28	-
5.0	11.65	113.32
15.0	19.43	95.58
25.0	31.51	104.06
Average		104.32 ± 8.87

Precision

Repeatability (within day) was evaluated by assaying each standard concentration at 5.0, 10.0, 20.0, 30.0, 40.0, and 50.0 µg/spot on the same day. The intermediate precision (between days) was studied by comparing the assay on the different days (3 days). The % RSD of repeatability of scopolamine contents were 7.88%, 2.13%, 3.78%, 4.20%, 4.35% and 4.47%, respectively (**Table. 21**). The % RSD of intermediate precision (between days) of scopolamine contents were 2.25%, 5.51%, 3.20%, 4.27%, 4.87% and 4.62%, respectively (**Table. 22**).

Table 21. The repeatability (within day) of scopolamine by TLC image analysis (n=3)

Concentration (µg/spot)	No.	Concentration calculated from peak area (µg/spot)
5.0	1	5.60
	2	5.12
	3	4.79
	Average	5.17
	SD	0.41
	%RSD	7.88
10.0	1	9.62
	2	9.24
	3	9.32
	Average	9.39
	SD	0.20
	%RSD	2.13
20.0	1	19.54
	2	20.83
	3	20.92
	Average	20.43
	SD	0.77
	%RSD	3.78
30.0	1	30.09
	2	32.16
	3	29.80
	Average	30.68
	SD	1.29
	%RSD	4.20
40.0	1	39.21
	2	41.30
	3	37.90
	Average	39.47
	SD	1.72
	%RSD	4.35
	1	48.03
	2	51.67

50.0	3	47.72
	Average	49.14
	SD	2.20
	%RSD	4.47

Table 22. The intermediate precision (between days) of scopolamine by TLC image analysis (n=3)

Concentration (µg/spot)	**Day**	**Concentration calculated from peak area (µg/spot)**
5.0	1	5.17
	2	4.99
	3	4.96
	Average	5.04
	SD	0.11
	%RSD	2.25
10.0	1	9.40
	2	10.47
	3	10.17
	Average	10.01
	SD	0.55
	%RSD	5.51
20.0	1	20.43
	2	20.11
	3	19.20
	Average	19.91
	SD	0.64
	%RSD	3.20
30.0	1	30.68
	2	28.17
	3	29.40
	Average	29.42
	SD	1.26
	%RSD	4.27
40.0	1	39.47
	2	41.30
	3	43.50
	Average	41.42
	SD	2.02
	%RSD	4.87
50.0	1	49.14
	2	47.83
	3	52.30
	Average	49.76
	SD	2.30
	%RSD	4.62

Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ values of this study were determined based on estimated standard deviation of the response and the slope. The slope and standard deviation of the response were estimated from 7 calibration curve. The slope value and standard deviation of the response were 10827.001 and 1994.273, respectively. The LOD value was 0.61 µg/spot which was the lowest amount of analyzing in sample that can be detected but not necessary quantitated as an exact value. LOQ for scopolamine was 1.84 µg/spot which was the lowest concentration of sample, accurately detected and integrated by TLC image using Image J software.

Determination of scopolamine content by HPLC method

The scopolamine contents of each sample (leaf, flower and fruit) of *D. metel* L. var. *metel* and *D. metel* L.var. *fastuosa* from six different locations were evaluated by HPLC analysis using LC solution workstation software (**Figure 42**). The yields of scopolamine content in each sample of six locations were shown in **Table 23**.

Table 23. Scopolamine contents of each sample of *D. metel* L. var. *metel* and *D. metel* L.var. *fastuosa* from six different locations by HPLC method

Sample	Location	Part used	Scopolamine content of dried sample (mg/g dry weight)			
			No.1	No.2	No.3	Mean ± SD
D. metel L. var. *metel*	Rayong (LPK081001)	Leaf	0.154	0.164	0.161	0.160 ± 0.005
		Flower	3.270	3.262	3.258	3.263 ± 0.006
		Fruit	0.726	0.769	0.765	0.753 ± 0.024
	Singburi (LPK011103)	Leaf	0.275	0.272	0.275	0.274 ± 0.002
		Flower	1.662	1.611	1.687	1.653 ± 0.039
		Fruit	2.060	2.134	2.111	2.102 ± 0.038
	Nakhonpathom (LPK091002)	Leaf	1.259	1.245	1.239	1.248 ± 0.010
		Flower	1.199	1.189	1.184	1.191 ± 0.008
		Fruit	1.263	1.262	1.262	1.262 ± 0.002
D. metel L.var. *fastuosa*	Bangkok (KSL071001)	Leaf	0.032	0.031	0.030	0.031 ± 0.001
		Flower	1.990	1.967	1.971	1.976 ± 0.012
		Fruit	8.305	8.262	8.255	8.274 ± 0.027
	Chonburi (KSL051105)	Leaf	0.633	0.612	0.610	0.619 ± 0.013
		Flower	1.385	1.346	1.370	1.367 ± 0.020
		Fruit	0.401	0.398	0.492	0.430 ± 0.054
	Chachoengsao (KSL041104)	Leaf	0.124	0.121	0.120	0.122 ± 0.002
		Flower	2.884	2.876	2.893	2.885 ± 0.008
		Fruit	3.102	3.128	3.147	3.125 ± 0.023

Method validation

Linearity and detection range

Each of different concentration of standards was injected three times. The peak areas obtained for three analyses were averaged at each concentration. The average of peak areas was plotted versus concentration. A linear response between peak area and concentration range from 50 – 500 µg/ml of standards were shown in **Table 24**. The correlation coefficient (r^2) of standard curve was 0.9999 and linear regression equation was y = 9068x + 10316 (**Figure 37**).

Where, y = concentration of scopolamine (µg/ml)

x = area under peak of standard scopolamine

Table 24. The linear data of scopolamine by HPLC analysis (n=3)

Concentration (µg/ml)	No. (n)	Peak area	Average	SD	% RSD
50.0	1	442451			
	2	443263			
	3	443132	442949	435.941	0.098
100.0	1	921153			
	2	921193			
	3	920166	920837	581.736	0.063
200.0	1	1834161			
	2	1834440			
	3	1834927	1834509	387.678	0.021
300.0	1	2748350			
	2	2747339			
	3	2748916	2748202	798.896	0.029
400.0	1	3660438			
	2	3658083			
	3	3629740	3649420	17084.295	0.468
500.0	1	4521616			
	2	4522036			
	3	4520666	4521439	701.878	0.016

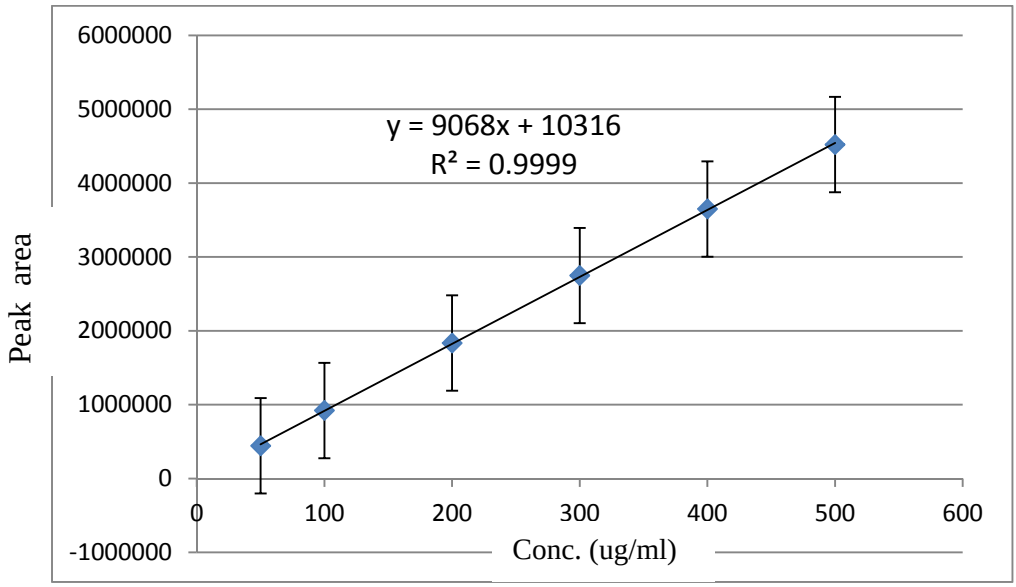


Figure 37. Calibration curve of standard scopolamine by HPLC analysis

Accuracy

The recovery of scopolamine content from crude extracted sample was performed on samples spiked with three different concentrations of scopolamine standard (50.0, 150.0, and 250.0 µg/ml). The accuracy of scopolamine content was determined and the average of % recovery was found to be 98.64 ± 3.76 (**Table 25**.)

Table 25. The recovery of scopolamine by HPLC analysis (n=3)

Amount of scopolamine added (µg/ml)	Amount of scopolamine detected (µg/ml)	Recovery (%)
0.0	93.02	-
50.0	141.64	97.24
150.0	236.71	95.79
250.0	350.27	102.90
Average		98.64 ± 3.76

Precision

Repeatability intraday (within day) was evaluated by assaying each standard at 100.0, 300.0, and 500.0 µg/ml on the same day. The inter-day precision (between days) was studied by comparing the assay on the different days (3 days). The % RSD of repeatabilitys of scopolamine contents were 4.93%, 1.17%, and 0.65%, respectively

(**Table 26**). The % RSD of intermediate of scopolamine contents were 2.56%, 0.42%, and 0.78%, respectively (**Table 27**).

Table 26. The repeatability (within day) of scopolamine by HPLC analysis (n=3)

Concentration (µg/ml)	No.	Concentration calculated from peak area (µg/ml)
100.0	1	93.02
	2	101.64
	3	101.25
	Average	98.64
	SD	4.87
	% RSD	4.93
300.0	1	299.94
	2	304.81
	3	306.84
	Average	303.86
	SD	3.55
	%RSD	1.17
500.0	1	498.95
	2	501.18
	3	505.38
	Average	501.84
	SD	3.26
	%RSD	0.65

Table 27. The intermediate precision (between days) of scopolamine by HPLC analysis (n=3)

Concentration (µg/ml)	Day	Concentration calculated from peak area (µg/ml)
100.0	1	98.64
	2	101.70
	3	103.81
	Average	101.38
	SD	2.60
	% RSD	2.56
300.0	1	303.86
	2	305.50
	3	302.99
	Average	304.12
	SD	1.27
	% RSD	0.42
500.0	1	506.54
	2	503.02
	3	498.72
	Average	502.76
	SD	3.92
	% RSD	0.78

Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ values of this study were determined based on estimated standard deviation of the response and the slope. The slop and standard deviation of the response were estimated from the calibration curve. The slope value and standard deviation of the response were 9068.038, and 14555.087, respectively. The LOD value was 5.30 µg/ml, it was the lowest amount of analyzing in sample that can be detected but not necessary quantitated as an exact value. The LOQ value was 16.05 µg/ml, it was the lowest concentration of sample, and accurately detected by HPLC method.

Method comparison between TLC image analysis and HPLC-DAD analysis

The scopolamine contents of 18 samples (leaves, flowers and fruits) of *D. metel* L. var. *metel* and *D. metel* L.var. *fastuosa* analysed by TLC image method using Image J software and the HPLC method were compared as shown in **Table 28** and the analytical data of both methods were shown in **Table 29.**

Table 28. Comparison of scopolamine contents of *D. metel* L. var. *metel* and *D. metel* L. var. *fastuosa* from six different locations by TLC image method and HPLC method

Sample	**Location**	**Part used**	**Scopolamine content of dried sample (mg/g dry weight)**	
			TLC image (n=3)	**HPLC (n=3)**
D. metel L. var. *metel*	Rayong (LPK081001)	Leaf	0.201± 0.005	0.160 ± 0.005
		Flower	3.086± 0.141	3.263 ± 0.006
		Fruit	0.882± 0.082	0.753 ± 0.024
	Singburi (LPK011103)	Leaf	0.291± 0.010	0.274 ± 0.002
		Flower	1.664± 0.009	1.653 ± 0.039
		Fruit	1.820± 0.012	2.102 ± 0.038
	Nakhonpathom (LPK091002)	Leaf	1.348± 0.074	1.248 ± 0.010
		Flower	1.585± 0.139	1.191 ± 0.008
		Fruit	1.057± 0.070	1.262 ± 0.002
D. metel L.var. *fastuosa*	Bangkok (KSL071001)	Leaf	0.100± 0.005	0.031 ± 0.001
		Flower	2.027± 0.264	1.976 ± 0.012
		Fruit	8.439± 0.389	8.274 ± 0.027
	Chonburi (KSL051105)	Leaf	1.247± 0.128	0.619 ± 0.013
		Flower	1.538± 0.149	1.367 ± 0.020
		Fruit	0.333± 0.013	0.430 ± 0.054
	Chachoengsao (KSL041104)	Leaf	0.215± 0.012	0.122 ± 0.002
		Flower	3.264± 0.190	2.885 ± 0.008
		Fruit	3.430± 0.132	3.125 ± 0.023

Table 29. Paired samples *t*-test of TLC image method and HPLC method

1. Paired samples statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	TLC image analysis	1.80706	18	1.955502	.460916
	HPLC analysis	1.70750	18	1.932073	.455394

Paired samples statistics shows for each variable the number of cases, the mean, the standard deviation, and the standard error of the mean.

2. Paired samples correlations

		N	Correlation	Sig.
Pair 1	TLC image analysis & HPLC analysis	18	.993	.000

Paired samples correlations shows the correlation between the two variables. The two variables are positively correlated, $r = 0.993$ (N=18), $p = 0.000$.

3. Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	TLC image & HPLC analysis	.099556	.226401	.053363	-.013031	.212142	1.866	17	.079

Paired samples test shows the *t* statistics for the paired differences. Compare between content of scopolamine determine by TLC image analysis using Image J software and HPLC analysis. The mean was less difference, 0.099556, t (17) = 1.866, $p = 0.079$.

- TLC image analysis = scopolamine content determine by TLC image analysis using Image J software
- HPLC analysis = scopolamine content determine by High Performance Liquid Chromatography analysis

CHAPTER V
DICUSSION AND CONCLUSION

The increased use of medicinal plants has the needs for methods to control the safety and administration of plants for the effective prevention and treatment of human diseases. The identification of plant is a great importance to ensure the highest efficacy (Ernst, 2006). There are a number of analytical tools available for medicinal plant authentication. Most of the regulatory guidelines and pharmacopoeias suggest macroscopic and microscopic evaluation and chemical profiling of herbal materials for quality control and standardization (WHO, 1998). Therefore, additional of DNA technologies have been applied for medicinal plant authentication (Sucher and Carles, 2008). Combinations of various analytical methods have been employed for quality assurance, control and authentication of medicinal plant species in herbal drug technology development.

Morphological assessment is an effective tool for determining the identity of plant material as it fast and inexpensive. Based on the morphological characteristics, macroscopic observations of the entire plant require highly skilled or well trained individuals whereas microscopic observations require smaller sections of the plant such as its leaf measurement index which require only simple sample preparation and standard laboratory instruments. Microscopic technique has been widely used for plant authentication by examination the palisade ratio, stomatal number, stomatal index, veinislet number, and vein-islet termination number (Trease and Evan, 2009; Roonyamarai et al., 2011). According to the results, macroscopic and microscopic analysis of *D. metel* L. var. *metel*. and *D. metel* L.var. *fastuosa* revealed the different morphology but contained almost similar cell components. Leave measurement index (stomatal number, stomata index and palisade ratio), the important property for species identification, showed different constant numbers espectially stomatal number in upper epidermis of *D. metel* L.var. *fastuosa* that found twofold higher than *D. metel* L. var. *metel*. In addition, molecular techniques have been also introduced for DNA fingerprinting. Analysis of the DNA that is present in all organisms is a suitable method for identifying plant materials because the genetic composition is unique for each

individual organism. DNA extracted from leaves, stems or roots of plants all carry the same genetic information without being affected by physiological conditions and environmental factors (Sucher and Carles, 2008).

Recently, several molecular markers have been developed and increasingly used as modern techniques to distinguish genotypes of organisms. The DNA fragment markers, such as Simple Sequence Repeat (SSR) (Morgante and Olivieri, 1993), Inter-Simple Sequence Repeat (ISSR) (Zietkiewicz et al., 1994), Random Amplified Polymorphic DNA (RAPD) (Williams et al., 1990), and Amplified Fragment Length Polymorphism (AFLP) (Vos et al., 1995), have been successfully used in polymorphism analysis and phylogenetic evaluation in many plants. Each marker technique has its own advantages and disadvantages. Common benefits from most markers include rapid analyses, highly informative results, and being independent on environmental factors. However, DNA fragment amplification markers also have some limitations in the data analysing step. For instance, DNA band results may not be clear enough for the analysis and some PCR amplified fragments may not be repeatable due to a low quality of the genomic DNA. To avoid such problems, DNA sequencing technique would rather be used as an alternative molecular marker than DNA fragment markers (Nantharat et al., 2009).

The species-specific regions in nuclear DNA, mitochondrial DNA and chloroplast DNA have been used for the identification of each individual species. Plant nuclear genome, ITS (internal transcribed spacer) region is now perhaps the most widely sequenced DNA region in fungi, gymnosperm and angiosperm due to the relatively small size (<700 base pairs) and high copy number of the ribosomal DNA gene which enable easy amplification even from small quantities of DNA or from herbarium materials and due to a high degree of variation, even between closely related species which makes the ITS region an interesting site for phylogenetic investigations (Bisbal et al., 2009, Sukrong et al., 2007). It has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races) (Baldwin et al., 1995). Because of its higher degree of variation than other genetic regions of rDNA, variation among individual rDNA repeats can sometimes be observed within both the ITS and IGS regions (Hunter et al., 1997). In addition, plant chloroplast genomes have also been proved to be a primary source of data for molecular genetic relationship studies. They are

now used routinely as a tool to investigate evolutionary processes in plants. Many early publications usually focused on several coding-regions of chloroplast DNA (cpDNA) sequences such as *rbc*L, *mat*K, *atp*B and *ndh*F genes to elucidate genetic relationships (Chase et al. 1993; Olmstead and Sweere, 1994; Steele and Vilgalys, 1994). Due to its relatively low average rate of evolution most previous studies have used cpDNA sequence variation to examine plant systematics and evolution above the species level (Palmer et al., 1988; Soltis and Miligan, 1992). In this recent study, the ITS, *rbc*L, and *atp*B region were investigated in *D. metel* L. The sequence comparisons of three regions showed 94-100% similarity in all three regions. The ITS region of *D.metel* L. showed the intra-species specific within the species, while the *rbc*L and *atp*B regions are highly conserved within the species and genus level. It revealed no distinguishing characteristic between two varieties of *D.metel* L. Two polymorphisms were found within the 5.8S region, and four polymorphisms within the ITS2. The two positions of single nucleotide polymorphism (SNP) were shown at positions 512 and 614 of ITS2 region. According to the SNP at position 512, restriction enzyme HpyCH41V (ACGT) and Mae II (ACGT) can be used for identification these closely related plant when performed for PCR-RFLP. Based on the results, the ITS2 region can be potentially used as a standard DNA barcode to identify the medicinal plants and their closely related species (Chen et al., 2010).

Chromatographic fingerprint is an analytical method for establishing a characteristic chemical pattern for a plant material fraction or extracts. TLC and HPLC are routinely used as valuable tools for qualitative determination of small amounts of impurities. TLC fingerprint analysis is an easy operating and time-saving method with low cost while HPLC has been used for analysis of a wide range of compounds and become the most widely applied effective separation and analysis tool for herbal products but require high cost of instrument. Several methods including GC, GC/MS, HPLC, LC/MS, and CE (Drager, 2002; Elisabetta et al., 2001; Ceyhan et al., 2001; Steenkamp et al., 2004; Cataldi and Bianco, 2008) were also used for identification and quantitative determination of scopolamine in herbal plants and herbal products. Even though the major advantages of these methods have been claimed for their being highly sensitivity and specific, the analytical instruments are quite costly and expertise is usually required. Unlike those, the use of simple and inexpensive TLC method can overcome these drawbacks and being more accessible to many local authorities and small

laboratories. Furthermore, based on a combination with simple computer technology and image analysis software, TLC-image analysis method has been developed and applied for quantitative assay with good accuracy and precision (Prosek and Vovk, 2003). Therefore, the aim of this study was to develop an economic, accurate, reproducible and convenient TLC-image analysis method for rapid determination and quantitative analysis of scopolamine contents of two varieties *D. metel* L. The proposed method was validated in compliance with ICH guidelines and compared with HPLC method.

In order to evaluate its accuracy and precision, TLC-image analysis was compared with high performance liquid chromatography (HPLC) analysis. In this study, a number of leaves, flowers and fruits of two varieties *D.metel* L. from six different locations were analyzed for their scopolamine content using two methods and the results were compared. The result indicated that scopolamine content in eighteen samples determine by TLC image method (Mean= 1.80706, SD=1.955502) were closed to that determine by HPLC method (Mean= 1.70750, SD=1.932073). The two variables are positive correlated, r = 0.993, p = 0.000 and there were not significantly different (t (17) = 1.866, p = 0.079). The amount of scopolamine from flower part of *D. metel* L. var. *metel* contained high scopolamine content whereas the fruit part of *D. metel* L.var. *fastuosa* showed high scopolamine content. Because of many environmental factors can influence the scopolamine content including soil composition, soil fertilization, salinity, climate and altitude, application of plant growth regulators and hormones, insect herbivory, and plant health (Afsharypuor et al., 1995; Shonle and Bergelson, 2000). The different of scopolamine contents of *D.metel* L.var. *fastuosa* in leaves part when compare with two methods could be ascribed to the contrast of matrix effect between scopolamine and background due to the impurities (such as ; chlorophyll). These will be the limitation of TLC image analysis for this part. Previous study of Gupta, et al. (1973) revealed scopolamine in *D. metel* L. var. *fastuosa* usually was the principal alkaloid in root to leaf and up to pre-flowering stages, later on hyoscyamine content increases. Very young fruits were found to possess maximum alkaloids. (Gupta et al., 1973). This information may be of immense value for commercial exploitation of this drug plant grown for its alkaloids.

Because of tropane alkaloids possess poor chromophores (maximum wavelength of UV absorption is about 205 nm), therefore, in majority of proposed detection and

quantitation systems derivatization with Dragendorff reagent was applied at first for increasing the sensitivity and the orange bands of alkaloids could be further scanned by absorption densitometric method at 520-530 nm (Mroczek, 2008). These would be captured by digital camera or scanner machine, and then analysed by image analysis software. In 2004, Berkov and Pavlov proposed a rapid and convenient TLC image method for preparation and simultaneous densitometric quantification of hyoscyamine and scopolamine by derivatization with Dragendorff reagent and quantification by QuantiScan image analysis software. The quantitative results of these were compared with gas chromatography (GC) method, and the result showed good correlation with each other (Berkov and Pavlov, 2004).

In conclusion, based on the result from this study, the combination of macroscopic, microscopic and molecular method are able to authenticate the closely related plants between *D. metel* L. var. *metel* and *D. metel* L.var. *fastuosa*. Both of them have a potency to be a source of scopolamine production.

Future study

1. There are many interesting of other chloroplast genomes, that may be used to investigate the two closely related plants, such as *mat*K, *atp*B-*rbc*L intergenic spacer, or intergenic region of *trn*H-*psb*A region.
2. In order to eliminate the impurity from samples, the solid phase extraction technique or standard addition method may be used for this purpose.

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APPENDICES

APPENDIX A

Part I. Microscopic evaluation

(Constant values of leaves)

Table 30. Stomatal number and stomatal index of *D. metel* L. var. *metel* (upper epidermis)

Location: The Somdej Phra Thepraratana Rajsuda Medicinal Plants Garden, Petroleum Authority of Thailand, Rayong province

Position	Number of stomata (1 sq.mm.)	Number of epidermal cell (1 sq.mm.)	Stomatal index
1	94	592	13.70
2	94	652	12.60
3	86	598	12.57
4	116	754	13.33
5	114	746	13.26
6	96	680	12.37
7	106	644	14.13
8	92	634	12.67
9	88	618	12.46
10	98	678	12.63
11	116	686	14.46
12	100	602	14.25
13	110	648	14.51
14	102	606	14.41
15	126	712	15.04
16	116	750	13.39
17	108	756	12.50
18	96	674	12.47
19	120	766	13.54
20	134	740	15.33
21	114	792	12.58
22	110	788	12.25
23	110	712	13.38
24	102	690	12.88
25	110	768	12.53
26	106	746	12.44
27	118	754	13.53
28	106	764	12.18
29	104	730	12.47
30	102	734	12.20
Mean	**106.47**	**700.47**	**13.20**
SD	**11.10**	**61.73**	**0.91**
Range	**92-126**	**592-792**	**12.18-15.33**

Table 31. Stomatal number and stomatal index of *D. metel* L. var. *metel* (lower epidermis)

Location: The Somdej Phra Thepraratana Rajsuda Medicinal Plants Garden, Petroleum Authority of Thailand, Rayong province

Position	Number of stomata (1 sq.mm.)	Number of epidermalcell (1 sq.mm.)	Stomatal index
1	218	912	19.29
2	196	840	18.92
3	198	896	18.10
4	188	840	18.29
5	214	868	19.78
6	210	884	19.20
7	194	802	19.48
8	202	818	19.80
9	192	790	19.55
10	206	812	20.24
11	204	898	18.51
12	184	854	17.73
13	212	906	18.96
14	222	950	18.94
15	194	838	18.80
16	218	978	18.23
17	218	986	18.11
18	222	992	18.29
19	204	934	17.93
20	216	964	18.31
21	200	896	18.25
22	204	892	18.61
23	186	818	18.53
24	188	832	18.43
25	198	842	19.04
26	162	756	17.65
27	180	804	18.29
28	208	876	19.19
29	178	774	18.70
30	218	944	18.76
Mean	**201.13**	**873.20**	**18.73**
SD	**14.74**	**64.89**	**0.64**
Range	**178-222**	**756-992**	**17.65-20.24**

Table 32. Stomatal number and stomatal index of *D. metel* L. var. *metel* (upper epidermis)

Location: Sirirukkachat garden, Faculty of Pharmacy, Mahidol University, Salaya, Nakhonpathom province

Position	Number of stomata (1 sq.mm.)	Number of epidermal cell (1 sq.mm.)	Stomatal index
1	74	418	15.04
2	72	454	13.69
3	56	402	12.23
4	64	420	13.22
5	60	388	13.39
6	76	458	14.23
7	62	400	13.42
8	62	386	13.84
9	66	364	15.35
10	64	386	14.22
11	64	380	14.41
12	58	420	12.13
13	52	380	12.04
14	58	398	12.72
15	80	376	17.54
16	70	458	13.26
17	56	368	13.21
18	56	332	14.43
19	82	440	15.71
20	70	478	12.77
21	82	578	12.42
22	74	478	13.41
23	80	484	14.18
24	64	458	12.26
25	60	428	12.30
26	58	422	12.08
27	60	388	13.39
28	78	474	14.13
29	62	384	13.90
30	68	404	14.41
Mean	**66.27**	**420.13**	**13.64**
SD	**8.72**	**49.31**	**1.24**
Range	**52-82**	**332-578**	**12.04-17.54**

Table 33. Stomatal number and stomatal index of *D. metel* L. var. *metel* (lower epidermis)

Location: Sirirukkachat garden, Faculty of Pharmacy, Mahidol University, Salaya, Nakhonpathom province

Position	Number of stomata (1 sq.mm.)	Number of epidermal cell (1 sq.mm.)	Stomatal index
1	216	864	20.00
2	216	840	20.45
3	212	860	19.78
4	178	792	18.35
5	210	834	20.11
6	184	820	18.33
7	202	770	20.78
8	190	788	19.43
9	204	792	20.48
10	200	798	20.04
11	254	1034	19.72
12	250	980	20.33
13	218	980	18.20
14	226	986	18.65
15	248	984	20.13
16	222	1006	18.08
17	260	1068	19.58
18	214	1038	17.09
19	226	1028	18.02
20	230	1084	17.50
21	218	1036	17.38
22	232	976	19.21
23	212	900	19.06
24	206	964	17.61
25	220	934	19.06
26	244	950	20.44
27	234	930	20.10
28	218	1018	17.64
29	216	930	18.85
30	226	908	19.93
Mean	**219.53**	**929.73**	**19.14**
SD	**19.49**	**93.89**	**1.10**
Range	**178-260**	**770-1084**	**17.38-20.78**

Table 34. Stomatal number and stomatal index of *D. metel* L. var. *metel* (upper epidermis)

Location: Bang Ra Jan district, Singburi province

Position	Number of stomata (1 sq.mm.)	Number of epidermal cell (1 sq.mm.)	Stomatal index
1	86	536	13.83
2	90	552	14.02
3	86	506	14.53
4	82	480	14.59
5	74	500	12.89
6	76	460	14.18
7	96	626	13.30
8	76	484	13.57
9	84	512	14.09
10	84	486	14.74
11	74	496	12.98
12	76	460	14.18
13	78	470	14.23
14	84	520	13.91
15	82	516	13.71
16	72	494	12.72
17	78	452	14.72
18	96	562	14.59
19	76	474	13.82
20	92	602	13.26
21	98	592	14.20
22	98	600	14.04
23	84	476	15.00
24	82	546	13.06
25	86	534	13.87
26	84	520	13.91
27	82	568	12.62
28	96	582	14.16
29	88	494	15.12
30	80	500	13.79
Mean	**84.00**	**520.00**	**13.92**
SD	**7.56**	**47.36**	**0.66**
Range	**72-98**	**452-626**	**12.72-15.12**

Table 35. Stomatal number and stomatal index of *D. metel* L. var. *metel* (lower epidermis)

Location: Bang Ra Jan district, Singburi province

Position	Number of stomata (1 sq.mm.)	Number of epidermal cell (1 sq.mm.)	Stomatal index
1	192	852	18.39
2	186	790	19.06
3	196	830	19.10
4	198	790	20.04
5	176	764	18.72
6	182	732	19.91
7	192	768	20.00
8	180	740	19.57
9	180	764	19.07
10	182	714	20.31
11	182	784	18.84
12	190	756	20.08
13	174	784	18.16
14	200	786	20.28
15	226	830	21.40
16	208	852	19.62
17	248	892	21.75
18	228	938	19.55
19	168	714	19.05
20	276	1018	21.33
21	182	740	19.74
22	162	736	18.04
23	160	696	18.69
24	150	664	18.43
25	172	716	19.37
26	194	806	19.40
27	178	806	18.09
28	212	944	18.34
29	196	914	17.66
30	218	920	19.16
Mean	**192.93**	**801.33**	**19.37**
SD	**26.43**	**83.86**	**1.01**
Range	**150-276**	**714-1018**	**17.66-21.75**

Table 36. Palisade ratio of *D. metel* L. var. *metel*

Location: The Somdej Phra Thepraratana Rajsuda Medicinal Plants Garden, Petroleum Authority of Thailand, Rayong province

Position	Number of Palisade cell*	Palisade ratio
1	19.5	4.88
2	18	4.50
3	24	6.00
4	20	5.00
5	23.5	5.88
6	20.5	5.13
7	20	5.00
8	20.5	5.13
9	18	4.50
10	20.5	5.13
11	22	5.50
12	22	5.50
13	22	5.50
14	23	5.75
15	19	4.75
16	16	4.00
17	17	4.25
18	19	4.75
19	17.5	4.38
20	21	5.25
21	18	4.50
22	24	6.00
23	22	5.50
24	24	6.00
25	23.5	5.88
26	22.5	5.63
27	24	6.00
28	24	6.00
29	23	5.75
30	19	4.75
Mean	**20.90**	**5.23**
S.D	**2.41**	**0.6**
Range	**16-24**	**4.00-6.00**

*Number of Palisade cell beneath 4 epidermal cells

Table 37. Palisade ratio of *D. metel* L. var. *metel*

Location: Sirirukkachat garden, Faculty of Pharmacy, Mahidol University, Salaya, Nakhonpathom province

Position	Number of Palisade cell*	Palisade ratio
1	18.5	4.63
2	19	4.75
3	21.5	5.38
4	21	5.25
5	23.5	5.88
6	24	6.00
7	20	5.00
8	21.5	5.38
9	22.5	5.63
10	23.5	5.88
11	17.5	4.38
12	22	5.50
13	18	4.50
14	24	6.00
15	20	5.00
16	20	5.00
17	21	5.25
18	17.5	4.38
19	18	4.50
20	21	5.25
21	21.5	5.38
22	20	5.00
23	16	4.00
24	19	4.75
25	22.5	5.63
26	19	4.75
27	23.5	5.88
28	24	6.00
29	23.5	5.88
30	21.5	5.38
Mean	**20.82**	**5.20**
S.D	**2.25**	**0.56**
Range	**16-24**	**4.00-6.00**

*Number of Palisade cell beneath 4 epidermal cells

Table 38. Palisade ratio of *D. metel* L. var. *metel*

Location: Bang Ra Jan district, Singburi province

Position	Number of Palisade cell*	Palisade ratio
1	18.5	4.63
2	21.5	5.38
3	20	5.00
4	22	5.50
5	19	4.75
6	20	5.00
7	21	5.25
8	20	5.00
9	21.5	5.38
10	19	4.75
11	20.5	5.13
12	21.5	5.38
13	17	4.25
14	20	5.00
15	20.5	5.13
16	19	4.75
17	20.5	5.13
18	19	4.75
19	20	5.00
20	18.5	4.63
21	16.5	4.13
22	18	4.50
23	19.5	4.88
24	20.5	5.13
25	18	4.50
26	21	5.25
27	16	4.00
28	20	5.00
29	17.5	4.38
30	21	5.25
Mean	**19.57**	**4.89**
S.D	**1.55**	**0.39**
Range	**16.5-21.5**	**4.00-5.50**

*Number of Palisade cell beneath 4 epidermal cells

Table 39. Stomatal number and stomatal index of *D. metel* L. var. *fastuosa* (upper epidermis)

Location: Chatuchak Plant Market, Bangkaen district, Bangkok province

Position	Number of stomata (1 sq.mm.)	Number of epidermal cell (1 sq.mm.)	Stomatal index
1	174	776	18.32
2	158	684	18.76
3	140	714	16.39
4	154	610	20.16
5	174	680	20.37
6	150	608	19.79
7	162	640	20.20
8	150	700	17.65
9	152	688	18.10
10	168	660	20.29
11	164	736	18.22
12	190	684	21.74
13	172	680	20.19
14	164	692	19.16
15	146	616	19.16
16	172	652	20.87
17	160	668	19.32
18	170	636	21.09
19	172	660	20.67
20	172	704	19.63
21	202	778	20.61
22	210	846	19.89
23	220	918	19.33
24	234	948	19.80
25	192	802	19.32
26	204	848	19.39
27	186	788	19.10
28	178	778	18.62
29	158	746	17.48
30	196	796	19.76
Mean	**174.80**	**724.53**	**19.45**
SD	**22.74**	**87.59**	**1.16**
Range	**140-234**	**608-948**	**16.39-21.74**

Table 40. Stomatal number and stomatal index of *D. metel* L. var. *fastuosa* (lower epidermis)

Location : Chatuchak Plant Market, Bangkaen district, Bangkok province

Position	Number of stomata (1 sq.mm.)	Number of epidermal cell (1 sq.mm.)	Stomatal index
1	186	758	19.70
2	202	740	21.44
3	154	644	19.30
4	172	688	20.00
5	174	714	19.59
6	152	632	19.39
7	206	794	20.60
8	182	682	21.06
9	296	1014	22.60
10	324	1082	23.04
11	322	1070	23.13
12	246	928	20.95
13	254	1082	19.01
14	228	860	20.96
15	242	838	22.41
16	234	846	21.67
17	252	890	22.07
18	238	866	21.56
19	242	838	22.41
20	252	906	21.76
21	204	884	18.75
22	238	890	21.10
23	248	956	20.60
24	268	950	22.00
25	278	940	22.82
26	258	948	21.39
27	278	936	22.90
28	288	986	22.61
29	242	868	21.80
30	212	860	19.78
Mean	**235.73**	**869.67**	**21.21**
SD	**44.93**	**122.82**	**1.30**
Range	**152-324**	**18.75-23.13**	**632-1082**

Table 41. Stomatal number and stomatal index of *D. metel* L. var. *fastuosa* (upper epidermis)

Location : Bang Nam Priao district, Chachoengsao province

Position	Number of stomata (1 sq.mm.)	Number of epidermal cell (1 sq.mm.)	Stomatal index
1	168	730	18.71
2	174	732	19.21
3	188	780	19.42
4	186	760	19.66
5	174	778	18.28
6	180	856	17.37
7	174	800	17.86
8	200	844	19.16
9	180	764	19.07
10	170	778	17.93
11	176	782	18.37
12	172	732	19.03
13	192	800	19.35
14	166	706	19.04
15	162	748	17.80
16	172	756	18.53
17	196	810	19.48
18	204	768	20.99
19	190	752	20.17
20	182	752	19.49
21	174	758	18.67
22	166	712	18.91
23	180	822	17.96
24	174	712	19.64
25	178	718	19.87
26	182	728	20.00
27	184	760	19.49
28	192	720	21.05
29	182	778	18.96
30	180	728	19.82
Mean	**179.93**	**762.13**	**19.11**
SD	**10.23**	**38.65**	**0.88**
Range	**162-204**	**706-856**	**17.37-21.05**

Table 42. Stomatal number and stomatal index of *D. metel* L. var. *fastuosa* (lower epidermis)

Location : Bang Nam Priao district, Chachoengsao province

Position	Number of stomata (1 sq.mm.)	Number of epidermal cell (1 sq.mm.)	Stomatal index
1	218	864	20.15
2	218	840	20.60
3	212	860	19.78
4	190	792	19.35
5	212	800	20.95
6	210	834	20.11
7	198	816	19.53
8	206	770	21.11
9	242	856	22.04
10	230	834	21.62
11	198	788	20.08
12	204	788	20.56
13	200	798	20.04
14	254	1034	19.72
15	250	980	20.33
16	226	968	18.93
17	230	982	18.98
18	262	972	21.23
19	248	984	20.13
20	230	980	19.01
21	260	1068	19.58
22	230	980	19.01
23	232	976	19.21
24	218	900	19.50
25	220	934	19.06
26	244	950	20.44
27	234	930	20.10
28	216	920	19.01
29	226	908	19.93
30	216	840	20.45
Mean	**224.47**	**898.20**	**20.02**
SD	**19.00**	**83.36**	**0.82**
Range	**190-262**	**770-1068**	**19.01-22.04**

Table 43. Stomatal number and stomatal index of *D. metel* L. var. *fastuosa* (upper epidermis)

Location : Muang district, Chonburi province

Position	Number of stomata (1 sq.mm.)	Number of epidermal cell (1 sq.mm.)	Stomatal index
1	204	804	20.24
2	168	718	18.96
3	166	686	19.48
4	184	778	19.13
5	174	766	18.51
6	198	850	18.89
7	220	862	20.33
8	226	928	19.58
9	210	922	18.55
10	228	932	19.66
11	226	1034	17.94
12	264	1084	19.58
13	230	900	20.35
14	232	970	19.30
15	246	984	20.00
16	274	1000	21.51
17	232	922	20.10
18	226	932	19.52
19	224	914	19.68
20	204	932	17.96
21	238	954	19.97
22	258	1022	20.16
23	212	938	18.43
24	264	1100	19.35
25	246	962	20.36
26	196	868	18.42
27	204	876	18.89
28	186	870	17.61
29	194	856	18.48
30	210	928	18.45
Mean	**218.13**	**909.73**	**19.31**
SD	**28.37**	**96.34**	**0.89**
Range	**166-274**	**686-1100**	**17.61-21.51**

Table 44. Stomatal number and stomatal index of *D. metel* L. var. *fastuosa* (lower epidermis)

Location : Muang district, Chonburi province

Position	Number of stomata (1 sq.mm.)	Number of epidermal cell (1 sq.mm.)	Stomatal index
1	244	838	22.55
2	228	848	21.19
3	246	856	22.32
4	226	882	20.40
5	252	900	21.88
6	236	884	21.07
7	252	932	21.28
8	258	918	21.94
9	250	920	21.37
10	232	914	20.24
11	258	936	21.61
12	278	1020	21.42
13	232	888	20.71
14	236	890	20.96
15	224	912	19.72
16	216	820	20.85
17	242	930	20.65
18	248	1002	19.84
19	252	958	20.83
20	242	918	20.86
21	248	968	20.39
22	288	964	23.00
23	276	914	23.19
24	268	934	22.30
25	228	866	20.84
26	200	810	19.80
27	236	866	21.42
28	268	960	21.82
29	264	998	20.92
30	296	1092	21.33
Mean	**247.47**	**917.93**	**21.22**
SD	**21.26**	**61.42**	**0.88**
Range	**200-296**	**810-1092**	**19-72-23.19**

Table 45. Palisade ratio of *D. metel* L. var. *fastuosa*

Location: Chatuchak Plant Market, Bangkaen district, Bangkok province

Position	Number of Palisade cell*	Palisade ratio
1	20	5.00
2	24	6.00
3	23.5	5.88
4	20	5.00
5	25	6.25
6	20.5	5.13
7	26	6.50
8	24	6.00
9	21	5.25
10	29	7.25
11	25.5	6.38
12	22	5.50
13	27	6.75
14	24	6.00
15	29.5	7.38
16	25.5	6.38
17	21	5.25
18	24	6.00
19	24.5	6.13
20	30	7.50
21	29.5	7.38
22	25	6.25
23	25.5	6.38
24	23	5.75
25	31	7.75
26	31.5	7.88
27	27	6.75
28	24	6.00
29	29	7.25
30	25	6.25
Mean	**25.22**	**6.30**
S.D	**3.25**	**0.81**
Range	**20-31.5**	**5.00-7.88**

*Number of Palisade cell beneath 4 epidermal cells

Table 46. Palisade ratio of *D. metel* L. var. *fastuosa*

Location: Bang Nam Priao district, Chachoengsao province

Position	Number of Palisade cell*	Palisade ratio
1	28.5	7.13
2	23	5.75
3	21.5	5.38
4	29.5	7.38
5	30	7.50
6	30	7.50
7	26.5	6.63
8	29	7.25
9	21.5	5.38
10	25	6.25
11	28	7.00
12	28	7.00
13	25	6.25
14	25.5	6.38
15	29	7.25
16	29	7.25
17	21.5	5.38
18	26.5	6.63
19	22	5.50
20	22.5	5.63
21	25.5	6.38
22	22.5	5.63
23	28	7.00
24	26.5	6.63
25	25.5	6.38
26	24.5	6.13
27	21	5.25
28	26.5	6.63
29	23.5	5.88
30	29	7.25
Mean	**25.80**	**6.45**
S.D	**2.91**	**0.73**
Range	**21-30**	**5.25-7.50**

*Number of Palisade cell beneath 4 epidermal cells

Table 47. Palisade ratio of *D. metel* L. var. *fastuosa*

Location: Muang district, Chonburi province

Position	Number of Palisade cell*	Palisade ratio
1	25.5	6.38
2	23.5	5.88
3	27	6.75
4	24.5	6.13
5	25	6.25
6	23.5	5.88
7	29	7.25
8	25	6.25
9	22.5	5.63
10	26.5	6.63
11	23	5.75
12	26	6.50
13	22	5.50
14	24	6.00
15	22.5	5.63
16	24.5	6.13
17	25	6.25
18	24	6.00
19	25.5	6.38
20	26	6.50
21	23.5	5.88
22	24.5	6.13
23	28	7.00
24	28.5	7.13
25	25.5	6.38
26	27.5	6.88
27	25	6.25
28	24.5	6.13
29	24	6.00
30	24.5	6.13
Mean	**25.00**	**6.25**
S.D	**1.75**	**0.44**
Range	**22-28.5**	**5.50-7.25**

*Number of Palisade cell beneath 4 epidermal cells

APPENDIX B

Part Il. Molecular evaluation

(Sequence Alignment)

1. Alignments of ITS sequences of *D.metel* L. var. *metel, D.metel* L. var. *fastuosa,* and hybrid *D.metel* L.

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KSL071001       GAAACCTGCAAAGCAGAACGACCCGCGAACCCGTTCAAACACTTGGGGAGCCGCGCGGGC 60
KSL071002       GAAACCTGCAAAGCAGAACGACCCGCGAACCCGTTCAAACACTTGGGGAGCCGCGCGGGC 60
LHB071001       GAAACCTGCAAAGCAGAACGACCCGCGAACCCGTTCAAACACTTGGGGAGCCGCGCGGGC 60
KSL031103       GAA-CCTGCAAAGCAGAACGACCCGCGAACCCGTTCAAACACTTGGGGAGCCGCGCGGGC 59
LHB031107       GAA-CCTGCAAAGCAGAACGACCCGCGAACCCGTTCAAACACTTGGGGAGCCGCGCGGGC 59
KSL041104       GAA-CCTGCAAAGCAGAACGACCCGCGAACCCGTTCAAACACTTGGGGAGCCGCGCGGGC 59
KSL051105       GAA-CCTGCAAAGCAGAACGACCCGCGAACCCGTTCAAACACTTGGGGAGCCGCGCGGGC 59
LHB071109       GAA-CCTGCAAAGCAGAACGACCCGCGAACCCGTTCAAACACTTGGGGAGCCGCGCGGGC 59
LHB081002       GAAACCTGCAAAGCAGAACGACCCGCGAACCCGTTCAAACACTTGGGGAGCCGCGCGGGC 60
LHB081111       GAAACCTGCAAAGCAGAACGACCCGCGAACCCGTTCAAACACTTGGGGAGCCGCGCGGGC 60
LHB081110       GAAACCTGCAAAGCAGAACGACCCGCGAACCCGTTCAAACACTTGGGGAGCCGCGCGGGC 60
LHB021106       GAAACCTGCAAAGCAGAACGACCCGCGAACCCGTTCAAACACTTGGGGAGCCGCGCGGGC 60
LHB101004       GAAACCTGCAAAGCAGAACGACCCGCGAACCCGTTCAAACACTTGGGGAGCCGCGCGGGC 60
LHB091003       GAAACCTGCAAAGCAGAACGACCCGCGAACCCGTTCAAACACTTGGGGAGCCGCGCGGGC 60
LPK091002       GAAACCTGCAAAGCAGAACGACCCGCGAACCCGTTCAAACACTTGGGGAGCCGCGCGGGC 60
LPK081001       GAAACCTGCAAAGCAGAACGACCCGCGAACCCGTTCAAACACTTGGGGAGCCGCGCGGGC 60
LHB101005       GAA-CCTGCAAAGCAGAACGACCCGCGAACCCGTTCAAACACTTGGGGAGCCGCGCGGGC 59
LPK011103       GAA-CCTGCAAAGCAGAACGACCCGCGAACCCGTTCAAACACTTGGGGAGCCGCGCGGGC 59
LHB061108       GAA-CCTGCAAAGCAGAACGACCCGCGAACCCGTTCAAACACTTGGGGAGCCGCGCGGGC 59
LHB091112       GAA-CCTGCAAAGCAGAACGACCCGCGAACCCGTTCAAACACTTGGGGAGCCGCGCGGGC 59
TNF111001       GAA-CCTGCAGAGCAGAACGACCCGCGAACCTGTTCAAACACTGGGGTGGCCGCGCGGGG 59
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KSL071001       GGGGCGCTTCGGCCCTCATCCGTGCGTCTCCCTCCCGTCCCCGGCGCGCGCTCGCGGGCG 120
KSL071002       GGGGCGCTTCGGCCCTCATCCGTGCGTCTCCCTCCCGTCCCCGGCGCGCGCTCGCGGGCG 120
LHB071001       GGGGCGCTTCGGCCCTCATCCGTGCGTCTCCCTCCCGTCCCCGGCGCGCGCTCGCGGGCG 120
KSL031103       GGGGCGCTTCGGCCCTCATCCGTGCGTCTCCCTCCCGTCCCCGGCGCGCGCTCGCGGGCG 119
LHB031107       GGGGCGCTTCGGCCCTCATCCGTGCGTCTCCCTCCCGTCCCCGGCGCGCGCTCGCGGGCG 119
KSL041104       GGGGCGCTTCGGCCCTCATCCGTGCGTCTCCCTCCCGTCCCCGGCGCGCGCTCGCGGGCG 119
KSL051105       GGGGCGCTTCGGCCCTCATCCGTGCGTCTCCCTCCCGTCCCCGGCGCGCGCTCGCGGGCG 119
LHB071109       GGGGCGCTTCGGCCCTCATCCGTGCGTCTCCCTCCCGTCCCCGGCGCGCGCTCGCGGGCG 119
LHB081002       GGGGCGCTTCGGCCCTCATCCGTGCGTCTCCCTCCCGTCCCCGGCGCGCGCTCGCGGGCG 120
LHB081111       GGGGCGCTTCGGCCCTCATCCGTGCGTCTCCCTCCCGTCCCCGGCGCGCGCTCGCGGGCG 120
LHB081110       GGGGCGCTTCGGCCCTCATCCGTGCGTCTCCCTCCCGTCCCCGGCGCGCGCTCGCGGGCG 120
LHB021106       GGGGCGCTTCGGCCCTCATCCGTGCGTCTCCCTCCCGTCCCCGGCGCGCGCTCGCGGGCG 120
LHB101004       GGGGCGCTTCGGCCCTCATCCGTGCGTCTCCCTCCCGTCCCCGGCGCGCGCTCGCGGGCG 120
LHB091003       GGGGCGCTTCGGCCCTCATCCGTGCGTCTCCCTCCCGTCCCCGGCGCGCGCTCGCGGGCG 120
LPK091002       GGGGCGCTTCGGCCCTCATCCGTGCGTCTCCCTCCCGTCCCCGGCGCGCGCTCGCGGGCG 120
LPK081001       GGGGCGCTTCGGCCCTCATCCGTGCGTCTCCCTCCCGTCCCCGGCGCGCGCTCGCGGGCG 120
LHB101005       GGGGCGCTTCGGCCCTCATCCGTGCGTCTCCCTCCCGTCCCCGGCGCGCGCTCGCGGGCG 119
LPK011103       GGGGCGCTTCGGCCCTCATCCGTGCGTCTCCCTCCCGTCCCCGGCGCGCGCTCGCGGGCG 119
LHB061108       GGGGCGCTTCGGCCCTCATCCGTGCGTCTCCCTCCCGTCCCCGGCGCGCGCTCGCGGGCG 119
LHB091112       GGGGCGCTTCGGCCCTCATCCGTGCGTCTCCCTCCCGTCCCCGGCGCGCGCTCGCGGGCG 119
TNF111001       GGGGTGCTTCGGCCCCCTTCCGCGCGTCACCCTCCCGTCCCCGGCGTGCGCCAGCG--CG 117
                **** ********** * **** ***** ***************** ****  ***  **

KSL071001       CGCCGGGTGATGAACTAACCCCGGCGCGGAAAGCGCCAAGGAATACTAAATTGATAGCCT 180
KSL071002       CGCCGGGTGATGAACTAACCCCGGCGCGGAAAGCGCCAAGGAATACTAAATTGATAGCCT 180
LHB071001       CGCCGGGTGATGAACTAACCCCGGCGCGGAAAGCGCCAAGGAATACTAAATTGATAGCCT 180
KSL031103       CGCCGGGTGATGAACTAACCCCGGCGCGGAAAGCGCCAAGGAATACTAAATTGATAGCCT 179
LHB031107       CGCCGGGTGATGAACTAACCCCGGCGCGGAAAGCGCCAAGGAATACTAAATTGATAGCCT 179
KSL041104       CGCCGGGTGATGAACTAACCCCGGCGCGGAAAGCGCCAAGGAATACTAAATTGATAGCCT 179
KSL051105       CGCCGGGTGATGAACTAACCCCGGCGCGGAAAGCGCCAAGGAATACTAAATTGATAGCCT 179
LHB071109       CGCCGGGTGATGAACTAACCCCGGCGCGGAAAGCGCCAAGGAATACTAAATTGATAGCCT 179
LHB081002       CGCCGGGTGATGAACTAACCCCGGCGCGGAAAGCGCCAAGGAATACTAAATTGATAGCCT 180
LHB081111       CGCCGGGTGATGAACTAACCCCGGCGCGGAAAGCGCCAAGGAATACTAAATTGATAGCCT 180
LHB081110       CGCCGGGTGATGAACTAACCCCGGCGCGGAAAGCGCCAAGGAATACTAAATTGATAGCCT 180
LHB021106       CGCCGGGTGATGAACTAACCCCGGCGCGGAAAGCGCCAAGGAATACTAAATTGATAGCCT 180
LHB101004       CGCCGGGTGATGAACTAACCCCGGCGCGGAAAGCGCCAAGGAATACTAAATTGATAGCCT 180
LHB091003       CGCCGGGTGATGAACTAACCCCGGCGCGGAAAGCGCCAAGGAATACTAAATTGATAGCCT 180
LPK091002       CGCCGGGTGATGAACTAACCCCGGCGCGGAAAGCGCCAAGGAATACTAAATTGATAGCCT 180
LPK081001       CGCCGGGTGATGAACTAACCCCGGCGCGGAAAGCGCCAAGGAATACTAAATTGATAGCCT 180
LHB101005       CGCCGGGTGATGAACTAACCCCGGCGCGGAAAGCGCCAAGGAATACTAAATTGATAGCCT 179
LPK011103       CGCCGGGTGATGAACTAACCCCGGCGCGGAAAGCGCCAAGGAATACTAAATTGATAGCCT 179
LHB061108       CGCCGGGTGATGAACTAACCCCGGCGCGGAAAGCGCCAAGGAATACTAAATTGATAGCCT 179
LHB091112       CGCCGGGTGATGAACTAACCCCGGCGCGGAAAGCGCCAAGGAATACTAAATTGATAGCCT 179
TNF111001       CGTCGGGTGATTAACGAACCCCGGCGCGGAAAGCGCCAAGGAATACTAAACTGACAGCCT 177
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KSL071001       GCCTCTCGCGCC-CCGTTCGCGGTGCGCGCGGGAGGGCCTGTGCTTCTTTTGAAACAAAA 239
KSL071002       GCCTCTCGCGCC-CCGTTCGCGGTGCGCGCGGGAGGGCCTGTGCTTCTTTTGAAACAAAA 239
LHB071001       GCCTCTCGCGCC-CCGTTCGCGGTGCGCGCGGGAGGGCCTGTGCTTCTTTTGAAACAAAA 239
KSL031103       GCCTCTCGCGCC-CCGTTCGCGGTGCGCGCGGGAGGGCCTGTGCTTCTTTTGAAACAAAA 238
LHB031107       GCCTCTCGCGCC-CCGTTCGCGGTGCGCGCGGGAGGGCCTGTGCTTCTTTTGAAACAAAA 238
KSL041104       GCCTCTCGCGCC-CCGTTCGCGGTGCGCGCGGGAGGGCCTGTGCTTCTTTTGAAACAAAA 238
KSL051105       GCCTCTCGCGCC-CCGTTCGCGGTGCGCGCGGGAGGGCCTGTGCTTCTTTTGAAACAAAA 238
LHB071109       GCCTCTCGCGCC-CCGTTCGCGGTGCGCGCGGGAGGGCCTGTGCTTCTTTTGAAACAAAA 238
LHB081002       GCCTCTCGCGCC-CCGTTCGCGGTGCGCGCGGGAGGGCCTGTGCTTCTTTTGAAACAAAA 239
LHB081111       GCCTCTCGCGCC-CCGTTCGCGGTGCGCGCGGGAGGGCCTGTGCTTCTTTTGAAACAAAA 239
LHB081110       GCCTCTCGCGCC-CCGTTCGCGGTGCGCGCGGGAGGGCCTGTGCTTCTTTTGAAACAAAA 239
LHB021106       GCCTCTCGCGCC-CCGTTCGCGGTGCGCGCGGGAGGGCCTGTGCTTCTTTTGAAACAAAA 239
LHB101004       GCCTCTCGCGCC-CCGTTCGCGGTGCGCGCGGGAGGGCCTGTGCTTCTTTTGAAACAAAA 239
LHB091003       GCCTCTCGCGCC-CCGTTCGCGGTGCGCGCGGGAGGGCCTGTGCTTCTTTTGAAACAAAA 239
LPK091002       GCCTCTCGCGCC-CCGTTCGCGGTGCGCGCGGGAGGGCCTGTGCTTCTTTTGAAACAAAA 239
LPK081001       GCCTCTCGCGCC-CCGTTCGCGGTGCGCGCGGGAGGGCCTGTGCTTCTTTTGAAACAAAA 239
LHB101005       GCCTCTCGCGCC-CCGTTCGCGGTGCGCGCGGGAGGGCCTGTGCTTCTTTTGAAACAAAA 238
LPK011103       GCCTCTCGCGCC-CCGTTCGCGGTGCGCGCGGGAGGGCCTGTGCTTCTTTTGAAACAAAA 238
LHB061108       GCCTCTCGCGCC-CCGTTCGCGGTGCGCGCGGGAGGGCCTGTGCTTCTTTTGAAACAAAA 238
LHB091112       GCCTCTCGCGCC-CCGTTCGCGGTGCGCGCGGGAGGGCCTGTGCTTCTTTTGAAACAAAA 238
TNF111001       GCCTCCCGCGCCGCCGTCCGCGGTGCGCGCGGG-GGTCCTGTGCTTCTTTTGTAACCGAA 236
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KSL071001       ACGACTCTCGGCAACGGATATCTGGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGA 299
KSL071002       ACGACTCTCGGCAACGGATATCTGGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGA 299
LHB071001       ACGACTCTCGGCAACGGATATCTGGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGA 299
KSL031103       ACGACTCTCGGCAACGGATATCTGGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGA 298
LHB031107       ACGACTCTCGGCAACGGATATCTGGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGA 298
KSL041104       ACGACTCTCGGCAACGGATATCTGGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGA 298
KSL051105       ACGACTCTCGGCAACGGATATCTGGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGA 298
LHB071109       ACGACTCTCGGCAACGGATATCTGGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGA 298
LHB081002       ACGACTCTCGGCAACGGATATCTGGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGA 299
LHB081111       ACGACTCTCGGCAACGGATATCTGGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGA 299
LHB081110       ACGACTCTCGGCAACGGATATCTGGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGA 299
LHB021106       ACGACTCTCGGCAACGGATATCTGGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGA 299
LHB101004       ACGACTCTCGGCAACGGATATCTGGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGA 299
LHB091003       ACGACTCTCGGCAACGGATATCTGGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGA 299
LPK091002       ACGACTCTCGGCAACGGATATCTGGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGA 299
LPK081001       ACGACTCTCGGCAACGGATATCTGGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGA 299
LHB101005       ACGACTCTCGGCAACGGATATCTGGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGA 298
LPK011103       ACGACTCTCGGCAACGGATATCTGGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGA 298
LHB061108       ACGACTCTCGGCAACGGATATCTGGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGA 298
LHB091112       ACGACTCTCGGCAACGGATATCTGGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGA 298
TNF111001       ACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGA 296
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KSL071001       TACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCC 359
KSL071002       TACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCC 359
LHB071001       TACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCC 359
KSL031103       TACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCC 358
LHB031107       TACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCC 358
KSL041104       TACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCC 358
KSL051105       TACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCC 358
LHB071109       TACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCC 358
LHB081002       TACTTGGTGTGAATTGCAGAATCCCGTGAACCATCCAGTCTTTGAACGCATGTTGCGCCC 359
LHB081111       TACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCC 359
LHB081110       TACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCC 359
LHB021106       TACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCC 359
LHB101004       TACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCC 359
LHB091003       TACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCC 359
LPK091002       TACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCC 359
LPK081001       TACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCC 359
LHB101005       TACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCC 358
LPK011103       TACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCC 358
LHB061108       TACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCC 358
LHB091112       TACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCC 358
TNF111001       TACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCC 356
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KSL071001       AAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCC-GCAC 418
KSL071002       AAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCC-GCAC 418
LHB071001       AAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCC-GCAC 418
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KSL031103       AAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCC-GCAC 417
LHB031107       AAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCC-GCAC 417
KSL041104       AAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCC-GCAC 417
KSL051105       AAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCC-GCAC 417
LHB071109       AAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCC-GCAC 417
LHB081002       AAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCC-GCAC 418
LHB081111       AAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCC-GCAC 418
LHB081110       AAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCC-GCAC 418
LHB021106       AAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCC-GCAC 418
LHB101004       AAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCC-GCAC 418
LHB091003       AAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCC-GCAC 418
LPK091002       AAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCC-GCAC 418
LPK081001       AAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCC-GCAC 418
LHB101005       AAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCC-GCAC 417
LPK011103       AAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCC-GCAC 417
LHB061108       AAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCC-GCAC 417
LHB091112       AAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCC-GCAC 417
TNF111001       GAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCCCGCAC 416
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KSL071001       TCCGCGCCAAAAACCTTGGCTGCGGTTGTGTCGGGGGACGGATACTGGCCTCCCGTGAGC 478
KSL071002       TCCGCGCCAAAAACCTTGGCTGCGGTTGTGTCGGGGGACGGATACTGGCCTCCCGTGAGC 478
LHB071001       TCCGCGCCAAAAACCTTGGCTGCGGTTGTGTCGGGGGACGGATACTGGCCTCCCGTGAGC 478
KSL031103       TCCGCGCCAAAAACCTTGGCTGCGGTTGTGTCGGGGGACGGATACTGGCCTCCCGTGAGC 477
LHB031107       TCCGCGCCAAAAACCTTGGCTGCGGTTGTGTCGGGGGACGGATACTGGCCTCCCGTGAGC 477
KSL041104       TCCGCGCCAAAAACCTTGGCTGCGGTTGTGTCGGGGGACGGATACTGGCCTCCCGTGAGC 477
KSL051105       TCCGCGCCAAAAACCTTGGCTGCGGTTGTGTCGGGGGACGGATACTGGCCTCCCGTGAGC 477
LHB071109       TCCGCGCCAAAAACCTTGGCTGCGGTTGTGTCGGGGGACGGATACTGGCCTCCCGTGAGC 477
LHB081002       TCCGCGCCAAAAACCTTGGCTGCGGTTGTGTCGGGGGACGGATACTGGCCTCCCGTGAGC 478
LHB081111       TCCGCGCCAAAAACCTTGGCTGCGGTTGTGTCGGGGGACGGATACTGGCCTCCCGTGAGC 478
LHB081110       TCCGCGCCAAAAACCTTGGCTGCGGTTGTGTCGGGGGACGGATACTGGCCTCCCGTGAGC 478
LHB021106       TCCGCGCCAAAAACCTTGGCTGCGGTTGTGTCGGGGGACGGATACTGGCCTCCCGTGAGC 478
LHB101004       TCCGCGCCAAAAACCTTGGCTGCGGTTGTGTCGGGGGACGGATACTGGCCTCCCGTGAGC 478
LHB091003       TCCGCGCCAAAAACCTTGGCTGCGGTTGTGTCGGGGGACGGATACTGGCCTCCCGTGAGC 478
LPK091002       TCCGCGCCAAAAACCTTGGCTGCGGTTGTGTCGGGGGACGGATACTGGCCTCCCGTGAGC 478
LPK081001       TCCGCGCCAAAAACCTTGGCTGCGGTTGTGTCGGGGGACGGATACTGGCCTCCCGTGAGC 478
LHB101005       TCCGCGCCAAAAACCTTGGCTGCGGTTGTGTCGGGGGACGGATACTGGCCTCCCGTGAGC 477
LPK011103       TCCGCGCCAAAAACCTTGGCTGCGGTTGTGTCGGGGGACGGATACTGGCCTCCCGTGAGC 477
LHB061108       TCCGCGCCAAAAACCTTGGCTGCGGTTGTGTCGGGGGACGGATACTGGCCTCCCGTGAGC 477
LHB091112       TCCGCGCCAAAAACCTTGGCTGCGGTTGTGTCGGGGGACGGATACTGGCCTCCCGTGAGC 477
TNF111001       AGCGCGCCCAAATTCTTGGCCGCGGCAGTGTCGTGGGACGGATACTGGCCTCCCGTGCGC 476
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KSL071001       CCCCGAGCCCGCGGCTGGCCTAAATGCGAGTCCACGTCGACGGACGTCACGGCAAGTGGT 538
KSL071002       CCCCGAGCCCGCGGCTGGCNAAAATGCAAGTCCACGTCGACGGACGTCACGGCAAGTGGT 538
LHB071001       CCCCGAGCCCGCGGCTGGCCTAAATGCGAGTCCACGTCGACGGACGTCACGGCAAGTGGT 538
KSL031103       CCCCGAGCCCGCGGCTGGCCTAAATGCGAGTCCACGTCGACGGACGTCACGGCAAGTGGT 537
LHB031107       CCCCGAGCCCGCGGCTGGCCTAAATGCGAGTCCACGTCGACGGACGTCACGGCAAGTGGT 537
KSL041104       CCCCGAGCCCGCGGCTGGCCTAAATGCGAGTCCACGTCGACGGACGTCACGGCAAGTGGT 537
KSL051105       CCCCGAGCCCGCGGCTGGCCTAAATGCGAGTCCACGTCGACGGACGTCACGGCAAGTGGT 537
LHB071109       CCCCGAGCCCGCGGCTGGCCTAAATGCGAGTCCACGTCGACGGACGTCACGGCAAGTGGT 537
LHB081002       CCCCGAGCCCGCGGCTGGCCTAAATGCGAGTCCCCGTCGACGGACGTCACGGCAAGTGGT 538
LHB081111       CCCCGAGCCCGCGGCTGGCCTAAATGCGAGTCCCCGTCGACGGACGTCACGGCAAGTGGT 538
LHB081110       CCCCGAGCCCGCGGCTGGCCTAAATGCGAGTCCCCGTCGACGGACGTCACGGCAAGTGGT 538
LHB021106       CCCCGAGCCCGCGGCTGGCCTAAATGCGAGTCCCCGTCGACGGACGTCACGGCAAGTGGT 538
LHB101004       CCCCGAGCCCGCGGCTGGCCTAAATGCGAGTCCCCGTCGACGGACGTCACGGCAAGTGGT 538
LHB091003       CCCCGAGCCCGCGGCTGGCCTAAATGCGAGTCCCCGTCGACGGACGTCACGGCAAGTGGT 538
LPK091002       CCCCGAGCCCGCGGCTGGCCTAAATGCGAGTCCCCGTCGACGGACGTCACGGCAAGTGGT 538
LPK081001       CCCCGAGCCCGCGGCTGGCCTAAATGCGAGTCCCCGTCGACGGACGTCACGGCAAGTGGT 538
LHB101005       CCCCGAGCCCGCGGCTGGCCTAAATGCGAGTCCCCGTCGACGGACGTCACGGCAAGTGGT 537
LPK011103       CCCCGAGCCCGCGGCTGGCCTAAATGCGAGTCCCCGTCGACGGACGTCACGGCAAGTGGT 537
LHB061108       CCCCGAGCCCGCGGCTGGCCTAAATGCGAGTCCCCGTCGACGGACGTCACGGCAAGTGGT 537
LHB091112       CCCCGAGCCCGCGGCTGGCCTAAATGCGAGTCCCCGTCGACGGACGTCACGGCAAGTGGT 537
TNF111001       CTC-GAGCTCGCGGCTGGCCTAAATGCGAGTCCACGTCGACGGACGTCACGGCAGGTGGT 535
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KSL071001       GGTTGGAACTCAACTCTCGTAATGTCGTGGCTACAGCCCGTCGCTCGTTTGTGCTCCTAG 598
KSL071002       GGTTGGAACTCAACTCTCGTAATGTCGTGGCTACAGCCCGTCGCTCGTTTGTGCTCCTAG 598
LHB071001       GGTTGGAACTCAACTCTCGTAATGTCGTGGCTACAGCCCGTCGCTCGTTTGTGCTCCTAG 598
KSL031103       GGTTGGAACTCAACTCTCGTAATGTCGTGGCTACAGCCCGTCGCTCGTTTGTGCTCCTAG 597
LHB031107       GGTTGGAACTCAACTCTCGTAATGTCGTGGCTACAGCCCGTCGCTCGTTTGTGCTCCTAG 597
KSL041104       GGTTGGAACTCAACTCTCGTAATGTCGTGGCTACAGCCCGTCGCTCGTTTGTGCTCCTAG 597
KSL051105       GGTTGGAACTCAACTCTCGTAATGTCGTGGCTACAGCCCGTCGCTCGTTTGTGCTCCTAG 597
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LHB071109       GGTTGGAACTCAACTCTCGTAATGTCGTGGCTACAGCCCGTCGCTCGTTTGTGCTCCTAG 597
LHB081002       GGTTGGAACTCAACTCTCGTAATGTCGTGGCTACAGCCCGTCGCTCGTTTGTGCTCCTAG 598
LHB081111       GGTTGGAACTCAACTCTCGTAATGTCGTGGCTACAGCCCGTCGCTCGTTTGTGCTCCTAG 598
LHB081110       GGTTGGAACTCAACTCTCGTAATGTCGTGGCTACAGCCCGTCGCTCGTTTGTGCTCCTAG 598
LHB021106       GGTTGGAACTCAACTCTCGTAATGTCGTGGCTACAGCCCGTCGCTCGTTTGTGCTCCTAG 598
LHB101004       GGTTGGAACTCAACTCTCGTAATGTCGTGGCTACAGCCCGTCGCTCGTTTGTGCTCCTAG 598
LHB091003       GGTTGGAACTCAACTCTCGTAATGTCGTGGCTACAGCCCGTCGCTCGTTTGTGCTCCTAG 598
LPK091002       GGTTGGAACTCAACTCTCGTAATGTCGTGGCTACAGCCCGTCGCTCGTTTGTGCTCCTAG 598
LPK081001       GGTTGGAACTCAACTCTCGTAATGTCGTGGCTACAGCCCGTCGCTCGTTTGTGCTCCTAG 598
LHB101005       GGTTGGAACTCAACTCTCGTAATGTCGTGGCTACAGCCCGTCGCTCGTTTGTGCTCCTAG 597
LPK011103       GGTTGGAACTCAACTCTCGTAATGTCGTGGCTACAGCCCGTCGCTCGTTTGTGCTCCTAG 597
LHB061108       GGTTGGAACTCAACTCTCGTAATGTCGTGGCTACAGCCCGTCGCTCGTTTGTGCTCCTAG 597
LHB091112       GGTTGGAACTCAACTCTCGTAATGTCGTGGCTACAGCCCGTCGCTCGTTTGTGCTCCTAG 597
TNF111001       GGTTGAAACTCAGCTCTCGTAGTGCCGTGGCCGCAGACCGTCGGCAGTTTGGGCTCCTAG 595
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KSL071001       ACCCTTCACGCGCTTGGGCGCTCCGACCGCGACCCCAGGTCAGGCGGGATCACCCGCTGA 658
KSL071002       ACCCTTCACGCGCTTGGGCGCTCCGACCGCGACCCCAGGTCAGGCGGGATCACCCGCTGA 658
LHB071001       ACCCTTCACGCGCTTGGGCGCTCCGACCGCGACCCCAGGTCAGGCGGGATCACCCGCTGA 658
KSL031103       ACCCTTCACGCGCTTGGGCGCTCCGACCGCGACCCCAGGTCAGGCGGGATCACCCGCTGA 657
LHB031107       ACCCTTCACGCGCTTGGGCGCTCCGACCGCGACCCCAGGTCAGGCGGGATCACCCGCTGA 657
KSL041104       ACCCTTCACGCGCTTGGGCGCTCCGACCGCGACCCCAGGTCAGGCGGGATCACCCGCTGA 657
KSL051105       ACCCTTCACGCGCTTGGGCGCTCCGACCGCGACCCCAGGTCAGGCGGGATCACCCGCTGA 657
LHB071109       ACCCTTCACGCGCTTGGGCGCTCCGACCGCGACCCCAGGTCAGGCGGGATCACCCGCTGA 657
LHB081002       ACCCTTCACGCGCTTAGGCGCTCCGACCGCGACCCCAGGTCAGGCGGGATCACCCGCTGA 658
LHB081111       ACCCTTCACGCGCTTAGGCGCTCCGACCGCGACCCCAGGTCAGGCGGGATCACCCGCTGA 658
LHB081110       ACCCTTCACGCGCTTAGGCGCTCCGACCGCGACCCCAGGTCAGGCGGGATCACCCGCTGA 658
LHB021106       ACCCTTCACGCGCTTAGGCGCTCCGACCGCGACCCCAGGTCAGGCGGGATCACCCGCTGA 658
LHB101004       ACCCTTCACGCGCTTAGGCGCTCCGACCGCGACCCCAGGTCAGGCGGGATCACCCGCTGA 658
LHB091003       ACCCTTCACGCGCTTAGGCGCTCCGACCGCGACCCCAGGTCAGGCGGGATCACCCGCTGA 658
LPK091002       ACCCTTCACGCGCTTAGGCGCTCCGACCGCGACCCCAGGTCAGGCGGGATCACCCGCTGA 658
LPK081001       ACCCTTCACGCGCTTAGGCGCTCCGACCGCGACCCCAGGTCAGGCGGGATCACCCGCTGA 658
LHB101005       ACCCTTCACGCGCTTAGGCGCTCCGACCGCGACCCCAGGTCAGGCGGGATCACCCGCTGA 657
LPK011103       ACCCTTCACGCGCTTAGGCGCTCCGACCGCGACCCCAGGTCAGGCGGGATCACCCGCTGA 657
LHB061108       ACCCTTCACGCGCTTAGGCGCTCCGACCGCGACCCCAGGTCAGGCGGGATCACCCGCTGA 657
LHB091112       ACCCTTCACGCGCTTAGGCGCTCCGACCGCGACCCCAGGTCAGGCGGGATCACCCGCTGA 657
TNF111001       ACCCTTCGTGCGCTTAGGCGCTCCGACCGCGACCCCAGGTCAGGCGGGATTACCCGCTGA 655
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KSL071001       GTTTAAGCATAT 670
KSL071002       GTTTAAGCATAT 670
LHB071001       GTTTAAGCATAT 670
KSL031103       GTTTAAGCATAT 669
LHB031107       GTTTAAGCATAT 669
KSL041104       GTTTAAGCATAT 669
KSL051105       GTTTAAGCATAT 669
LHB071109       GTTTAAGCATAT 669
LHB081002       GTTTAAGCATAT 670
LHB081111       GTTTAAGCATAT 670
LHB081110       GTTTAAGCATAT 670
LHB021106       GTTTAAGCATAT 670
LHB101004       GTTTAAGCATAT 670
LHB091003       GTTTAAGCATAT 670
LPK091002       GTTTAAGCATAT 670
LPK081001       GTTTAAGCATAT 670
LHB101005       GTTTAAGCATAT 669
LPK011103       GTTTAAGCATAT 669
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LHB091112       GTTTAAGCATAT 669
TNF111001       GTTTAAGCATAT 667
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Sequence type explicitly set to DNA
Sequence format is Pearson
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Sequence 2: KSL071002 670 bp
Sequence 3: LHB071001 670 bp
Sequence 4: LHB081002 670 bp
Sequence 5: LPK081001 670 bp
Sequence 6: LPK091002 670 bp

Sequence 7: LHB091003 670 bp
Sequence 8: LHB101004 670 bp
Sequence 9: LHB101005 669 bp
Sequence 10: TNF111001 667 bp
Sequence 11: LPK011103 669 bp
Sequence 12: LHB021106 670 bp
Sequence 13: KSL031103 669 bp
Sequence 14: LHB031107 669 bp
Sequence 15: KSL041104 669 bp
Sequence 16: KSL051105 669 bp
Sequence 17: LHB061108 669 bp
Sequence 18: LHB071109 669 bp
Sequence 19: LHB081110 670 bp
Sequence 20: LHB081111 670 bp
Sequence 21: LHB091112 669 bp
Start of Pairwise alignments
Aligning...

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Sequences (1:4) Aligned. Score: 99
Sequences (1:5) Aligned. Score: 99
Sequences (1:6) Aligned. Score: 99
Sequences (1:7) Aligned. Score: 99
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Sequences (1:9) Aligned. Score: 99
Sequences (1:10) Aligned. Score: 91
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Sequences (1:12) Aligned. Score: 99
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Sequences (2:5) Aligned. Score: 99
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Sequences (14:20) Aligned. Score: 99
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Sequences (16:21) Aligned. Score: 99
Sequences (17:18) Aligned. Score: 99
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Sequences (18:21) Aligned. Score: 99
Sequences (19:20) Aligned. Score: 100
Sequences (19:21) Aligned. Score: 99
Sequences (20:21) Aligned. Score: 99

There are 20 groups
Start of Multiple Alignment

Aligning...
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Group 3: Sequences: 2 Score:12711
Group 4: Sequences: 3 Score:12711
Group 5: Sequences: 4 Score:12711
Group 6: Sequences: 5 Score:12711
Group 7: Sequences: 8 Score:12687
Group 8: Sequences: 2 Score:12692
Group 9: Sequences: 3 Score:12711
Group 10: Sequences: 4 Score:12717
Group 11: Sequences: 5 Score:12720
Group 12: Sequences: 6 Score:12722
Group 13: Sequences: 7 Score:12723
Group 14: Sequences: 8 Score:12724
Group 15: Sequences: 2 Score:12711
Group 16: Sequences: 3 Score:12711
Group 17: Sequences: 4 Score:12711
Group 18: Sequences: 12 Score:12696
Group 19: Sequences: 20 Score:12667
Group 20: Sequences: 21 Score:11778
Alignment Score 1092794

Figure 38. Comparison of nucleotide sequence of ITS (ITS1-5.8S- ITS2) region of rDNA gene of *D.metel* L. var. *metel*, *D.metel* L. var. *fastuosa*, and hybrid *D.metel* L. Highlight indicate 5.8S region, * indicate clustal consensus, - indicate indels, (TNF111001 was assigned as outgroup sample)

2. Alignments of *rbc*L sequences of *D.metel* L. var. *metel, D.metel* L. var. *fastuosa,* and hybrid *D.metel* L.

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LHB081002       GCTGGTGTTAAAGAGTACAAATTGACTTATTATACTCCTGAGTACCAAACCAAGGATACT 60
KSL041104       GCTGGTGTTAA-GAGTACAAATTGACTTATTATACTCCTGAGTACCAAACCAAGGATACT 59
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LHB081111       GCTGGTGTTAA-GAGTACAAATTGACTTATTATACTCCTGAGTACCAAACCAAGGATACT 59
LHB091003       GCTGGTGTTAA-GAGTACAAATTGACTTATTATACTCCTGAGTACCAAACCAAGGATACT 59
KSL051105       GCTGGTGTTAA-GAGTACAAATTGACTTATTATACTCCTGAGTACCAAACCAAGGATACT 59
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LHB071109       GCTGGTGTTAA-GAGTACAAATTGACTTATTATACTCCTGAGTACCAAACCAAGGATACT 59
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KSL071002       GCTGGTGTTAAAGAGTACAAATTGACTTATTATACTCCTGAGTACCAAACCAAGGATACT 60
TNF111001       GCTGGTGTTAAAGAGTACAAATTGACTTATTATACTCCTCAGTACCAAACCAAGGATACT 60
LPK091002       GCTGGTGTTAAAGAGTACAAATTGACTTATTATACTCCTGAGTACCAAACCAAGGATACT 60
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LHB101005       GCTGGTGTTAAAGAGTACAAATTGACTTATTATACTCCTGAGTACCAAACCAAGGATACT 60
LHB081110       GCTGGTGTTAA-GAGTACAAATTGACTTATTATACTCCTGAGTACCAAACCAAGGATACT 59
LHB091112       GCTGGTGTTAA-GAGTACAAATTGACTTATTATACTCCTGAGTACCAAACCAAGGATACT 59
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LHB071001       GATATATTGGCAGCATTCCGAGTAACTCCTCAACCTGGAGTTCCACCTGAAGAAGCAGGG 120
LHB081002       GATATATTGGCAGCATTCCGAGTAACTCCTCAACCTGGAGTTCCACCTGAAGAAGCAGGG 120
KSL041104       GATATATTGGCAGCATTCCGAGTAACTCCTCAACCTGGAGTTCCACCTGAAGAAGCAGGG 119
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TNF111001       GCCGCGGTAGCTGCCGAATCTTCTACTGGTACATGGACAACTGTATGGACCGATGGACTT 180
LPK091002       GCCGCGGTAGCTGCCGAATCTTCTACTGGTACATGGACAACTGTATGGACCGATGGACTT 180
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LHB101005       GCCGCGGTAGCTGCCGAATCTTCTACTGGTACATGGACAACTGTATGGACCGATGGACTT 180
LHB081110       GCCGCGGTAGCTGCCGAATCTTCTACTGGTACATGGACAACTGTATGGACCGATGGACTT 179
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LPK081001       ACCAGTCTTGATCGTTACAAAGGGCGATGCTACCGCATCGAGCGTGTTGTTGGAGAAAAA 240
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LHB091003       ACCAGTCTTGATCGTTACAAAGGGCGATGCTACCGCATCGAGCGTGTTGTTGGAGAAAAA 239
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TNF111001       ACCAGTCTTGATCGTTACAAAGGGCGATGCTACCGCATCGAGCGTGTTGTTGGAGAAAAA 240
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LHB101005       GATCAATATATTG-CTTATGTAGCTTACCCTTTAGACCTTTTTGAAGAAGGTTCCGTTAC 299
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KSL041104       CGTCTGGAAGATCTGCGAATCCCTCCTGCTTATATTAAAACTTTCCAGGGT-CCGCCTCA 415
LPK081001       CGTCTGGAAGATCTGCGAATCCCTCCTGCTTATATTAAAACTTTCCAGGGT-CCGCCTCA 416
LHB081111       CGTCTGGAAGATCTGCGAATCCCTCCTGCTTATATTAAAACTTTCCAGGGT-CCGCCTCA 415
LHB091003       CGTCTGGAAGATCTGCGAATCCCTCCTGCTTATATTAAAACTTTCCAGGGT-CCGCCTCA 415
KSL051105       CGTCTGGAAGATCTGCGAATCCCTCCTGCTTATATTAAAACTTTCCAGGGT-CCGCCTCA 415
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LHB071109       CGTCTGGAAGATCTGCGAATCCCTCCTGCTTATATTAAAACTTTCCAGGGT-CCGCCTCA 416
LPK011103       CGTCTGGAAGATCTGCGAATCCCTCCTGCTTATATTAAAACTTTCCAGGGT-CCGCCTCA 415
KSL071002       CGTCTGGAAGATCTGCGAATCCCTCCTGCTTATATTAAAACTTTCCAGGGT-CCGCCTCA 417
TNF111001       CGTCTGGAAGATCTGCGAATCCCTCCTGCTTATATTAAAACTTTCCAGGGT-CCGCCTCA 416
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KSL071001       CGTCTGGAAGATCTGCGAATCCCTCCTGCTTATATTAAAACTTTCCAGGGT-CCGCCTCA 417
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LHB101005       CGTCTGGAAGATCTGCGAATCCCTCCTGCTTATATTAAAACTTTCCAGGGT-CCGCCTCA 416
LHB081110       CGTCTGGAAGATCTGCGAATCCCTCCTGCTTATATTAAAACTTTCCAGGGT-CCGCCTCA 415
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LHB081002       TGGGATCCAAGTTGAAAGAGATAAATTGAACAAGTATGGTCGTCCCCTG-TTGGGATGTA 476
KSL041104       TGGGATCCAAGTTGAAAGAGATAAATTGAACAAGTATGGTCGTCCCCCTGTTGGGATGTA 475
LPK081001       TGGGATCCAAGTTGAAAGAGATAAATTGAACAAGTATGGTCGTCCCC-TGTTGGGATGTA 475
LHB081111       TGGGATCCAAGTTGAAAGAGATAAATTGAACAAGTATGGTCGTTCCCCTGTTGGGATGTA 475
LHB091003       TGGGATCCAAGTTGAAAGAGATAAATTGAACAAGTATGGTCGTTCCCCTGTTGGGATGTA 475
KSL051105       TGGGATCCAAGTTGAAAGAGATAAATTGAACAAGTATGGTCGT-CCCCTGTTGGGATGTA 474
LHB021106       TGGGATCCAAGTTGAAAGAGATAAATTGAACAAGTATGGTCGT-CCCCTGTTGGGATGTA 475
LHB071109       TGGGATCCAAGTTGAAAGAGATAAATTGAACAAGTATGGTCGT-CCCCTGTTGGGATGTA 475
LPK011103       TGGGATCCAAGTTGAAAGAGATAAATTGAACAAGTATGGTCGT-CCCCYGTTGGGATGTA 474
KSL071002       TGGGATCCAAGTTGAAAGAGATAAATTGAACAAGTATGGTCGT-CCCCTGTTGGGATGTA 476
TNF111001       TGGGATCCAAGTTGAAAGAGATAAATTGAACAAGTATGGTCGT-CCCCTGTTGGGATGTA 475
LPK091002       TGGGATCCAAGTTGAAAGAGATAAATTGAACAAGTATGGTCGT-CCCCTGTTGGGATGTA 475
KSL071001       TGGGATCCAAGTTGAAAGAGATAAATTGAACAAGTATGGTCGT-CCCCTGTTGGGATGTA 476
LHB101004       TGGGATCCAAGTTGAAAGAGATAAATTGAACAAGTATGGTCGT-CCCCTGTTGGGATGTA 475
LHB101005       TGGGATCCAAGTTGAAAGAGATAAATTGAACAAGTATGGTCGT-CCCCTGTTGGGATGTA 475
LHB081110       TGGGATCCAAGTTGAAAGAGATAAATTGAACAAGTATGGTCGT-CCCCTGTTGGGATGTA 474
LHB091112       TGGGATCCAAGTTGAAAGAGATAAATTGAACAAGTATGGTCGT-CCCCTGTTGGGATGTA 474
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KSL031103       CTATT-AAACCTAAATTGGGGTTATCT-GCTAAAAAYTACGGTAGAGCTGTTTATGAATG 533
LHB031107       CTATTTAAACCTAAATTGGGGTTATCT-GCTAAAAACTACGGTAGAGCTGTTTATGAATG 535
LHB071001       CTATT-AAACCTAAATTGGGGTTATCT-GCTAAAAACTACGGTAGAGCTGTTTATGAATG 533
LHB081002       CTATT-AAACCTAAATTGGGGTTATCT-GCTAAAAACTACGGTAGAGCTGTTTATGAATG 534
KSL041104       CTATT-AAACCTAAATTGGGGTTATCT-GCTAAAAACTACGGTAGAGCTGTTTATGAATG 533
LPK081001       CTATT-AAACCTAAATTGGGGTTATCT-GCTAAAAACTACGGTAGAGCTGTTTATGAATG 533
LHB081111       CTATT-AAACCTAAATTGGGGTTATCT-GCTAAAAACTACGGTAGAGCTGTTTATGAATG 533
LHB091003       CTATT-AAACCTAAATTGGGGTTATCT-GCTAAAAACTACGGTAGAGCTGTTTATGAATG 533
KSL051105       CTATT-AAACCTAAATTGGGGTTATCT-GCTAAAAACTACGGTAGAGCTGTTTATGAATG 532
LHB021106       CTATT-AAACCTAAATTGGGGTTATCT-GCTAAAAACTACGGTAGAGCTGTTTATGAATG 533
LHB071109       CTATT-AAACCTAAATTGGGGTTATCT-GCTAAAAACTACGGTAGAGCTGTTTATGAATG 533
LPK011103       CTATT-AAACCTAAATTGGGGTTATCT-GCTAAAAACTACGGTAGAGCTGTTTATGAATG 532
KSL071002       CTATT-AAACCTAAATTGGGGTTATCT-GCTAAAAACTACGGTAGAGCTGTTTATGAATG 534
TNF111001       CTATT-AAACCTAAATTGGGGTTATCT-GCTAAAAACTACGGTAGAGCTGTTTATGAATG 533
LPK091002       CTATT-AAACCTAAATTGGGGTTATCT-GCTAAAAACTACGGTAGAGCTGTTTATGAATG 533
KSL071001       CTATT-AAACCTAAATTGGGGTTATCT-GCTAAAAACTACGGTAGAGCTGTTTATGAATG 534
LHB101004       CTATT-AAACCTAAATTGGGGTTATCT-GCTAAAAACTACGGTAGAGCTGTTTATGAATG 533
LHB101005       CTATT-AAACCTAAATTGGGGTTATCT-GCTAAAAACTACGGTAGAGCTGTTTATGAATG 533
LHB081110       CTATT-AAACCTAAATTGGGGTTATCTTGCTAAAAACTACGGTAGAGCTGTTTATGAATG 533
LHB091112       CTATT-AAACCTAAATTGGGGTTATCT-GCTAAAAACTACGGTAGAGCTGTTTATGAATG 532
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KSL031103       TCTT-CGCGGTGGACTTGATTTTACCAAAGATGATGAGAACGTGAACTCACAACCATTTA 592
LHB031107       TCTTTCGCGGTGGACTTGATTTTACCAAAGATGATGAGAACGTGAACTCACAACCATTTA 595
LHB071001       TCTT-CGCGGTGGACTTGATTTTACCAAAGATGATGAGAACGTGAACTCACAACCATTTA 592
LHB081002       TCTT-CGCGGTGGACTTGATTTTACCAAAGATGATGAGAACGTGAACTCACAACCATTTA 593
KSL041104       TCTT-YGCGGTGGACTTGATTTTACCAAAGATGATGAGAACGTGAACTCACAACCATTTA 592
LPK081001       TCTT-CGCGGTGGACTTGATTTTACCAAAGATGATGAGAACGTGAACTCACAACCATTTA 592
LHB081111       TCTT-CGCGGTGGACTTGATTTTACCAAAGATGATGAGAACGTGAACTCACAACCATTTA 592
LHB091003       TCTT-CGCGGTGGACTTGATTTTACCAAAGATGATGAGAACGTGAACTCACAACCATTTA 592
KSL051105       TCTT-CGCGGTGGACTTGATTTTACCAAAGATGATGAGAACGTGAACTCACAACCATTTA 591
LHB021106       TCTT-CGCGGTGGACTTGATTTTACCAAAGATGATGAGAACGTGAACTCACAACCATTTA 592
LHB071109       TCTT-CGCGGTGGACTTGATTTTACCAAAGATGATGAGAACGTGAACTCACAACCATTTA 592
LPK011103       TCTT-CGCGGTGGACTTGATTTTACCAAAGATGATGAGAACGTGAACTCACAACCATTTA 591
KSL071002       TCTT-CGCGGTGGACTTGATTTTACCAAAGATGATGAGAACGTGAACTCACAACCATTTA 593
TNF111001       TCTT-CGCGGTGGACTTGATTTTACCAAAGATGATGAGAACGTGAACTCACAACCATTTA 592
LPK091002       TCTT-CGCGGTGGACTTGATTTTACCAAAGATGATGAGAACGTGAACTCACAACCATTTA 592
KSL071001       TCTT-CGCGGTGGACTTGATTTTACCAAAGATGATGAGAACGTGAACTCACAACCATTTA 593
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LHB101004       TCTT-CGCGGTGGACTTGATTTTACCAAAGATGATGAGAACGTGAACTCACAACCATTTA 592
LHB101005       TCTT-CGCGGTGGACTTGATTTTACCAAAGATGATGAGAACGTGAACTCACAACCATTTA 592
LHB081110       TCTT-CGCGGTGGACTTGATTTTACCAAAGATGATGAGAACGTGAACTCACAACCATTTA 592
LHB091112       TCTT-CGCGGTGGACTTGATTTTACCAAAGATGATGAGAACGTGAACTCACAACCATTTA 591
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KSL031103       TGCGTTGGAGAGATCGTTTCTTATTTTGTGCCGAAGCACTTTTT-AAAGCACAGGTTGAA 651
LHB031107       TGCGTTGGAGAGATCGTTTCTTATTTTGTGCCGAAGCACTTTTTTAAAGCACAGGTTGAA 655
LHB071001       TGCGTTGGAGAGATCGTTTCTTATTTTGTGCCGAAGCACTTTTT-AAAGCACAGGTTGAA 651
LHB081002       TGCGTTGGAGAGATCGTTTCTTATTTTGTGCCGAAGCACTTTTT-AAAGCACAGGTTGAA 652
KSL041104       TGCGTTGGAGAGATCGTTTCTTATTTTGTGCCGAAGCACTTTTT-AAAGCACAGGTTGAA 651
LPK081001       TGCGTTGGAGAGATCGTTTCTTATTTTGTGCCGAAGCACTTTTT-AAAGCACAGGTTGAA 651
LHB081111       TGCGTTGGAGAGATCGTTTCTTATTTTGTGCCGAAGCACTTTTT-AAAGCACAGGTTGAA 651
LHB091003       TGCGTTGGAGAGATCGTTTCTTATTTTGTGCCGAAGCACTTTTT-AAAGCACAGGTTGAA 651
KSL051105       TGCGTTGGAGAGATCGTTTCTTATTTTGTGCCGAAGCACTTTTT-AAAGCACAGGTTGAA 650
LHB021106       TGCGTTGGAGAGATCGTTTCTTATTTTGTGCCGAAGCACTTTTT-AAAGCACAGGTTGAA 651
LHB071109       TGCGTTGGAGAGATCGTTTCTTATTTTGTGCCGAAGCACTTTTT-AAAGCACAGGTTGAA 651
LPK011103       TGCGTTGGAGAGATCGTTTCTTATTTTGTGCCGAAGCACTTTTT-AAAGCACAGGTTGAA 650
KSL071002       TGCGTTGGAGAGATCGTTTCTTATTTTGTGCCGAAGCACTTTTT-AAAGCACAGGTTGAA 652
TNF111001       TGCGTTGGAGAGATCGTTTCTTATTTTGTGCCGAAGCACTGTTT-AAAGCACAGACTGAA 651
LPK091002       TGCGTTGGAGAGATCGTTTCTTATTTTGTGCCGAAGCACTTTTT-AAAGCACAGGTTGAA 651
KSL071001       TGCGTTGGAGAGATCGTTTCTTATTTTGTGCCGAAGCACTTTTT-AAAGCACAGGTTGAA 652
LHB101004       TGCGTTGGAGAGATCGTTTCTTATTTTGTGCCGAAGCACTTTTT-AAAGCACAGGTTGAA 651
LHB101005       TGCGTTGGAGAGATCGTTTCTTATTTTGTGCCGAAGCACTTTTT-AAAGCACAGGTTGAA 651
LHB081110       TGCGTTGGAGAGATCGTTTCTTATTTTGTGCCGAAGCACTTTTT-AAAGCACAGGTTGAA 651
LHB091112       TGCGTTGGAGAGATCGTTTCTTATTTTGTGCCGAAGCACTTTTT-AAAGCACAGGTTGAA 650
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KSL031103       ACAGGTGAAATCAAAGGGCATTACTTGAATGCTACTGCAGGTACATGCGAAGAAATGATC 711
LHB031107       ACAGGTGAAATCAAAGGGCATTACTTGAATGCTACTGCAGGTACATGCGAAGAAATGATC 715
LHB071001       ACAGGTGAAATCAAAGGGCATTACTTGAATGCTACTGCAGGTACATGCGAAGAAATGATC 711
LHB081002       ACAGGTGAAATCAAAGGGCATTACTTGAATGCTACTGCAGGTACATGCGAAGAAATGATC 712
KSL041104       ACAGGTGAAATCAAAGGGCATTACTTGAATGCTACTGCAGGTACATGCGAAGAAATGATC 711
LPK081001       ACAGGTGAAATCAAAGGGCATTACTTGAATGCTACTGCAGGTACATGCGAAGAAATGATC 711
LHB081111       ACAGGTGAAATCAAAGGGCATTACTTGAATGCTACTGCAGGTACATGCGAAGAAATGATC 711
LHB091003       ACAGGTGAAATCAAAGGGCATTACTTGAATGCTACTGCAGGTACATGCGAAGAAATGATC 711
KSL051105       ACAGGTGAAATCAAAGGGCATTACTTGAATGCTACTGCAGGTACATGCGAAGAAATGATC 710
LHB021106       ACAGGTGAAATCAAAGGGCATTACTTGAATGCTACTGCAGGTACATGCGAAGAAATGATC 711
LHB071109       ACAGGTGAAATCAAAGGGCATTACTTGAATGCTACTGCAGGTACATGCGAAGAAATGATC 711
LPK011103       ACAGGTGAAATCAAAGGGCATTACTTGAATGCTACTGCAGGTACATGCGAAGAAATGATC 710
KSL071002       ACAGGTGAAATCAAAGGGCATTACTTGAATGCTACTGCAGGTACATGCGAAGAAATGATC 712
TNF111001       ACAGGTGAAATCAAAGGGCATTACTTGAATGCTACTGCAGGTACATGCGAAGAAATGATG 711
LPK091002       ACAGGTGAAATCAAAGGGCATTACTTGAATGCTACTGCAGGTACATGCGAAGAAATGATC 711
KSL071001       ACAGGTGAAATCAAAGGGCATTACTTGAATGCTACTGCAGGTACATGCGAAGAAATGATC 712
LHB101004       ACAGGTGAAATCAAAGGGCATTACTTGAATGCTACTGCAGGTACATGCGAAGAAATGATC 711
LHB101005       ACAGGTGAAATCAAAGGGCATTACTTGAATGCTACTGCAGGTACATGCGAAGAAATGATC 711
LHB081110       ACAGGTGAAATCAAAGGGCATTACTTGAATGCTACTGCAGGTACATGCGAAGAAATGATC 711
LHB091112       ACAGGTGAAATCAAAGGGCATTACTTGAATGCTACTGCAGGTACATGCGAAGAAATGATC 710
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KSL031103       AAAAGAGCTGTATTTGCTAGAGAATTAGGCGTTCC-GATCGTAATGCATGACTACTTAAC 770
LHB031107       AAAAGAGCTGTATTTGCTAGAGAATTAGGCGTTCC-GATCGTAATGCATGACTACTTAAC 774
LHB071001       AAAAGAGCTGTATTTGCTAGAGAATTAGGCGTTCC-GATCGTAATGCATGACTACTTAAC 770
LHB081002       AAAAGAGCTGTATTTGCTAGAGAATTAGGCGTTCC-GATCGTAATGCATGACTACTTAAC 771
KSL041104       AAAAGAGCTGTATTTGCTAGAGAATTAGGCGTTCC-GATCGTAATGCATGACTACTTAAC 770
LPK081001       AAAAGAGCTGTATTTGCTAGAGAATTAGGCGTTCC-GATCGTAATGCATGACTACTTAAC 770
LHB081111       AAAAGAGCTGTATTTGCTAGAGAATTAGGCGTTCC-GATCGTAATGCATGACTACTTAAC 770
LHB091003       AAAAGAGCTGTATTTGCTAGAGAATTAGGCGTTCC-GATCGTAATGCATGACTACTTAAC 770
KSL051105       AAAAGAGCTGTATTTGCTAGAGAATTAGGCGTTCC-GATCGTAATGCATGACTACTTAAC 769
LHB021106       AAAAGAGCTGTATTTGCTAGAGAATTAGGCGTTCC-GATCGTAATGCATGACTACTTAAC 770
LHB071109       AAAAGAGCTGTATTTGCTAGAGAATTAGGCGTTCC-GATCGTAATGCATGACTACTTAAC 770
LPK011103       AAAAGAGCTGTATTTGCTAGAGAATTAGGCGTTCC-GATCGTAATGCATGACTACTTAAC 769
KSL071002       AAAAGAGCTGTATTTGCTAGAGAATTAGGCGTTCC-GATCGTAATGCATGACTACTTAAC 771
TNF111001       AAAAGAGCTGTATTTGCTAGAGAATTGGGCGTTCC-GATCGTAATGCATGACTACTTAAC 770
LPK091002       AAAAGAGCTGTATTTGCTAGAGAATTAGGCGTTCC-GATCGTAATGCATGACTACTTAAC 770
KSL071001       AAAAGAGCTGTATTTGCTAGAGAATTAGGCGTTCC-GATCGTAATGCATGACTACTTAAC 771
LHB101004       AAAAGAGCTGTATTTGCTAGAGAATTAGGCGTTCC-GATCGTAATGCATGACTACTTAAC 770
LHB101005       AAAAGAGCTGTATTTGCTAGAGAATTAGGCGTTCC-GATCGTAATGCATGACTACTTAAC 770
LHB081110       AAAAGAGCTGTATTTGCTAGAGAATTAGGCGTTCC-GATCGTAATGCATGACTACTTAAC 770
LHB091112       AAAAGAGCTGTATTTGCTAGAGAATTAGGCGTTCCCGATCGTAATGCATGACTACTTAAC 770
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KSL031103       GGGGGG-ATTCACCG-CAAATACTAGC-TTGGCTC-ATTA-TTGCC-GAGATAATGG-TC 823
LHB031107       GGGGGG-ATTCACCG-CAAATACTAGC-TTGGCTC-ATTA-TTGCC-GAGATAATGG-TC 827
LHB071001       GGGGGG-ATTCACCG-CAAATACTAGC-TTGGCTC-ATTA-TTGCC-GAGATAATGG-TC 823
LHB081002       GGGGGG-ATTCACCG-CAAATACTAGC-TTGGCTC-ATTA-TTGCC-GAGATAATGG-TC 824
KSL041104       GGGGGG-ATTCACCG-CAAATACTAGC-TTGGCTC-ATTA-TTGCC-GAGATAATGG-TC 823
LPK081001       GGGGGG-ATTCACCG-CAAATACTAGC-TTGGCTC-ATTA-TTGCC-GAGATAATGG-TC 823
LHB081111       GGGGGGGATTCACCGGCAAATACTAGCCTTGGCTC-ATTA-TTGCCCGAGATAATGGGTC 828
LHB091003       GGGGGGGATTCACCGGCAAATACTAGCCTTGGCTC-ATTA-TTGCCCGAGATAATGGGTC 828
KSL051105       GGGGGG-ATTCACCG-CAAATACTAGC-TTGGCTC-ATTA-TTGCC-GAGATAATGG-TC 822
LHB021106       GGGGGG-ATTCACCG-CAAATACTAGC-TTGGCTC-ATTA-TTGCC-GAGATAATGG-TC 823
LHB071109       GGGGGGGATTCACCG-CAAATACTAGC-TTGGCTC-ATTAATTGCC-GAGATAATGGGTC 826
LPK011103       GGGGGG-ATTCACCG-CAAATACTAGC-TTGGCTC-ATTA-TTGCC-GAGATAATGG-TC 822
KSL071002       GGGGGG-ATTCACCG-CAAATACTAGC-TTGGCTC-ATTA-TTGCC-GAGATAATGG-TC 824
TNF111001       GGGGGG-ATTCACCG-CAAATACTACC-TTGGCTC-ATTA-TTGCC-GAGATAATGG-TC 823
LPK091002       GGGGGG-ATTCACCG-CAAATACTAGC-TTGGCTC-ATTA-TTGCC-GAGATAATGG-TC 823
KSL071001       GGGGGG-ATTCACCG-CAAATACTAGC-TTGGCTC-ATTA-TTGCC-GAGATAATGG-TC 824
LHB101004       GGGGGG-ATTCACCG-CAAATACTAGC-TTGGCTC-ATTA-TTGCC-GAGATAATGG-TC 823
LHB101005       GGGGGG-ATTCACCG-CAAATACTAGC-TTGGCTC-ATTA-TTGCC-GAGATAATGG-TC 823
LHB081110       GGGGGGGATTCACCGGCAAATACTAGCTTTGGCTTCATTAATTGCCGAAGATAATGGGTC 830
LHB091112       GGGGGG-ATTCACCGGCAAATACTAGCCTTGGCTCAATTATTTGCCGAGATAAATGGGTC 829
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KSL031103       TACT--TCTTCACATC-CACCGT-GCAA-TGCAT-GC-GGTTATT-GA-TAGAC-AGAAG 873
LHB031107       TACT--TCTTCACATC-CACCGT-GCAA-TGCAT-GC-GGTTATT-GA-TAGAC-AGAAG 877
LHB071001       TACT--TCTTCACATC-CACCGT-GCAA-TGCAT-GC-GGTTATT-GA-TAGAC-AGAAG 873
LHB081002       TACT--TCTTCACATC-CACCGT-GCAA-TGCAT-GC-GGTTATT-GA-TAGAC-AGAAG 874
KSL041104       TACT--TCTTCACATC-CACCGT-GCAA-TGCAT-GC-GGTTATT-GA-TAGAC-AGAAG 873
LPK081001       TACT--TCTTCACATC-CACCGT-GCAA-TGCAT-GC-GGTTATT-GA-TAGAC-AGAAG 873
LHB081111       TACT--TCTTCACATC-CACCGTTGCAA-TGCAT-GCGGGTTATT-GA-TAGAC-AGAAG 880
LHB091003       TACT--TCTTCACATC-CACCGTTGCAA-TGCAT-GCGGGTTATT-GA-TAGAC-AGAAG 880
KSL051105       TACT--TCTTCACATC-CACCGT-GCAA-TGCAT-GCGG-TTATT-GA-TAGAC-AGAAG 872
LHB021106       TACT--TCTTCACATC-CACCGTG-CAA-TGCAT-GC-GGTTATT-GA-TAGAC-AGAAG 873
LHB071109       TACTC-TCTTCACATCGCACCGTGGCAA-TGCAT-GCCGGTTATTTGA-TAGACTAGAAG 882
LPK011103       TACT--TCTTCACATC-CACCGT-GCAA-TGCAT-GC-GGTTATT-GA-TAGAC-AGAAG 872
KSL071002       TACT--TCTTCACATC-CACCGT-GCAA-TGCAT-GC-GGTTATT-GA-TAGAC-AGAAG 874
TNF111001       TACT--TCTTCACATC-CACCGT-GCAA-TGCAT-GC-GGTTATT-GA-TAGAC-AGAAG 873
LPK091002       TACT--TCTTCACATC-CACCGT-GCAA-TGCAT-GC-GGTTATT-GA-TAGAC-AGAAG 873
KSL071001       TACT--TCTTCACATC-CACCGT-GCAA-TGCAT-GC-GGTTATT-GA-TAGAC-AGAAG 874
LHB101004       TACT--TCTTCACATC-CACCGT-GCAA-TGCAT-GC-GGTTATT-GA-TAGAC-AGAAG 873
LHB101005       TACT--TCTTCACATC-CACCGT-GCAA-TGCAT-GC-GGTTATT-GA-TAGAC-AGAAG 873
LHB081110       TACTTTCTTTAACATTCCACCGTTGCCAATGCATTGCCGGTTATTTGAATAGAC-AGAAG 889
LHB091112       TACTT-CTTTCACATTCCACCGTGGCAATTGCAT-GCCGGTTATTTGATTAGA--ACAAG 885
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KSL031103       AA-----TCATGGT-A-TCCA-CTT-CCGGGTA-TTAGC-AAAAGC-GTTACGT-ATG-T 919
LHB031107       AA-----TCATGGT-A-TCCA-CTT-CCGGGTA-TTAGC-AAAAGC-GTTACGT-ATG-T 923
LHB071001       AA-----TCATGGT-A-TCCA-CTT-CCGGGTA-TTAGC-AAAAGC-GTTACGT-ATG-T 919
LHB081002       AA-----TCATGGT-A-TCCA-CTT-CCGGGTA-TTAGC-AAAAGC-GTTACGT-ATG-T 920
KSL041104       AA-----TCATGGT-A-TCCACTTT-CCGGGTA-TTAGCAAAAAGC-GTTACGT-ATG-T 921
LPK081001       AA-----TCATGGT-A-TCCACTT--CCGGGTA-TTAGCAAAA-GC-GTTACGT-ATG-T 919
LHB081111       AAA----TCATGGT-AATCCAACTT-CCGGGGTATTAGCCAAAAGCCGTTACGT-ATGGT 933
LHB091003       AAA----TCATGGT-AATCCAACTT-CCGGGGTATTAGCCAAAAGCCGTTACGT-ATGGT 933
KSL051105       AA-----TCATGGT-A-TCCA-CTT-CCGGGTAATTAGC-AAAAGC-GTTACGT-ATG-T 919
LHB021106       AAT----CA-TGGT-A-TCCACTT--CCGGGTATT-AGCAAAAGG--GTTACGT-ATG-T 919
LHB071109       AAT----CAATGGT-AATCCACTTT-CCGGGTATTTAGCAAAAGGC-GTTACGTTATG-T 934
LPK011103       AA-----TCATGGT-A-TCCA-CTT-CCGGGTA-TTAGCAAAAAGC-GTTACGTAATG-T 920
KSL071002       AA-----TCATGGT-A-TCCA-CTT-CCGGGTA-TTAGC-AAAAGC-GTTACGT-ATG-T 920
TNF111001       AA-----TCATGGT-A-TCCA-CTT-CCGGGTA-TTAGC-AAAAGC-GTTACGT-ATG-T 919
LPK091002       AA-----TCATGGT-A-TCCA-CTT-CCGGGTA-TTAGC-AAAAGC-GTTACGT-ATG-T 919
KSL071001       AA-----TCATGGT-A-TCCA-CTT-CCGGGTA-TTAGC-AAAAGC-GTTACGT-ATG-T 920
LHB101004       AA-----TCATGGT-A-TCCA-CTT-CCGGGTA-TTAGC-AAAAGC-GTTACGT-ATG-T 919
LHB101005       AA-----TCATGGT-A-TCCA-CTT-CCGGGTA-TTAGC-AAAAGC-GTTACGT-ATG-T 919
LHB081110       AA-----TCATGGT-ATTC-AMTT--CCGGGT-ATTAGCA-AAAGC-GTTACGT-ATG-T 935
LHB091112       AAGGAATTCATGGTTATCCCACTTTCCCGGGTTATTAGCACAAAGC-GTTACGT-ATG-T 942
                **        **** *  * *  *  *****   * ***  ** *  ******* *** *

KSL031103       CTGG-TGGAGA-TCATATTC-ACTCTGG-TACC--GTAGTA-GGTAAA-CTTG-AAGGTG 970
LHB031107       CTGG-TGGAGA-TCATATTC-ACTCTGG-TACC--GTAGTA-GGTAAA-CTTG-AAGGTG 974
LHB071001       CTGG-TGGAGA-TCATATTC-ACTCTGG-TACC--GTAGTA-GGTAAA-CTTG-AAGGTG 970
LHB081002       CTGG-TGGAGA-TCATATTC-ACTCTGG-TACC--GTAGTA-GGTAAA-CTTG-AAGGTG 971
KSL041104       CTTGGTGGAGA-TCATATTC-ACTCTGG-TACC-GTAAGTA-GGTAAA-CTTGGAAGGTG 975
LPK081001       CT-GGTGGAGA-TCATATTC-ACTCTGG-TACC-GTA-GTA-GGTAAA-CTTG-AAGGTG 970
LHB081111       CTGGGTGGAGAATCATATTTCACTCTGGGTACCCGTAAGWA-GGTAAAACTTGAAAGGTG 992
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LHB091003       CTGGGTGGAGAATCATATTTCACTCTGGGTACCCGTAAGWA-GGTAAAACTTGAAAGGTG 992
KSL051105       CTGG-TGGAGA-TCATATT-CACTCTGGGAACC--KWAKTA-GGTAAA-CTTGAAAGGTG 972
LHB021106       CTGG-TGGAGA-TCATATTC-ACTCTGG-TACC--GTAGTAGG--TAAACTTGAA-GGTG 970
LHB071109       CTGGGTGGAGA-TCATATTCCACTCTGGGTACC--GTAGKAAGGTCAAACTTGAAAGGTG 991
LPK011103       CTGGGTGGAAGATCATATTCACTTCTGGTTACC-GKAATTAAGGTAAACTTTGAAAGGTG 979
KSL071002       CTGG-TGGAGA-TCATATTC-ACTCTGG-TACC--GTAGTA-GGTAAA-CTTG-AAGGTG 971
TNF111001       CTGG-TGGAGA-TCATATTC-ACTCTGG-TACC--GTAGTA-GGTAAA-CTTG-AAGGTG 970
LPK091002       CTGG-TGGAGA-TCATATTC-ACTCTGG-TACC--GTAGTA-GGTAAA-CTTG-AAGGTG 970
KSL071001       CTGG-TGGAGA-TCATATTC-ACTCTGG-TACC--GTAGTA-GGTAAA-CTTG-AAGGTG 971
LHB101004       CTGG-TGGAGA-TCATATTC-ACTCTGG-TACC--GTAGTA-GGTAAA-CTTG-AAGGTG 970
LHB101005       CTGG-TGGAGA-TCATATTC-ACTCTGG-TACC--GTAGTA-GGTAAA-CTTG-AAGGTG 970
LHB081110       CTGG-TGGAGA-TCATATTC-ACTCTGG-TACC--GTAGTA-GGTAAA-CTTG-AAGGTG 986
LHB091112       CTGG-TGGAGA-TCATATTC-ACTCTGG-TACC--GTAGTA-GGTAAA-CTTG-AAGGTG 993
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KSL031103       AAA--GAGACATAACTTTGGG---CTTTGTTGA-TTTAC-TG-CGTGATGA--TTTTGTT 1020
LHB031107       AAA--RAGACATAACTTTGGG---CTTTGTTGA-TTTAC-TG-CGTGATGA--TTTTGTT 1024
LHB071001       AAA--GAGACATAACTTTGGG---CTTTGTTGA-TTTAC-TG-CGTGATGA--TTTTGTT 1020
LHB081002       AAA--GAGACATAACTTTGGG---CTTTGTTGA-TTTAC-TG-CGTGATGA--TTTTGTT 1021
KSL041104       AAA-GAAGACATAACTTTKGG--CTTTTGTTGAATTTAC-TGGCGTGATGGATTTTTGTT 1031
LPK081001       AAA-GA-GACATAACTTTGGG--CTTT-GTTGA-TTTAC-TG-CGTGATGA--TTTTGTT 1020
LHB081111       AAA-AGAGAMATAAACTTTTGGGCCTTTGTTGA-ATTAACTGCCGGGATGAA-TTTTGTT 1049
LHB091003       AAA-AGAGAMATAAACTTTTGGGCCTTTGTTGA-ATTAACTGCCGGGATGAA-TTTTGTT 1049
KSL051105       AAA-GARAACATAAACTTTGGGGCTTTTGTTGA-TTTACCTGCCGTGATGAA-TTTTGTT 1029
LHB021106       AAA-GAGA-CATAACTTTGGG---CTTTGTTGA-TTTAC-TGC-GTGATGAT-TTT-GTT 1020
LHB071109       AAAAGAGAACATAACTTTTGGTG-YTTTGTTGAATTTACCTGCCGTGATGAT-TTTTGTT 1049
LPK011103       AAAAGAGACAATAA-CTTTGGGG-CTTTGTTGAATTTAC-TGCCGTGATGAA-TTTTGTT 1035
KSL071002       AAA--GAGACATAACTTTGGG---CTTTGTTGA-TTTAC-TG-CGTGATGA--TTTTGTT 1021
TNF111001       AAA--GAGACATAACTTTGGG---CTTTGTTGA-TTTAC-TG-CGTGATGA--TTTTGTT 1020
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LHB101004       AAA--GAGACATAACTTTGGG---CTTTGTTGA-TTTAC-TG-CGTGATGA--TTTTGTT 1020
LHB101005       AAA--GAGACATAACTTTGGG---CTTTGTTGA-TTTAC-TG-CGTGATGA--TTTTGTT 1020
LHB081110       AAA--GAGACATAACTTTGGG---CTTTGTTGA-TTTAC-TG-CGTGATGA--TTTTGTT 1036
LHB091112       AAA--GAGACATAACTTTGGG---CTTTGTTGA-TTTAC-TG-CGTGATGA--TTTTGTT 1043
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KSL031103       GAACAARA---TCGAAG--TCGCG-GGTATTTATTT-CACTC-AAGATTGGGGTCTCTTT 1072
LHB031107       GAACAAGA---TCGAAG--TCGCC-GGTATTTATTT-CACTC-AAGATTGGGGTCTCTTT 1076
LHB071001       GAACAAGA---TCGAAG--TCGC--GGTATTTATTT-CACTC-AAGATTGGG-TCTCTTT 1070
LHB081002       GAACAAGA---TCGAAG--TCGC--GGTATTTATTT-CACTC-AAGATTGGG-TCTCTTT 1071
KSL041104       GAACAARATATCGGAARTTCCGC--GGTATTTATTT-CACTC-AAGATTGGG-TCTCTTT 1086
LPK081001       GAACAAGATC---GAAGT--CGC--GGTATTTATTT-CACTC-AAGATTGGG-TCTCTTT 1070
LHB081111       GAAACAAG-AWCCGAAAG-TCGCCGGGAATTTATTT-CACTC-AAGATTGGG-TCTCTTT 1104
LHB091003       GAAACAAG-AWCCGAAAG-TCGCCGGGAATTTATTT-CACTC-AAGATTGGG-TCTCTTT 1104
KSL051105       GAA-CAAG-AWCCGAAR--TCGC-GGGAATTTATTT-CACTCCAAGATTGGG-TCTCTTT 1082
LHB021106       GAACAAG---ATCGAAG--TCGC--GGTATTTATTT-CACTC-AAGATTGGG-TCTCTTT 1070
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TNF111001       ACCAGGTGTTCTACC-TGTGGCTTC-AGGAGGTA-TTCACGTTTGGCATAT-GCCTGCTC 1126
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LHB101005       AGCTCGTAATGAAGGACGTGATCTTGCTCGGGAAGGTAATGAGATTATTCGCGAGGCTTC 1304
LHB081110       AGCTCGTAATGAAGGACGTGATCTTGCTCGGGAAGGTAATGAGATTATTCGCGAGGCTTC 1320
LHB091112       AGCTCGTAATGAAGGACGTGATCTTGCTCGGGAAGGTAATGAGATTATTCGCGAGGCTTC 1327
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KSL031103       CAAATGGAGCCCGGAACTAGCTGCTGCTTGTGAGGTATGGAAAGAGATCGTATTTAATTT 1369
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LHB071001       CAAATGGAGCCCGGAACTAGCTGCTGCTTGTGAGGTATGGAAAGAGATCGTATTTAATTT 1364
LHB081002       CAAATGGAGCCCGGAACTAGCTGCTGCTTGTGAGGTATGGAAAGAGATCGTATTTAATTT 1365
KSL041104       CAAATGGAGCCCGGAACTAGCTGCTGCTTGTGAGGTATGGAAAGAGATCGTATTTAATTT 1380
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LHB081111       CAAATGGAGCCCGGAACTAGCTGCTGCTTGTGAGGTATGGAAAGAGATCGTATTTAATTT 1398
LHB091003       CAAATGGAGCCCGGAACTAGCTGCTGCTTGTGAGGTATGGAAAGAGATCGTATTTAATTT 1398
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Sequence format is Pearson
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Sequence 2: KSL041104 1504 bp
Sequence 3: KSL051105 1503 bp

Sequence 4: KSL071001 1489 bp
Sequence 5: KSL071002 1488 bp
Sequence 6: LHB021106 1486 bp
Sequence 7: LHB031107 1503 bp
Sequence 8: LHB071001 1486 bp
Sequence 9: LHB071109 1518 bp
Sequence 10: LHB081002 1491 bp
Sequence 11: LHB081110 1502 bp
Sequence 12: LHB081111 1523 bp
Sequence 13: LHB091003 1523 bp
Sequence 14: LHB091112 1511 bp
Sequence 15: LHB101004 1489 bp
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Start of Pairwise alignments
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Sequences (6:9) Aligned. Score: 97
Sequences (6:10) Aligned. Score: 97
Sequences (6:11) Aligned. Score: 96
Sequences (6:12) Aligned. Score: 97
Sequences (6:13) Aligned. Score: 97
Sequences (6:14) Aligned. Score: 97
Sequences (6:15) Aligned. Score: 97
Sequences (6:16) Aligned. Score: 98
Sequences (6:17) Aligned. Score: 97
Sequences (6:18) Aligned. Score: 98
Sequences (6:19) Aligned. Score: 97
Sequences (6:20) Aligned. Score: 97
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Sequences (7:9) Aligned. Score: 95
Sequences (7:10) Aligned. Score: 98
Sequences (7:11) Aligned. Score: 96
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Sequences (7:15) Aligned. Score: 98
Sequences (7:16) Aligned. Score: 98
Sequences (7:17) Aligned. Score: 96
Sequences (7:18) Aligned. Score: 98
Sequences (7:19) Aligned. Score: 98
Sequences (7:20) Aligned. Score: 96
Sequences (8:9) Aligned. Score: 97
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Sequences (8:11) Aligned. Score: 96
Sequences (8:12) Aligned. Score: 97
Sequences (8:13) Aligned. Score: 97
Sequences (8:14) Aligned. Score: 97
Sequences (8:15) Aligned. Score: 98
Sequences (8:16) Aligned. Score: 98
Sequences (8:17) Aligned. Score: 97
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Sequences (9:10) Aligned. Score: 97
Sequences (9:11) Aligned. Score: 95
Sequences (9:12) Aligned. Score: 96
Sequences (9:13) Aligned. Score: 96
Sequences (9:14) Aligned. Score: 95
Sequences (9:15) Aligned. Score: 97
Sequences (9:16) Aligned. Score: 97
Sequences (9:17) Aligned. Score: 96
Sequences (9:18) Aligned. Score: 97
Sequences (9:19) Aligned. Score: 97
Sequences (9:20) Aligned. Score: 96
Sequences (10:11) Aligned. Score: 96

Sequences (10:12) Aligned. Score: 96
Sequences (10:13) Aligned. Score: 96
Sequences (10:14) Aligned. Score: 97
Sequences (10:15) Aligned. Score: 98
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Sequences (10:17) Aligned. Score: 97
Sequences (10:18) Aligned. Score: 97
Sequences (10:19) Aligned. Score: 98
Sequences (10:20) Aligned. Score: 96
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Sequences (11:13) Aligned. Score: 95
Sequences (11:14) Aligned. Score: 96
Sequences (11:15) Aligned. Score: 96
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Sequences (12:14) Aligned. Score: 95
Sequences (12:15) Aligned. Score: 97
Sequences (12:16) Aligned. Score: 97
Sequences (12:17) Aligned. Score: 96
Sequences (12:18) Aligned. Score: 97
Sequences (12:19) Aligned. Score: 97
Sequences (12:20) Aligned. Score: 95
Sequences (13:14) Aligned. Score: 95
Sequences (13:15) Aligned. Score: 97
Sequences (13:16) Aligned. Score: 97
Sequences (13:17) Aligned. Score: 96
Sequences (13:18) Aligned. Score: 97
Sequences (13:19) Aligned. Score: 97
Sequences (13:20) Aligned. Score: 95
Sequences (14:15) Aligned. Score: 97
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Sequences (14:17) Aligned. Score: 96
Sequences (14:18) Aligned. Score: 97
Sequences (14:19) Aligned. Score: 97
Sequences (14:20) Aligned. Score: 96
Sequences (15:16) Aligned. Score: 98
Sequences (15:17) Aligned. Score: 97
Sequences (15:18) Aligned. Score: 98
Sequences (15:19) Aligned. Score: 98
Sequences (15:20) Aligned. Score: 97
Sequences (16:17) Aligned. Score: 97
Sequences (16:18) Aligned. Score: 98
Sequences (16:19) Aligned. Score: 98
Sequences (16:20) Aligned. Score: 97
Sequences (17:18) Aligned. Score: 97
Sequences (17:19) Aligned. Score: 97
Sequences (17:20) Aligned. Score: 96
Sequences (18:19) Aligned. Score: 98

Sequences (18:20) Aligned. Score: 97
Sequences (19:20) Aligned. Score: 97

There are 19 groups
Start of Multiple Alignment

Aligning...
Group 1: Sequences: 2 Score:28004
Group 2: Sequences: 2 Score:28154
Group 3: Sequences: 4 Score:27967
Group 4: Sequences: 2 Score:27881
Group 5: Sequences: 2 Score:28937
Group 6: Sequences: 3 Score:27786
Group 7: Sequences: 2 Score:27582
Group 8: Sequences: 5 Score:27670
Group 9: Sequences: 6 Score:27503
Group 10: Sequences: 8 Score:27348
Group 11: Sequences: 12 Score:27059
Group 12: Sequences: 2 Score:27971
Group 13: Sequences: 3 Score:28042
Group 14: Sequences: 4 Score:28014
Group 15: Sequences: 5 Score:28028
Group 16: Sequences: 17 Score:26928
Group 17: Sequences: 18 Score:27271
Group 18: Sequences: 2 Score:27912
Group 19: Sequences: 20 Score:27072
Alignment Score 1791301

Figure 39. Comparison of nucleotide sequence of *rbc*L gene of *D.metel* L. var. *metel, D.metel* L. var. *fastuosa*, and hybrid *D.metel* L., (TNF111001 was assigned as outgroup sample)
* indicate clustal consensus, - indicate indels

3. Alignments of *atp*B sequences of *D.metel* L. var. *metel*, *D.metel* L. var. *fastuosa*, and hybrid *D.metel* L.

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KSL041104       --AAAAAAAACC-GGGGCGTGTCGTCCAAATCATCGGTCCGGTACTAGATGTAGCCTTTC 57
LHB081110       -AAAAAAAAACC-GGGGCGTGTCGTCCAAATCATCGGTCCGGTACTAGATGTAGCCTTTC 58
KSL031103       -AAAAAAAAACC-GGGGCGTGTCGTCCAAATCATCGGTCCGGTACTAGATGTAGCCTTTC 58
LHB031107       ----AAAAAACC-GGGGCGTGTCGTCCAAATCATCGGTCCGGTACTAGATGTAGCCTTTC 55
LHB021106       ----AAAAAACC-GGGGCGTGTCGTCCAAATCATCGGTCCGGTACTAGATGTAGCCTTTC 55
LPK091002       -AAAAAAAAACC-GGGGCGTGTCGTCCAAATCATCGGTCCGGTACTAGATGTAGCCTTTC 58
LHB071009       ----AAAAAACC-GGGGCGTGTCGTCCAAATCATCGGTCCGGTACTAGATGTAGCCTTTC 55
LHB081111       ---AAAAAAACC-GGGGCGTGTCGTCCAAATCATCGGTCCGGTACTAGATGTAGCCTTTC 56
LHB091112       ---AAAAAAACC-GGGGCGTGTCGTCCAAATCATCGGTCCGGTACTAGATGTAGCCTTTC 56
LHB061108       ---AAAAAAACC-GGGGCGTGTCGTCCAAATCATCGGTCCGGTACTAGATGTAGCCTTTC 56
KSL051105       AAAAAAAAAACC-GGGGCGTGTCGTCCAAATCATCGGTCCGGTACTAGATGTAGCCTTTC 59
LHB071001       AAAAAAAAAACC-GGGGCGTGTCGTCCAAATCATCGGTCCGGTACTAGATGTAGCCTTTC 59
KSL071001       -AAAAAAAAACC-GGGGCGTGTCGTCCAAATCATCGGTCCGGTACTAGATGTAGCCTTTC 58
LHB081002       -AAAAAAAAACC-GGGGCGTGTCGTCCAAATCATCGGTCCGGTACTAGATGTAGCCTTTC 58
LHB091003       -AAAAAAAAACC-GGGGCGTGTCGTCCAAATCATCGGTCCGGTACTAGATGTAGCCTTTC 58
TNF111001       -AAAAAAAAACCTGGGGCGTGTCGTCCAAATCATCGGTCCGGTACTAGATGTAGCCTTTC 59
LHB101005       AAAAAAAAAACC-GGGGCGTGTCGTCCAAATCATCGGTCCGGTACTAGATGTAGCCTTTC 59
KSl071002       --AAAAAAAACC-GGGGCGTGTCGTCCAAATCATCGGTCCGGTACTAGATGTAGCCTTTC 57
LPK081001       --AAAAAAAACC-GGGGCGTGTCGTCCAAATCATCGGTCCGGTACTAGATGTAGCCTTTC 57
LHB101004       -AAAAAAAAACC-GGGGCGTGTCGTCCAAATCATCGGTCCGGTACTAGATGTAGCCTTTC 58
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LHB081110       CCCCGGGCAAGATGCCGAA-TATTTATAACGCTCTGGTAGTTCAAGGTCGAGATAGTGTT 117
KSL031103       CCCCGGGCAAGATGCCGAAATATTTATAACGCTCTGGTAGTTCAAGGTCGAGATAGTGTT 118
LHB031107       CCCCGGGCAAGATGCCGAA-TATTTATAACGCTCTGGTAGTTCAAGGTCGAGATAGTGTT 114
LHB021106       CCCCGGGCAAGATGCCGAA-TATTTATAACGCTCTGGTAGTTCAAGGTCGAGATAGTGTT 114
LPK091002       CCCCGGGCAAGATGCCGAA-TATTTATAACGCTCTGGTAGTTCAAGGTCGAGATAGTGTT 117
LHB071009       CCCCGGGCAAGATGCCGAA-TATTTATAACGCTCTGGTAGTTCAAGGTCGAGATAGTGTT 114
LHB081111       CCCCGGGCAAGATGCCGAA-TATTTATAACGCTCTGGTAGTTCAAGGTCGAGATAGTGTT 115
LHB091112       CCCCGGGCAAGATGCCGAA-TATTTATAACGCTCTGGTAGTTCAAGGTCGAGATAGTGTT 115
LHB061108       CCCCGGGCAAGATGCCGAA-TATTTATAACGCTCTGGTAGTTCAAGGTCGAGATAGTGTT 115
KSL051105       CCCCGGGCAAGATGCCGAA-TATTTATAACGCTCTGGTAGTTCAAGGTCGAGATAGTGTT 118
LHB071001       CCCCGGGCAAGATGCCGAA-TATTTATAACGCTCTGGTAGTTCAAGGTCGAGATAGTGTT 118
KSL071001       CCCCGGGCAAGATGCCGAA-TATTTATAACGCTCTGGTAGTTCAAGGTCGAGATAGTGTT 117
LHB081002       CCCCGGGCAAGATGCCGAA-TATTTATAACGCTCTGGTAGTTCAAGGTCGAGATAGTGTT 117
LHB091003       CCCCGGGCAAGATGCCGAA-TATTTATAACGCTCTGGTAGTTCAAGGTCGAGATAGTGTT 117
TNF111001       CCCCGGGCAAGATGCCGAA-TATTTATAACGCTCTGGTAGTTCAAGGTCGAGATAGTGTT 118
LHB101005       CCCCGGGCAAGATGCCGAA-TATTTATAACGCTCTGGTAGTTCAAGGTCGAGATAGTGTT 118
KSl071002       CCCCGGGCAAGATGCCGAA-TATTTATAACGCTCTGGTAGTTCAAGGTCGAGATAGTGTT 116
LPK081001       CCCCGGGCAAGATGCCGAA-TATTTATAACGCTCTGGTAGTTCAAGGTCGAGATAGTGTT 116
LHB101004       CCCCGGGCAAGATGCCGAA-TATTTATAACGCTCTGGTAGTTCAAGGTCGAGATAGTGTT 117
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KSL031103       GGTCAACCAATTAATGTGGCTTGTGAGGTACAGCAATTATTAGGAAATAATCGAGTTAGG 178
LHB031107       GGTCAACCAATTAATGTGGCTTGTGAGGTACAGCAATTATTAGGAAATAATCGAGTTAGG 174
LHB021106       GGTCAACCAATTAATGTGGCTTGTGAGGTACAGCAATTATTAGGAAATAATCGAGTTAGG 174
LPK091002       GGTCAACCAATTAATGTGGCTTGTGAGGTACAGCAATTATTAGGAAATAATCGAGTTAGG 177
LHB071009       GGTCAACCAATTAATGTGGCTTGTGAGGTACAGCAATTATTAGGAAATAATCGAGTTAGG 174
LHB081111       GGTCAACCAATTAATGTGGCTTGTGAGGTACAGCAATTATTAGGAAATAATCGAGTTAGG 175
LHB091112       GGTCAACCAATTAATGTGGCTTGTGAGGTACAGCAATTATTAGGAAATAATCGAGTTAGG 175
LHB061108       GGTCAACCAATTAATGTGGCTTGTGAGGTACAGCAATTATTAGGAAATAATCGAGTTAGG 175
KSL051105       GGTCAACCAATTAATGTGGCTTGTGAGGTACAGCAATTATTAGGAAATAATCGAGTTAGG 178
LHB071001       GGTCAACCAATTAATGTGGCTTGTGAGGTACAGCAATTATTAGGAAATAATCGAGTTAGG 178
KSL071001       GGTCAACCAATTAATGTGGCTTGTGAGGTACAGCAATTATTAGGAAATAATCGAGTTAGG 177
LHB081002       GGTCAACCAATTAATGTGGCTTGTGAGGTACAGCAATTATTAGGAAATAATCGAGTTAGG 177
LHB091003       GGTCAACCAATTAATGTGGCTTGTGAGGTACAGCAATTATTAGGAAATAATCGAGTTAGG 177
TNF111001       GGTCAACCAATTAATGTGGCTTGTGAGGTACAGCAATTATTAGGAAATAATAGAGTTAGG 178
LHB101005       GGTCAACCAATTAATGTGGCTTGTGAGGTACAGCAATTATTAGGAAATAATCGAGTTAGG 178
KSl071002       GGTCAACCAATTAATGTGGCTTGTGAGGTACAGCAATTATTAGGAAATAATCGAGTTAGG 176
LPK081001       GGTCAACCAATTAATGTGGCTTGTGAGGTACAGCAATTATTAGGAAATAATCGAGTTAGG 176
LHB101004       GGTCAACCAATTAATGTGGCTTGTGAGGTACAGCAATTATTAGGAAATAATCGAGTTAGG 177
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LHB081110       GCTGTAGCTATGAGTGCTACAGACGGTCTAACGAGAGG-AATGGAAGTGATTGACACAGG 236
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LHB021106       GCTGTAGCTATGAGTGCTACAGACGGTCTAACGAGAGG-AATGGAAGTGATTGACACAGG 233
LPK091002       GCTGTAGCTATGAGTGCTACAGACGGTCTAACGAGAGG-AATGGAAGTGATTGACACAGG 236
LHB071009       GCTGTAGCTATGAGTGCTACAGACGGTCTAACGAGAGG-AATGGAAGTGATTGACACAGG 233
LHB081111       GCTGTAGCTATGAGTGCTACAGACGGTCTAACGAGAGG-AATGGAAGTGATTGACACAGG 234
LHB091112       GCTGTAGCTATGAGTGCTACAGACGGTCTAACGAGAGG-AATGGAAGTGATTGACACAGG 234
LHB061108       GCTGTAGCTATGAGTGCTACAGACGGTCTAACGAGAGG-AATGGAAGTGATTGACACAGG 234
KSL051105       GCTGTAGCTATGAGTGCTACAGACGGTCTAACGAGAGG-AATGGAAGTGATTGACACAGG 237
LHB071001       GCTGTAGCTATGAGTGCTACAGACGGTCTAACGAGAGG-AATGGAAGTGATTGACACAGG 237
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LHB081002       GCTGTAGCTATGAGTGCTACAGACGGTCTAACGAGAGG-AATGGAAGTGATTGACACAGG 236
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TNF111001       GCTGTAGCTATGAGTGCTACAGACGGTCTAACGAGAGGGAATGGAAGTGATTGACACAGG 238
LHB101005       GCTGTAGCTATGAGTGCTACAGACGGTCTAACGAGAGG-AATGGAAGTGATTGACACAGG 237
KSl071002       GCTGTAGCTATGAGTGCTACAGACGGTCTAACGAGAGGGAATGGAAGTGATTGACACAGG 236
LPK081001       GCTGTAGCTATGAGTGCTACAGACGGTCTAACGAGAGG-AATGGAAGTGATTGACACAGG 235
LHB101004       GCTGTAGCTATGAGTGCTACAGACGGTCTAACGAGAGG-AATGGAAGTGATTGACACAGG 236
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LHB081110       AGCTCCTA-TAAGTGTTCCGGTCGGGGGAGCGACTCTGGGACGAATTTTT-AACGTG-CT 293
KSL031103       AGCTCCTA-TAAGTGTTCCGGTCGGGGGAGCGACTCTGGGACGAATTTTT-AACGTG-CT 294
LHB031107       AGCTCCTA-TAAGTGTTCCGGTCGGGGGAGCGACTCTGGGACGAATTTTT-AACGTG-CT 290
LHB021106       AGCTCCTA-TAAGTGTTCCGGTCGGGGGAGCGACTCTGGGACGAATTTTT-AACGTG-CT 290
LPK091002       AGCTCCTA-TAAGTGTTCCGGTCGGGGGAGCGACTCTGGGACGAATTTTT-AACGTG-CT 293
LHB071009       AGCTCCTA-TAAGTGTTCCGGTCGGGGGAGCGACTCTGGGACGAATTTTT-AACGTG-CT 290
LHB081111       AGCTCCTA-TAAGTGTTCCGGTCGGGGGAGCGACTCTGGGACGAATTTTT-AACGTG-CT 291
LHB091112       AGCTCCTA-TAAGTGTTCCGGTCGGGGGAGCGACTCTGGGACGAATTTTT-AACGTG-CT 291
LHB061108       AGCTCCTA-TAAGTGTTCCGGTCGGGGGAGCGACTCTGGGACGAATTTTTTAACGTGTCT 293
KSL051105       AGCTCCTA-TAAGTGTTCCGGTCGGGGGAGCGACTCTGGGACGAATTTTT-AACGTG-CT 294
LHB071001       AGCTCCTA-TAAGTGTTCCGGTCGGGGGAGCGACTCTGGGACGAATTTTT-AACGTG-CT 294
KSL071001       AGCTCCTA-TAAGTGTTCCGGTCGGGGGAGCGACTCTGGGACGAATTTTT-AACGTG-CT 293
LHB081002       AGCTCCTTATAAGTGTTCCGGTCGGGGGAGCGACTCTGGGACGAATTTTT-AACGTG-CT 294
LHB091003       AGCTCCTA-TAAGTGTTCCGGTCGGGGGAGCGACTCTGGGACGAATTTTT-AACGTG-CT 293
TNF111001       AGCTCCTA-TAAGTGTTCCGGTCGGGGGAGCGACTCTGGGACGAATTTTT-AACGTG-CT 295
LHB101005       AGCTCCTA-TAAGTGTTCCGGTCGGGGGAGCGACTCTGGGACGAATTTTT-AACGTG-CT 294
KSl071002       AGCTCCTA-TAAGTGTTCCGGTCGGGGGAGCGACTCTGGGACGAATTTTT-AACGTG-CT 293
LPK081001       AGCTCCTA-TAAGTGTTCCGGTCGGGGGAGCGACTCTGGGACGAATTTTT-AACGTG-CT 292
LHB101004       AGCTCCTA-TAAGTGTTCCGGTCGGGGGAGCGACTCTGGGACGAATTTTT-AACGTG-CT 293
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LHB081110       CGGAGAGCCT-GTTGATAATTTAGGG--CCTGTA-GATACT--AGTA-CAACG-TCTCCC 345
KSL031103       CGGAGAGCCT-GTTGATAATTTAGGGGCCCTGTA-GATACTTTAGTA-CAACGGTCTTCC 351
LHB031107       CGGAGAGCCT-GTTGATAATTTAGGG-CCCTGTA-GATACT--AGTA-CAACG--TYTCC 342
LHB021106       CGGAGAGCCT-GTTGATAATTTAGGG--CCTGTA-GATACT--AGTA-CAACG--TCTCC 341
LPK091002       CGGAGAGCCT-GTTGATAATTTAGGG--CCTGTA-GATACT--AGTA-CAACG--TCTCC 344
LHB071009       CGGAGAGCCT-GTTGATAATTTAGGG--CCTGTA-GATACT--AGTA-CAACG--TCTCC 341
LHB081111       CGGAGAGCCT-GTTGATAATTTAGGG--CCTGTA-GATACT--AGTA-CAACG--TCTCC 342
LHB091112       CGGAGAGCCT-GTTGATAATTTAGGG--CCTGTA-GATACT--AGTA-CAACG--TCTCC 342
LHB061108       CGGAGAGCCTTGTTGATAATTTAAGG-GCCTGTAGAATACT--AGTAACAACG-TTCTCC 349
KSL051105       CGGAGAGCCT-GTTGATAATTTAGGG--CCTGTA-GATACT--AGTA-CAACG--TCTCC 345
LHB071001       CGGAGAGCCT-GTTGATAATTTAGGG--CCTGTA-GATACT--AGTA-CAACG--TYTCC 345
KSL071001       CGGAGAGCCT-GTTGATAATTTAGGG--CCTGTA-GATACT--AGTA-CAACG--TYTCC 344
LHB081002       CGGAGAGCCT-GTTGATAATTTAGGG--CCTGTA-GATACT--AGTA-CAACG--TCTCC 345
LHB091003       CGGAGAGCCT-GTTGATAATTTAGGG--CCTGTA-GATACT--AGTA-CAACG--TCTCC 344
TNF111001       CGGAGAGCCT-GTTGATAATTTAGGG--CCTGTA-GATACT--AGTA-CAATG--TCTCC 346
LHB101005       CGGAGAGCCT-GTTGATAATTTAGGG--CCTGTA-GATACT--AGTA-CAACG--TCTCC 345
KSl071002       CGGAGAGCCT-GTTGATAATTTAGGG--CCTGTA-GATACT--AGTA-CAACG--TYTCC 344
LPK081001       CGGAGAGCCT-GTTGATAATTTAGGG--CCTGTA-GATACT--AGTA-CAACG--TCTCC 343
LHB101004       CGGAGAGCCT-GTTGATAATTTAGGG--CCTGTA-GATACT--AGTA-CAACG--TCTCC 344
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KSL041104       T-ATTCA-TAGAT-CCGCGCCCC-GCCTTTATAC--AGTTGG-ATACAAAATTATCTW-T 397
LHB081110       T-ATTCA-TAGATTCCGCGCCC--GCCTTTATACC-AGTTGG-ATACAAAATTATCTW-T 398
KSL031103       TTATTCAATAGATTCCGCGCCCCCGCCTTTATATCAAGTTGGGATACAAAATTATCTTAT 411
LHB031107       T-ATTCA-TAGAT-CCGCGCCCC-GCCTTTATAAC-AGTTGG-ATACAAAATTATCTA-- 394
LHB021106       T-ATTCA-TAGAT-CCGCGCCC--GCCTTTATAC--AGTTGG-ATACAAAATTATCTA-- 391
LPK091002       T-ATTCA-TAGAT-CCGCGCCC--GCCTTTATAC--AGTTGG-ATACAAAATTATCTA-- 394
LHB071009       T-ATTCA-TAGAT-CCGCGCCC--GCCTTTATAC--AGTTGG-ATACAAAATTATCTA-- 391
LHB081111       T-ATTCA-TAGAT-CCGCGCCC--GCCTTTATAC--AGTTGG-ATACAAAATTATCTA-- 392
LHB091112       T-ATTCA-TAGAT-CCGCGCCC--GCCTTTATAC--AGTTGG-ATACAAAATTATCTA-- 392
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LHB061108       T-ATTCA-TAGATCCGGCGCCC--GCCTTTATAC--AGTTGG-ATACAAAATTATCTW-- 400
KSL051105       T-ATTCA-TAGAT-CCGCGCCC--GCCTTTATAC--AGTTGG-ATACAAAATTATCTA-- 395
LHB071001       T-ATTCA-TAGAT-CCGCGCCC--GCCTTTATAC--AGTTGG-ATACAAAATTATCTA-- 395
KSL071001       T-ATTCA-TAGAT-CCGCGCCC--GCCTTTATAC--AGTTGG-ATACAAAATTATCTA-- 394
LHB081002       T-ATTCA-TAGAT-CCGCGCCC--GCCTTTATAC--AGTTGG-ATACAAAATTATCTA-- 395
LHB091003       T-ATTCA-TAGAT-CCGCGCCC--GCCTTTATAC--AGTTGG-ATACAAAATTATCTA-- 394
TNF111001       T-ATTCA-TAGAT-CTGCGCCC--GCCTTTATAC--AGTTGG-ATACAAAATTATCTA-- 396
LHB101005       T-ATTCA-TAGAT-CCGCGCCC--GCCTTTATAC--AGTTGG-ATACAAAATTATCTA-- 395
KSl071002       T-ATTCA-TAGAT-CCGCGCCC--GCCTTTATAC--AGTTGG-ATACAAAATTATCTA-- 394
LPK081001       T-ATTCA-TAGAT-CCGCGCCC--GCCTTTATAC--AGTTGG-ATACAAAATTATCTA-- 393
LHB101004       T-ATTCA-TAGAT-CCGCGCCC--GCCTTTATAC--AGTTGG-ATACAAAATTATCTA-- 394
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LHB081110       TTTTTGAAACAAGGAATT-AAAGTAG-TAGATTCTTTTAGCCCCTTAATCGCCGTGG-AG 455
KSL031103       TTTTTGAAACAGGGAATTTAAAGTAGGTAGATCTTTTTAGCCCCTTAATCGCCGTGGGAG 471
LHB031107       TTTTTGAAACA-GGAATT-AAAGTAG-TAGATC-TTTTAGCCCCTTA-TCGCCGTGG-AG 448
LHB021106       TTTTTGAAACA-GGAATT-AAAGTAG-TAGATC-TTTTAGCCCCTTA-TCGCCGTGG-AG 445
LPK091002       TTTTTGAAACA-GGAATT-AAAGTAG-TAGATC-TTTTAGCCCCTTA-TCGCCGTGG-AG 448
LHB071009       TTTTTGAAACA-GGAATT-AAAGTAG-TAGATC-TTTTAGCCCCTTA-TCGCCGTGG-AG 445
LHB081111       TTTTTGAAACA-GGAATT-AAAGTAG-TAGATC-TTTTAGCCCCTTA-TCGCCGTGG-AG 446
LHB091112       TTTTTGAAACA-GGAATT-AAAGTAG-TAGATC-TTTTAGCCCCTTA-TCGCCGTGG-AG 446
LHB061108       TTTTTGAAACA-GGAATT-AAAGTAG-TAGATC-TTTTAGCCCCTTA-TCGCCGTGG-AG 454
KSL051105       TTTTTGAAACA-GGAATT-AAAGTAG-TAGATC-TTTTAGCCCCTTA-TCGCCGTGG-AG 449
LHB071001       TTTTTGAAACA-GGAATT-AAAGTAG-TAGATC-TTTTAGCCCCTTA-TCGCCGTGG-AG 449
KSL071001       TTTTTGAAACA-GGAATT-AAAGTAG-TAGATC-TTTTAGCCCCTTA-TCGCCGTGG-AG 448
LHB081002       TTTTTGAAACA-GGAATT-AAAGTAG-TAGATC-TTTTAGCCCCTTA-TCGCCGTGG-AG 449
LHB091003       TTTTTGAAACA-GGAATT-AAAGTAG-TAGATC-TTTTAGCCCCTTA-TCGCCGTGG-AG 448
TNF111001       TTTTTGAAACA-GGAATT-AAAGTAG-TAGATC-TTTTAGCCCCTTA-TCGCCGTGG-AG 450
LHB101005       TTTTTGAAACA-GGAATT-AAAGTAG-TAGATC-TTTTAGCCCCTTA-TCGCCGTGG-AG 449
KSl071002       TTTTTGAAACA-GGAATT-AAAGTAG-TAGATC-TTTTAGCCCCTTA-TCGCCGTGG-AG 448
LPK081001       TTTTTGAAACA-GGAATT-AAAGTAG-TAGATC-TTTTAGCCCCTTA-TCGCCGTGG-AG 447
LHB101004       TTTTTGAAACA-GGAATT-AAAGTAG-TAGATC-TTTTAGCCCCTTA-TCGCCGTGG-AG 448
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KSL041104       GAAAAAT-CGGACTATT-CGGGGG-AGCTGG-AGTGGGTAAAAC-AGTACTC-ATTATGG 506
LHB081110       GAAAAAT-CGGACTATTTCGGGGG-AGCTGG-AGTGGGTAAAAC-AGTACTC-ATTATGG 510
KSL031103       GAAAAATTCGGACTATTTCGGGGGGAGCTGGGAGTGGGTAAAACCAGTACTCCATTATGG 531
LHB031107       GAAAAAT-CGGACTATT-CGGGGG-AGCTGG-AGTGGGTAAAAC-AGTACTC-ATTATGG 502
LHB021106       GAAAAAT-CGGACTATT-CGGGGG-AGCTGG-AGTGGGTAAAA-CAGTACT-CATTATGG 499
LPK091002       GAAAAAT-CGGACTATT-CGGGGG-AGCTGG-AGTGGGTAAAA-CAGTACT-CATTATGG 502
LHB071009       GAAAAAT-CGGACTATT-CGGGGG-AGCTGG-AGTGGGTAAAA-CAGTACT-CATTATGG 499
LHB081111       GAAAAAT-CGGACTATT-CGGGGG-AGCTGG-AGTGGGTAAAAACAGTACTTCATTATGG 502
LHB091112       GAAAAAT-CGGACTATT-CGGGGG-AGCTGG-AGTGGGTAAAA-CAGTACT-CATTATGG 500
LHB061108       GAAAAAT-CGGACTATT-CGGGGG-AGCTGG-AGTGGGTAAAA-CAGTACTC-ATTATGG 508
KSL051105       GAAAAAT-CGGACTATT-CGGGGG-AGCTGG-AGTGGGTAAAA-CAGTACTC-ATTATGG 503
LHB071001       GAAAAAT-CGGACTATT-CGGGGG-AGCTGG-AGTGGGTAAAA-CAGTACTC-ATTATGG 503
KSL071001       GAAAAAT-CGGACTATT-CGGGGG-AGCTGG-AGTGGGTAAAA-CAGTACTC-ATTATGG 502
LHB081002       GAAAAAT-CGGACTATT-CGGGGG-AGCTGG-AGTGGGTAAAA-CAGTACTC-ATTATGG 503
LHB091003       GAAAAAT-CGGACTATT-CGGGGG-AGCTGG-AGTGGGTAAAA-CAGTACTC-ATTATGG 502
TNF111001       GAAAAAT-CGGACTATT-CGGGGG-AGCTGG-AGTGGGTAAAA-CAGTACTC-ATTATGG 504
LHB101005       GAAAAAT-CGGACTATT-CGGGGG-AGCTGG-AGTGGGTAAAA-CAGTACTC-ATTATGG 503
KSl071002       GAAAAAT-CGGACTATT-CGGGGG-AGCTGG-AGTGGGTAAAA-CAGTACTC-ATTATGG 502
LPK081001       GAAAAAT-CGGACTATT-CGGGGG-AGCTGG-AGTGGGTAAAA-CAGTACTC-ATTATGG 501
LHB101004       GAAAAAT-CGGACTATT-CGGGGG-AGCTGG-AGTGGGTAAAA-CAGTACTC-ATTATGG 502
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KSL041104       AA-TTGATTAACAA-TATTGC-TAAAGCT-CACGGGGG-CGTA-TCCG-TATTT-GGTGG 558
LHB081110       AA-TTGATTAACAA-TATTGC-TAAAGCT-CACGGGGG-CGTA-TCCG-TATTT-GGTGG 562
KSL031103       AA-TTGATTAACAA-TATTGC-TAAAGCT-CACGGGGGGCGTA-TCCG-TATTTTGGTGG 585
LHB031107       AA-TTGATTAACAA-TATTGC-TAAAGCT-CACGGGGG-CGTA-TCCG-TATTT-GGTGG 554
LHB021106       AA-TTGATTAACAA-TATTGC-TAAAGCTTCACGGGAGGCGTAATCCG-TATTTTGGTGG 555
LPK091002       AA-TTGATTAACAA-TATTGC-TAAAGCT-CACGGG-GGCGTA-TCCG-TATTT-GGTGG 554
LHB071009       AA-TTGATTAACAA-TATTGC-TAAAGCTC-ACGGGGG-CGTA-TCCG-TATTT-GGTGG 551
LHB081111       AAATTGATTAACAAATATTGCCTAAAGCTCCACGGGGGGCGTA-TCCGGTATTT-GGTGG 560
LHB091112       AA-TTGATTAACAA-TATTGC-TAAAGCT-CACGGG-GGCGTA-TCCG-TATTT-GGTGG 552
LHB061108       AA-TTGATTAACAA-TATTGC-TAAAGCT-CACGGGGG-CGTA-TCCG-TATTT-GGTGG 560
KSL051105       AA-TTGATTAACAA-TATTGC-TAAAGCT-CACGGGGG-CGTA-TCCG-TATTT-GGTGG 555
LHB071001       AA-TTGATTAACAA-TATTGC-TAAAGCT-CACGGGGG-CGTA-TCCG-TATTT-GGTGG 555
KSL071001       AA-TTGATTAACAA-TATTGC-TAAAGCT-CACGGGGG-CGTA-TCCG-TATTT-GGTGG 554
LHB081002       AA-TTGATTAACAA-TATTGC-TAAAGCT-CACGGGGG-CGTA-TCCG-TATTT-GGTGG 555
LHB091003       AA-TTGATTAACAA-TATTGC-TAAAGCT-CACGGGGG-CGTA-TCCG-TATTT-GGTGG 554
TNF111001       AA-TTGATTAACAA-TATTGC-TAAAGCT-CACGGGGG-CGTA-TCCG-TATTT-GGTGG 556
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LHB101005       AA-TTGATTAACAA-TATTGC-TAAAGCT-CACGGGGG-CGTA-TCCG-TATTT-GGTGG 555
KSl071002       AA-TTGATTAACAA-TATTGC-TAAAGCT-CACGGGGG-CGTA-TCCG-TATTT-GGTGG 554
LPK081001       AA-TTGATTAACAA-TATTGC-TAAAGCT-CACGGGGG-CGTA-TCCG-TATTT-GGTGG 553
LHB101004       AA-TTGATTAACAA-TATTGC-TAAAGCT-CACGGGGG-CGTA-TCCG-TATTT-GGTGG 554
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KSL041104       -AGTGGG-TGAACG-TACTCGGG-AAGGAAATGATCTTT-ACATGGAAATGAAAGAATCT 613
LHB081110       -AGTGGG-TGAACG-TACTCGGG-AAGGAAATGATCTTT-ACATGGAAATGAAAGAATCT 617
KSL031103       -AGTGGG-TGAACG-TACTCGGG-AAGGAAATGATCTTT-ACATGGAAATGAAAGAATCT 640
LHB031107       -AGTGGG-TGAACG-TACTCGGG-AAGGAAATGATCTTT-ACATGGAAATGAAAGAATCT 609
LHB021106       -AGTGGGGTGAACGGTACTCGGGGAAGGAAATGATCTTTTACATGGAAATGAAAGAATCT 614
LPK091002       -AGTGGG-TGAACG-TACTCGGG-AAGGAAATGATCTTT-ACATGGAAATGAAAGAATCT 609
LHB071009       -AGTGGG-TGAACG-TACTCGGG-AAGGAAATGATCTTTTACATGGAAATGAAAGAATCT 607
LHB081111       GAGTGGG-TGAACG-TACTCGGG-AAGGAAATGATCTTT-ACATGGAAATGAAAGAATCT 616
LHB091112       -AGTGGG-TGAACG-TACTCGGG-AAGGAAATGATCTTT-ACATGGAAATGAAAGAATCT 607
LHB061108       -AGTGGG-TGAACG-TACTCGGG-AAGGAAATGATCTTT-ACATGGAAATGAAAGAATCT 615
KSL051105       -AGTGGG-TGAACG-TACTCGGG-AAGGAAATGATCTTT-ACATGGAAATGAAAGAATCT 610
LHB071001       -AGTGGG-TGAACG-TACTCGGG-AAGGAAATGATCTTT-ACATGGAAATGAAAGAATCT 610
KSL071001       -AGTGGG-TGAACG-TACTCGGG-AAGGAAATGATCTTT-ACATGGAAATGAAAGAATCT 609
LHB081002       -AGTGGG-TGAACG-TACTCGGG-AAGGAAATGATCTTT-ACATGGAAATGAAAGAATCT 610
LHB091003       -AGTGGG-TGAACG-TACTCGGG-AAGGAAATGATCTTT-ACATGGAAATGAAAGAATCT 609
TNF111001       -AGTGGG-TGAACG-TACTCGGG-AAGGAAATGATCTTT-ACATGGAAATGAAAGAATCT 611
LHB101005       -AGTGGG-TGAACG-TACTCGGG-AAGGAAATGATCTTT-ACATGGAAATGAAAGAATCT 610
KSl071002       -AGTGGG-TGAACG-TACTCGGG-AAGGAAATGATCTTT-ACATGGAAATGAAAGAATCT 609
LPK081001       -AGTGGG-TGAACG-TACTCGGG-AAGGAAATGATCTTT-ACATGGAAATGAAAGAATCT 608
LHB101004       -AGTGGG-TGAACG-TACTCGGG-AAGGAAATGATCTTT-ACATGGAAATGAAAGAATCT 609
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KSL041104       -GGAGTGATTAATGAAGAAAATATTGCAGAATCAAAAGTGGCCCTAGTTTACGG-TCAGA 671
LHB081110       -GGAGTGATTAATGAAGAAAATATTGCAGAATCAAAAGTGGCCCTAGTTTACGG-TCAGA 675
KSL031103       -GGAGTGATTAATGAAGAAAATATTGCAGAATCAAAAGTGGCCCTAGTTTACGG-TCAGA 698
LHB031107       -GGAGTGATTAATGAAGAAAATATTGCAGAATCAAAAGTGGCCCTAGTTTACGG-TCAGA 667
LHB021106       TGGAGTGATTAATGAAGAAAATATTGCAGAATCAAAAGTGGCCCTAGTTTACGG-TCAGA 673
LPK091002       -GGAGTGATTAATGAAGAAAATATTGCAGAATCAAAAGTGGCCCTAGTTTACGG-TCAGA 667
LHB071009       -GGAGTGATTAATGAAGAAAATATTGCAGAATCAAAAGTGGCCCTAGTTTACGG-TCAGA 665
LHB081111       -GGAGTGATTAATGAAGAAAATATTGCAGAATCAAAAGTGGCCCTAGTTTACGG-TCAGA 674
LHB091112       -GGAGTGATTAATGAAGAAAATATTGCAGAATCAAAAGTGGCCCTAGTTTACGGGTCAGA 666
LHB061108       -GGAGTGATTAATGAAGAAAATATTGCAGAATCAAAAGTGGCCCTAGTTTACGG-TCAGA 673
KSL051105       -GGAGTGATTAATGAAGAAAATATTGCAGAATCAAAAGTGGCCCTAGTTTACGG-TCAGA 668
LHB071001       -GGAGTGATTAATGAAGAAAATATTGCAGAATCAAAAGTGGCCCTAGTTTACGG-TCAGA 668
KSL071001       -GGAGTGATTAATGAAGAAAATATTGCAGAATCAAAAGTGGCCCTAGTTTACGG-TCAGA 667
LHB081002       -GGAGTGATTAATGAAGAAAATATTGCAGAATCAAAAGTGGCCCTAGTTTACGG-TCAGA 668
LHB091003       -GGAGTGATTAATGAAGAAAATATTGCAGAATCAAAAGTGGCCCTAGTTTACGG-TCAGA 667
TNF111001       -GGAGTGATTAATGAAGAAAATATTGCAGAATCAAAAGTGGCCCTAGTTTACGG-TCAGA 669
LHB101005       -GGAGTGATTAATGAAGAAAATATTGCAGAATCAAAAGTGGCCCTAGTTTACGG-TCAGA 668
KSl071002       -GGAGTGATTAATGAAGAAAATATTGCAGAATCAAAAGTGGCCCTAGTTTACGG-TCAGA 667
LPK081001       -GGAGTGATTAATGAAGAAAATATTGCAGAATCAAAAGTGGCCCTAGTTTACGG-TCAGA 666
LHB101004       -GGAGTGATTAATGAAGAAAATATTGCAGAATCAAAAGTGGCCCTAGTTTACGG-TCAGA 667
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KSL041104       TGAATGAACCACCGGGAGCTCGTATGAGAGTTGGTTTGACTGCCCTAACTATGGCGGAAT 731
LHB081110       TGAATGAACCACCGGGAGCTCGTATGAGAGTTGGTTTGACTGCCCTAACTATGGCGGAAT 735
KSL031103       TGAATGAACCACCGGGAGCTCGTATGAGAGTTGGTTTGACTGCCCTAACTATGGCGGAAT 758
LHB031107       TGAATGAACCACCGGGAGCTCGTATGAGAGTTGGTTTGACTGCCCTAACTATGGCGGAAT 727
LHB021106       TGAATGAACCACCGGGAGCTCGTATGAGAGTTGGTTTGACTGCCCTAACTATGGCGGAAT 733
LPK091002       TGAATGAACCACCGGGAGCTCGTATGAGAGTTGGTTTGACTGCCCTAACTATGGCGGAAT 727
LHB071009       TGAATGAACCACCGGGAGCTCGTATGAGAGTTGGTTTGACTGCCCTAACTATGGCGGAAT 725
LHB081111       TGAATGAACCACCGGGAGCTCGTATGAGAGTTGGTTTGACTGCCCTAACTATGGCGGAAT 734
LHB091112       TGAATGAACCACCGGGAGCTCGTATGAGAGTTGGTTTGACTGCCCTAACTATGGCGGAAT 726
LHB061108       TGAATGAACCACCGGGAGCTCGTATGAGAGTTGGTTTGACTGCCCTAACTATGGCGGAAT 733
KSL051105       TGAATGAACCACCGGGAGCTCGTATGAGAGTTGGTTTGACTGCCCTAACTATGGCGGAAT 728
LHB071001       TGAATGAACCACCGGGAGCTCGTATGAGAGTTGGTTTGACTGCCCTAACTATGGCGGAAT 728
KSL071001       TGAATGAACCACCGGGAGCTCGTATGAGAGTTGGTTTGACTGCCCTAACTATGGCGGAAT 727
LHB081002       TGAATGAACCACCGGGAGCTCGTATGAGAGTTGGTTTGACTGCCCTAACTATGGCGGAAT 728
LHB091003       TGAATGAACCACCGGGAGCTCGTATGAGAGTTGGTTTGACTGCCCTAACTATGGCGGAAT 727
TNF111001       TGAATGAACCGCCGGGAGCTCGTATGAGAGTTGGTTTGACTGCCCTAACTATGGCGGAAT 729
LHB101005       TGAATGAACCACCGGGAGCTCGTATGAGAGTTGGTTTGACTGCCCTAACTATGGCGGAAT 728
KSl071002       TGAATGAACCACCGGGAGCTCGTATGAGAGTTGGTTTGACTGCCCTAACTATGGCGGAAT 727
LPK081001       TGAATGAACCACCGGGAGCTCGTATGAGAGTTGGTTTGACTGCCCTAACTATGGCGGAAT 726
LHB101004       TGAATGAACCACCGGGAGCTCGTATGAGAGTTGGTTTGACTGCCCTAACTATGGCGGAAT 727
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KSL041104       ATTTCCGAGATGTTAATGAGCAAGACGTACTTCTATTTATTGACAATATCTTCCGTTTCG 791
LHB081110       ATTTCCGAGATGTTAATGAGCAAGACGTACTTCTATTTATTGACAATATCTTCCGTTTCG 795
KSL031103       ATTTCCGAGATGTTAATGAGCAAGACGTACTTCTATTTATTGACAATATCTTCCGTTTCG 818
LHB031107       ATTTCCGAGATGTTAATGAGCAAGACGTACTTCTATTTATTGACAATATCTTCCGTTTCG 787
LHB021106       ATTTCCGAGATGTTAATGAGCAAGACGTACTTCTATTTATTGACAATATCTTCCGTTTCG 793
LPK091002       ATTTCCGAGATGTTAATGAGCAAGACGTACTTCTATTTATTGACAATATCTTCCGTTTCG 787
LHB071009       ATTTCCGAGATGTTAATGAGCAAGACGTACTTCTATTTATTGACAATATCTTCCGTTTCG 785
LHB081111       ATTTCCGAGATGTTAATGAGCAAGACGTACTTCTATTTATTGACAATATCTTCCGTTTCG 794
LHB091112       ATTTCCGAGATGTTAATGAGCAAGACGTACTTCTATTTATTGACAATATCTTCCGTTTCG 786
LHB061108       ATTTCCGAGATGTTAATGAGCAAGACGTACTTCTATTTATTGACAATATCTTCCGTTTCG 793
KSL051105       ATTTCCGAGATGTTAATGAGCAAGACGTACTTCTATTTATTGACAATATCTTCCGTTTCG 788
LHB071001       ATTTCCGAGATGTTAATGAGCAAGACGTACTTCTATTTATTGACAATATCTTCCGTTTCG 788
KSL071001       ATTTCCGAGATGTTAATGAGCAAGACGTACTTCTATTTATTGACAATATCTTCCGTTTCG 787
LHB081002       ATTTCCGAGATGTTAATGAGCAAGACGTACTTCTATTTATTGACAATATCTTCCGTTTCG 788
LHB091003       ATTTCCGAGATGTTAATGAGCAAGACGTACTTCTATTTATTGACAATATCTTCCGTTTCG 787
TNF111001       ATTTCCGAGATGTTAATGAGCAAGACGTACTTCTATTTATTGACAATATCTTCCGTTTCG 789
LHB101005       ATTTCCGAGATGTTAATGAGCAAGACGTACTTCTATTTATTGACAATATCTTCCGTTTCG 788
KSl071002       ATTTCCGAGATGTTAATGAGCAAGACGTACTTCTATTTATTGACAATATCTTCCGTTTCG 787
LPK081001       ATTTCCGAGATGTTAATGAGCAAGACGTACTTCTATTTATTGACAATATCTTCCGTTTCG 786
LHB101004       ATTTCCGAGATGTTAATGAGCAAGACGTACTTCTATTTATTGACAATATCTTCCGTTTCG 787
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KSL041104       TCCAAGCAGGATCCGAAGTATC-GGCCTTATTGGGTA-GAATGCCTTCCS-CTGTGGGTT 848
LHB081110       TCCAAGCAGGATCCGAAGTATCCGGCCTTATTGGGTAAGAATGCCTTCCCGCTGTGGGTT 855
KSL031103       TCCAAGCAGGATCCGAAGTATC-GGCCTTATTGGGTA-GAATGCCTTCCG-CTGTGGGTT 875
LHB031107       TCCAAGCAGGATCCGAAGTATC-GGCCTTATTGGGTA-GAATGCCTTCCG-CTGTGGGTT 844
LHB021106       TCCAAGCAGGATCCGAAGTATC-GGCCTTATTGGGTA-GAATGCCTTCCG-CTGTGGGTT 850
LPK091002       TCCAAGCAGGATCCGAAGTATC-GGCCTTATTGGGTA-GAATGCCTTCCG-CTGTGGGTT 844
LHB071009       TCCAAGCAGGATCCGAAGTATC-GGCCTTATTGGGTA-GAATGCCTTCCG-CTGTGGGTT 842
LHB081111       TCCAAGCAGGATCCGAAGTATC-GGCCTTATTGGGTA-GAATGCCTTCCG-CTGTGGGTT 851
LHB091112       TCCAAGCAGGATCCGAAGTATC-GGCCTTATTGGGTA-GAATGCCTTCCG-CTGTGGGTT 843
LHB061108       TCCAAGCAGGATCCGAAGTATC-GGCCTTATTGGGTA-GAATGCCTTCCG-CTGTGGGTT 850
KSL051105       TCCAAGCAGGATCCGAAGTATC-GGCCTTATTGGGTA-GAATGCCTTCCG-CTGTGGGTT 845
LHB071001       TCCAAGCAGGATCCGAAGTATC-GGCCTTATTGGGTA-GAATGCCTTCCG-CTGTGGGTT 845
KSL071001       TCCAAGCAGGATCCGAAGTATC-GGCCTTATTGGGTA-GAATGCCTTCCG-CTGTGGGTT 844
LHB081002       TCCAAGCAGGATCCGAAGTATC-GGCCTTATTGGGTA-GAATGCCTTCCG-CTGTGGGTT 845
LHB091003       TCCAAGCAGGATCCGAAGTATC-GGCCTTATTGGGTA-GAATGCCTTCCG-CTGTGGGTT 844
TNF111001       TCCAAGCAGGATCCGAAGTATC-GGCCTTATTGGGTA-GAATGCCTTCCG-CTGTGGGTT 846
LHB101005       TCCAAGCAGGATCCGAAGTATC-GGCCTTATTGGGTA-GAATGCCTTCCG-CTGTGGGTT 845
KSl071002       TCCAAGCAGGATCCGAAGTATC-GGCCTTATTGGGTA-GAATGCCTTCCG-CTGTGGGTT 844
LPK081001       TCCAAGCAGGATCCGAAGTATC-GGCCTTATTGGGTA-GAATGCCTTCCG-CTGTGGGTT 843
LHB101004       TCCAAGCAGGATCCGAAGTATC-GGCCTTATTGGGTA-GAATGCCTTCCG-CTGTGGGTT 844
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KSL041104       ATCAACC-GACCCTAA-GTACCGAAA-TGGGGTTCTTTACAAAGAAARAATTACTTC-TA 904
LHB081110       ATCAACCCGACCCTAAAGTACCGAAAATGGGGTTCTTTACAAAGAAAGAATTACTTCCTA 915
KSL031103       ATCAACCCGACCCTAA-GTACCGAAA--TGGGTTCTTTACAA-GAAAGAATTACTTTCTA 931
LHB031107       ATCAACC-GACCCTAA-GTACCGAAA--TGGGTTCTTTACAA-GAAAGAATTACTTC-TA 898
LHB021106       ATCAACC-GACCCTAA-GTACCGAAA--TGGGTTCTTTACAA-GAAAGAATTACTTC-TA 904
LPK091002       ATCAACC-GACCCTAA-GTACCGAAA--TGGGTTCTTTACAA-GAAAGAATTACTTC-TA 898
LHB071009       ATCAACC-GACCCTAA-GTACCGAAA--TGGGTTCTTTACAA-GAAAGAATTACTTC-TA 896
LHB081111       ATCAACC-GACCCTAA-GTACCGAAA--TGGGTTCTTTACAA-GAAAGAATTACTTC-TA 905
LHB091112       ATCAACC-GACCCTAA-GTACCGAAA--TGGGTTCTTTACAA-GAAAGAATTACTTC-TA 897
LHB061108       ATCAACC-GACCCTAA-GTACCGAAA--TGGGTTCTTTACAARAAAAAAAATTACTTC-TA 905
KSL051105       ATCAACC-GACCCTAA-GTACCGAAA-TGGGGTTCTTTACAAGAAAAAAAATTACTTC-TA 901
LHB071001       ATCAACC-GACCCTAA-GTACCGAAA--TGGGTTCTTTACAARAAAAAAAATTACTTC-TA 900
KSL071001       ATCAACC-GACCCTAA-GTACCGAAA--TGGGTTCTTTACAA-GAAAGAATTACTTC-TA 898
LHB081002       ATCAACC-GACCCTAA-GTACCGAAA--TGGGTTCTTTACAA-GAAAGAATTACTTC-TA 899
LHB091003       ATCAACC-GACCCTAA-GTACCGAAA--TGGGTTCTTTACAA-GAAAGAATTACTTC-TA 898
TNF111001       ATCAACC-GACCCTAA-GTACCGAAA--TGGGTTCTTTACAA-GAAAGAATTACTTC-TA 900
LHB101005       ATCAACC-GACCCTAA-GTACCGAAA--TGGGTTCTTTACAA-GAAAGAATTACTTC-TA 899
KSl071002       ATCAACC-GACCCTAA-GTACCGAAA--TGGGTTCTTTACAA-GAAAGAATTACTTC-TA 898
LPK081001       ATCAACC-GACCCTAA-GTACCGAAA--TGGGTTCTTTACAA-RAAARAATTACTTC-TA 897
LHB101004       ATCAACC-GACCCTAA-GTACCGAAA--TGGGTTCTTTACAA-GAAAGAATTACTTC-TA 898
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KSL041104       CCAAARAAAGGGTCC-ATAACCYC-TATT-CAAGC-AGTTTATGTACCCGC-AGAACGAT 959
LHB081110       CCAAARAAAGGGTCCCATAACCYCCTATT-CAAGCCAGTTTATGTACCCGC-AGA-CGAT 972
KSL031103       CCAAAAGAAGGGTCC-ATAACCCTCTATT-CAAGCARTTTTATGTACCCGCCAGAACGAT 989
LHB031107       CCAAA-GAAGGGTCC-ATAACCTC-TATT-CAAGC-AGTTTATGTACCCGC-AGA-CGAT 951
LHB021106       CCAAA-GAAGGGTCC-ATAACCTC-TATT-CAAGC-AGTTTATGTACCCGC-AGA-CGAT 957
LPK091002       CCAAA-GAAGGGTCC-ATAACCTC-TATT-CAAGC-AGTTTATGTACCCGC-AGA-CGAT 951
LHB071009       CCAAA-GAAGGGTCC-ATAACCTC-TATT-CAAGC-AGTTTATGTACCCGC-AGA-CGAT 949
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LHB081111       CCAAA-GAAGGGTCC-ATAACCTC-TATT-CAAGC-AGTTTATGTACCCGC-AGA-CGAT 958
LHB091112       CCAAA-GAAGGGTCC-ATAACCTC-TATT-CAAGC-AGTTTATGTACCCGC-AGA-CGAT 950
LHB061108       CCAAGAGAAGGGTCC-ATAACCCTCTATT-CAAGC-AGTTTATGTACCCGC-AGAACGAT 961
KSL051105       CCAAARAAAGGGTCC-ATAACCTC-TATTTCAAGCAGTTTTATGTACCCGC-AGAACGAT 958
LHB071001       CCAAA-GAAGGGTCC-ATAACCTC-TATT-CAAGC-AGTTTATGTACCCGC-AGA-CGAT 953
KSL071001       CCAAA-GAAGGGTCC-ATAACCTC-TATT-CAAGC-AGTTTATGTACCCGC-AGA-CGAT 951
LHB081002       CCAAA-GAAGGGTCC-ATAACCTC-TATT-CAAGC-AGTTTATGTACCCGC-AGA-CGAT 952
LHB091003       CCAAA-GAAGGGTCC-ATAACCTC-TATT-CAAGC-AGTTTATGTACCCGC-AGA-CGAT 951
TNF111001       CTAAA-GAAGGGTCC-ATAACCTC-TATT-CAAGC-AGTTTATGTACCCGC-AGA-CGAT 953
LHB101005       CCAAA-GAAGGGTCC-ATAACCTC-TATT-CAAGC-AGTTTATGTACCCGC-AGA-CGAT 952
KSl071002       CCAAA-GAAGGGTCC-ATAACCTC-TATT-CAAGC-AGTTTATGTACCCGC-AGA-CGAT 951
LPK081001       CCAAA-GAAGGGTCC-ATAACCTC-TATT-CAAGC-AGTTTATGTACCCGC-AGA-CGAT 950
LHB101004       CCAAA-RAAGGGTCC-ATAACCTC-TATT-CAAGC-AGTTTATGTACCCGC-AGA-CGAT 951
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KSL041104       TTGACCGA-CCCTG-CTCCTG-CTACGA-CATTTGCACA--TTTAGA-TGCTACTACC-G 1011
LHB081110       TTGACCGA-CCCTG-CTCCTG-CTACGA-CATTTGCACA--TTTAGA-TGCTACTACC-G 1024
KSL031103       TTGACCSAACCCTG-CTCCTGGCTACGA-CATTTGCACCATTTTAGA-TGCTACTACCCG 1046
LHB031107       TTGACCGA-CCCTG-CTCCTG-CTACGA-CATTTGCACA--TTTAGA-TGCTACTACC-G 1003
LHB021106       TTGACCGA-CCCTG-CTCCTG-CTACGA-CATTTGCACA--TTTAGA-TGCTACTACC-G 1009
LPK091002       TTGACCGA-CCCTG-CTCCTG-CTACGA-CATTTGCACA--TTTAGA-TGCTACTACC-G 1003
LHB071009       TTGACCGA-CCCTG-CTCCTG-CTACGA-CATTTGCACA--TTTAGA-TGCTACTACC-G 1001
LHB081111       TTGACCGA-CCCTG-CTCCTG-CTACGA-CATTTGCACA--TTTAGA-TGCTACTACC-G 1010
LHB091112       TTGACCGA-CCCTG-CTCCTG-CTACGA-CATTTGCACA--TTTAGA-TGCTACTACC-G 1002
LHB061108       TTGACCSAACCCTG-CTCCCTGCTACGAACATTTGCACA--TTTAGAATGCTACTACCCG 1018
KSL051105       TTGACCCGACCCTGGCTCCTG-CTACGAACATTTGCACA--TTTAGAATGCTACTACCCG 1015
LHB071001       TTGACCGA-CCCTG-CTCCTG-CTACGA-CATTTGCACA--TTTAGA-TGCTACTACC-G 1005
KSL071001       TTGACCGA-CCCTG-CTCCTG-CTACGA-CATTTGCACA--TTTAGA-TGCTACTACC-G 1003
LHB081002       TTGACCGA-CCCTG-CTCCTG-CTACGA-CATTTGCACA--TTTAGA-TGCTACTACC-G 1004
LHB091003       TTGACCGA-CCCTG-CTCCTG-CTACGA-CATTTGCACA--TTTAGA-TGCTACTACC-G 1003
TNF111001       TTGACCGA-CCCTG-CTCCTG-CTACGA-CATTTGCACA--TTTAGA-TGCTACTACC-G 1005
LHB101005       TTGACCGA-CCCTG-CTCCTG-CTACGA-CATTTGCACA--TTTAGA-TGCTACTACC-G 1004
KSl071002       TTGACCGA-CCCTG-CTCCTG-CTACGA-CATTTGCACA--TTTAGA-TGCTACTACC-G 1003
LPK081001       TTGACCGA-CCCTG-CTCCTG-CTACGA-CATTTGCACA--TTTAGA-TGCTACTACC-G 1002
LHB101004       TTGACCGA-CCCTG-CTCCTG-CTACGA-CATTTGCACA--TTTAGA-TGCTACTACC-G 1003
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KSL041104       TACTATC-AAGAGGA-TTGGCTGCC--AAAGGTATTTAT-CCAGCAGTAGATCCTTTAGA 1066
LHB081110       TACTATC-AAGAGGA-TTGGCTGCC--AAAGGTATTTAT-CCAGCAGTAGATCCTTTAGA 1079
KSL031103       TACTATCCAARAGGA-TTGGCTGGCCAAAAGGTATTTATTCCAGCAGTAGATCCTTTAGA 1105
LHB031107       TACTATC-AAGAGGA-TTGGCTGCC--AAAGGTATTTAT-CCAGCAGTAGATCCTTTAGA 1058
LHB021106       TACTATC-AAGAGGA-TTGGCTGCC--AAAGGTATTTAT-CCAGCAGTAGATCCTTTAGA 1064
LPK091002       TACTATC-AAGAGGA-TTGGCTGCC--AAAGGTATTTAT-CCAGCATTAAATCCTTTAAA 1058
LHB071009       TACTATC-AAGAGGA-TTGGCTGCC--AAAGGTATTTAT-CCAGCAGTAGATCCTTTAGA 1056
LHB081111       TACTATC-AAGAGGA-TTGGCTGCC--AAAGGTATTTAT-CCAGCAGTAGATCCTTTAGA 1065
LHB091112       TACTATC-AAGAGGA-TTGGCTGCC--AAAGGTATTTAT-CCAGCAGTAGATCCTTTAGA 1057
LHB061108       TACTATCAARAAGGAATTGGCTGCC--AAAGGTATTTAT-CCAGCAGTAGATCCTTTAGA 1075
KSL051105       TACTATTCAARAAGAATTGGCTGCC--AAAGGTATTTAT-CCAGCAGTAGATCCTTTAGA 1072
LHB071001       TACTATCAARAAGGA-TTGGCTGCC--AAAGGTATTTAT-CCAGCAGTAGATCCTTTAGA 1061
KSL071001       TACTATCAAGA-GGA-TTGGCTGCC--AAAGGTATTTATCC-AGCAGTARATCCTTTARA 1058
LHB081002       TACTATCAARA-RGA-TTGGCTGCC--AAAGGTATTTATCC-AGCARTARATCCTTTARA 1059
LHB091003       TACTATCAAGA-GGA-TTGGCTGCC--AAAGGTATTTATCC-AGCAKTARATCCTTTAGA 1058
TNF111001       TACTATCAAGA-GGA-TTGGCTGCC--AAAGGTATTTATCC-AGCAKTARATCCTTTARA 1060
LHB101005       TACTATCAAGA-GGA-TTGGCTGCC--AAAGGTATTTATCC-AGCAGTARATCCTTTAGA 1059
KSl071002       TACTATCAARAAGGA-TTGGCTGCC--AAAGGTATTTATCCCAGCARTARATCCTTTARA 1060
LPK081001       TACTATCAAGA-GGA-TTGGCTGCC--AAAGGTATTTATCC-AGCAKTARATCCTTTARA 1057
LHB101004       TACTATCAARA-GGA-TTGGCTGCC--AAAGGTATTTATCC-AGCAKTARATCCTTTARA 1058
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KSL041104       TTCAACGTCAACCATGCTTCAACCTCGGATCGTTGGTGAGGAACATTACGAAACCGCCC- 1125
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KSL031103       TTCAACGTCAACCATGCTTCAACCTCGGATCGTTGGTGAGGAACATTACGAAACCGCCC- 1164
LHB031107       TTCAACGTCAACCATGCTTCAACCTCGGATCGTTGGTGAGGAACATTACGAAACCGCCC- 1117
LHB021106       TTCAACGTCAACCATGCTTCAACCTCGGATCGTTGGTGAGGAACATTACGAAACCGCCC- 1123
LPK091002       TTCAACGTCAACCATGCTTCAACCTCCGATCGTTGGTGAGGAACATTACGAAACCGCCC- 1117
LHB071009       TTCAACGTCAACCATGCTTCAACCTCGGATCGTTGGTGAGGAACATTACGAAACCGCCC- 1115
LHB081111       TTCAACGTCAACCATGCTTCAACCTCGGATCGTTGGTGAGGAACATTACGAAACCGCCC- 1124
LHB091112       TTCAACGTCAACCATGCTTCAACCTCGGATCGTTGGTGAGGAACATTACGAAACCGCCC- 1116
LHB061108       TTCAACGTCAACCATGCTTCAACCTCGGATCGTTGGTGAGGAACATTACGAAACCGCCC- 1134
KSL051105       TTCAACGTCAACCATGCTTCAACCTCGGATCGTTGGTGAGGAACATTACGAAACCGCCC- 1131
LHB071001       TTCAACGTCAACCATGCTTCAACCTCGGATCGTTGGTGAGGAACATTACGAAACCGCCC- 1120
KSL071001       TTCAACGTCAACCATGCTTCAACCTCGGATCGTTGGTGAGGAACATTACGAAACCGCCC- 1117
LHB081002       TTCAACGTCAACCATGCTTCAACCTCGGATCGTTGGTGAGGAACATTMCGAAACCGCCCC 1119
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LHB091003       TTCAACGTCAACCATGCTTCAACCTCGGATCGTTGGTGAGGAACATTACGAAACCSCCC- 1117
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LHB101005       TTCAACGTCAACCATGCTTCAACCTCGGATCGTTGGTGAGGAACATTACGAAACCGCCC- 1118
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KSL041104       AAAGAGTTAAGCAAACTTTAC-AACGTTACAAAGAACTTCA-GGACATT-ATAGCTATCC 1182
LHB081110       AAAGAGTTAAGCAAACTTTAC-AACGTTACAAAGAACTTCA-GGACATT-ATAGCTATCC 1195
KSL031103       AAAGAGTTAAGCAAACTTTAC-AACGTTACAAAGAACTTCA-GGACATT-ATAGCTATCC 1221
LHB031107       AAAGAGTTAAGCAAACTTTAC-AACGTTACAAAGAACTTCA-GGACATT-ATAGCTATCC 1174
LHB021106       AAAGAGTTAAGCAAACTTTAC-AACGTTACAAAGAACTTCA-GGACATT-ATAGCTATCC 1180
LPK091002       AAAAAGTTAAGCAAACTTTAA-CACCTTTCCAAAAACTTCA-GGACTTT-A-AGCTATCC 1173
LHB071009       AAAGAGTTAAGCAAACTTTAC-AACGTTACAAAGAACTTCA-GGACATT-ATAGCTATCC 1172
LHB081111       AAAGAGTTAAGCAAACTTTAC-AACGTTACAAAGAACTTCA-GGACATT-ATAGCTATCC 1181
LHB091112       AAAGAGTTAAGCAAACTTTAC-AACGTTACAAAGAACTTCA-GGACATT-ATAGCTATCC 1173
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KSL051105       AAAGAGTTAAGCAAACTTTAC-AACGTTACAAAGAACTTCA-GGACATT-ATAGCTATCC 1188
LHB071001       AAAGAGTTAAGCAAACTTTAC-AACGTTACAAAGAACTTCA-GGACATT-ATAGCTATCC 1177
KSL071001       AAAGAGTTAAGCAAACTTTAC-AACGTTACAAAGAACTTCA-GGACWTT-ATAGCTATCC 1174
LHB081002       AAAGAGTTAAGCAAACTTTACCAACGTTACAAAGAACTTCA-GGACATTTAWAGCTATCC 1178
LHB091003       AAAGAGTTAAGCAAACTTTAC-AACGTTACAAAGAACTTCA-GGACWTT-ATAGCTATCC 1174
TNF111001       AAAGAGTTAAGCAAACTTTAC-AACGTTACAAAGAACTTCA-GGACATT-ATAGCTATCC 1176
LHB101005       AAAGAGTTAAGCAAACTTTAC-AACGTTACAAAGAACTTCA-GGACWTT-ATAGCTATCC 1175
KSl071002       AAAGAGTTAAGCAAACTTTAC-AACGTTACAAARAACTTCA-GGACATT-AWAGCTATCC 1176
LPK081001       AAAGAGTTAAGCAAACTTTACCAACGTTACAAARAACTTCAAGGACWTT-AWAGCTATCC 1176
LHB101004       AAAGAGTTAAGCAAACTTTAC-AACGTTACAAAGAACTTCA-GGACATT-ATAGCTATCC 1174
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KSL041104       TTGGATTGGACGAATTATCC-GAAGA-GGATCGTTTACTCGTAGCAAGAGCGCGAAAAAT 1240
LHB081110       TTGGATTGGACGAATTATCC-GAAGA-GGATCGTTTACTCGTAGCAAGAGCGCGAAAAAT 1253
KSL031103       TTGGATTGGACGAATTATCC-GAAGA-GGATCGTTTACTCGTAGCAAGAGCGCGAAAAAT 1279
LHB031107       TTGGATTGGACGAATTATCC-GAAGA-GGATCGTTTACTCGTAGCAAGAGCGCGAAAAAT 1232
LHB021106       TTGGATTGGACGAATTATCC-GAAGA-GGATCGTTTACTCGTAGCAAGAGCGCGAAAAAT 1238
LPK091002       TTGGATTGGAC---------------------------------------------- 1184
LHB071009       TTGGATTGGACGAATTATCC-GAAGA-GGATCGTTTACTCGTAGCAAGAGCGCGAAAAAT 1230
LHB081111       TTGGATTGGACGAATTATCC-GAAGA-GGATCGTTTACTCGTAGCAAGAGCGCGAAAAAT 1239
LHB091112       TTGGATTGGACGAATTATCC-GAAGA-GGATCGTTTACTCGTAGCAAGAGCGCGAAAAAT 1231
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KSL051105       TTGGATTGGACGAATTATCC-GAAGA-GGATCGTTTACTCGTAGCAAGAGCGCGAAAAAT 1246
LHB071001       TTGGATTGGACGAATTATCC-GAAGA-GGATCGTTTACTCGTAGCAAGAGCGCGAAAAAT 1235
KSL071001       TTGGATTGGACGAATTATCC-GAAGA-GGATCGTTTACTCGTAGCAAGAGCGCGAAAAAT 1232
LHB081002       TTGGATTGGACGAATTATCC-GAAGA-GGATCGTTTACTCGTAGCAAGAGCGCGAAAAAT 1236
LHB091003       TTGGATTGGACGAATTATCC-GAAGA-GGATCGTTTACTCGTAGCAAGAGCGCGAAAAAT 1232
TNF111001       TTGGATTGGACGAATTATCC-GAAGAAGGATCGTTTACTCGTAGCAAGAGCGCGAAAAAT 1235
LHB101005       TTGGATTGGACGAATTAYCC-RAAGA-GGATCGTTTACYCGTAGCAAGAGCGCGAAAAAT 1233
KSl071002       TTGGATTGGACGAATTATCC-GAAGA-GGATCGTTTACTCGTAGCAAGAGCGCGAAAAAT 1234
LPK081001       TTGGATTGGACGAATTATCCCGAAGA-GGATCGTTTACTCGTAGCAAGAGCGCGAAAAAT 1235
LHB101004       TTGGATTGGACGAATTATCC-GAAGA-GGATCGTTTACTCGTAGCAAGAGCGCGAAAAAT 1232
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KSL041104       TGAGCGTTTCTTATCACAACCCTTTTTCGTAGCAGAAGTATTTACCGGTTCTCCAGGGAA 1300
LHB081110       TGAGCGTTTCTTATCACAACCCTTTTTCGTAGCAGAAGTATTTACCGGTTCTCCAGGGAA 1313
KSL031103       TGAGCGTTTCTTATCACAACCCTTTTTCGTAGCAGAAGTATTTACCGGTTCTCCAGGGAA 1339
LHB031107       TGAGCGTTTCTTATCACAACCCTTTTTCGTAGCAGAAGTATTTACCGGTTCTCCAGGGAA 1292
LHB021106       TGAGCGTTTCTTATCACAACCCTTTTTCGTAGCAGAAGTATTTACCGGTTCTCCAGGGAA 1298
LPK091002       ------------------------------------------------------------
LHB071009       TGAGCGTTTCTTATCACAACCCTTTTTCGTAGCAGAAGTATTTACCGGTTCTCCAGGGAA 1290
LHB081111       TGAGCGTTTCTTATCACAACCCTTTTTCGTAGCAGAAGTATTTACCGGTTCTCCAGGGAA 1299
LHB091112       TGAGCGTTTCTTATCACAACCCTTTTTCGTAGCAGAAGTATTTACCGGTTCTCCAGGGAA 1291
LHB061108       TGAGCGTTTCTTATCACAACCCTTTTTCGTAGCAGAAGTATTTACCGGTTCTCCAGGGAA 1309
KSL051105       TGAGCGTTTCTTATCACAACCCTTTTTCGTAGCAGAAGTATTTACCGGTTCTCCAGGGAA 1306
LHB071001       TGAGCGTTTCTTATCACAACCCTTTTTCGTAGCAGAAGTATTTACCGGTTCTCCAGGGAA 1295
KSL071001       TGAGCGTTTCTTATCACAACCCTTTTTCGTAGCAGAAGTATTTACCGGTTCTCCAGGGAA 1292
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LHB091003       TGAGCGTTTCTTATCACAACCCTTTTTCGTAGCAGAAGTATTTACCGGTTCTCCAGGGAA 1292
TNF111001       TGAGCGTTTCTTATCACAACCCTTTTTCGTAGCAGAAGTATTTACCGGTTCTCCAGGGAA 1295
LHB101005       TGAGCGTTTCTTATCACAACCCTTTTTCGTAGCAGAAGTATTTACCGGTTCTCCAGGGAA 1293
KSl071002       TGAGCGTTTCTTATCACAACCCTTTTTCGTAGCAGAAGTATTTACCGGTTCTCCAGGGAA 1294
LPK081001       TGAGCGTTTCTTATCACAACCCTTTTTCGTAGCAGAAGTATTTACCGGTTCTCCAGGGAA 1295
LHB101004       TGAGCGTTTCTTATCACAACCCTTTTTCGTAGCAGAAGTATTTACCGGTTCTCCAGGGAA 1292
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KSL041104       ATATGTTGGTCTAGCGGAAACAATTCGAGGATTTCAATTGATCCTTTCCGGAGAATTAGA 1360
LHB081110       ATATGTTGGTCTAGCGGAAACAATTCGAGGATTTCAATTGATCCTTTCCGGAGAATTAGA 1373
KSL031103       ATATGTTGGTCTAGCGGAAACAATTCGAGGATTTCAATTGATCCTTTCCGGAGAATTAGA 1399
LHB031107       ATATGTTGGTCTAGCGGAAACAATTCGAGGATTTCAATTGATCCTTTCCGGAGAATTAGA 1352
LHB021106       ATATGTTGGTCTAGCGGAAACAATTCGAGGATTTCAATTGATCCTTTCCGGAGAATTAGA 1358
LPK091002       ------------------------------------------------------------
LHB071009       ATATGTTGGTCTAGCGGAAACAATTCGAGGATTTCAATTGATCCTTTCCGGAGAATTAGA 1350
LHB081111       ATATGTTGGTCTAGCGGAAACAATTCGAGGATTTCAATTGATCCTTTCCGGAGAATTAGA 1359
LHB091112       ATATGTTGGTCTAGCGGAAACAATTCGAGGATTTCAATTGATCCTTTCCGGAGAATTAGA 1351
LHB061108       ATATGTTGGTCTAGCGGAAACAATTCGAGGATTTCAATTGATCCTTTCCGGAGAATTAGA 1369
KSL051105       ATATGTTGGTCTAGCGGAAACAATTCGAGGATTTCAATTGATCCTTTCCGGAGAATTAGA 1366
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KSL071001       ATATGTTGGTCTAGCGGAAACAATTCGAGGATTTCAATTGATCCTTTCCGGAGAATTAGA 1352
LHB081002       ATATGTTGGTCTAGCGGAAACAATTCGAGGATTTCAATTGATCCTTTCCGGAGAATTAGA 1356
LHB091003       ATATGTTGGTCTAGCGGAAACAATTCGAGGATTTCAATTGATCCTTTCCGGAGAATTAGA 1352
TNF111001       ATATGTTGGTCTAGCGGAAACAATTCGAGGATTTCAATTGATCCTTTCCGGAGAATTAGA 1355
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KSl071002       ATATGTTGGTCTAGCGGAAACAATTCGAGGATTTCAATTGATCCTTTCCGGAGAATTAGA 1354
LPK081001       ATATGTTGGTCTAGCGGAAACAATTCGAGGATTTCAATTGATCCTTTCCGGAGAATTAGA 1355
LHB101004       ATATGTTGGTCTAGCGGAAACAATTCGAGGATTTCAATTGATCCTTTCCGGAGAATTAGA 1352


KSL041104       TGGTCTTCCTGAACAGTCCTTTTATTTGGTAGGTAATATCGATGAAGCTACCGCGAAGGC 1420
LHB081110       TGGTCTTCCTGAACAGTCCTTTTATTTGGTAGGTAATATCGATGAAGCTACCGCGAAGGC 1433
KSL031103       TGGTCTTCCTGAACAGTCCTTTTATTTGGTAGGTAATATCGATGAAGCTACCGCGAAGGC 1459
LHB031107       TGGTCTTCCTGAACAGTCCTTTTATTTGGTAGGTAATATCGATGAAGCTACCGCGAAGGC 1412
LHB021106       TGGTCTTCCTGAACAGTCCTTTTATTTGGTAGGTAATATCGATGAAGCTACCGCGAAGGC 1418
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LHB071009       TGGTCTTCCTGAACAGTCCTTTTATTTGGTAGGTAATATCGATGAAGCTACCGCGAAGGC 1410
LHB081111       TGGTCTTCCTGAACAGTCCTTTTATTTGGTAGGTAATATCGATGAAGCTACCGCGAAGGC 1419
LHB091112       TGGTCTTCCTGAACAGTCCTTTTATTTGGTAGGTAATATCGATGAAGCTACCGCGAAGGC 1411
LHB061108       TGGTCTTCCTGAACAGTCCTTTTATTTGGTAGGTAATATCGATGAAGCTACCGCGAAGGC 1429
KSL051105       TGGTCTTCCTGAACAGTCCTTTTATTTGGTAGGTAATATCGATGAAGCTACCGCGAAGGC 1426
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KSL071001       TGGTCTTCCTGAACAGTCCTTTTATTTGGTAGGTAATATCGATGAAGCTACCGCGAAGGC 1412
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LHB101005       TGGTCTTCCTGAACAGTCCTTTTATTTGGTAGGTAATATCGATGAAGCTACCGCGAAGGC 1413
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LHB081110       TATGAATTA-GAGATGCAAACCAATTTT---TTTT--- 1464
KSL031103       TATGAACTA-GAGATGCAAACCAATTTT---TTTTTTT 1493
LHB031107       TATGAATTA-GAGATGCATCCCATTTTT---TTTTT-- 1444
LHB021106       TATGAATTA-GAGAT--CA-CCATTTTT---TTTTA-- 1447
LPK091002       -----------------------------------
LHB071009       TATGAATTA-GAGA--CCA-CTATTTTT---TTTTT-- 1439
LHB081111       TATGAATTA-GAAATGCAA-CCAATTTT---TTTTTT- 1451
LHB091112       TATGAATTA-GAGATGCAT-CCTATTTT---TTTT--- 1441
LHB061108       TATGAATTA-GAGATCCAA-CCTATTTT---TTTT--- 1459
KSL051105       TATGAACTA-GAAATGAGA--CCATTTTAAATTTT--- 1458
LHB071001       TATGAACTAAGAAATGCAAACCAATTAT---TTTTTTT 1450
KSL071001       TATGAACTTAGAAATGGAGAGCAATTGAGATTTTTT-- 1448
LHB081002       TATGAACT-AGAAATGGAGAGCAATTTATTTATTT--- 1450
LHB091003       TATGAACTTAGAAATGGAGAGCAATTAATTAATTT--- 1447
TNF111001       TATGAACTTAGAAATGGAGAGCAATTTAAAAAAT---- 1449
LHB101005       TATGAACTTAGAAATGCAAACCAAATTAATTTTTTT-- 1449
KSl071002       TATGAATTTAGAAAATTCAATCCTATTATTTTTTTT-- 1450
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Sequence type explicitly set to DNA
Sequence format is Pearson
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Sequence 2: KSL041104 1452 bp
Sequence 3: KSL051105 1458 bp

Sequence 4: KSL071001 1448 bp
Sequence 5: KSL071002 1450 bp
Sequence 6: LHB021106 1447 bp
Sequence 7: LHB031107 1444 bp
Sequence 8: LHB061108 1459 bp
Sequence 9: LHB071001 1450 bp
Sequence 10: LHB071009 1439 bp
Sequence 11: LHB081002 1450 bp
Sequence 12: LHB081110 1464 bp
Sequence 13: LHB081111 1451 bp
Sequence 14: LHB091003 1447 bp
Sequence 15: LHB091112 1441 bp
Sequence 16: LHB101004 1447 bp
Sequence 17: LHB101005 1449 bp
Sequence 18: LPK081001 1451 bp
Sequence 19: LPK091002 1184 bp
Sequence 20: TNF111001 1449 bp
Start of Pairwise alignments
Aligning...

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Sequences (1:5) Aligned. Score: 94
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Sequences (9:14) Aligned. Score: 97
Sequences (9:15) Aligned. Score: 98
Sequences (9:16) Aligned. Score: 97
Sequences (9:17) Aligned. Score: 97
Sequences (9:18) Aligned. Score: 97
Sequences (9:19) Aligned. Score: 97
Sequences (9:20) Aligned. Score: 96
Sequences (10:11) Aligned. Score: 96

Sequences (10:12) Aligned. Score: 97
Sequences (10:13) Aligned. Score: 97
Sequences (10:14) Aligned. Score: 97
Sequences (10:15) Aligned. Score: 98
Sequences (10:16) Aligned. Score: 97
Sequences (10:17) Aligned. Score: 96
Sequences (10:18) Aligned. Score: 96
Sequences (10:19) Aligned. Score: 97
Sequences (10:20) Aligned. Score: 96
Sequences (11:12) Aligned. Score: 95
Sequences (11:13) Aligned. Score: 96
Sequences (11:14) Aligned. Score: 97
Sequences (11:15) Aligned. Score: 97
Sequences (11:16) Aligned. Score: 97
Sequences (11:17) Aligned. Score: 96
Sequences (11:18) Aligned. Score: 96
Sequences (11:19) Aligned. Score: 97
Sequences (11:20) Aligned. Score: 96
Sequences (12:13) Aligned. Score: 96
Sequences (12:14) Aligned. Score: 96
Sequences (12:15) Aligned. Score: 98
Sequences (12:16) Aligned. Score: 96
Sequences (12:17) Aligned. Score: 96
Sequences (12:18) Aligned. Score: 95
Sequences (12:19) Aligned. Score: 97
Sequences (12:20) Aligned. Score: 95
Sequences (13:14) Aligned. Score: 96
Sequences (13:15) Aligned. Score: 98
Sequences (13:16) Aligned. Score: 96
Sequences (13:17) Aligned. Score: 96
Sequences (13:18) Aligned. Score: 96
Sequences (13:19) Aligned. Score: 97
Sequences (13:20) Aligned. Score: 95
Sequences (14:15) Aligned. Score: 97
Sequences (14:16) Aligned. Score: 98
Sequences (14:17) Aligned. Score: 97
Sequences (14:18) Aligned. Score: 96
Sequences (14:19) Aligned. Score: 97
Sequences (14:20) Aligned. Score: 97
Sequences (15:16) Aligned. Score: 97
Sequences (15:17) Aligned. Score: 97
Sequences (15:18) Aligned. Score: 97
Sequences (15:19) Aligned. Score: 98
Sequences (15:20) Aligned. Score: 96
Sequences (16:17) Aligned. Score: 97
Sequences (16:18) Aligned. Score: 97
Sequences (16:19) Aligned. Score: 97
Sequences (16:20) Aligned. Score: 97
Sequences (17:18) Aligned. Score: 96
Sequences (17:19) Aligned. Score: 97
Sequences (17:20) Aligned. Score: 96
Sequences (18:19) Aligned. Score: 97

Sequences (18:20) Aligned. Score: 95
Sequences (19:20) Aligned. Score: 97

There are 19 groups
Start of Multiple Alignment

Aligning...
Group 1: Sequences: 2 Score:27042
Group 2: Sequences: 3 Score:26694
Group 3: Sequences: 4 Score:26535
Group 4: Sequences: 2 Score:22047
Group 5: Sequences: 2 Score:27042
Group 6: Sequences: 4 Score:24493
Group 7: Sequences: 5 Score:26968
Group 8: Sequences: 9 Score:26293
Group 9: Sequences: 10 Score:26356
Group 10: Sequences: 11 Score:26367
Group 11: Sequences: 12 Score:26409
Group 12: Sequences: 2 Score:27297
Group 13: Sequences: 2 Score:27242
Group 14: Sequences: 4 Score:27115
Group 15: Sequences: 5 Score:27213
Group 16: Sequences: 2 Score:27283
Group 17: Sequences: 3 Score:27258
Group 18: Sequences: 8 Score:26979
Group 19: Sequences: 20 Score:25951
Alignment Score 1719367

Figure 40. Comparison of nucleotide sequence of *atp*B gene of *D.metel* L. var. *metel*, *D.metel* L. var. *fastuosa*, and hybrid *D.metel* L., (TNF111001 was assigned as outgroup sample)
* indicate clustal consensus, - indicate indels

Reagent and buffers for agarose gel electrophoresis

1. **Loading dye (10x)**

   Ten time concentrate loading dye (10x) consisted of 50% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanole FF

2. **TBE buffer (10x)**

   To prepare the 10x TBE buffer, the following ingredients were mixed:

Tris-base	104.0 g
Boric acid	55.0 g
EDTA-2H_2O	9.3 g
Deionized water	700.0 ml

   The solution was adjusted pH to 8.3 with concentrate HCl before the volume was made to 1,000 ml. This buffer was sterilized by autoclaving.

3. **Working TBE buffer (1x)**

   The 10x TBE (100 ml) was added to 900 ml of deionized water. This solution can be reused three times.

4. **Ethidium bromide solution**

   To prepare stock ethidium bromide solution, a Tablet of ethidium bromide was dissolved in 1 ml of deionized water to obtain a concentration of 10 mg/ml**.** Fifty microliters of the stock solution was then added to 100 ml of the buffer to make of 0.5 µg/ml working concentration. The solution was kept protected from light.

5. **Agarose (1.5%) gel preparation**

   Agarose 0.45 g was added to 30 ml of either 1x TBE buffer and dissolved by heating. Molten agarose was allowed to cool down to 50-60 ºC at 25 ºC before poring in a gel casting apparatus.

APPENDIX C

Part IlI. Scopolamine evaluation

Determination of scopolamine content by TLC image method

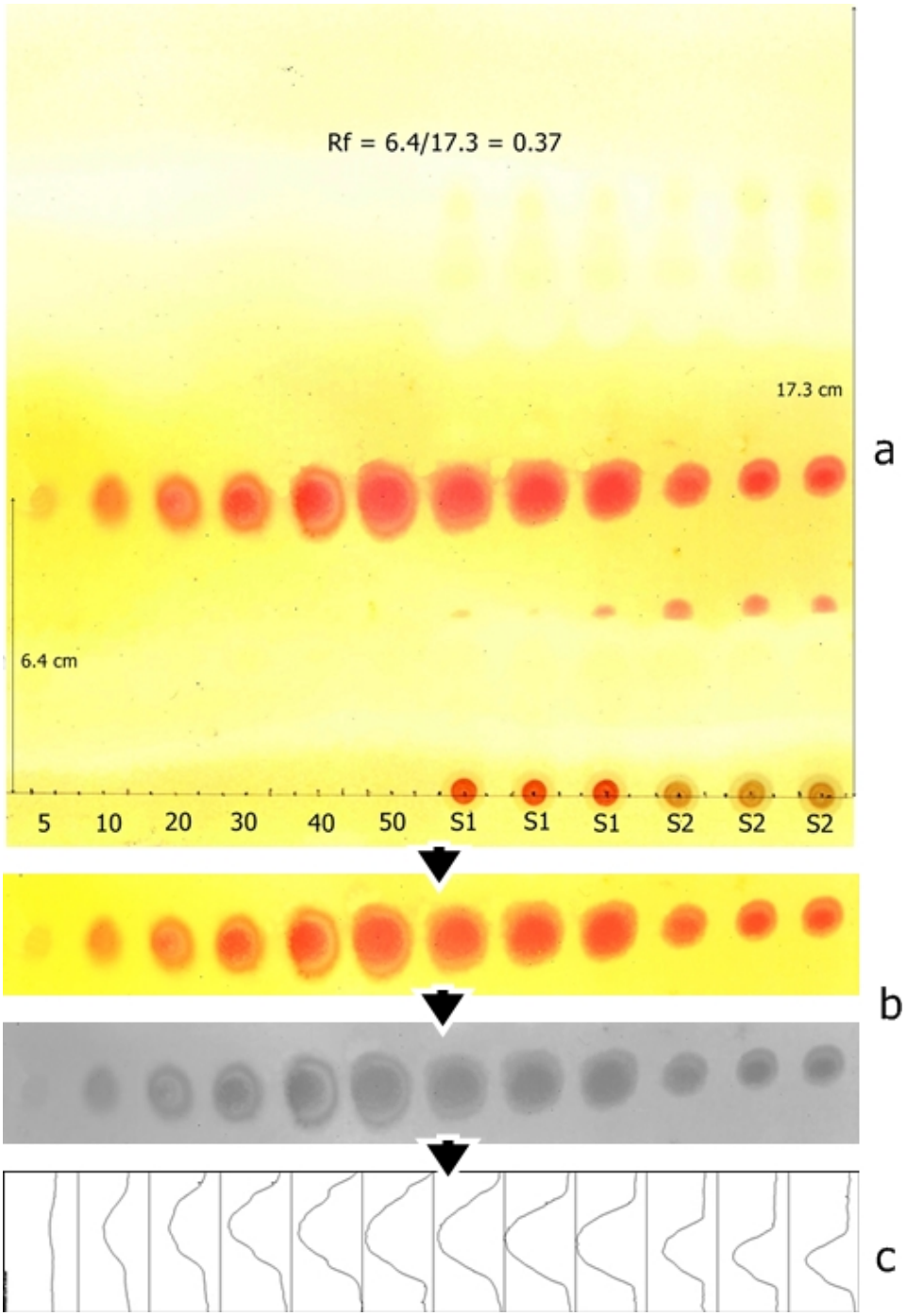

Figure 41. Processing of TLC image analysis by image J software: (a) TLC chromatogram; (b) converting the image to greyscale; (c) chromatogram profiles obtained from the converting image. (From left to right lanes: standard scopolamines 5-50 µg/spot and triplicate of two samples.) TLC chromatogram showed the spot of scopolamine at the retention factor (R_f) of 0.37, which developed in toluene: ethyl acetate: diethylamine (7: 2: 1 v/v)

Reagent

- **Dragendorff's reagent**

Solution A: Dissolve 1.7 g bismuth (III) nitrate in a mixture of 20 mL glacial acetic acid and 80 mL of water (4:1).

Solution B: Dissolve 40 g potassium iodide in 100 mL of water.

Spray solution: Mix equal parts of solution A and solution B, before use. Store this solution in refrigerater and discard after 2 weeks.

Determination of scopolamine content by HPLC method

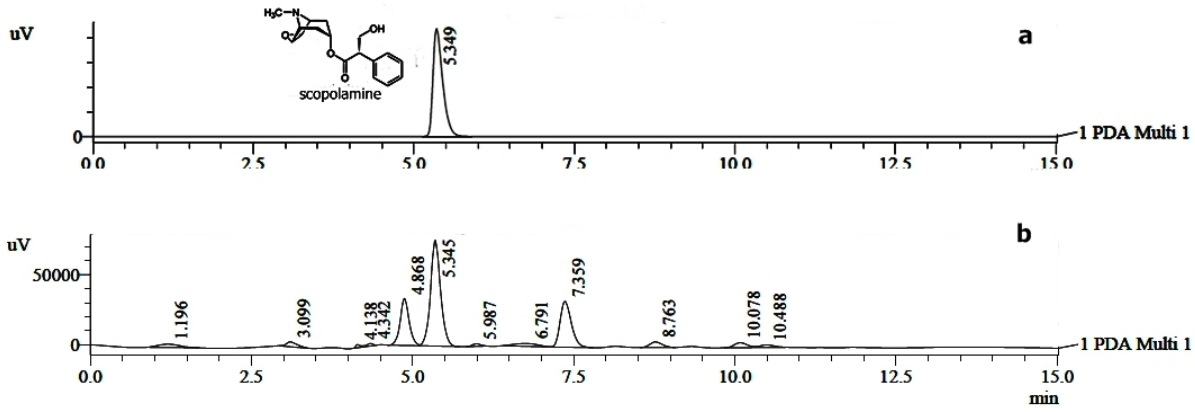

Figure 42. HPLC chromatogram pattern of standard scopolamine hydrocloride (a) and crude extracted of *D.metel* L. (b) HPLC chromatogram showed scopolamine peak at the retention time (R_t) of 5.34 min

Reagent and buffer

- **50 mM Potassium dihydrogen orthophosphate buffer**

To prepare 50 mM KH_2PO_4, the following ingredients were mixed:

KH_2PO_4	6.804 g
Deionized water	800.0 ml

The solution was adjusted pH to 3.0 with orthophosphoric acid before the volume was made to 1,000 ml. This buffer was filtered through a 0.45 µm filter before use.

VITA

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Publication

Issaravanich, S., Rungsihirunrat, K., and Ruangrungsi, N. Nucleotide sequence of the internal transcribed spacer (ITS) region of *Datura metel* L. var. *fastuosa* in Thailand. Proceedings of the 9th Joint Seminar Natural Medicine Research for the Next Decade: New Challenges and Future Collaboration, pp. 305-306. Bangkok, 2010.

Scholarships

1. Research Fund; 90 th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund).
2. The Herbal Remedies and Alternative Medicine Task Force of STAR: Special Task Force for Activating Research under 100 Years Chulalongkorn University Fund.