วิวัฒนาการร่วมของยีนโทโพไอโซเมอเรส I กับการสร้างแคมป์โทเธซินในพืชสกุล *Ophiorrhiza*

นางสาว วราลี วิราพร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชเวท ภาควิชาเภสัชเวทและเภสัชพฤกษศาสตร์
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COEVOLUTION OF *TOPOISOMERASE* I AND CAMPTOTHECIN PRODUCTION

IN *OPHIORRHIZA* PLANTS

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A Thesis Submitted in Partial Fulfillment of the Requirements

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วราลี วิราพร : วิวัฒนาการร่วมของยีนโทโพไอโซเมอเรส I กับการสร้างแคมป์โทเธซินในพืชสกุล *Ophiorrhiza.* (COEVOLUTION OF *TOPOISOMERASE* I AND CAMPTOTHECIN PRODUCTION IN *OPHIORRHIZA* PLANTS)
อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ดร. สุชาดา สุขหร่อง,
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แคมป์โทเธซินเป็นสารในกลุ่ม indole alkaloid ซึ่งพบในธรรมชาติ และเป็นสารตั้งต้นในกระบวนการผลิตยาเคมีบำบัดที่มีใช้กันอย่างแพร่หลายทั่วโลก ความต้องการในการใช้ยากลุ่มนี้มีเพิ่มมากขึ้น ปัจจุบันแคมป์โทเธซินยังคงได้มาจากการสกัดจากพืช ได้แก่ *Camptotheca acuminata* และ *Nothapodytes foetida* มีรายงานว่าพืชสกุล *Ophiorrhiza* บางชนิดสามารถสร้างแคมป์โทเธซิน และพบว่าพืชหลายชนิดที่สร้างแคมป์โทเธซินมีเอนไซม์โทโพไอโซเมอเรส I ที่เกิดการกลายพันธุ์ในหลายตำแหน่ง ทำให้แคมป์โทเธซินไม่สามารถเข้าจับกับเอนไซม์ โทโพไอโซเมอเรส I ได้ ส่งผลให้พืชเหล่านั้นทนทานต่อพิษของแคมป์โทเธซินที่พืชสร้างขึ้นมาเอง งานวิจัยนี้เป็นการศึกษาวิวัฒนาการร่วมของยีนโทโพไอโซเมอเรส I กับการสร้างแคมป์โทเธซินของพืชสกุล *Ophiorrhiza* 8 ชนิดในประเทศไทย พบว่ามีพืช 5 ชนิดที่สร้างแคมป์โทเธซินและอนุพันธ์ของแคมป์โทเธซินในบริเวณใบหรือราก เมื่อศึกษาลำดับนิวคลีโอไทด์ของของยีนแม็ตเคและยีนโทโพไอโซเมอเรส I ของพืชสกุล *Ophiorrhiza* เพื่อจัดจำแนกกลุ่มพืชและศึกษาวิวัฒนาการ พบว่าวงศ์วานวิวัฒนาการเชิงโมเลกุลของยีนแม็ตเค, ยีนโทโพไอโซเมอเรส I, และทั้งสองยีนสามารถแบ่งกลุ่มพืช *Ophiorrhiza* เป็นสองกลุ่มตามคุณสมบัติในการสร้างแคมป์โทเธซินและอนุพันธ์ของแคมป์โทเธซิน สรุปว่าพันธุกรรมมีบทบาทสำคัญในการกำหนดความสามารถในการสร้างสารกลุ่มแคมป์โทเธซินในพืชสกุลนี้ และพืชสกุล *Ophiorrhiza* มีวิวัฒนาการร่วมระหว่างยีนแม็ตเคและยีนโทโพไอโซเมอเรส I กับการสร้างแคมป์โทเธซินและอนุพันธ์ ดังนั้นจึงสามารถใช้ยีนแม็ตเคและยีนโทโพไอโซเมอเรส I เพื่อช่วยในการทำนายการสร้างแคมป์โทเธซินและอนุพันธ์ของแคมป์โทเธซินของพืชในสกุล *Ophiorrhiza* ได้ นอกจากนั้นยังพบการแทนที่ของกรดอะมิโนหลายตำแหน่งในเอนไซม์โทโพไอโซเมอเรส I ที่สามารถใช้เป็นเครื่องหมายในการจำแนกกลุ่มพืชที่สร้างและกลุ่มพืชที่ไม่สร้างแคมป์โทเธซินซึ่งใช้เป็นข้อมูลพื้นฐานในการทำนายการดื้อยาในผู้ป่วยโรคมะเร็งในอนาคต

ภาควิชา เภสัชเวทและเภสัชพฤกษศาสตร์ ลายมือชื่อนิสิต..
สาขาวิชา เภสัชเวท..................ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก..................................
ปีการศึกษา 2553...................ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.................................

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VARALEE VIRAPORN: COEVOLUTION OF *TOPOISOMERASE* I AND CAMPTOTHECIN PRODUCTION IN *OPHIORRHIZA* PLANTS. THESIS ADVISOR: ASSISTANT PROFESSOR SUCHADA SUKRONG, Ph.D., THESIS CO-ADVISOR: TAKSINA CHUANASA, Ph.D., 124 pp.

Camptothecin (CPT), a naturally occurring indole alkaloid, is an essential precursor of semi-synthetic chemotherapeutic agents for cancers throughout the world. In spite of the rapid growth of market demand, CPT raw material is still harvested by extraction from *Camptotheca acuminata* and *Nothapodytes foetida*. Previous study found that many CPT-producing plants, including some of *Ophiorrhiza* spp., have topoisomerase I (TopI) enzymes with several point-mutations that confer resistance to CPT to avoid CPT toxicity. The purpose of this thesis is to study the coevolution between *Top*I gene and CPT production in *Ophiorrhiza* plants. Eight species of the genus *Ophiorrhiza* in Thailand were examined as novel alternative sources of CPT. CPT and its derivatives were differently detected in five species in leaf and root extracts. Chloroplast *mat*K and nuclear *Top*I genes of eight species were investigated in order to classify and study the coevolution in this genus. The molecular phylogenetic trees of both separated and combined *mat*K and *Top*I nucleotide sequences revealed two major clades of *Ophiorrhiza* taxa correlated with the productions of CPT and CPT derivatives. We conclude that *Ophiorrhiza* plants have *mat*K and *Top*I coevolved with CPT production. Thus, *mat*K and *Top*I gene sequences could be utilized for the prediction of CPT and CPT derivatives production ability of any members of *Ophiorrhiza*. We also proposed that several unique amino acid substitutions in TopI of CPT-producing *Ophiorrhiza* plants could be used as amino acid markers and provide useful information toward recognition of the point mutations in CPT-resistant cancer patients in the future.

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Finally, this thesis and this degree are dedicated to my well-beloved family.

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LIST OF ABBREVIATIONS

%	= percent (part per 100); percentage
A, T, C, G	= nucleotide containing the base adenine, thymine, cytosine, and guanine, respectively
bp	= base pair
cDNA	= complementary deoxyribonucleic acid
CI	= consistency index
°C	= degree celsius
DMF	= dimethylformamide
DNA	= deoxyribonucleic acid
dNTPs	= deoxyribonucleotide triphosphates (dATP, dTTP, dGTP, dCTP)
g	= gram(s)
HOAc	= acetic acid
HPLC-DAD	= high-performance liquid chromatography-diode array detection
hr	= hour(s)
HRMS	= high resolution mass spectrometry
H_2O	= water
ITS	= internal transcribed spacer
kb	= kilobase
kDa	= kilodalton
L	= liter(s)
LB-Amp	= lysogeny broth containing the antibiotic ampicillin
LC-MS/MS	= liquid chromatography-mass spectrometry/ mass spectrometry
M	= molar
MeOH	= methanol
$MgCl_2$	= magnesium chloride
min	= minute(s)
mL	= milliliter
mM	= millimolar

MPT(s)	= maximum parsimonious tree(s)
N	= northern
NE	= north eastern
ng	= nanogram(s)
nm	= nanometer
PAUP	= phylogenetic analysis using parsimony
PCR	= polymerase chain reaction
RC	= rescaled consistency index
RI	= retention index
pH	= the negative logarithm of the concentration of hydrogen ions
RNA	= ribonucleic acid
rRNA	= ribosomal ribonucleic acid
rpm	= revolution per minute
s	= second(s)
SE	= south eastern
SW	= south western
SD	= standard deviation
sp.	= species (singular)
spp.	= species (plural)
TAE buffer	= tris-acetate and EDTA buffer
TIA(s)	= monoterpenoid indole-alkaloid(s)
Trp	= tryptophan (amino acid)
UV	= ultraviolet
µg	= microgram(s)
µL	= microliter(s)
µM	= micromolar
WS	= west southern

Amino acid abbreviations

A	/ Ala	= alanine
C	/ Cys	= cysteine
D	/ Asp	= aspartic acid
E	/ Glu	= glutamic acid
F	/ Phe	= phenylalanine
G	/ Gly	= glycine
H	/ His	= histidine
I	/ Ile	= isoleucine
K	/ Lys	= lysine
L	/ Leu	= leucine
M	/ Met	= methionine
N	/ Asn	= asparagine
P	/ Pro	= proline
Q	/ Gln	= glutamine
R	/ Arg	= arginine
S	/ Ser	= serine
T	/ Thr	= threonine
V	/ Val	= valine
W	/ Trp	= tryptophan
Y	/ Tyr	= tyrosine

CHAPTER I

INTRODUCTION

Camptothecin (CPT), a naturally occurring pentacyclic indole alkaloid, exhibits an anticancer activity due to its ability to inhibit topoisomerase I (TopI) enzyme involving in DNA topology (Hsiang *et al*., 1985). Knowledge of the structure-activity relationship of CPT has led to the development of CPT derivatives to increase solubility, stability, and bioavailability with manageable toxicities (Dancey and Eisenhauer, 1996). Two semi-synthetic CPT analogs, topotecan (Hycamtin®) and irinotecan (Camptosar®) are currently used throughout the world for various cancer treatments, such as ovarian cancer, lung cancer, colon cancer, and over a dozen more CPT analogs are at various stages of clinical development (Lorence and Nessler, 2004). In spite of the rapid growth of market demand, CPT raw material is still harvested by extraction from *Camptotheca acuminata* and *Nothapodytes foetida* since its total synthesis is not cost effective. As a result, this could lead to a lack or an extinction of CPT-producing plants in the future. Plants reported to contain CPT are *C. acuminata* (Nyssaceae) (Wall *et al*., 1966), *N. foetida* (Icacinaceae) (Govindachari and Viswanathan, 1972), *Ervatamia heyneana* (Apocynaceae) (Gunasekera *et al*., 1979), and some species in the genus *Ophiorrhiza* (Rubiaceae) (Lorence and Nessler, 2004). Recently, CPT-producing *Ophiorrhiza* plants have become interesting as alternative sources for CPT production in tissue cultures (Martin *et al*., 2008; Roja, 2008).

Ophiorrhiza L. is a predominantly herbaceous genus belonging to the family Rubiaceae and comprising about 400 species (Schanzer, 2005). Since the genus is taxonomically complex and has high morphological variability (Chou, Yang, and Liao 2006; Darwin, 1976; Kudoh *et al*., 2001), few studies have attempted to resolve this taxonomic problem using molecular phylogenetic systematics (Nakamura *et al*., 2006, 2007). Several DNA regions, such as ITS, *atp*B-*rbc*L and *trn*K/*mat*K have been utilized to determine the species and varieties within *Ophiorrhiza*. Previous study found that many CPT-producing plants including *Camptotheca acuminata*, *N. foetida*, *O. pumila* and *O.*

liukiuensis have TopI enzymes with several point-mutations that confer resistance to CPT to avoid CPT toxicity. This could be inferred as a self-resistance mechanism coevolved with the production of CPT (Sirikantaramas, Yamazaki, and Saito, 2008).

This thesis aims to study the coevolution between *Top*I gene and CPT production in *Ophiorrhiza* plants. The research consists of two main parts. The first part is to study CPT-producing ability of *Ophiorrhiza* plants. The methanol extracts of *Ophiorrhiza* species were analyzed for CPT compound using HPLC/DAD/ESI/MS. Standard solutions of CPT, CPT derivatives, and other chemical compounds involved in CPT biosynthesis pathway were also analyzed using the same method. In the second part, the molecular phylogenetic trees of chloroplast *mat*K and nuclear *Top*I nucleotide sequences were reconstructed to classify and study an evolution in this genus. Furthermore, amino acid sequences of TopI enzymes were analyzed to detect point mutations in the CPT-producing *Ophiorrhiza* spp. In conclusion, the molecular phylogenetic trees and the CPT-producing ability were concurrently analyzed to define the coevolution of *TopI* and CPT production in *Ophiorrhiza* plants.

In this study, five out of eight species of *Ophiorrhiza* were discovered as novel alternative sources of CPT and CPT derivatives. We found that both *mat*K and *Top*I of *Ophiorrhiza* plants coevolved with CPT production. Thus, *mat*K and *Top*I gene sequences could be utilized for the prediction of CPT- and CPT derivatives-producing ability of members of *Ophiorrhiza*. We also proposed that several unique amino acid substitutions in TopI of CPT-producing *Ophiorrhiza* plants could be used as useful markers and provide information toward recognition of the point mutations in CPT-resistant cancer patients in the future.

CHAPTER II

LITERATURE REVIEW

2.1 Camptothecin (CPT)

2.1.1 Structure and biosynthesis pathway of CPT

CPT (Figure 2.1) is a plant-originated pentacyclic indole alkaloid (Yamazaki *et al*., 2004). The pentacyclic ring system includes a pyztolo[3,4-b]quinoline (ring A, B and C), a conjugated pyridine (ring D), and six-membered lactone (ring E) with a chiral center at position C-20 (Narkunan *et al*., 2009). The structural features of CPT that are essential for activity include the 20(*S*)-hydroxyl (Wang, Zhou, and Hecht, 1999), the pyridone moiety, the lactone, and the planarity of the five-membered ring system (Carbonero and Supko, 2002). Since CPT is a weak acid, the lactone ring is highly susceptible to ring opening by hydrolysis, forming carboxylate (Figure 2.2). At physiological pH, a labile E-ring lactone is hydrolyzed to an inactive hydroxy acid, which binds to human serum albumin. This reaction is reversible at acidic pH, as it is in cancer cell microenvironment, regenerating the active compound. Due to the unique chemistry of the CPT molecule, this physiology results in environmental conditions that may provide tumor site-specific enhancement of CPT activity (Flowers *et al*., 2003). However, the low aqueous solubility of CPT in the lactone form greatly limited the practical clinical utility of the drug because prohibitively large volumes of fluid have to be administered to the subject in order to provide an effective dose of the drug (Narkunan *et al*., 2009).

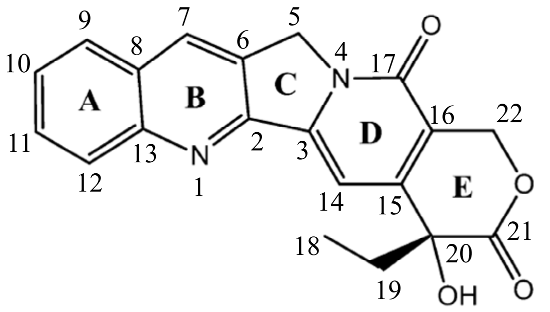


Figure 2.1 Chemical structure of camptothecin.

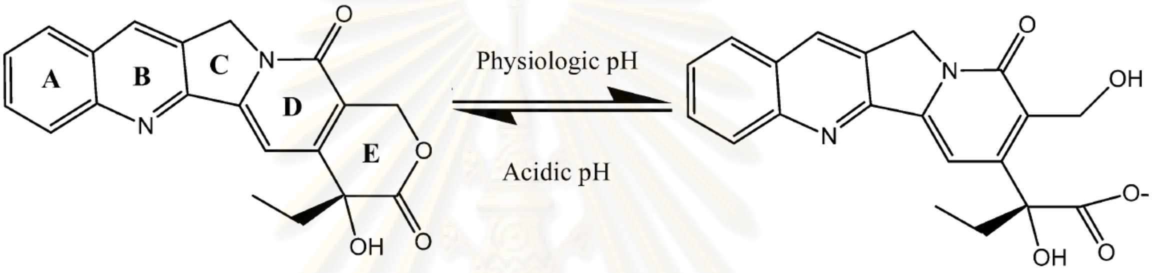


Figure 2.2 The pH-dependent dynamic equilibrium between the lactone and carboxylic acid forms of camptothecin (Flowers *et al.*, 2003).

CPT is a product of secondary metabolism from monoterpenoid indole-alkaloids (TIAs) (Lu *et al.*, 2008) derived from amino acid tryptophan (Trp) and terpenoid precursors (Lorence and Nessler, 2004). Camptothecin is formed by the combination of the 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway and the shikimate pathway, which involves many distinct enzymatic steps (Figure 2.3). The common intermediate, from which a variety of TIAs are formed, is strictosidine. Strictosidine is formed by the condensation of tryptophan-derivative tryptamine with the iridoid glucoside, secologanin. This condensation is catalyzed by a key enzyme, strictosidine synthase. Subsequently, intramolecular cyclization of strictosidine yields strictosamide, a penultimate precursor of camptothecin formation (Yamazaki *et al.*, 2003, 2004). 3(*S*)-pumiloside and 3(*S*)-deoxy pumiloside are thought to be biogenetic intermediates in the formation of CPT from strictosamide.

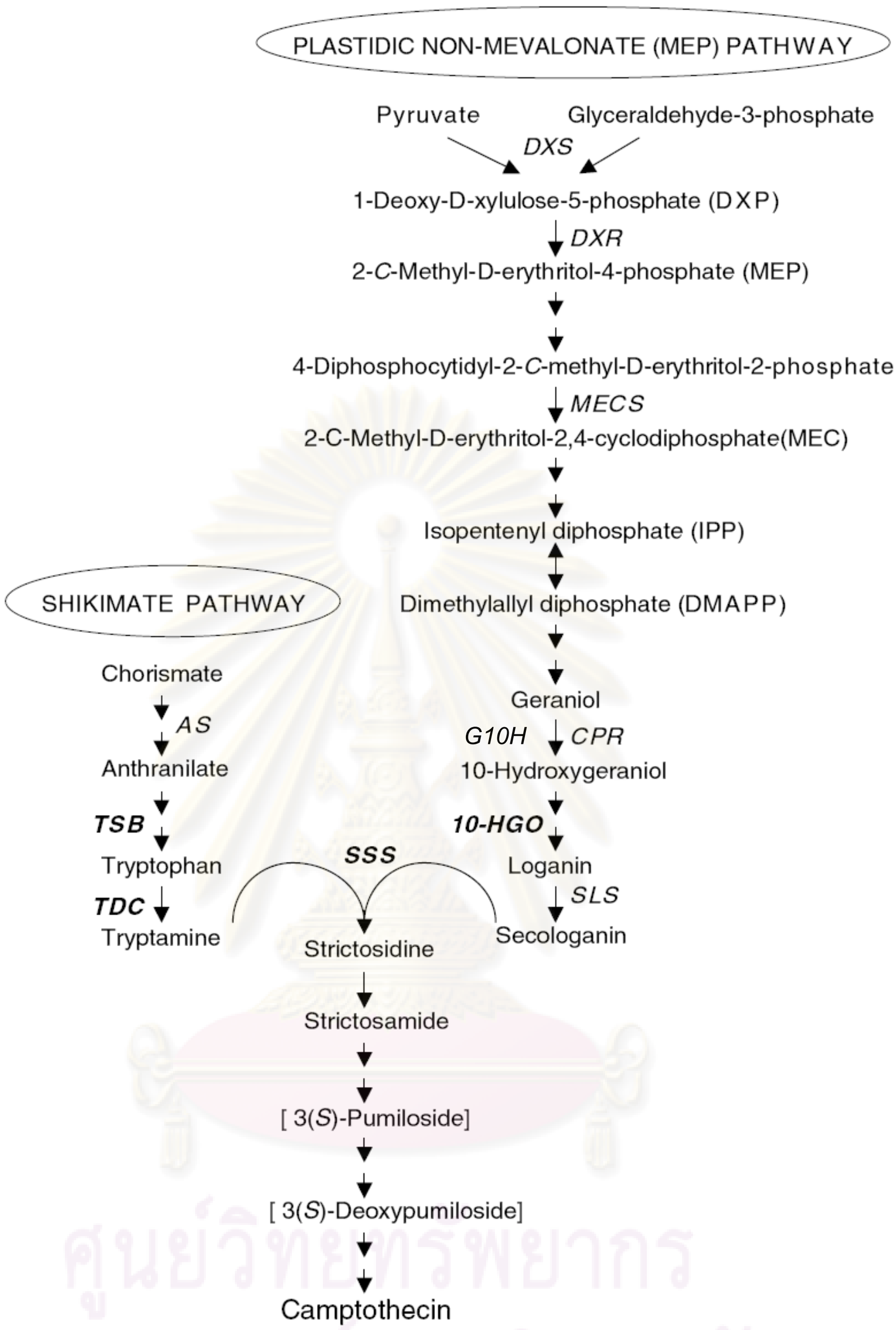


Figure 2.3 Biosynthetic pathway for TIAs in CPT-producing plants. Multiple arrows indicate multiple steps between intermediates. The enzymes involve in the pathways: DXP synthase (DXS); DXP reductoisomerase (DXR); 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase (MECS); geraniol-10-hydroxylase (G10H); secologanin synthase (SLS); TSB (b-subunit of tryptophan synthase); TDC (tryptophan decarboxylase); SSS (strictosidine synthase), and 10-HGO (10-hydroxygeraniol oxidoreductase (source: Yamazaki *et al*., 2004).

2.1.2 Anticancer CPT analogs

By the early 1970's, CPT had reach Phase I and Phase II clinical trials. Although CPT was found to possess antitumor activity, there were numerous side-effects including hemorrhagic cystitis, leucopenia and thrombocytopenia which were dose-limiting toxicities (Muggia *et al*., 1972). In addition, CPT is extremely poor water soluble and easily hydrolysable due to the closed E-ring lactone. Thus, knowledge of the structure–activity relationship of CPT has led to the development of CPT derivatives to increase solubility, stability and bioavailability with manageable toxicities (Dancey and Eisenhauer, 1996). Originally, CPT was delivered as the sodium salt of the carboxylate to help overcome solubility issues, however, the poor efficacy created a need for new alternatives (Hsiang *et al*., 1989). The modifications of the quinoline ring provided increased solubility, lactone stability (Chourpa *et al*., 1998), and antitumor activity (Vladu *et al*., 2000). Modifications to the 7, 9, 10, and 11 positions of the A-ring and B-ring, are generally well tolerated and in many cases enhance the potency of the CPT analog in both *in vivo* and *in vitro* studies (Redinbo *et al*., 1998)

Two semi-synthetic water-soluble CPT analogs (Figure 2.4), topotecan (Hycamtin®) and irinotecan (Camptosar®) were approved for use by the USFDA in 1966, and over a dozen other CPT analogs are at various stages of clinical development (Lorence and Nessler, 2004). Topotecan gains its increase solubility and greater *in vivo* activity due to a tertiary amine at the 9-position, while irinotecan presents its improvement through the 10-hydroxyl moiety. Topotecan is currently approved for use in the USA as second-line therapy in ovarian and small cell lung cancer. Irinotecan is a pro-drug that undergoes enzymatic conversion to the biologically active metabolite 7-ethyl-10-hydroxy-CPT. Its approved indications were cancers of the lung (small cell and non-small cell), cervix, ovaries, and also colon cancer as a second-line agent. It is presently the treatment of choice when used in combination with fluoropyrimidines for patients with advanced colorectal cancer or as a single agent after failure of 5-fluorouracil-based chemotherapy (Carbonero and Supko, 2002).

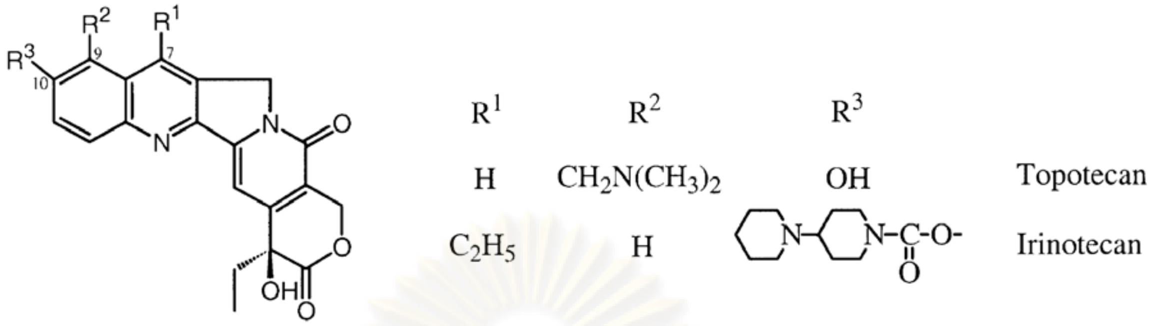


Figure 2.4 Clinically used semi-synthetic CPT analogs, topotecan and irinotecan (Yamazaki *et al*., 2004).

Hydroxy CPT and methoxy CPT are a naturally occurring CPT derivative isolated from many CPT-producing plants (Wani and Wall, 1969; Yamazaki *et al*., 2004). 10-hydroxy CPT (10-HCPT) has been found to be more potent and less toxic than CPT (Zhang *et al*., 1998). Many water-soluble aminoalkyl CPT analogs, including topotecan, were prepared by oxidation of CPT to 10-HCPT followed by additional modifications (Kingsbury *et al*., 1991). Irinotecan is also a 10-HCPT analog synthesized by bonding phenolic hydroxyl group of 7-ethyl-10-HCPT with diamines (Sawada *et al*., 1991). Besides, long-chain fatty acid esters of 10-HCPT derivatives could be useful as prodrug-type anticancer agents (Takayama *et al*., 1998). The methoxy analog was found to be more active than CPT (Tafur *et al*., 1976). 9-methoxy camptothecin (9-MCPT) has been reported as a starting material for a synthesis of 9-methoxy mappicine which has antiviral activity (Das and Madhusudhan, 1999).

2.1.3 Distribution of CPT and its derivatives

CPT is first isolated from extracts of *Camptotheca acuminata* (Nyssaceae), a deciduous tree native to China and Tibet (Wall *et al*., 1966). Plants which have been reported containing CPT belong to the following unrelated orders and families: Order Cornales (Nyssaceae): *C. acuminata*: Order Celastrales (Icacinaceae): *Nothapodytes foetida* (Aiyama *et al*., 1988), *Pyrenacantha klaineana* (Zhou *et al*., 2000), *Merrilliodendron megacarpum* (Arisawa *et al*., 1981): Order Gentianales (Rubiaceae): some species of the genus *Ophiorrhiza*, Family Apocynaceae: *Ervatamia heyneana* (Dai, Cardellina, and Boyd, 1999), and Family Gelsemiaceae: *Mostuea brunonis* (Gunasekera *et al*., 1979). It is likely that the genes encoding enzymes involved in their biosynthesis evolved early during evolution. These genes were presumably not lost during evolution but might have been ‘‘switched off’’ during a certain period of time and ‘‘switched on’’ again at some later point (Wink, 2003).

The information regarding the sites of accumulation of CPT and CPT derivatives including their concentration in multiple natural sources are summarized in Table 2.1. The most abundant natural CPT derivatives are 10-HCPT and 9-MCPT. *N. foetida* was found to produce CPT by an endophyte at shake flask and bioreactor (Rehman *et al*., 2009). 10-HCPT, 9-MCPT, and 10-methoxy camptothecin (10-MCPT) were also produced by endophytic fungi, *Fusarium solani,* isolated from CPT-producing plants (Shweta *et al*., 2010). *C. acuminata* was detected the 5-6 fold of the CPT content in young leaves compared to mature ones (López-Meyer, Nessler, and McKnight, 1994). In fact, the immature leaves are attractive to herbivory and pathogen. Although the role of CPT as a defense chemical has not been directly tested, there are indirect lines of evidence indicating its involvement in plant defense (Lorence and Nessler, 2004). According to the previous study of *N. foetida* (Roja, 2006), an increase in the level of 9-MCPT in the mature plant suggests that the accumulation of the 9-MCPT may probably be associated with the maturation of the plant.

Table 2.1 Sites of accumulation of CPT and CPT derivatives in natural sources.

Species	Tissue analyzed	Sample origin	Camptothecinoids content (µg/g dry weight)	Reference
Camptotheca acuminata Decaisne	Young leaves	Texas, USA	CPT 4000–5000 10-HCPT 20–30	López-Meyer *et al*., 1994
	Seeds		CPT 3000 10-HCPT 25	
	Bark		CPT 1800–2000 10-HCPT 2–90	
	Roots		CPT 400 10-HCPT 13–20	
	Young leaves	Texas, USA	CPT 2421–3022	Li *et al*., 2002
	Old leaves		CPT 482	
	Young fruit		CPT 842	
	Old fruit		CPT 2362	
	Hairy roots	Texas, USA	CPT 1000 10-HCPT 150	Lorence, Medina-Bolivar, and Nessler, 2004
	Callus	Shangai, China	CPT 2040–2360 10-HCPT 80–100	Wiedenfeld *et al*., 1997
	Cell cultures		CPT 2.5 –4	Sakato *et al*., 1974; van Hengel *et al*., 1992
Camptotheca lowreyana Li	Young leaves	Texas, USA	CPT 3913–5537	Li *et al*., 2002
	Old leaves		CPT 909–1184	
Camptotheca yunnanensis Dode	Young leaves	Texas, USA	CPT 2592–4494	Li *et al*., 2002
	Old leaves		CPT 590	
Ervatamia heyneana (Wall) T. Cooke	Wood and stem bark	India	CPT 1300 9-MCPT 400	Gunasekera *et al*., 1979
Nothapodytes foetida (Wight) Sleumer	Stem wood	Okinawa, Japan	CPT 1400–2400 dCPT 19	Aiyama *et al*., 1988
	Stem	Taiwan	ACPT 0.24	Wu *et al*., 1995
	Shoot	Mahabaleshwar, India	CPT 750 9-MCPT 130	Roja and Heble, 1994
	Plantlet culture		9-MCPT 7	
	Callus		9-MCPT 1	
	Stem	Godavari, India	MACPT 2.5	Srinivas and Das, 2003
	Callus	Ooty, India	CPT 9.5 9-MCPT traces	Ciddi and Shuler, 2000
	Cell culture	Satara, India	CPT 1.1 9-MCPT 0.81	Fulzele *et al*., 2001
Merriliodendron megacarpum (Hemsl.) Sleumer	Leaves and stem	Guam	CPT 530 9-MCPT 170	Arisawa *et al*., 1981
Mostuea brunonis Didr.	Entire plant	Lope, Gabon	CPT-20-*O*-b-glucoside 100 DPMI 100 Strictosamide 600	Dai *et al*., 1999

Table 2.1 (continued)

Species	Tissue analyzed	Sample origin	Camptothecinoids content (µg/g dry weight)	Reference
Ophiorrhiza fistipula	Leaves	-	7-MCPT	Arbain, Putra, and Sargent, 1993
Ophiorrhiza kuroiwae Makino	tissue cultures	Okinawa, Japan	CPT 55 10-MCPT 2	Asano *et al*., 2009
Ophiorrhiza liukiuensis Hayata	Whole plants	Okinawa, Japan	CPT 127 9-MCPT 126 10-MCPT 30	Kitajima *et al*., 2005
Ophiorrhiza mungos Linn.	Entire plant	Colombo, Ceylan	CPT 12 9-MCPT 10.41	Tafur *et al*., 1976
	Shoots	Kerala, India	CPT 96 9-MCPT traces	Roja, 2006
	Roots		CPT 176 9-MCPT traces	
Ophiorrhiza pumila Champ.	Leaves	Japan	CPT 300–400	Saito *et al*., 2001
	Young roots		CPT 1000	
	Hairy roots		CPT 1000	
	Entire plant	Kagoshima, Japan	CPT 300–510 9-MCPT 70–140 Chaboside 300–690	Yamazaki *et al*., 2003
	Hairy roots		CPT 240	
	Cell cultures	Japan	None	Kitajima *et al*., 1998
Ophiorrhiza rugosa	Shoots	Kerala, India	CPT 10 9-MCPT traces	Roja, 2006
	Roots		CPT 20 9-MCPT traces	
Ophiorrhiza trichocarpon Blume	Whole plant	Satun, Thailand	CPT MDOCPT	Klausmeyer *et al*., 2007
Pyrenacantha klaineana Pierre ex Exell & Mendoca	Stems	Ankasa Game Reserve, Ghana	CPT 4.8 9-MCPT 1.6	Zhou *et al*., 2000

CPT = camptothecin; ACPT = *O*-acetyl-CPT; dCPT = (*20S*)-18,19-dehydro CPT;

10-HCPT = 10-hydroxy CPT; MACPT = 9-methoxy-20-*O*-acetyl-CPT; 9-MCPT = 9-methoxy CPT;

7-MCPT = 7-methoxy CPT, 10-MCPT = 10-methoxy CPT; DPMI = deoxy pumiloside;

MDOCPT = 9,10-methylenedioxy-(*20S*)-CPT

2.2 The genus *Ophiorrhiza*

2.2.1 Botanical aspects of *Ophiorrhiza*

Ophiorrhiza L. is a predominantly herbaceous genus and comprising about 400 species (Schanzer, 2005). The genus *Ophiorrhiza* belongs to the family Rubiaceae, the subfamily Rubioideae, the tribe Ophiorrhizeae. This genus is distributed from eastern India to the western Pacific and from southern China to northern Australia. About 44 species have been recorded from Thailand (Puff, 2007). Since the systematic knowledge of this genus is still inadequate, recent regional revisions are available only for marginal parts of its area: the Pacific, China, and the Indian subcontinent. Many herbarium collections of *Ophiorrhiza* for the coming treatment for Flora of Thailand revealed a number of specimens that could not be assigned to any of the species described so far.

The characteristics of the genus *Ophiorrhiza* were described in Rubiaceae of Thailand (Chamchumroon, Chayamarit and Puff, 2005) and Flora of Thailand: Rubiaceae (Puff, 2007). *Ophiorrhiza* L. is distinctly herbaceous plant; prostrate or erect perennial or annual, uncommonly subshrubby; stem sometimes succulent. Leaves are opposite (decussate), rarely slightly anisophyllous, and blades mostly membranous; leaf-like stipules entire or fimbriate. Inflorescence terminal often consist of helicoid or scorpioid cymes, sometimes congested and head-like; bracts well developed or absent. Flowers are 5-merous, hermaphrodite, heterostylous or isostylous, sometimes cleistogamous; calyx lobes often very small; corolla typically narrowly infundibular to hypocrateriform, tube inside glabrous or hairy, base of tube occasionally distinctly bulbous, lobes valvate in bud, ascending to reflexed in open flowers; stamen inserted at different levels in corolla tube (usually high up in short-styled, but in the low part in long-styled morphs), filaments long or short, anthers included or exerted; style filiform, with 2-lobed stigma, included or exerted (above the level of the anthers in long-styled morphs but not necessarily exserted); ovary 2-celled, each locule with numerous ovules on placenta attached to lower half of septum; roof of ovary with conspicuous 2-lobed disk. Fruits are strongly laterally compressed, capsular, obcordate, usually much broader than high, loculicidally dehiscent; seeds very numerous, small, rhomboid, vivipary sometimes observed (Tan and Rao, 1981).

2.2.2 Phylogenetic systematic of *Ophiorrhiza*

According to the taxonomically complex and the high morphological variability of the genus *Ophiorrhiza* (Chou *et al*., 2006; Darwin, 1976; Kudoh *et al*., 2001), few studies have attempted to resolve this taxonomic problem using molecular phylogenetic systematics (Nakamura *et al*., 2006, 2007, 2010). Several DNA regions, such as ITS, *atp*B-*rbc*L and *trn*K*/mat*K have been utilized to determine the species and the varieties within *Ophiorrhiza.* Four species of *Ophiorrhiza*, which comprise all species of this genus distributed in Taiwan and Japan, were examined. The molecular phylogenetic analyses conducted with these four species revealed two major clades in the trees (Figure 2.5). The genus *Hayataella* was considered to be synonymous with *Ophiorrhiza* and also included in the *Ophiorrhiza* clade.

In plant chloroplasts, the tRNA^{Lys}(UUU) gene (*trn*K) contains a intron which encodes the *mat*K open reading frame (ORF). The *trn*K intron and its encoded *mat*K ORF have generated substantial interest in the fields of plant evolution and molecular biology (Hausner *et al*., 2006; Hilu *et al*., 2003). Based on assessments of recoverability, sequence quality, and levels of species discrimination, *rbc*L and *mat*K genes were recommended to be used as the plant barcode to identify specimens and contribute toward the discovery of overlooked species of land plants (CBOL Plant Working Group, 2009).

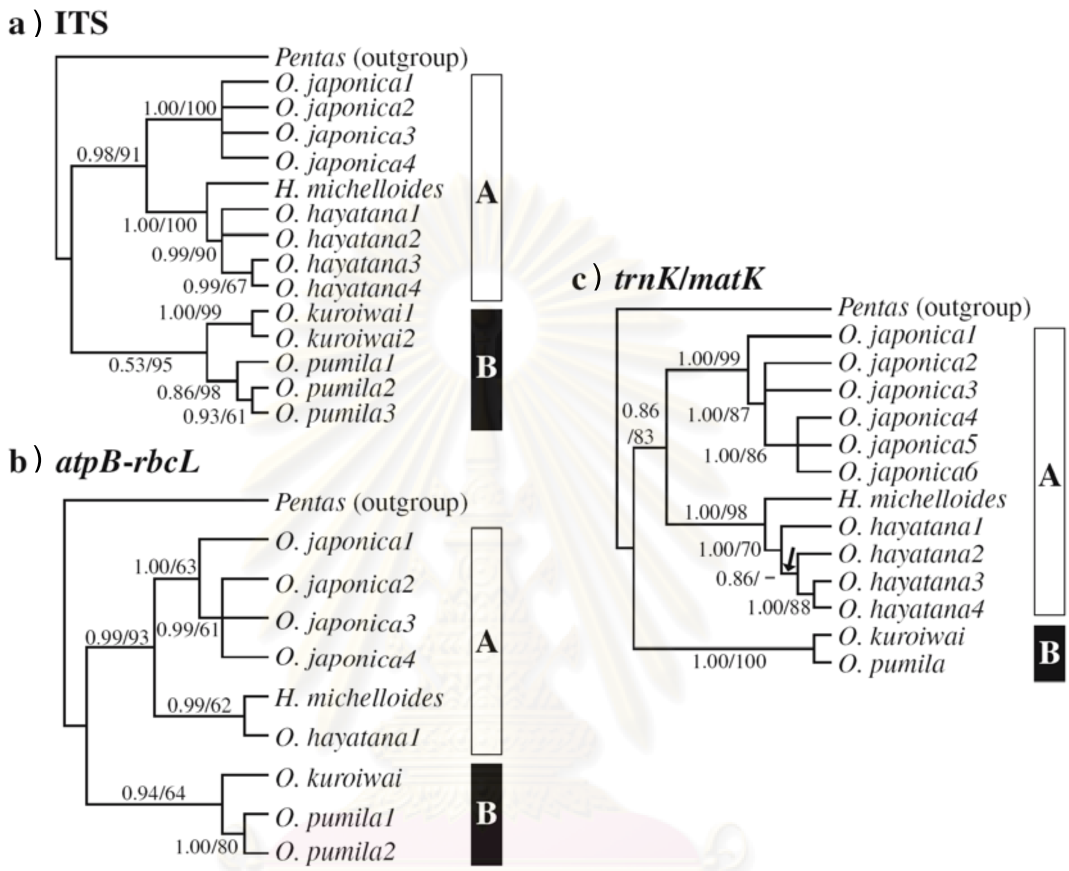


Figure 2.5 Bayesian trees based on a) ITS, b) *atp*B-*rbc*L, and c) *trn*K/*mat*K regions of *Ophiorrhiza* spp. The topologies of the strict consensus trees of the most parsimonious trees were consistent with the Bayesian trees in each region, except the branch indicated by an arrow in tree c, which collapsed in the strict consensus tree. Numerals indicate Bayesian posterior probabilities (left) and bootstrap percentages in the maximum parsimony analyses (right) (source: Nakamura *et al*., 2006).

2.2.3 Chemical constituents of *Ophiorrhiza*

CPT was isolated and identified from entire plant of *O. mungos* (Tafur *et al*., 1976). Later, publications on isolation of the constituents from the other *Ophiorrhiza* spp. were reported including *O. fistipula*, *O. kuroiwae, O. liukiuensis, O. pumila, O. rugosa*, and *O. trichocarpon* (Table 2.1). CPT, its derivatives, and CPT-related alkaloids isolated from these plants are summarized in Table 2.2.

At the cellular level, the previous study of the distribution of CPT in different tissues of *O. pumila* suggested that the highest levels of CPT accumulation were found in flower buds, young leaves, and roots (Yamazaki *et al*., 2003). At the subcellular level, the study of hairy roots of *O. pumila* indicated that CPT is biosynthesized at the endoplasmic reticulum and transported to accumulate in a vacuole *via* vesicles (Sirikantaramas *et al*., 2007) (Figure 2.6). It has been proposed that the lipophilic form of alkaloids is protonated to the hydrophilic form in the acidic conditions of the vacuole (Matile, 1976). As a result, the protonated form cannot move across a tonoplast membrane. After CPT is formed, it is partly stored in the vacuole, a site for avoiding self-toxicity. However, some part of the compound can diffuse freely inside the cytoplasm before excretion due to its lipophilicity (Figure 2.6). The cytoplasmic CPT might be expected to interfere with TopI in the nucleus.

Table 2.2 Chemical constituents and structures found in *Ophiorrhiza* spp.

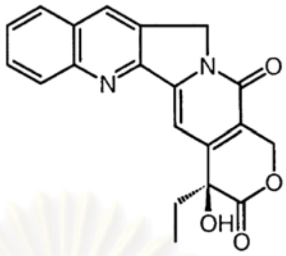
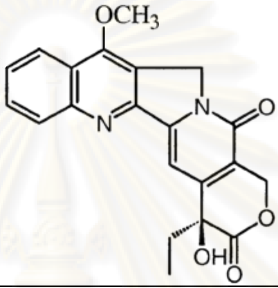
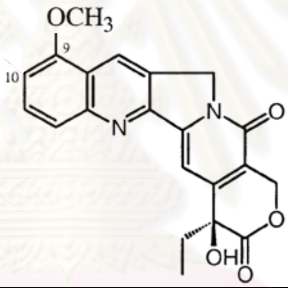
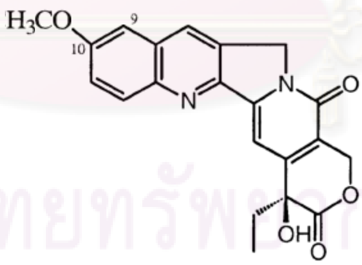
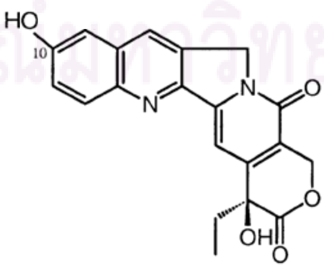
Chemical compound	Chemical structure	Reference
Camptothecin		Asano *et al*., 2009 Kitajima *et al*., 2005 Klausmeyer *et al*., 2007 Roja, 2006 Saito *et al*., 2001 Tafur *et al*., 1976 Yamazaki *et al*., 2003 Zhou *et al*., 2000
7-Methoxy CPT		Arbain *et al*., 1993
9-Methoxy CPT		Kitajima *et al*., 2005 Roja, 2006 Tafur *et al*., 1976 Yamazaki *et al*., 2003 Zhou *et al*., 2000
10-Methoxy CPT		Asano *et al*., 2009 Kitajima *et al*., 2005
10-Hydroxy CPT		Yamazaki *et al*., 2003

Table 2.2 (continued)

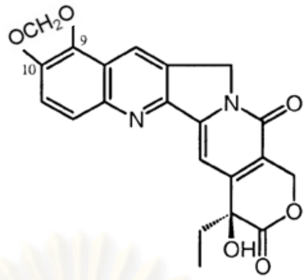
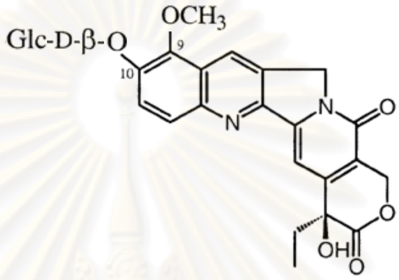
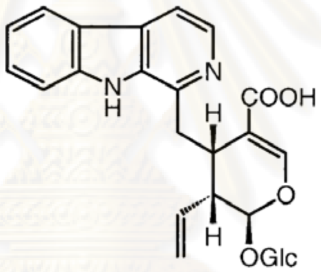
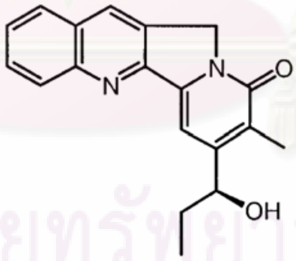
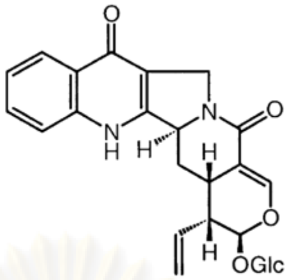
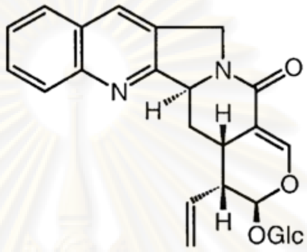
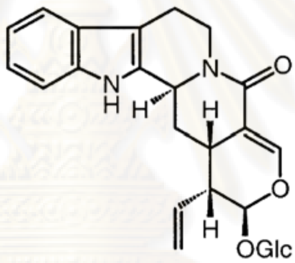
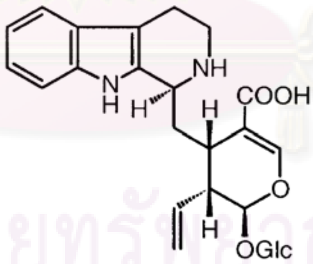
Chemical compound	Chemical structure	Reference
9,10-Methylenedioxy CPT		Klausmeyer *et al*., 2007
Chaboside		Yamazaki *et al*., 2003
Lyalosidic acid		Kitajima *et al*., 2005
Mappicine		Yamazaki *et al*., 2003

Table 2.2 (continued)

Chemical compound	Chemical structure	Reference
Pumiloside		Kitajima *et al*., 2005 Yamazaki *et al*., 2003
Deoxy pumiloside		Kitajima *et al*., 2005 Yamazaki *et al*., 2003
Strictosamide		Kitajima *et al*., 2005
Strictosidinic acid		Yamazaki *et al*., 2003

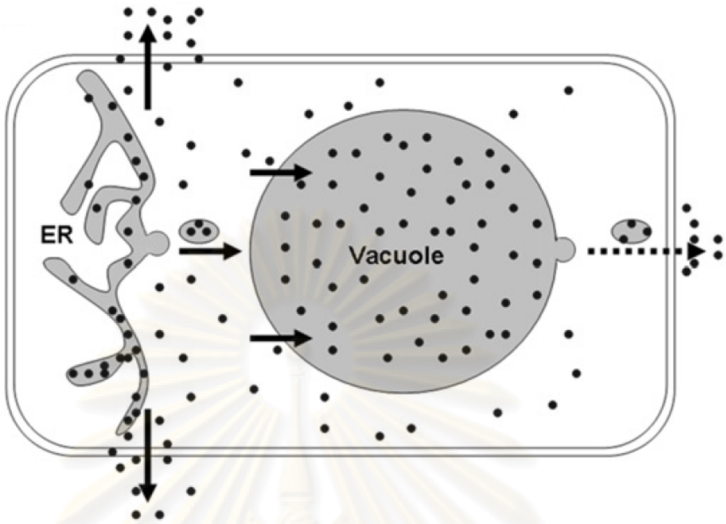


Figure 2.6 Proposed models for CPT transport, accumulation, and excretion in the hairy root cell of *O. pumila*. Dots represent CPT molecules. Black arrows indicate CPT trafficking pathways. Dots in circles represent vesicle-mediated CPT transport. Dashed arrow indicates possible outward transport from the vacuole (source: Sirikantaramas *et al*., 2007).

2.2.4 *Ophiorrhiza* distributed in Thailand

Ophiorrhiza existing in Thailand has been recorded for 44 species (Puff, 2007). Only some species have local name in Thai (Smitinand, 2001; Puff and Chayamarit, 2006). Many species of *Ophiorrhiza* were recorded as the endemic and rare plants of Thailand (Santisuk *et al*., 2006). Since Flora of Thailand (Rubiaceae) is on a process of revision, many species are still not fully resolved (Chamchumroon and Puff, 2003). The *Ophiorrhiza* spp. distributed in Thailand were described in Table 2.3.

Table 2.3 *Ophiorrhiza* spp. distributed in Thailand.

Botanical name	Local name	Distribution	Reference
O. alata Craib	ผักหลอดดอกขาว	South eastern: Chantaburi	Smitinand, 2001
O. ankae Craib	สร้อยกะจับ	Northern: Chiang Mai, Nan, Tak	Smitinand, 2001 Chamchumroon *et al*., 2005
O. brachycarpa	–	Koh Chang, Trat	Chamchumroon and Puff, 2003
O. communis Ridl.	เป็นเบรัคนาสิ	Malay-Yala	Smitinand, 2001 Chamchumroon *et al*., 2005
O. harrisiana	–	Koh Chang, Trat	Chamchumroon and Puff, 2003
O. hispidula Wall. ex G.Don	หญ้าตีนมือตุ๊ดตู่	Surat Thani	Smitinand, 2001
O. hispidula Wall. ex G.Don var. *hispidula*	–	Doi Chiang Dao, Chiang Mai	Putiyanan and Maxwell, 2007
O. kratensis Craib	กะเสิมหิน	Trat	Smitinand, 2001
O. larseniorum Schanzer	–	Peninsular: Surat Thani	Schanzer, 2005
O. longifloriformis Schanzer	–	Koh Chang, Trat	Schanzer, 2005
O. pedunculata Schanzer (*O. hispidula* B. Heyne ex Hook. f. var. *longipedunculata* Craib)	–	Northern: Mae Hong Son, Chiang Mai, Chiang Rai; South western: Thong Pha Phum, Kanchanaburi	Schanzer, 2004
O. pseudofasciculata Schanzer	–	Northern: Chang Mai, Nan, Chiang Rai, Lampang	Chamchumroon *et al*., 2005 Schanzer, 2005
O. ripicola Craib	แดงก่อนจาก	Doi Inthanon National Park, Chiang Mai	Smitinand, 2001 Chamchumroon *et al*., 2005 Puff and Chayamarit, 2007
O. rugosa	–	Koh Chang, Trat	Chamchumroon and Puff, 2003
O. schmidtiana Craib	ผักพรหมมิ	South eastern	Smitinand, 2001
O. trichocarpon Blume	ผักสามชาย	Khao Yai National Park, Northern: Chiang Mai, Chiang Rai, Phayao	Puff and Chayamarit, 2006 Chamchumroon *et al*., 2005 Schanzer, 2004
O. trichocarpon Blume var. *glabra* Schanzer	–	South eastern: Sa Kaeo	Schanzer, 2004
O. villosa Roxb	–	Western, Northern: Doi Chiang Dao, Chiang Mai	Putiyanan and Maxwell, 2007 Schanzer, 2004

2.3 Topoisomerase I enzyme (TopI)

2.3.1 Cellular role and structure of TopI

DNA topoisomerases are nuclear enzymes that make transient strand breaks in DNA to allow a cell to manipulate its topology (Osheroff, 1998). Every cell type so far examined contains DNA topoisomerases for cell growth. During DNA replication, the two strands of the DNA must become completely unlinked by topoisomerases, and during transcription, the translocating RNA polymerase generates supercoiling tension in the DNA that must be relaxed (Wang, 1996). There are two classes of topoisomerase, known as type I and type II enzyme. Those enzymes that cleave only one strand of the DNA are defined as type I (Champoux, 2001). In contrast, type II enzyme make a transient double-stranded break in DNA and pass a separate double-stranded molecule through the break.

Eukaryotic type I topoisomerase (topoisomerase I, TopI) is classified as type IB subfamily members (formerly called typeI-3'), the TopI that cleaves the DNA by becoming covalently linked to the 3' DNA terminus (Pommier *et al.*, 1998). The 91-kDa human TopI protein has been subdivided into four distinct domains (Figure 2.7). The N-terminal domain contains putative signals for the enzyme’s nuclear localization. The core domain is essential for the relaxation of supercoiled DNA; it shows a high phylogenetic conservation, particularly in the residues closely interacting with the double helix. The C-terminal domain contains the active site enzyme tyrosine, which forms a transiently covalent phosphodiester bond between TopI and the DNA (González *et al.*, 2007). Strand scission occurs through a transesterification in which a tyrosine hydroxyl group of TopI is covalently linked to the 3' phosphate of a phosphodiester bond, liberating the 5' hydroxyl to generate a strand break (Champoux, 1981) (Figure 2.8). DNA religation occurs in the following step to release TopI from the cleavage complexes and to repair TopI-mediated DNA damage.

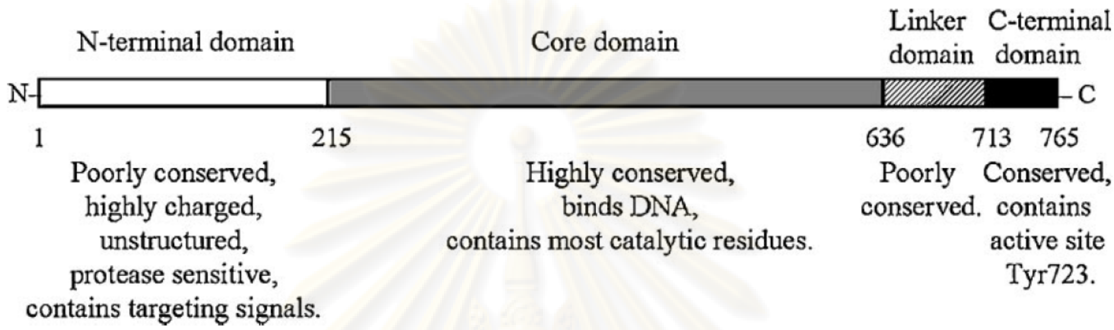


Figure 2.7 Domain structure of human TopI. Human TopI comprises an N-terminal domain (open box), a core domain (gray box), a linker domain (diagonally striped box), and a C-terminal domain (black box). The domain boundaries are based on sequence alignments, limited proteolysis studies, and the crystal structures of the protein (source: Champoux, 2001).

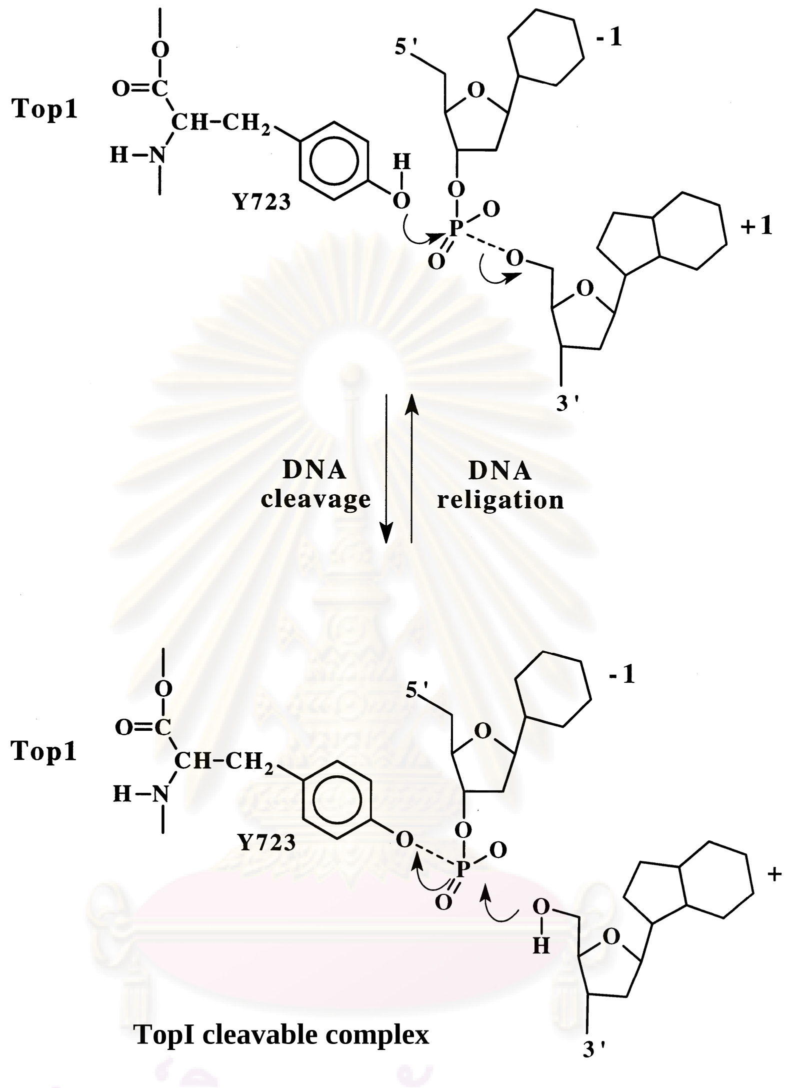


Figure 2.8 Human TopI-mediated DNA cleavage and religation. Y723 refers to the tyrosine involved in the transesterification reaction with the DNA. By convention, the bases flanking the top1 cleavage site are referred to as -1 and +1 for the bases at the 3' and 5' DNA termini, respectively (Pommier *et al.*, 1998).

2.3.2 Mechanism of TopI-targeted CPT

CPT and its derivatives exhibit antitumor activity due to their interacting with the cellular TopI (Hsiang *et al.*, 1985). This interaction damages the DNA, causing the cancer cell to be destroyed or preventing the cancer cell from growing and reproducing (Pommier, 1998). CPT are named topoisomerase "poisons" to distinguish them from conventional enzyme inhibitors. CPT does not bind to TopI alone but it stabilizes a covalent complex between TopI and the nicked DNA (Reid, Benedetti, and Bjornsti, 1998). Trapping of the cleavable complex and preventing DNA re-ligation could be poison into the cells (Figure 2.9). The collision of advancing replication forks with compound-stabilized intermediates appears to produce the cytotoxic DNA lesions that signal cell cycle arrest and cause cell death (Strumberg *et al.*, 2000). Therefore, inhibitory of TopI activity was a great harm to a cellular genome to develop a nuclear toxin that can efficiently kill cancer cells.

CPT and its derivatives have been studied as potent inhibitors of replication, transcription, and packing of double stranded DNA-containing adenoviruses, papovaviruses, and herpesviruses, and the single-stranded DNA-containing autonomous parvoviruses (Pantazis *et al.*, 1999). CPT was also shown to have promising activity against parasitic trypanosomes and Leishmania (Bodley and Shapiro, 1995). CPT, 9-MCPT, 10-MCPT and 9,10-methylenedioxy CPT showed functional inhibition of the hypoxia-inducible factor 1α (HIF-1α), a master regulator of the cancer cell's ability to survive under oxygen deprivation (Klausmeyer *et al.*, 2007). Hence, these drugs may have other desirable activities against solid tumors that are independent of TopI poisoning.

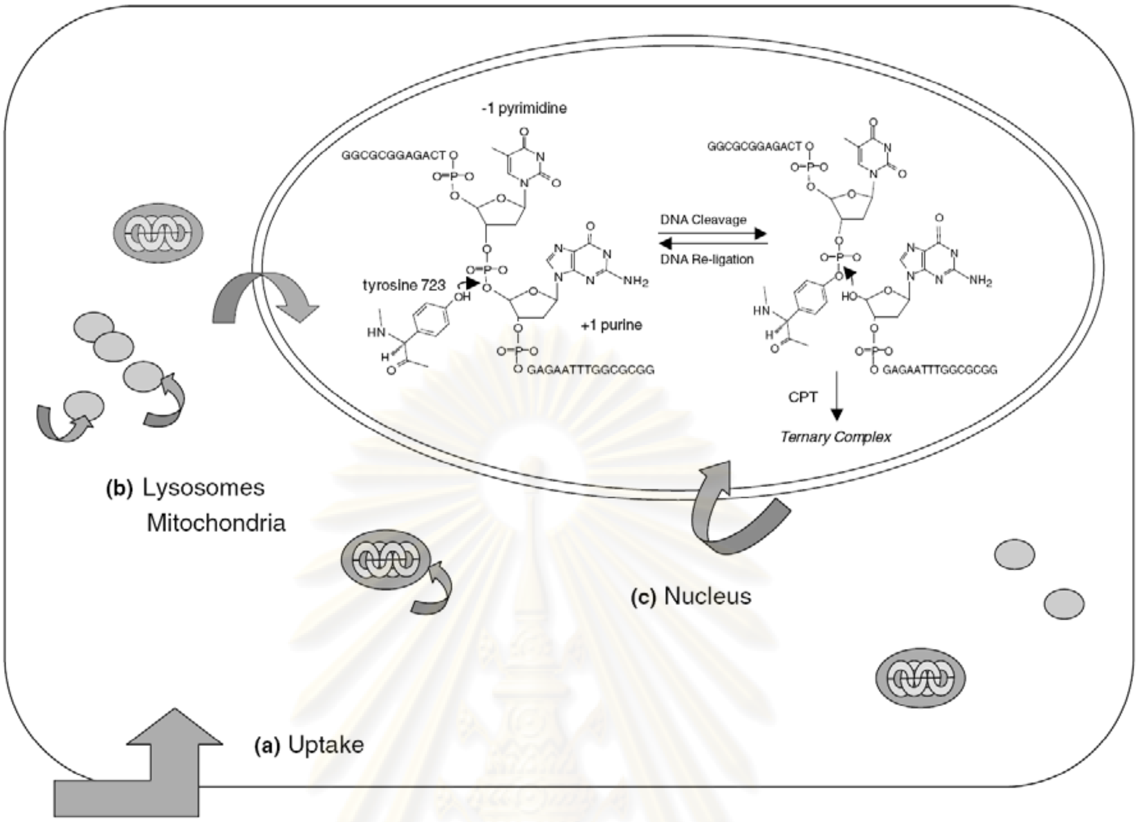


Figure 2.9 Mechanism of action of CPT. Relevant events in determining the cytotoxic potency of CPT and its derivatives are: (a) uptake, (b) lysosomal or mitochondrial sequestration and, (c) nuclear localization and stabilization of the "cleavable complex" (source: Lorence and Nessler 2004).

The selective cytotoxicity of CPT for tumor cells depends on the level of TopI activity and the rate of repair of the replication induced double-strand break (Gupta, Fujimori, and Pommier, 1995). Cell lines which have high levels of TopI enzyme are hypersensitive to CPT-induced cytotoxicity, such as colon adenocarcinoma, ovarian and esophageal carcinoma. Although TopI is expressed throughout the cell cycle, cells in S-phase are 1000 times more sensitive than cells in G_1 or G_2- phase reflecting the need for DNA replication for drug efficacy (Del Bino, Lassota, and Darzynkiewic, 1991).

2.3.3 TopI of CPT-resistant cancer cell

CPT analogs have proven to be effective anticancer drugs; however, resistance is still a critical clinical problem (Rasheed and Rubin, 2003). There are several different ways by which resistance to CPT could hamper the treatment in cancer patients, such as reduced drug uptake, overexpression of P-glycoprotein, and mutation in *Top*I gene which results in altered TopI structure or function leading to decrease in enzyme activity and ability of CPT to stabilize the cleavable complex (Gupta *et al*., 1995). Previous study found that normal cell can express both wild-type and mutant TopI, whereas CPT-resistant cancer cells express mutant TopI only (Wang *et al*., 1997). Besides, the cancer cells expressing mutant TopI contain similar level and activity of TopI, compared with the normal cell.

Most of the mutations are contained in well-conserved regions of the TopI, the core and the C-terminal domains, which are critical for catalytic activity and interaction with CPT (Gupta *et al*., 1995). The three-dimensional structure of human TopI suggests four regions that can be mutated to produce a CPT-resistant TopI (Redinbo *et al*., 1998) (Table 2.4). These residues may play a structural role in the proper packing of the C-terminal and core domains and may affect CPT efficacy by interfering with the positioning of catalytic or CPT-binding residues. Other residues and substitutions in human TopI which confer resistance in CPT-resistant cancer cells were summarized in Table 2.4. Some of these point mutations in human TopI were also found at the corresponding position in the CPT-resistant yeast and vaccinia viruses. For example, N726S and N726D substitutions in CPT-resistant yeast TopI are at the corresponding 722 position of human TopI (Fertala *et al*., 2000).

Table 2.4 Point mutations at residues of CPT-resistant human TopI.

No.	Residues and substitution	Reference
1	F361 to M370 region	Redinbo *et al*., 1998
2	F361S	Chrencik *et al*., 2004 Rubin *et al*., 1994
3	G363C	Benedetti *et al*., 1993
4	R364H	Urasaki *et al*., 2001
5	M370T	Gupta *et al*., 1995
6	G503S	Pommier *et al*., 1998
7	K532 to S534 region	Redinbo *et al*., 1998
8	D533N D533G	Rasheed and Rubin, 2003 Tamura *et al*., 1990
9	D583G	Pommier *et al*., 1998
10	G717 to N722 region	Redinbo *et al*., 1998
11	G717V	Wang *et al*., 1997
12	L721R	Gupta *et al*., 1995
13	N722S N722S, N722A	Chrencik *et al*., 2004 Gupta *et al*., 1995
14	Y723F	Woo *et al*., 2002
15	I725R	Rasheed and Rubin, 2003
16	N726S/A	Woo *et al*., 2002
	Y727F	Woo *et al*., 2002
17	T729A T729 T729I	Kubota *et al*., 1992 Redinbo *et al*., 1998 Wang *et al*., 1997
18	G737S	Rasheed and Rubin, 2003

2.3.4 TopI of CPT-producing plants

Many CPT-producing plants, including *C. acuminata*, *O. pumila* and *O. liukiuensis,* have TopI enzymes with several point-mutations that confer resistance to CPT (Sirikantaramas *et al*., 2008). Three amino acid substitutions that contribute to CPT resistance were identified: N421K, L530I, and N722S (numbered according to human TopI). The proposed amino acids that are involved in catalytic function or affect CPT binding are shown in Figure 2.10. Asp-533 and Ser-722 directly bind to CPT. Other residues that indirectly bind to CPT are important for the proper positioning of Asp-533 and Ser-722.

The crystal structure of human TopI in a covalent complex with duplex DNA containing topotecan suggests that mutations of these amino acids would disrupt drug binding (Sirikantaramas *et al*., 2008). These mutations suggest the effect of an endogenous toxic metabolite on the evolution of the target cellular component. A phylogenetic tree based on *Top*I sequences of CPT-producing and non-CPT-producing organisms revealed a close relationship of CPT-producing plants, *O. pumila* and *O. liukiuensis,* and a separation of *O. japonica*, a non-CPT-producing plant (Figure 2.11).

	Residue number																		
	532	590	632	723	361	363	364	367	418	420	421	502	503	530	533	619	722	725	729
	Catalytic function				Direct/Indirect CPT binding														
Hs	K	R	H	Y	F	G	R	H	E	I	Q	V	G	L	D	Y	N	D	T
At	K	R	H	Y	F	G	R	H	D	I	N	V	G	L	D	Y	N	D	T
Cr	K	R	H	Y	F	G	R	H	D	I	N	V	G	L	D	Y	N	D	T
Oj	K	R	H	Y	F	G	R	H	D	I	N	V	G	L	D	Y	N	D	T
Op	K	R	H	Y	F	G	R	H	D	V	N	V	G	I	D	Y	S	D	T
Ol	K	R	H	Y	F	G	R	H	D	I	N	V	G	I	D	Y	S	D	T
Ca	K	R	H	Y	F	G	R	H	D	I	K	V	G	L	D	Y	S	D	T

Figure 2.10 Amino acid polymorphism in TopI of CPT-producing plants and non-producing organisms. The numbering is based on human TopI. The red characters indicate the amino acid substitutions in TopI of CPT-producing plants. *Hs*, *Homo sapiens*; *At*, *Arabidopsis thaliana*; *Cr*, *Catharanthus roseus*; *Oj*, *Ophiorrhiza japonica*; *Op*, *Ophiorrhiza pumila*; *Ol*, *Ophiorrhiza liukiuensis*; *Ca*, *Camptotheca acuminata* (source: Sirikantaramas *et al.*, 2008).

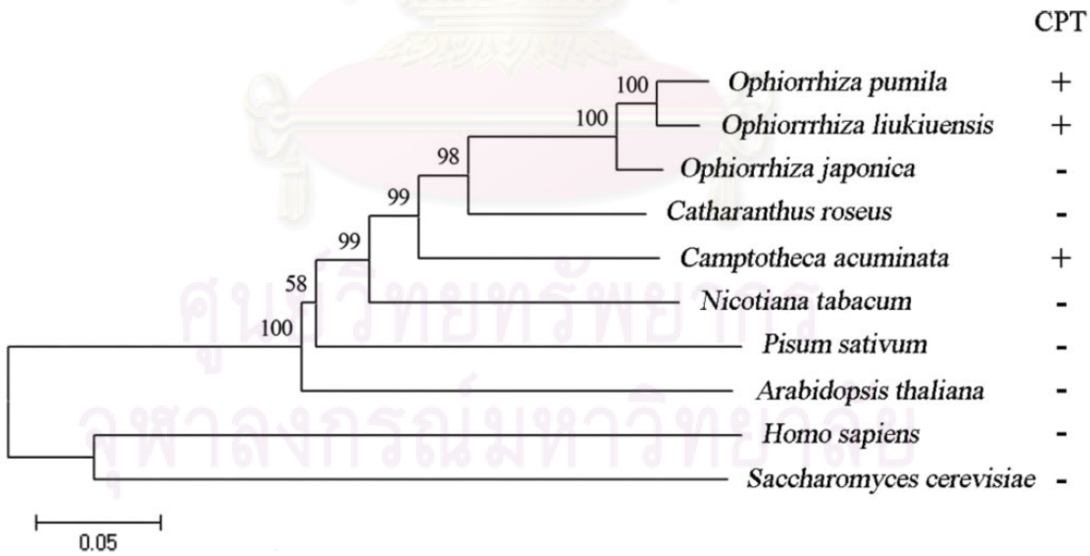


Figure 2.11 Neighbor-joining tree of *TopI* sequences of plants, yeast, and humans. The numbers indicate bootstrap values. CPT production is indicated by “+” or “–” (source: Sirikantaramas *et al.*, 2008).

2.4 Definition and previous studies of coevolution

The theory of coevolution was briefly described in the Origin of Species (Darwin, 1859) that populations evolve over the course of generations through a process of natural selection, and presented a body of evidence that the diversity of life arose through a branching pattern of evolution and common descent. Currently, the concept of coevolution (covariation/correlated mutation) is the change of a biological object triggered by the change of a related object (Yip *et al.*, 2008). Coevolution can occur at multiple levels of biology: it can be as microscopic as correlated mutations between amino acids in a protein, or as macroscopic as covarying traits between different species in an environment.

Species-level coevolution includes the evolution of a host species and its parasites, for instance, the coevolution between the resistance gene and the virulence gene of host plants and their fungal pathogens (Frank, 1993). The interdependent plant-vertebrate seed dispersal systems suggest the coevolution of plant-animal (Herrera, 1985). Genetic coevolution includes the coding genes of some interacting proteins are preserved or eliminated together in new species (Pellegrini *et al.*, 1999), or have similar phylogenetic trees (Goh *et al.*, 2000). At the amino acid level, some residues under physical or functional constraints exhibit correlated mutations (Gloor *et al.*, 2005; Socolich *et al.*, 2005; Süel *et al.*, 2003). A corresponding mutation in two position of the multiple sequence alignment may propose residues which are functionally or structurally important, or possibly key sites of interaction between the protein and its substrate (Martin *et al.*, 2008).

The mutation in TopI of CPT-producing plants suggests the coevolution between CPT biosynthetic pathway and self-resistance mechanism (Sirikantaramas, Yamazaki, and Saito, 2009). The *Ophiorrhiza* genus is composed of both CPT-producing and non-producing species. This provides a great benefit to follow the coevolution of the CPT biosynthetic pathway and TopI mutation as a self-resistance mechanism.

CHAPTER III

THE PRODUCTION OF CAMPTOTHECIN

In this study, CPT productions by Thai *Ophiorrhiza* plants were explored for the first time. Plant specimens were collected from various locations of Thailand in order to find numerous species for coevolution study and for novel alternative sources of CPT. The methanol extracts of plant samples were analyzed using HPLC/DAD/ESI/MS. Standard solutions of CPT, CPT derivatives, and chemical compounds involved in CPT biosynthesis pathway were also analyzed using the same method.

3.1 Materials and Methods

3.1.1 Plant specimen collection

Ophiorrhiza species distributed in Thailand were collected from previously reported provinces (Chamchumroon and Puff, 2003; Schanzer, 2004, 2005), including Trat, Kanchanaburi, Chiang Mai, Chaing Rai and Lampang. Phuket, Chantaburi and Nakorn Ratchasima were also investigated for *Ophiorrhiza* plants. Numbers of specimens collected from each locality depended on their abundance in the native habitats and their different features. The collected plants were then cultivated in the Medicinal Plant Garden of the Faculty of Pharmaceutical Sciences, Chulalongkorn University. All specimens were identified to species-level by Ivan A. Schanzer, Ph.D. from Herbarium Main Botanical Garden, Russia (Table 3.1). The specimens were deposited at the Museum of Natural Medicines, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok and Queen Sirikit Botanical Garden Herbarium, Chiang Mai Province, Thailand. Some of fresh *Ophiorrhiza* specimens are shown in appendix A.

Table 3.1 Eight *Ophiorrhiza* species in this study, their voucher numbers, and localities.

Species	Specimen No.	Voucher No.	Locality (area, province)	Part of Thailand
O. fucosa Hance	ophi 32-33	BH-090519-032, 033	Khao Soi Dao Nua, Chantaburi	SE
	ophi 34	VV-090523-034	Khao Soi Dao Tai, Chantaburi	SE
	ophi 64	VV-090924-064	Phlio National Park, Chantaburi	SE
	ophi 65	VV-090924-065	Khlong Narai waterfall, Chantaburi	SE
O. harrisiana B. Heyne ex Hook. f.	ophi 14-27	VV-090502-014-027	Than Mayom waterfall, Ko Chang, Trat	SE
O. pedunculata Schanzer (*O. hispidula* Wall. ex G.Don var. *longipedunculata* Craib)	ophi 28-31	VV-090925-028-031	Erawan National Park, Kanchanaburi	SW
	ophi 41-42	VV-090708-041	Mok Fa waterfall, Chiang Mai	N
	ophi 47	VV-090806-047	Tard Mok waterfall, Chiang Mai	N
	ophi 57	ISC-090919-057	Chae Son National Park, Lampang	N
	ophi 60	ISC-090919-060	Mae Yom National Park, Lampang	N
	ophi 66-67	VV-090925-066-067	Erawan National Park, Kanchanaburi	SW
O. plumbea Craib	ophi 1-13	VV-090421-001-013	Bangpae waterfall, Phuket	WS
O. pseudofasciculata Schanzer	ophi 37	BH-090726-037	Doi Suthep-Pui National Park, Chiang Mai	N
	ophi 54	ISC-090920-054	Chae Son National Park, Lampang	N
	ophi 62	ISC-090918-062	Khun Kon waterfall, Chiang Rai	N
	ophi 63	ISC-090917-063	Doi Pha Hom Pok National Park, Chiang Mai	N

Table 3.1 (continued)

Species	Specimen No.	Voucher No.	Locality (area, province)	Part of Thailand
O. ridleyana Craib	ophi 52-53	VV-090806-052, 053	Queen Sirikit Botanical Garden, Chiang Mai	N
	ophi 55-56	ISC-090919-055, 056	Chae Son National Park, Lampang	N
	ophi 61	ISC-090913-061	Mae Yom National Park, Lampang	N
O. trichocarpon Blume var. *glabra* Schanzer	ophi 44-45	VV-090806-044-045	Mok Fa waterfall, Chiang Mai	N
	ophi 46	VV-090806-046	Tard Mok waterfall, Chiang Mai	N
	ophi 51	VV-090808-051	Queen Sirikit Botanical Garden, Chiang Mai	N
	ophi 58	ISC-090919-58	Chae Son National Park, Lampang	N
	ophi 68	VV-090926-068	Khao Yai National Park, Nakorn Ratchasima	NE
Ophiorrhiza sp. 35	ophi 35-36	VV-090523-035, 036	Rambhai Barni Rajabhat University, Chantaburi	SE

3.1.2 Methanol extraction

According to the previous study of alkaloid accumulation in *O. pumila* (Yamazaki *et al.*, 2003), young leaves and roots which contained high amount of CPT were used as materials for CPT analysis in this study. Specimens from different localities of each species were examined for CPT analysis. Young leaves and roots of *Ophiorrhiza* samples were freeze-dried and ground using liquid nitrogen with Multi-beads shocker® (Yasui kikai Co, Japan) at 1500 rpm for 10 s and stored in a vacuum desiccator overnight. The dried specimens were weighed and extracted with MeOH 10 mg/1 mL (dry weight), following by ultrasonication for 30 min and kept at 4°C overnight. The crude extracts were centrifuged at 15000 g for 10 min. The supernatants were filtered through 0.45-μm filters (Millipore Co, USA) and analyzed by Agilent 1100 series HPLC/DAD/ESI/MS (Palo Alto, CA, USA).

3.1.3 HPLC-MS analysis

HPLC analyses were carried out using a Mightysil RP-18 column (5 mm, 250 mm × 4.6 mm, Kanto Chemical Co Inc, Japan) at a flow rate of 0.8 mL/min. Elution gradient was as follows: 0–35 min linear gradient from solvent A [H_2O:HOAc:MeOH (79.8:0.2:20)] to solvent B [H_2O:HOAc:MeOH (9.975:0.025:90)], 35–40 min isocratic at 100% of solvent B. Each examined sample was analyzed three times. All samples in each time analysis were randomly injected into a system. The HPLC-MS system was set to 4°C. The standard compounds in powder form were dissolved in MeOH to prepare standard solution (Appendix B). Standard solutions of CPT, 9-methoxy camptothecin (9-MCPT), 10-hydroxy camptothecin (10-HCPT), pumiloside (PMI), deoxy pumiloside (DPMI), chaboside and mappicine were analyzed using the same method (Yamazaki *et al.*, 2003). CPT and other compounds in *Ophiorrhiza* samples were identified by their MS spectra, UV spectra at 254-nm detection and retention times compared with those of standard compounds. The contents of compounds detected in each sample were calculated. Average contents and standard deviation (SD) of triplicate analyses were calculated.

3.2 Results

3.2.1 Species identification

Ophiorrhiza specimens collected in this study were identified into eight species (Table 3.1). Only one sample: *Ophiorrhiza* sp. 35, from Rambhai Barni Rajabhat University, Chantaburi Province, could not be determined a specific species. According to the morphological identification, Ophi 62 was not assured being *O. pseudofasciculata* (Appendix A, Figure A7).

3.2.2 HPLC-MS results

HPLC-DAD chromatograms monitored at 254 nm, UV spectra and mass spectra of standard compounds were shown in Appendix B. CPT, 9-MCPT, 10-HCPT, PMI, and DPMI were detected in the samples (Table 3.2). Chaboside and mappicine were not found in any samples. The average contents of detected compounds of five species, *O. fucosa*, *O. harrisiana*, *O. plumbea*, *O. ridleyana* and *Ophiorrhiza* sp. 35, were calculated (Figure 3.1, 3.2, 3.4–3.6).

HPLC-DAD chromatograms of some samples, including Ophi 64 (*O. fucosa*), revealed a peak at a retention time approximately 26.4 min eluted earlier than 9-MCPT peak at 26.9 min (Figure 3.8). This compound had UV and mass spectrum patterns similar to those of 9-MCPT. Thus, it was named 9-MCPT analog and was calculated for the content by comparing with 9-MCPT standard. The average contents of 9-MCPT analog in five *Ophiorrhiza* spp. were shown in Figure 3.3.

From eight species of collected *Ophiorrhiza*, CPT was found in the root extracts of four species: *O. fucosa*, *O. harrisiana*, *O. ridleyana* and *O. plumbea* and in the leaf extract of one species, *O. harrisiana*. 9-MCPT and 9-MCPT analog were detected in CPT-detected species and also in the leaf extract of *Ophiorrhiza* sp. 35. 10-HCPT was detected only in the root and leaf extracts of Ophi 56 (*O. ridleyana*). PMI and DPMI were detected in four CPT-detected species. Average contents of CPT and CPT derivatives in each species were calculated (Figure 3.7). The highest amounts of CPT and 9-MCPT analog were detected in the leaf extracts of *O. harrisiana*. The highest amounts of 9-MCPT was detected in the root extract of *O. harrisiana*.

Table 3.2 HPLC-MS results of compounds detected in the leaf (L) and root (R) extracts of eight *Ophiorrhiza* spp.

Species	Locality (area, province)	Specimen No.	Voucher No.	CPT		9-MCPT		9-MCPT analog		10-HCPT		PMI		DPMI	
				L	R	L	R	L	R	L	R	L	R	L	R
O. fucosa	Khao Soi Dao Nua, Chantaburi	Ophi 32	BH-090519-032	–	+	–	–	–	+	–	–	–	+	–	–
	Khao Soi Dao Tai, Chantaburi	Ophi 34	VV-090523-034	–	+	–	+	+	+	–	–	–	+	–	+
	Phlio National Park, Chantaburi	Ophi 64	VV-090924-064	–	+	–	+	–	+	–	–	–	+	–	+
	Khlong Narai waterfall, Chantaburi	Ophi 65	VV-090924-065	–	+	–	+	–	+	–	–	–	+	–	+
O. harrisiana	Than Mayom waterfall, Ko Chang, Trat	Ophi 18	VV-090502-018	+	+	+	+	+	+	–	–	–	+	–	+
		Ophi 25	VV-090502-025	+	+	+	+	+	+	–	–	–	+	–	+
		Ophi 26	VV-090502-026	+	+	–	–	+	+	–	–	+	+	–	–
		Ophi 27	VV-090502-027	+	+	+	–	+	+	–	–	+	+	–	–
O. pedunculata	Erawan National Park, Kanchanaburi	Ophi 31	VV-090925-031	–	–	–	–	–	–	–	–	–	–	–	–
		Ophi 67	VV-090925-067	–	–	–	–	–	–	–	–	–	–	–	–
	Mork Fa waterfall, Chiang Mai	Ophi 41	VV-090708-041	–	–	–	–	–	–	–	–	–	–	–	–
	Jae Son National Park, Lampang	Ophi 57	ISC-090919-057	–	–	–	–	–	–	–	–	–	–	–	–
O. plumbea	Bangpae waterfall, Phuket	Ophi 3	VV-090421-003	–	+	–	+	+	+	–	–	–	+	–	+
		Ophi 4	VV-090421-004	–	+	–	+	–	+	–	–	–	–	–	–
		Ophi 6	VV-090421-006	–	+	–	+	–	+	–	–	–	+	–	+
O. pseudofasciculata	Doi Suthep-Pui National Park, Chiang Mai	Ophi 37	BH-090726-037	–	–	–	–	–	–	–	–	–	–	–	–
	Jae Son National Park, Lampang	Ophi 54	ISC-090920-054	–	–	–	–	–	–	–	–	–	–	–	–
	Khun Kon waterfall, Chiang Rai	Ophi 62	ISC-090918-062	–	–	–	–	–	–	–	–	–	–	–	–
	Doi Pha Hom Pok National Park, Chiang Mai	Ophi 63	ISC-090917-063	–	–	–	–	–	–	–	–	–	–	–	–
O. ridleyana	Queen Sirikit Botanical Garden, Chiang Mai	Ophi 52	VV-090806-052	–	–	–	–	–	–	–	–	–	–	–	–
	Jae Son National Park, Lampang	Ophi 55	ISC-090919-055	–	+	+	+	–	+	–	–	–	+	–	+
		Ophi 56	ISC-090919-056	–	+	+	+	–	+	+	+	–	+	–	+
	Mae Yom National Park, Lampang	Ophi 61	ISC-090913-061	–	+	+	+	–	–	–	–	–	+	–	+
O. trichocarpon var. *glabra*	Queen Sirikit Botanical Garden, Chiang Mai	Ophi 51	VV-090808-051	–	–	–	–	–	–	–	–	–	–	–	–
	Jae Son National Park, Lampang	Ophi 58	ISC-090919-058	–	–	–	–	–	–	–	–	–	–	–	–
	Khao Yai National Park, Nakorn Ratchasima	Ophi 68	VV-090926-068	–	–	–	–	–	–	–	–	–	–	–	–
Ophiorrhiza sp. 35	Rambhai Barni Rajabhat University, Chantaburi	Ophi 35	VV-090523-035	–	–	+	+	–	–	–	–	–	–	–	–
		Ophi 36	VV-090523-036	–	–	+	+	–	–	–	–	–	–	–	–

'+' detected

'–' non-detected

37

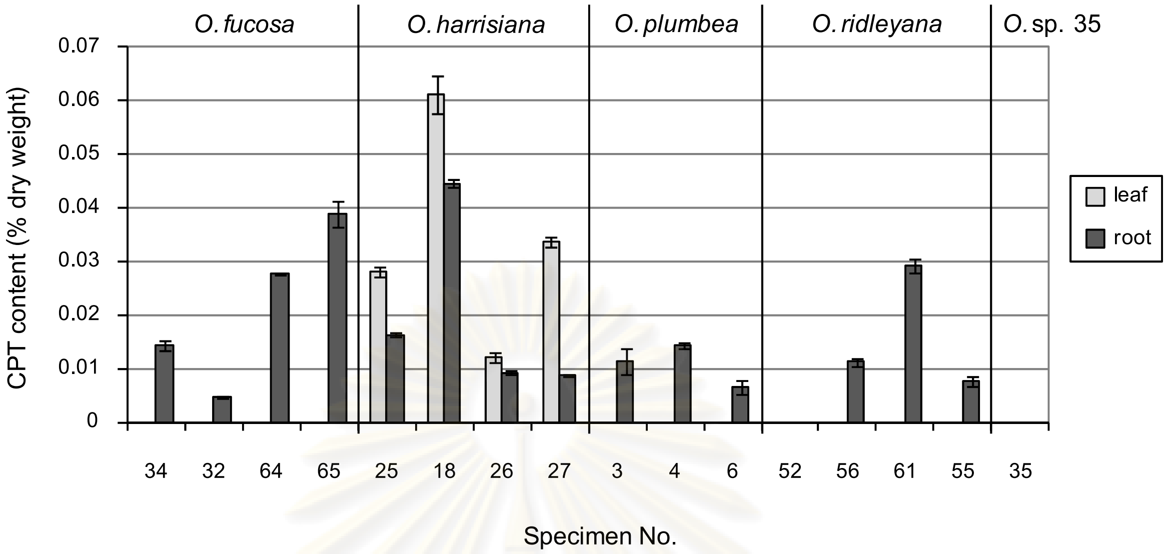


Figure 3.1 Camptothecin content (% dry weight) in the leaf and root extracts of each *Ophiorrhiza* samples. Each bar represents the mean ± SD of triplicate analyses.

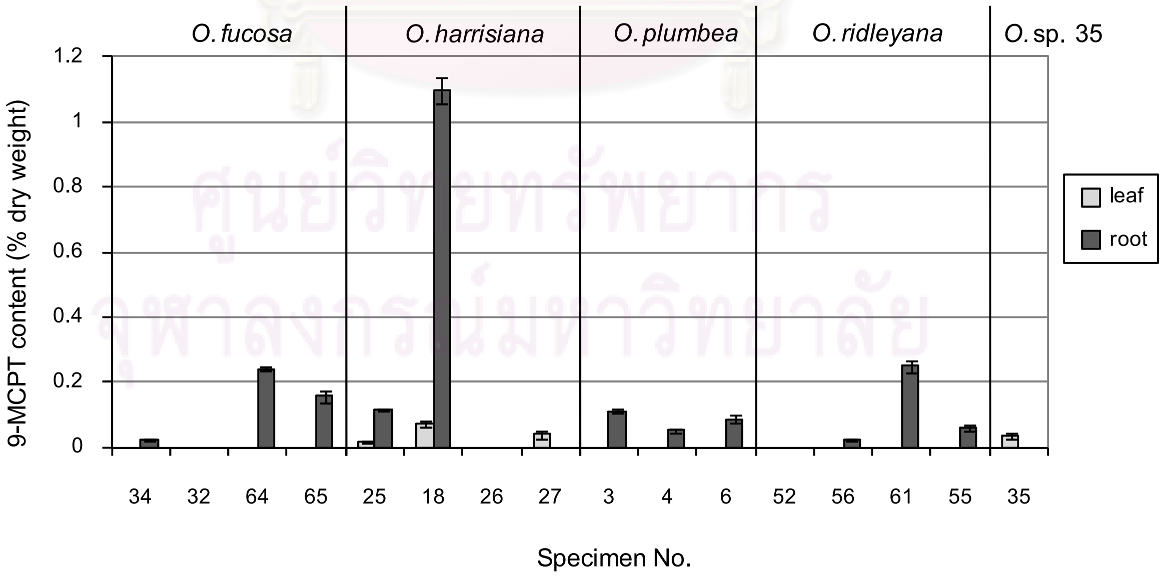


Figure 3.2 9-methoxy camptothecin content (% dry weight) in the leaf and root extracts of each *Ophiorrhiza* samples. Each bar represents the mean ± SD of triplicate analyses.

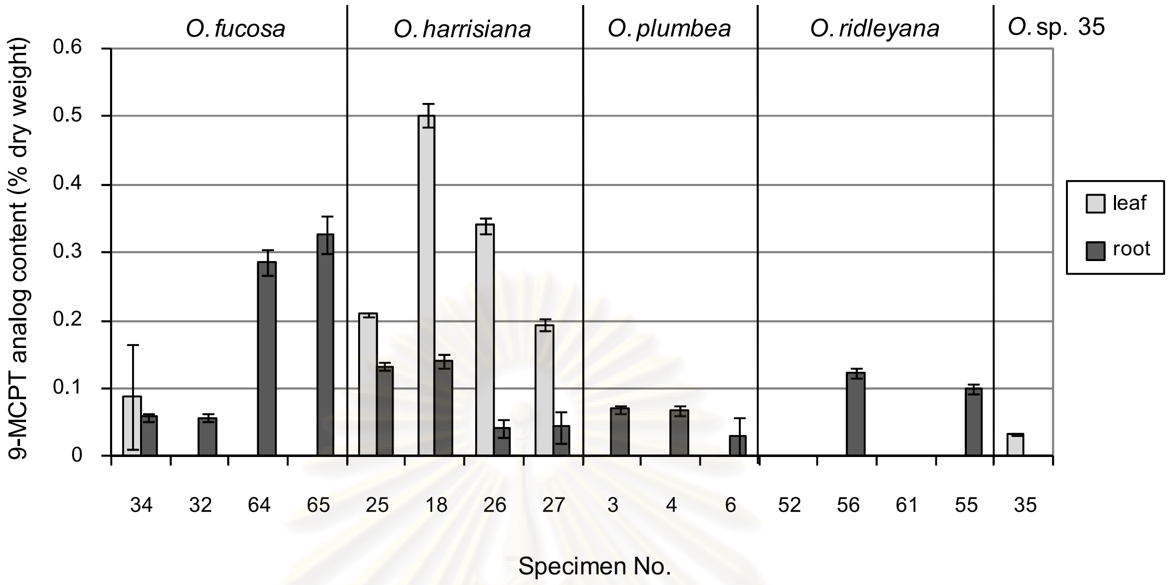


Figure 3.3 9-methoxy camptothecin analog content (% dry weight) in the leaf and root extracts of each *Ophiorrhiza* samples. Each bar represents the mean ± SD of triplicate analyses.

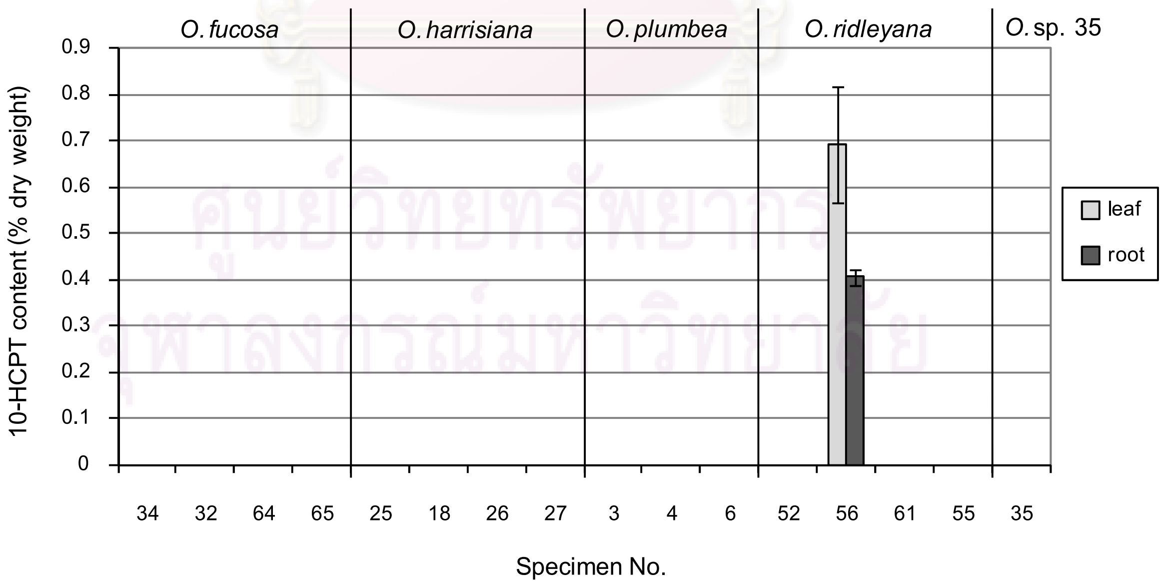


Figure 3.4 10-hydroxy camptothecin content (% dry weight) in the leaf and root extracts of each *Ophiorrhiza* samples. Each bar represents the mean ± SD of triplicate analyses.

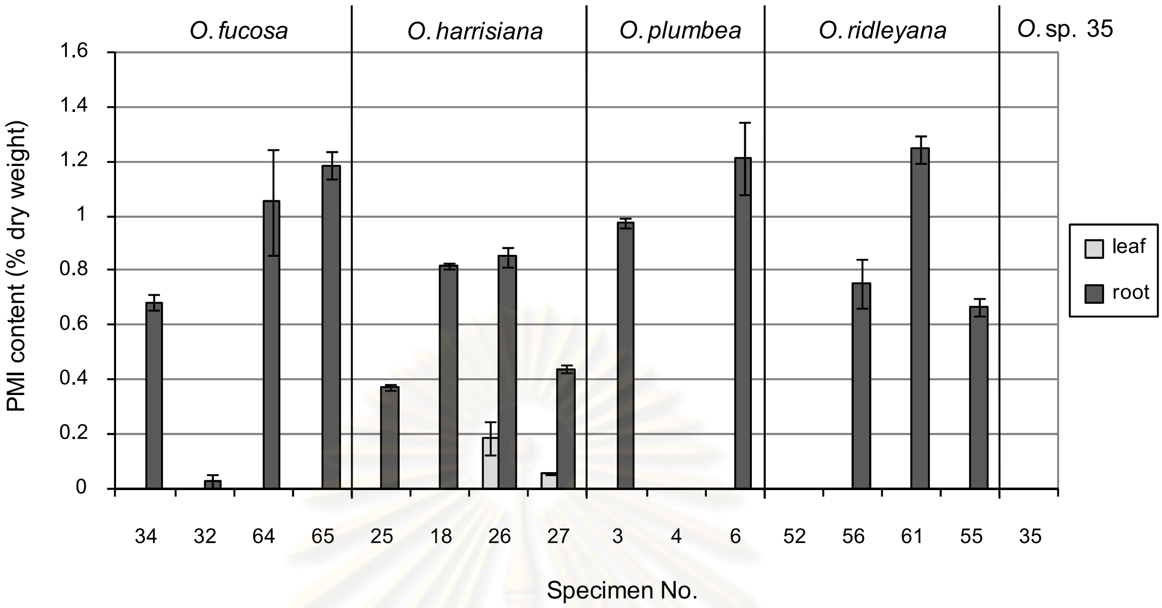


Figure 3.5 Pumiloside content (% dry weight) in the leaf and root extracts of each *Ophiorrhiza* samples. Each bar represents the mean ± SD of triplicate analyses.

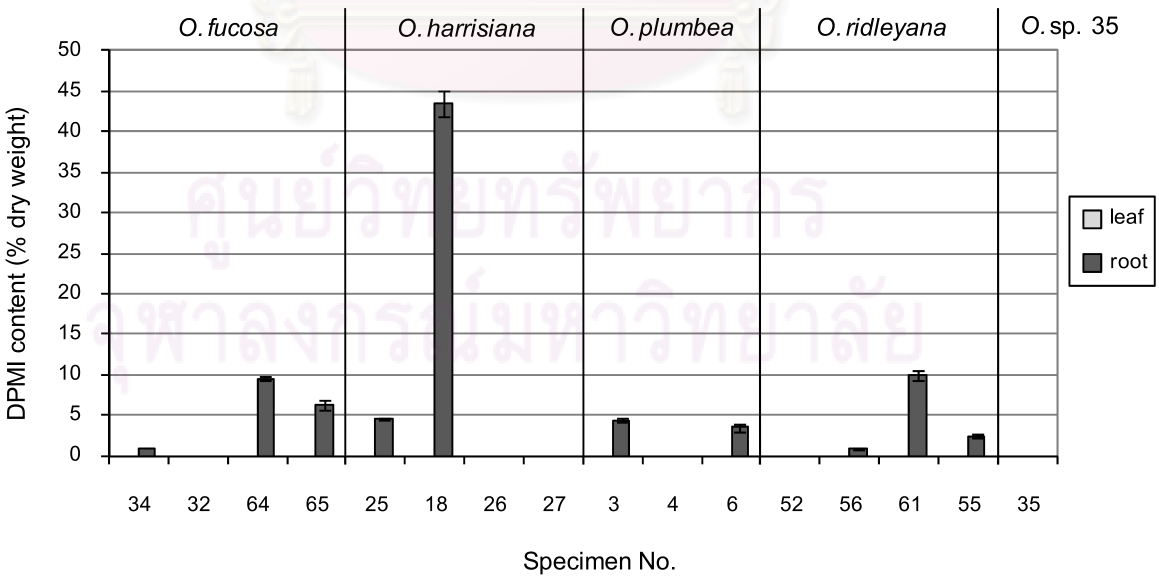


Figure 3.6 Deoxy pumiloside content (% dry weight) in the leaf and root extracts of each *Ophiorrhiza* samples. Each bar represents the mean ± SD of triplicate analyses.

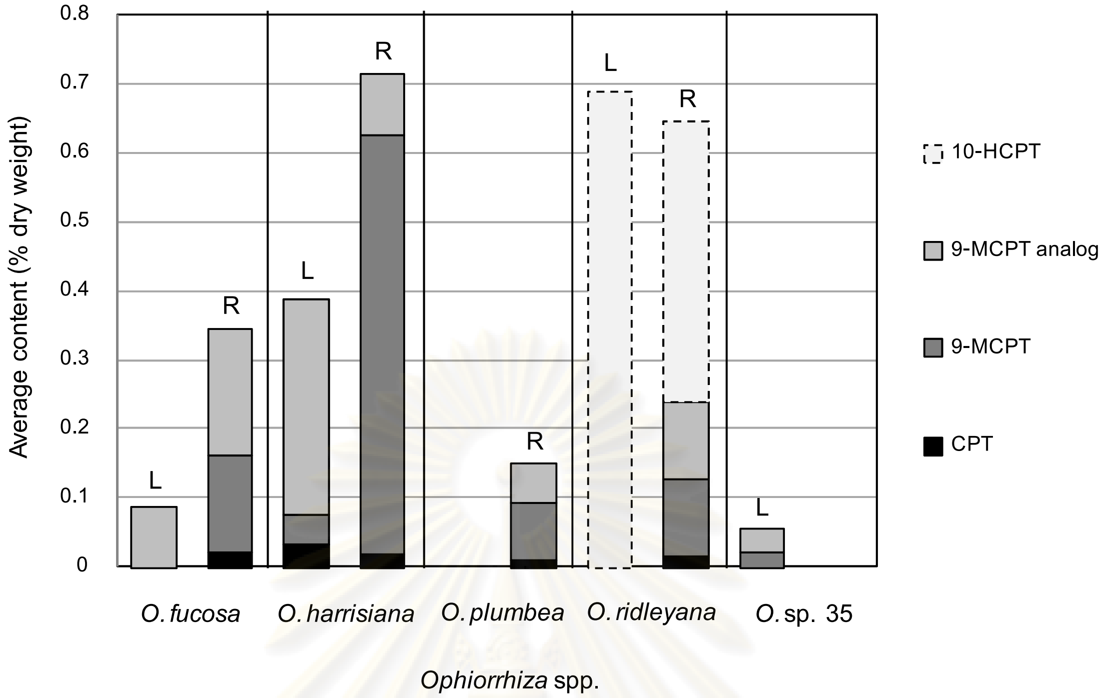


Figure 3.7 Average contents (% dry weight) of CPT and its derivatives in the leaf (L) and root (R) extracts of each *Ophiorrhiza* species.

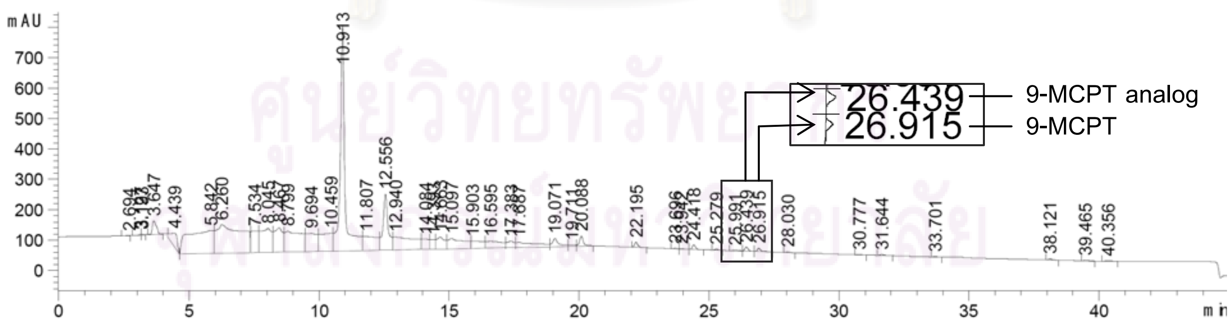


Figure 3.8 HPLC-DAD chromatogram of the root extract of Ophi 64 showing a peak of unknown compound, 9-MCPT analog, at a retention time 26.4 min and a peak of 9-MCPT at 26.9 min.

3.3 Discussion

The distribution of *Ophiorrhiza* spp. collected in this study (Table 3.1, Fig 3.9) showed some interesting viewpoints. *Ophiorrhiza* in northern part of Thailand had high species diversity. For instance, there were four species collected in one location, Chae Son National Park, Lampang that were *O. pedunculata, O. pseudofasciculata, O. ridleyana,* and *O. trichocarpon.* Even Mok Fa and Tard Mok waterfall in Chaing Mai Province are small areas, two species were found. *Ophiorrhiza* in other parts of Thailand had lower diversity comparing with the northern part. For instance, Chantaburi Province in south-eastern part was found two species in four locations. We can imply that the northern areas are appropriate for the growth of *Ophiorrhiza* plants. According to previous study (Schanzer, 2004) and our collecting experience, most *Ophiorrhiza* habitats were along streams and waterfalls on humus, open soil, wet rocks, in evergreen, mixed, or disturbed bamboo dominated forests. They required humid climate with shade, not directed sunlight. For intraspecies aspect, *O. pedunculata* and *O. trichocarpon* collected in this study had wide distribution comparing with *O. pseudofasciculata* and *O. ridleyana* which were found only in northern part of Thailand. *O. fucosa* which has never been reported in Thailand were found only in Chantaburi Province.

Although there was a fluctuation of CPT content, the presence or absence, and part of accumulation of CPT detected in samples within species, but Ophi 52, were congruent (Figure 3.1), despite their various localities of collection (Table 3.1). Conversely, different *Ophiorrhiza* spp. grown naturally in the same area had different CPT-producing abilities (e.g. *O. pseudofasciculata* and *O. ridleyana* from Chae Son National Park, Lampang). From these results, we propose that *Ophiorrhiza* in Thailand had CPT production abilities which were mainly related to species, not habitat. Based on our hypothesis, Ophi 52 should not be *O. ridleyana*; only its morphological characteristics looked similar to this species. Thus, we expected that *mat*K and *Top*I sequence analysis results can resolve this problem.

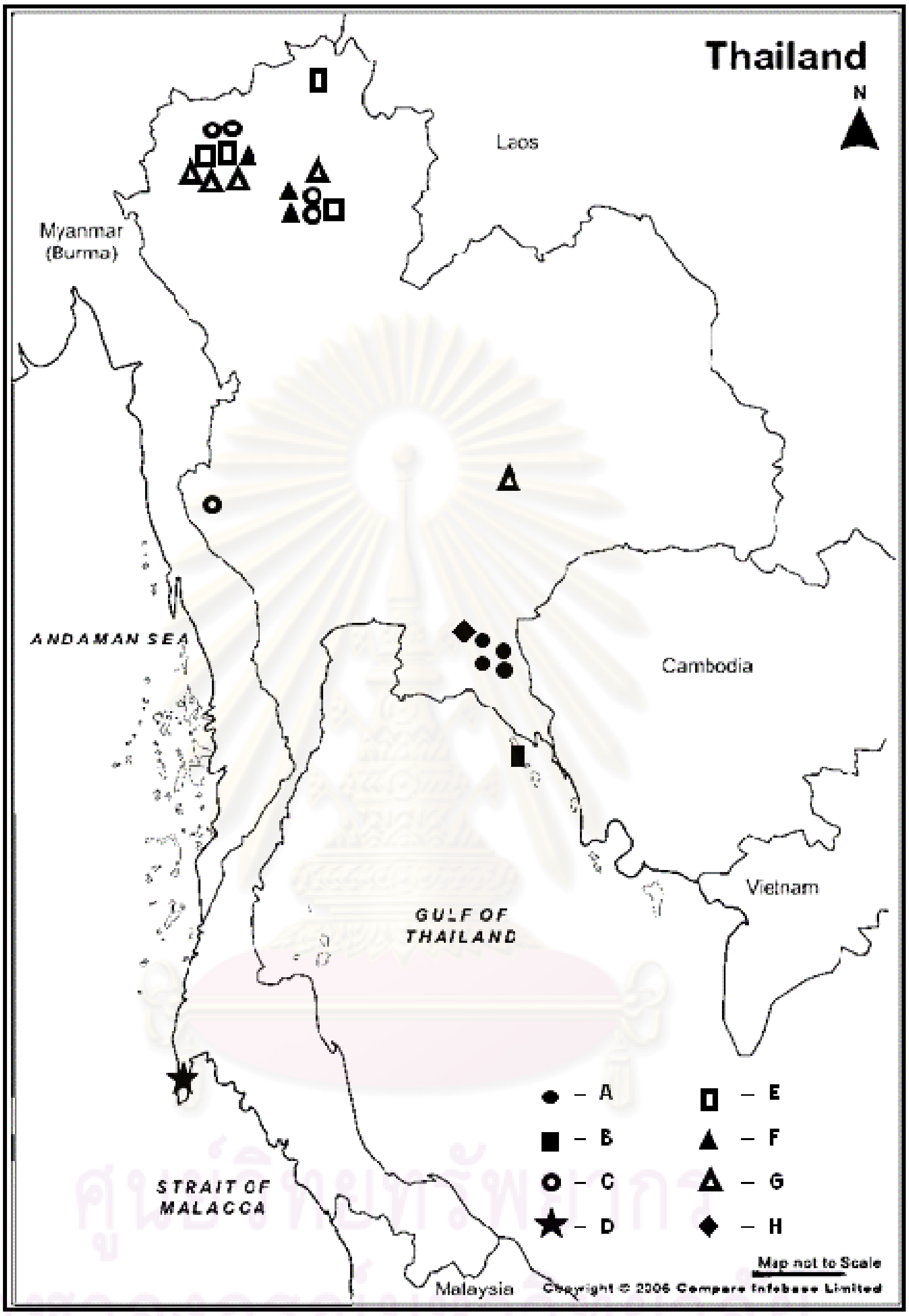


Figure 3.9 Distribution of *Ophiorrhiza* spp. collected in this study: A. *O. fucosa*; B. *O. harrisiana*; C. *O. pedunculata*; D. *O. plumbea*; E. *O. pseudofasciculata*; F. *O. ridleyana*; G. *O. trichocarpon*; H. *Ophiorrhiza* sp. 35. Black shapes represent CPT- and CPT derivatives-producing *Ophiorrhiza* spp. White-centered shapes represent non-CPT- producing *Ophiorrhiza* spp.

Unlike CPT, some compounds had incongruent detection results in samples within species. For instance, 9-MCPT was not detected in Ophi 32, among other *O. fucosa* plants and 9-MCPT analog was not detected in Ophi 61, among other *O. ridleyana*. However, it was noticeable that most samples showed relative contents of detected compounds within species (Figure 3.1-3.6). For instance, among *O. harrisiana,* Ophi 18 had the highest content of CPT, 9-MCPT, 9-MCPT analog, including PMI and DPMI. Among *O. fucosa,* Ophi 32 had the lowest content of CPT, 9-MCPT analog, and PMI, whereas 9-MCPT and DPMI were not detected. In fact, PMI and DPMI are the indicators of CPT production. This study, PMI and DPMI were detected in the CPT accumulating parts in CPT-producing species. From these results, we can imply that the plants which had relative contents of PMI, DPMI, and CPT might be in a CPT-production phase. From the relation of CPT and CPT derivative contents, we still cannot conclude that CPT derivatives are produced earlier of the CPT production.

From HPLC-MS results (Table 3.2), 9-MCPT analog was detected in all 9-MCPT-containing species. 9-MCPT analog were eluted earlier than 9-MCPT for 0.5 min approximately (26.4 min of 9-MCPT analog and 26.9 min of 9-MCPT). Mass spectra of these two compounds showed the major ion *m/z* 379.0 $[M + H]^+$ (Appendix B). A recent study (Shweta *et al*., 2010) reported an identification of 9-MCPT isomer, 10-methoxy camptothecin (10-MCPT) using LC-MS/MS and HRMS. Although solvents for mobile phase were not the same as our study, they used the same C18 column and used gradient elution of high polar to less polar mobile phase. Surprisingly, HRMS chromatograms revealed the retention times of 10-MCPT at 24.07 min and 9-MCPT at 24.53 min, which differed for 0.5 min similar to our results (Figure 3.8). 9-MCPT analog may possibly be 10-MCPT. To prove this assumption, we have to analyze the samples with standard compound of 10-MCPT, otherwise, it is necessary to use the higher techniques.

The HPLC-MS data confirms the species identification results. However, *O. pseudofasciculata* had not any HPLC-MS data to confirm that Ophi 62 is exactly this species. Among *O. harrisiana*, Ophi 18 had the remarkably high contents of 9-MCPT

(Figure 3.2) and DPMI (Figure 3.6). Ophi 56 (*O. ridleyana*) was the only 10-HCPT-detecting specimen in this study (Figure 3.4). Thus, Ophi 62, Ophi 18 and Ophi 56 should be analyzed for genetic characteristics to prove they were exactly the species previously identified.

The fluctuation of CPT and CPT derivative detections in this study was possibly because of the different age of examined plants and the instability of the compounds. Some growing plants might not ready to produce secondary metabolites or produce in trace amounts below the initial detection point of the equipment. In addition, the content of any compounds produced by plant can be affected by seasonal variability, plant elicitor, and part of the plant material. The example of this case may be *Ophiorrhiza* sp. 35 that was detected only 9-MCPT and its analog in quite low amount. Another factor affecting HPLC-MS analysis was the time used in one analysis for each sample which was about an hour. Lots of samples were stayed over-night in HPLC-MS system. An increasing of crude extract concentration or hydrolysis of the compounds may occur. We decreased these possible errors by setting a temperature of the HPLC-MS system to 4°C and random injection of all samples in each time. However, the content of some compounds were fluctuation in triplicate analyses such as 10-HCPT, especially in the leaf extract.

Average contents of CPT and CPT derivatives in each *Ophiorrhiza* spp. in Figure 3.7 demonstrated that *Ophiorrhiza* plants accumulated CPT mostly in roots and in derivative forms. For *O. fucosa* and *O. ridleyana*, CPT was detected only in roots, whereas, CPT derivatives were detected in leaves and roots. It is the fact that plants possess secondary compounds to defend themselves against herbivore attacks or the manifestation of microorganisms (Sirikantaramas *et al*., 2009). In this case, *Ophiorrhiza* plants might produce CPT mainly in root, and then CPT would be changed into water-soluble derivatives in order to be easily transported to protect the upper parts of the plants. This hypothesis may not included *O. harrisiana*, the only one species that produce CPT in both leaves and roots.

In conclusion, this is the first study which reports the detections of CPT, CPT derivatives, and chemical compounds involved in CPT biosynthesis pathway in *O. fucosa, O. harrisiana, O. plumbea, O. ridleyana,* and *Ophiorrhiza* sp. 35. These five *Ophiorrhiza* species could be alternative sources of CPT and CPT derivatives for anticancer research and pharmaceutical industrial production in the future. Subsequently research should focuses on a quantitative analysis of CPT and CPT derivatives production. Tissue culture technique is also interesting to be utilized for increasing the CPT-producing potential of *Ophiorrhiza* plants in Thailand.

CHAPTER IV

SEQUENCE ANALYSES OF *MAT*K AND *TOPOISOMERASE* I

In order to classify and study a coevolution in *Ophiorrhiza* spp., we constructed the molecular phylogenetic trees based on chloroplast *mat*K and nuclear *Top*I nucleotide sequences. Besides, amino acid sequences of TopI enzymes were analyzed to investigate point mutations in the CPT-producing *Ophiorrhiza* species.

4.1 Materials and Methods

From all specimens, samples from eight *Ophiorrhiza* species were examined (Table 4.1). Mostly, one sample was chosen as a representative for each species. Ophi 18, Ophi 56, and Ophi 62 were also analyzed to prove that they were exactly the species previously identified by their morphological characteristics.

4.1.1 RNA extraction and reverse transcription

Fresh leaves of each examined samples were rapidly ground with liquid nitrogen using mortar and pestle, and then extracted with RNeasy™ Plant Mini Kit (Qiagen, Germany), following the manufacturer's protocol. Total RNA was performed on 0.8% agarose gel electrophoresis stained by ethidium bromide and visualized under UV light. A Lambda DNA-Hind III Digest (New England BioLabs Inc., USA) was used as standard molecular size. The extracted RNA was promptly kept at -80°C. Total RNA of each examined sample was converted to cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen, USA) and oligo$(dT)_{20}$ primer, following the manufacturer's protocol. The total cDNA of each sample was then kept at -20°C for further use in PCR amplification. For qualification and quantification of RNA and cDNA samples, gel electrophoresis method was used. Samples were loaded in 0.8% agarose gel (Bio-Rad Laboratories, USA) and run on an electrophoresis apparatus filled with 1XTAE buffer. The gel was stained with ethidium bromide solution, destained and transferred to Gel Doc™ XR System (Bio-Rad

Laboratories, Inc., USA). The samples were visualized under UV light and photographed.

Table 4.1 Specimens of eight *Ophiorrhiza* spp. with accession numbers and size of their full-length *mat*K and *Top*I sequences.

Species	Locality (area, province)	Examined specimen	Accession No. (size)	
			*mat*K	*Top*I
O. fucosa	Phlio National Park, Chantaburi	Ophi 64	AB564412 (1518 bp)	AB564420 (2781 bp)
O. harrisiana	Than Mayom waterfall, Ko Chang, Trat	Ophi 27	AB564413 (1518 bp)	AB564421 (2766 bp)
		Ophi 18	✓ (1518 bp)	–
O. pedunculata	Mork-Fa waterfall, Chiangmai	Ophi 41	AB564414 (1518 bp)	AB564422 (2766 bp)
O. plumbea	Bangpae waterfall, Phuket	Ophi 6	AB564415 (1518 bp)	AB564423 (2766 bp)
O. pseudofasciculata	Doi Suthep-Pui National Park, Chiangmai	Ophi 37	AB564416 (1518 bp)	AB564424 (2778 bp)
	Khun Kon waterfall, Chiangrai	Ophi 62	✓ (1518 bp)	✓ (2854 bp)
O. ridleyana	Mae Yom National Park, Lampang	Ophi 61	AB564417 (1518 bp)	AB564425 (2781 bp)
	Chae Son National Park, Lampang	Ophi 56	✓ (1518 bp)	✓ (2832 bp)
	Queen Sirikit Botanical Garden, Chiang Mai	Ophi 52	✓ (1518 bp)	–
O. trichocarpon var. *glabra*	Tard Mok waterfall, Chiang Mai	Ophi 46	AB564418 (1518 bp)	AB564426 (2766 bp)
Ophiorrhiza sp. 35	Rambhai Barni Rajabhat University, Chantaburi	Ophi 35	AB564419 (1518 bp)	AB564427 (2766 bp)

✓ Full-length gene was sequenced but not submitted to GenBank.

– Full-length gene was not sequenced.

4.1.2 Primers design

4.1.2.1 *matK* primers

To amplify and sequence the *mat*K gene of *Ophiorrhiza*, four primers were designed. Nucleotide *mat*K sequences of *O. pumila* (accession no. AB247150), *O. kuroiwae* (AB247256), *O. japonica* (AB257123), and *O. hayatana* (AB247255) were obtained from DDBJ/EMBL/GenBank databases. All sequences were aligned and conserved regions were selected. Details of these primers are presented in Table 4.2 and the relative positions on *mat*K gene are shown in Figure 4.1. The designed primers were synthesized by Aitbiotech Pte Ltd, Singapore.

Table 4.2 PCR amplification primers and sequencing primers of *mat*K gene used in this study.

Primer name	Primer sequence (5' to 3')	Direction
matKOpu-560F	TCCGTCCCCGAGGTATCTATTC	Forward
matKOpu-1188F	TGCCTCTTCCTTGCATTTATTACG	Forward
matKOpu-1693R	GCACACTTGAAAGATAGCCCATAAA	Reverse
matKOpu-2227R	ATTTCCATTTACAAGGCCTCAGAA	Reverse

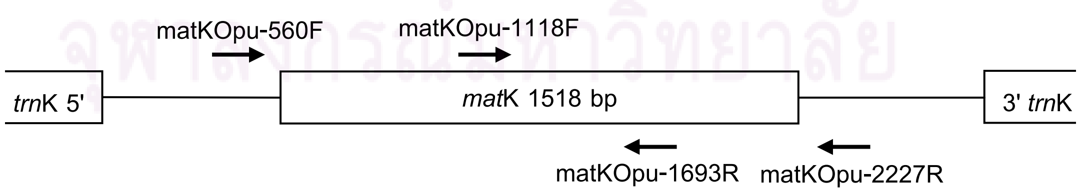


Figure 4.1 Relative positions of the PCR amplification primers and sequencing primers on *mat*K gene (1518 bp in length) of *Ophiorrhiza* spp. Arrows (⟶) represent forward primers. Arrows (⟵) represent reverse primers.

4.1.2.2 *Topoisomerase* I primers

Nine primers were newly designed. Nucleotide *TopI* sequences of *O. pumila* (AB372508), *O. liukiuensis* (AB372509) and *O. japonica* (AB372510) were aligned and conserved regions were selected. The designed primers were synthesized by Aitbiotech Pte Ltd, Singapore. Opstart primer was obtained from Graduate School of Pharmaceutical Sciences, Chiba University, Japan. Details of these primers are presented in Table 4.3 and the relative positions on *mat*K gene are shown in Figure 4.2.

Table 4.3 PCR amplification primers and sequencing primers of *TopI* gene used in this study.

Primer name	Primer sequence (5' to 3')	Direction
opstart	ATGGCTGTTGAGGCCTGTA	Forward
TopI-471F	GCTAGGACTTCTGGTTGCTCA	Forward
TopI-960F	CCAATATCCCAAAGAATCAAGAA	Forward
TopI-1518F	GGTGTCAAAGAGAAGGTCGGTA	Forward
TopI-2139F	CGAAGTGGGAAAGAGGGTAGT	Forward
TopI-696R	CATTTTGTTGAACTTTTGCTGC	Reverse
TopI-1078R	TAACAGAAGCTGGTGACTTC	Reverse
TopI-1518R	TACCGACCTTCTCTTTGACACC	Reverse
TopI-1831R	GCTTTCTCATATTTCTCCTTGTCA	Reverse
TopI-2753R	CATGGCCCAGGCAAACT	Reverse

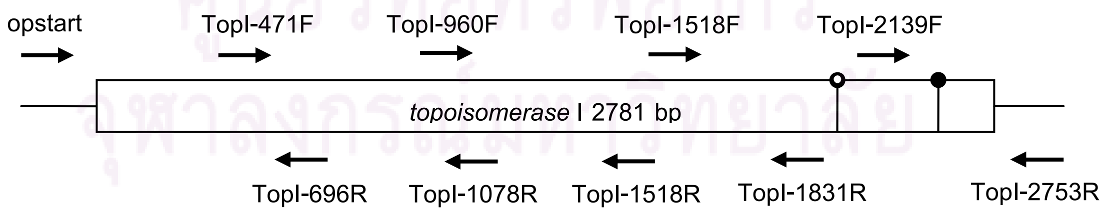


Figure 4.2 Relative positions of the PCR amplification primers and sequencing primers on *Top*I gene (2781 bp in length) of *O. pumila*. Arrows (→) represent forward primers. Arrows (←) represent reverse primers. Pin shape with white circle ( ) indicates amino acid mutation at position 530 based on *H. sapiens* TopI. Pin shape with black circle ( ) indicates mutation at position 722 based on *H. sapiens* TopI.

4.1.3 PCR amplification

The cDNA fragments encoding *mat*K and *Top*I were used as templates for PCR amplification of *mat*K and *Top*I genes using TaKaRa Ex Taq^{TM} Polymerase (Takara Bio Inc, Japan), following the manufacturer's protocol. PCR amplification was carried out in Bio-Rad Laboratories C1000 Thermal Cycler (Bio-Rad Laboratories, Inc., USA). The PCR products were run on a 1% agarose gel with Lambda DNA/*Pst*I marker and subsequently cloned into *E. coli*.

4.1.3.1 PCR of *mat*K gene

PCR amplification of *mat*K region was performed using 2 µL of cDNA template in 50 µL of reaction mixture consisting of 5 units/µL TaKaRa Ex Taq ^{TM}, 1X Ex Taq Buffer (including 2 mM of $MgCl_2$), 2.5 mM each of dNTPs mixture, and 0.2 µM of each matKOpu-560F and matKOpu-2227R primer. The PCR cycling program started with an initial denaturation step at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 2 min, and a final extension at 72°C for 5 min, then held at 4°C. An expected size of the PCR product was 1700 bp approximately.

4.1.3.2 PCR of *topoisomerase* I gene

PCR amplification was performed using 2 µL of cDNA template in 50 µL of reaction mixture consisting of 5 units/µL TaKaRa Ex Taq ^{TM}, 1X Ex Taq Buffer (including 2 mM of $MgCl_2$), 2.5 mM each of dNTPs mixture, and 0.2 µM of each primer. Initially, TopI-1518F and TopI-2753R primers were used to amplify a mutation region in *Top*I gene. The PCR cycling program started with an initial denaturation step at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 48°C for 30 s, extension at 72°C for 1.5 min, and a final extension at 72°C for 5 min, then held at 4°C. An expected size of the PCR product was 1200 bp approximately. Other couples of primers were used to complete a full-length gene of *Top*I using similar PCR condition with the modification of an extension time depended on fragment size.

4.1.4 Cloning and sequencing

PCR products of *mat*K and *Top*I fragments were cloned and transformed to *E. coli* DH5α competent cells with the pGEM®-T Easy Vector System (Promega Corp, USA). Each PCR product (3 µL) was ligated with pGEM-T Easy Vector (1 µL) using 1 µL of T4 DNA ligase enzyme in 5 µL of 2X ligation buffer. Ligation mixture was incubated at 4°C overnight and transformed to *E. coli* DH5α competent cells by heat shock method.

Competent cells was removed from the -80°C freezer and thawed on ice. After thawing, 100 µL of cells was mixed with 5 µL of ligation mixture and incubated on ice for 30 min. The cell mixture was heat shocked at 37°C for 60 s and rapidly placed on ice for 5 min and then 900 µL of SOC media (42°C preheated) was added to the cell mixture. The cell mixture was transferred into a 15-ml tube and shacked for 1 hr at 37°C.

The recombinant clones were selected using blue/white selection technique. The LB-Amp (Luria-Bertani medium with ampicillin) plates were prepared earlier. The mixture of 2% X-Gal in DMF (40 µL) and 100 mM IPTG (100 µL) was spread on LB-Amp plate to prepare an X-gal plate. The recombinant *E. coli* cell mixture was plated onto the X-gal plates and placed at 37°C overnight. White colonies were randomly chosen from the overnight plates and checked for corrected size of inserts by colony PCR.

Colony PCR was performed using small amount of white colony as a template in 10 µL of reaction mixture consisting of reagents in a proportion similar to that of typical PCR amplification with T7 and SP6 primers. The PCR cycling program was the same as that of PCR product amplification, with the modification of 5 min initial denaturation time. The products of colony PCR were determined the size by gel agarose electrophoresis. The colonies inserted with expected-sized PCR products were cultured in LB-Amp broth and shacked at 37°C overnight.

Recombinant *E. coli* culture was extracted for plasmid using GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich Corp, USA), following the manufacturer's protocol.

The purified plasmids were used as templates for nucleotide sequencing by Aitbiotech Pte Ltd, Singapore.

4.1.5 Phylogenetic tree construction

The obtained *mat*K and *Top*I sequences were assembled and their consensus sequences were constructed using SeqMan™ program (DNA Star Inc, USA). The nucleotide sequence data was submitted to the DDBJ/EMBL/GenBank nucleotide sequence databases with accession numbers (Table 4.1). The nucleotide sequence alignments of *mat*K and *Top*I were performed (Appendix C and D). Both separated and combined *mat*K and *Top*I sequence data-matrices were phylogenetically analyzed using PAUP* 4.0b10 program (Sinauer Assoc Inc, USA).

In the case of *mat*K sequence, *O. pumila* (accession no. AB247150), *O. kuroiwae* (AB247256), *O. japonica* (AB257123), and *O. hayatana* (AB247255) were included in the analysis with *Joosia umbellifera* (AY538396), belonging to the same family, added as an outgroup. Maximum parsimony (MP) analysis was performed using a branch-and-bound searching strategy. All characters were treated as unordered and equally weighted. Strict, semistrict and 50%-majority consensus trees of all equal MP trees were generated and compared together. Bootstrap analyses of 1000 replicates were performed with a branch-and-bound search.

MP trees of *Top*I and combined *mat*K and *Top*I data-matrices were also reconstructed with the same approach as *mat*K. *Top*I sequences of *O. pumila* (AB372508), *O. liukiuensis* (AB372509) and *O. japonica* (AB372510) were included in the *Top*I analysis with *Camptotheca acuminata* (AB372511) and *Catharanthus roseus* (AB372512) as outgroups. The combined *mat*K and *Top*I tree was midpoint-rooted without any outgroup added to the analysis.

4.1.6 Analysis of *topoisomerase* I amino acid

Nucleotide sequences of *topoisomerase* I of each *Ophiorrhiza* spp. were translated into amino acid sequences. Encoded amino acid sequences of *Ophiorrhiza* TopI protein were aligned using MegAlign^{TM} program (DNA Star Inc, USA) and were compared with those of other organisms retrieved from GenBank. These additional TopI sequences were from three CPT-producing plants, *O. pumila*, *O. liukiuensis* and *Camptotheca acuminata*, and three non-CPT-producing organisms, *O. japonica*, *Catharanthus roseus* and *Homo sapiens* (NM_003286).

4.2 Results

4.2.1 RNA determination

Total RNA extracted from leaf tissue of samples (Table 4.1) and Lambda DNA-*Hind* III marker were visualized under UV light and photographed. Figure 4.3 showed two bands of 28S and 18S ribosomal RNA (rRNA) and a smeared appearance of partially degraded RNA.

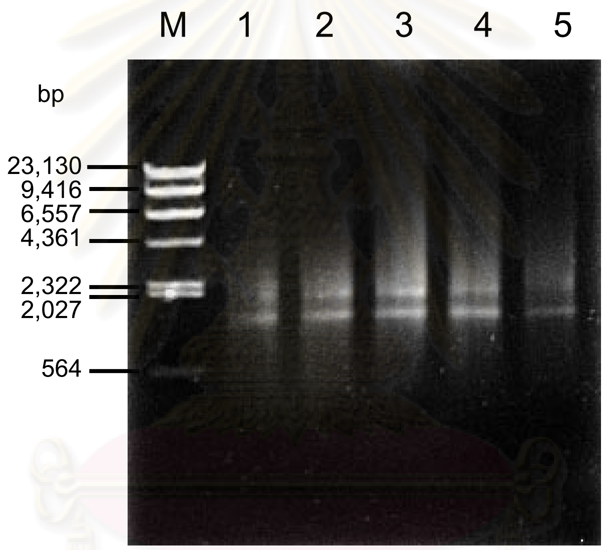


Figure 4.3 Agarose gel electrophoresis of total RNA extracted from *Ophiorrhiza* samples.

Lane M: Lambda DNA-*Hind* III marker

Lane 1: *O. fucosa* (Ophi 64)

Lane 2: *O. harrisiana* (Ophi 27)

Lane 3: *O. pedunculata* (Ophi 41)

Lane 4: *O. plumbea* (Ophi 6)

Lane 5: *O. pseudofasciculata* (Ophi 37)

4.2.2 PCR and colony PCR product determination

4.2.2.1 *mat*K gene

The PCR products of *mat*K amplified with matKOpu-560F and matKOpu-2227R primers were approximately 1700 bp in length. From agarose gel electrophoretogram, each sample showed one band of PCR product with corrected size (Figure 4.4). These were cloned and transformed to *E. coli*.

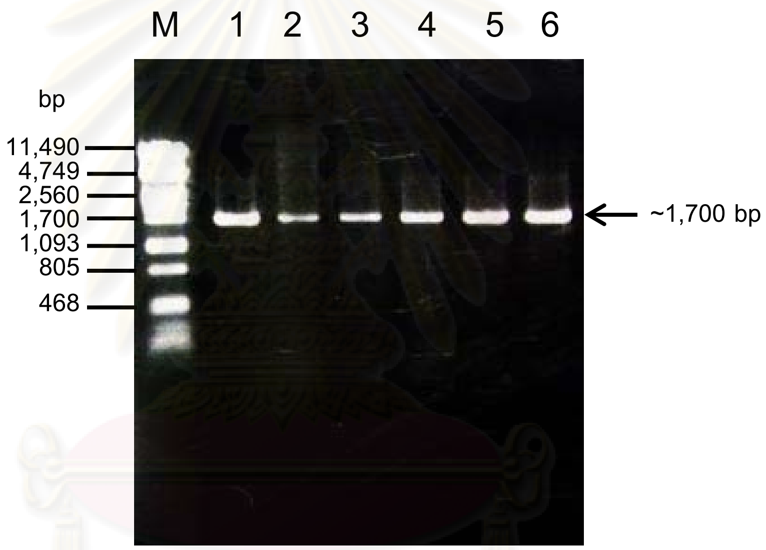


Figure 4.4 Agarose gel electrophoresis of 1700-bp *mat*K region amplified from cDNA of *Ophiorrhiza* samples.

Lane M: Lambda DNA/*Pst*I marker

Lane 1: *O. fucosa* (Ophi 64)

Lane 2: *O. harrisiana* (Ophi 27)

Lane 3: *O. pedunculata* (Ophi 41)

Lane 4: *O. plumbea* (Ophi 6)

Lane 5: *O. pseudofasciculata* (Ophi 37)

Lane 6: *O. ridleyana* (Ophi 61)

Eight randomly white colonies were picked from the overnight plates and checked for corrected insert size by colony PCR (Figure 4.5). The colonies inserted with 1700 bp PCR products were cultured. Plasmids were extracted using GenElute™ Plasmid Miniprep Kit.

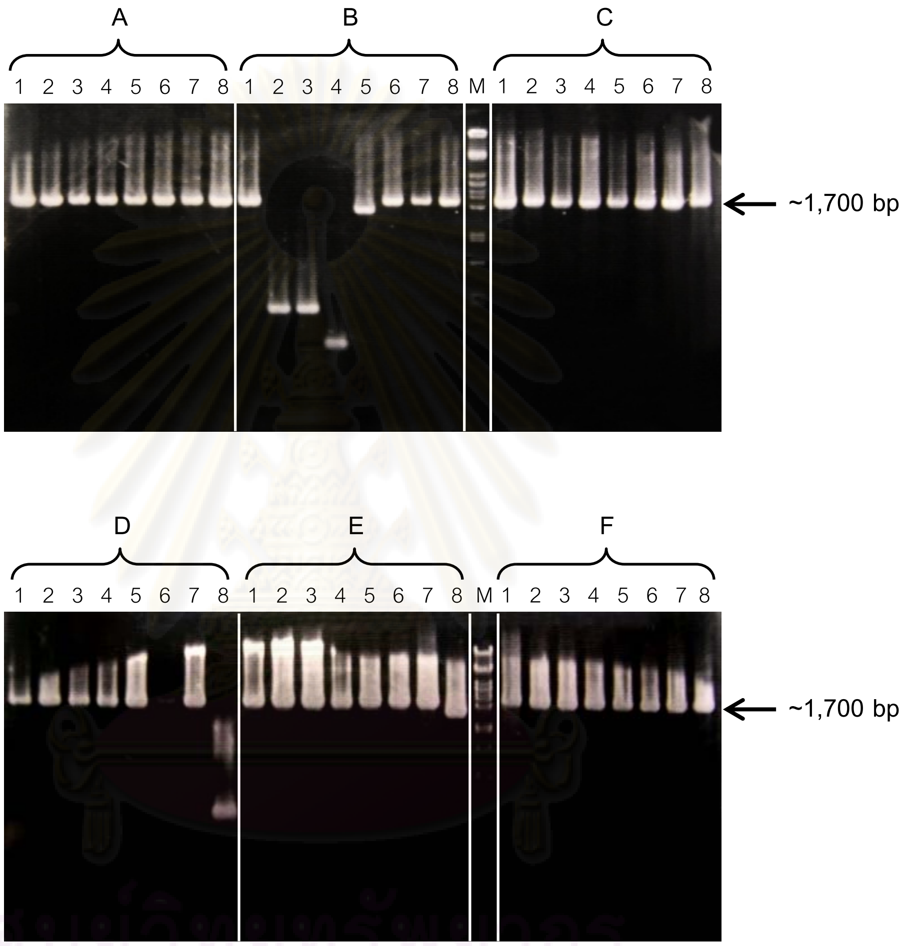


Figure 4.5 Colony screening for the 1700-bp size of *mat*K region inserts. The number above each lane indicates clone number of *Ophiorrhiza* spp.

Lane M: Lambda DNA/*Pst*I marker

A: *O. fucosa* (Ophi 64) D: *O. plumbea* (Ophi 6)

B: *O. harrisiana* (Ophi 27) E: *O. pseudofasciculata* (Ophi 37)

C: *O. pedunculata* (Ophi 41) F: *O. ridleyana* (Ophi 61)

4.2.2.2 *Topoisomerase* I gene

The PCR products of *Top*I amplified with TopI-1518F and TopI-2753R primers were approximately 1250 bp in length. From agarose gel electrophoretogram (Figure 4.6), most sample showed one band of PCR product with corrected size. *O. pseudofasciculata* (lane 5) also showed two non-specific bands which were 300 bp and 800 bp in length. These were cloned and transformed to *E. coli*.

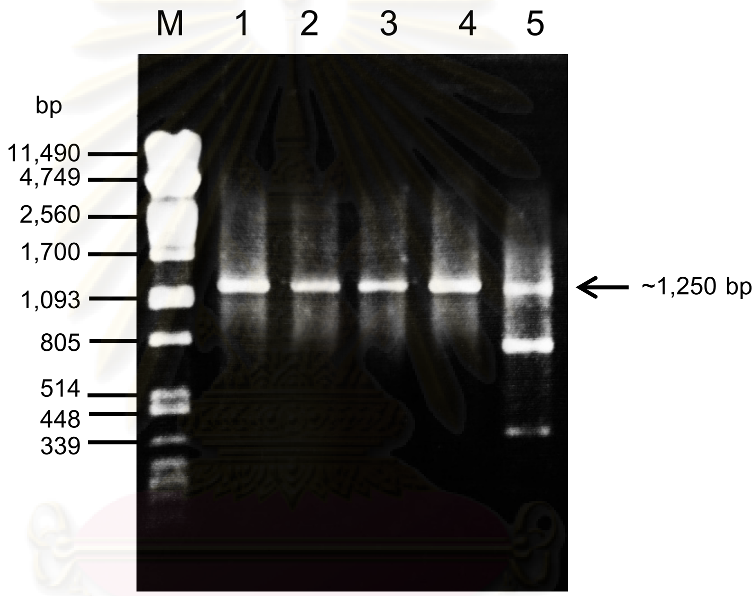


Figure 4.6 Agarose gel electrophoresis of 1250-bp *Top*I fragment amplified from cDNA of *Ophiorrhiza* samples.

Lane M: Lambda DNA/*Pst*I marker

Lane 1: *O. fucosa* (Ophi 64)

Lane 2: *O. harrisiana* (Ophi 27)

Lane 3: *O. pedunculata* (Ophi 41)

Lane 4: *O. plumbea* (Ophi 6)

Lane 5: *O. pseudofasciculata* (Ophi 37)

Eight randomly white colonies were picked from the overnight plates and checked for the size of inserts by colony PCR (Figure 4.7). The colonies inserted with 1250 bp PCR products were cultured. Plasmids were extracted using GenElute™ Plasmid Miniprep Kit.

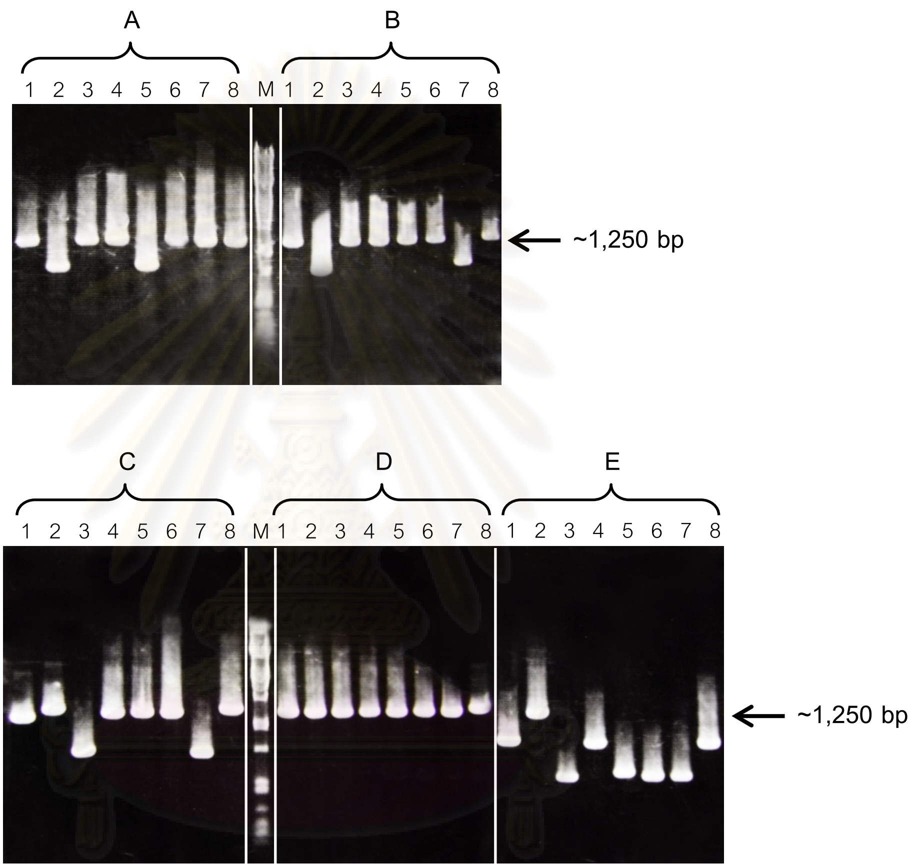


Figure 4.7 Colony screening for the 1250-bp size of *Top*I fragment inserts. The number above each lane indicates clone number of *Ophiorrhiza* spp.

Lane M: Lambda DNA/*Pst*I marker

A: *O. fucosa* (Ophi 64)

B: *O. harrisiana* (Ophi 27)

C: *O. pedunculata* (Ophi 41)

D: *O. plumbea* (Ophi 6)

E: *O. pseudofasciculata* (Ophi 37)

4.2.3 Phylogenetic tree of *mat*K gene

The *mat*K sequences of *O. pumila*, *O. kuroiwae*, *O. japonica*, and *O. hayatana* obtained from GenBank database were added in the analysis. *Joosia umbellifera*, belonging to the same family, was added as an outgroup. The nucleotide sequences of *mat*K were aligned using CLC Sequence Viewer program (CLC bio, Aarhus, Denmark) (Appendix C) and phylogenetically analyzed using PAUP* 4.0b10 program (Sinauer Assoc Inc, USA).

The obtained *mat*K data matrix was 1518 total characters and numbers of parsimony-informative characters were 22 (1.45%). The numbers of equally most parsimonious trees were eleven. One of the 11 maximum parsimonious trees (MPTs) was shown as a phylogram (Figure 4.8). The 50% majority consensus tree of 11 equally MPTs was constructed (Figure 4.9). The length of each MPT is 179; CI = 0.9609, RI = 0.9167 and RC = 0.8808. The 50% majority consensus tree classified *Ophiorrhiza* spp. into two major clades.

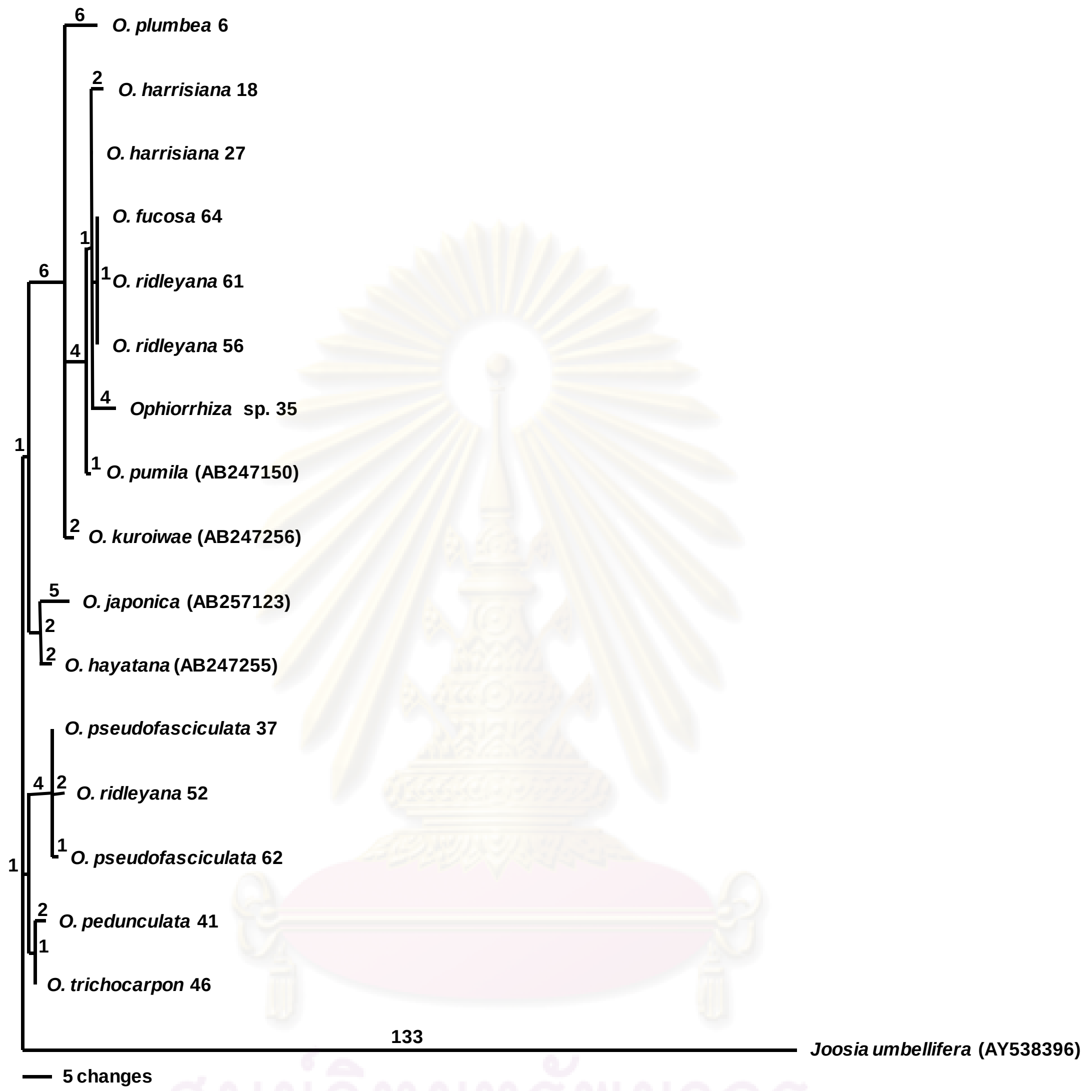


Figure 4.8 The maximum parsimonious phylogram of *mat*K gene. The length of each maximum parsimonious trees (MPTs) is 179; CI = 0.9609, RI = 0.9167 and RC = 0.8808. Numbers above the lines are branch lengths of the maximum parsimonious tree (MPT). Specimen numbers come after taxa name.

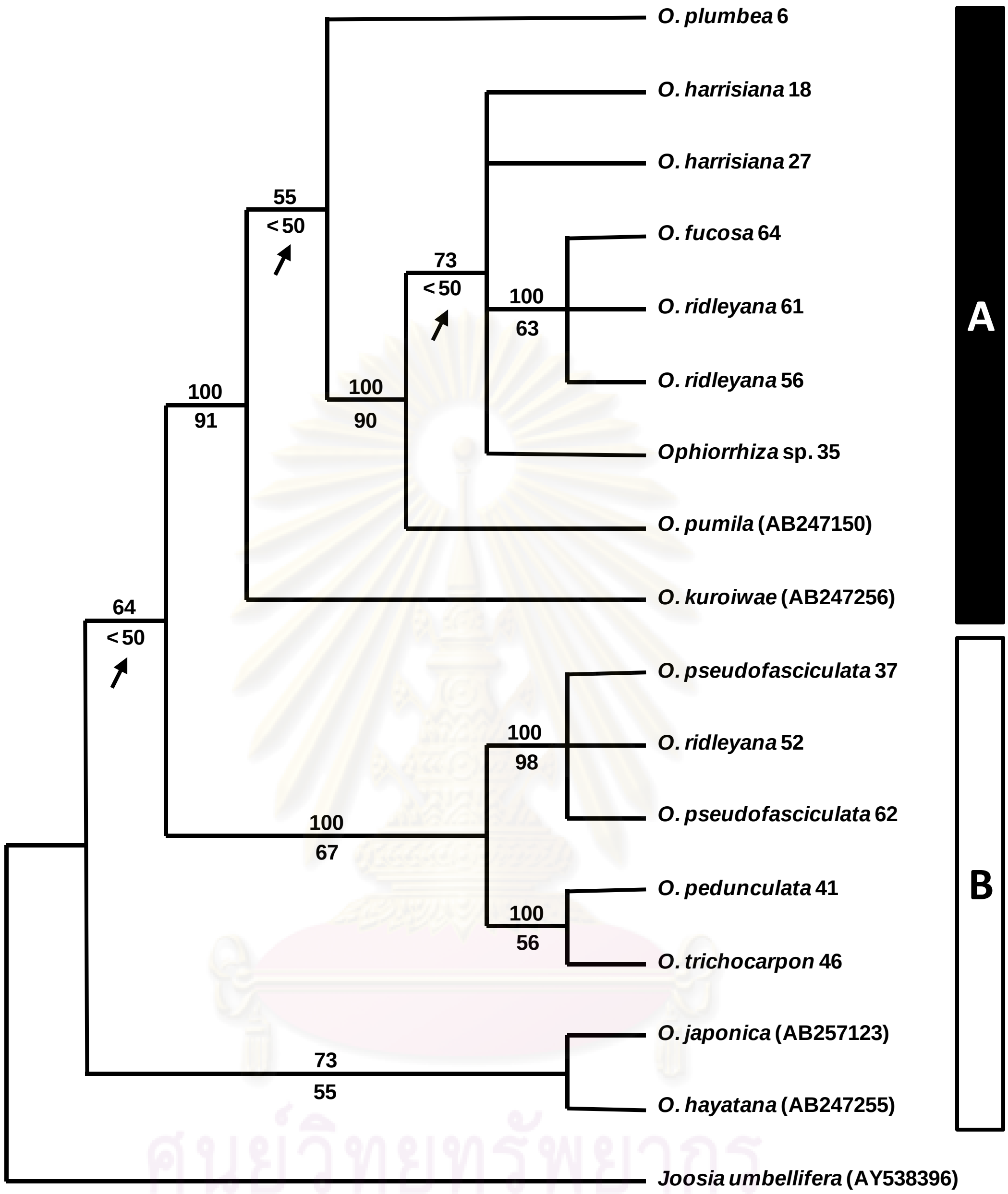


Figure 4.9 The 50% majority consensus tree of 11 equally MPTs based on the *mat*K gene. The length of each MPT is 179; CI = 0.9609, RI = 0.9167 and RC = 0.8808. Numbers above the lines are %majority between all MPTs. Numbers below the lines are %bootstrap values with 1000 replicates. Arrows indicate nodes collapsed in the strict consensus tree. Specimen numbers come after taxa name. ‘A’ and ‘B’ indicates the clade of CPT-producing and -non-producing plants.

4.2.4 Phylogenetic tree of *topoisomerase* I gene

The *Top*I sequences of *O. pumila*, *O. liukiuensis*, and *O. japonica* obtained from GenBank database were added in the analysis. *Camptotheca acuminata* and *Catharanthus roseus* were added as outgroups. The nucleotide sequences of *Top*I were aligned using CLC Sequence Viewer program (CLC bio, Aarhus, Denmark) (Appendix D) and phylogenetically analyzed using PAUP* 4.0b10 program (Sinauer Assoc Inc, USA).

The obtained *Top*I data matrix was 2932 total characters and numbers of parsimony-informative characters were 313 (10.68%). The numbers of equally most parsimonious trees were two. One of two MPTs was shown as a phylogram (Figure 4.10). The strict consensus tree of two equally MPTs was constructed (Figure 4.11). The length of each MPT is 1189; CI = 0.9058, RI = 0.8025 and RC = 0.7269.The strict consensus tree classified *Ophiorrhiza* spp. into two major clades.

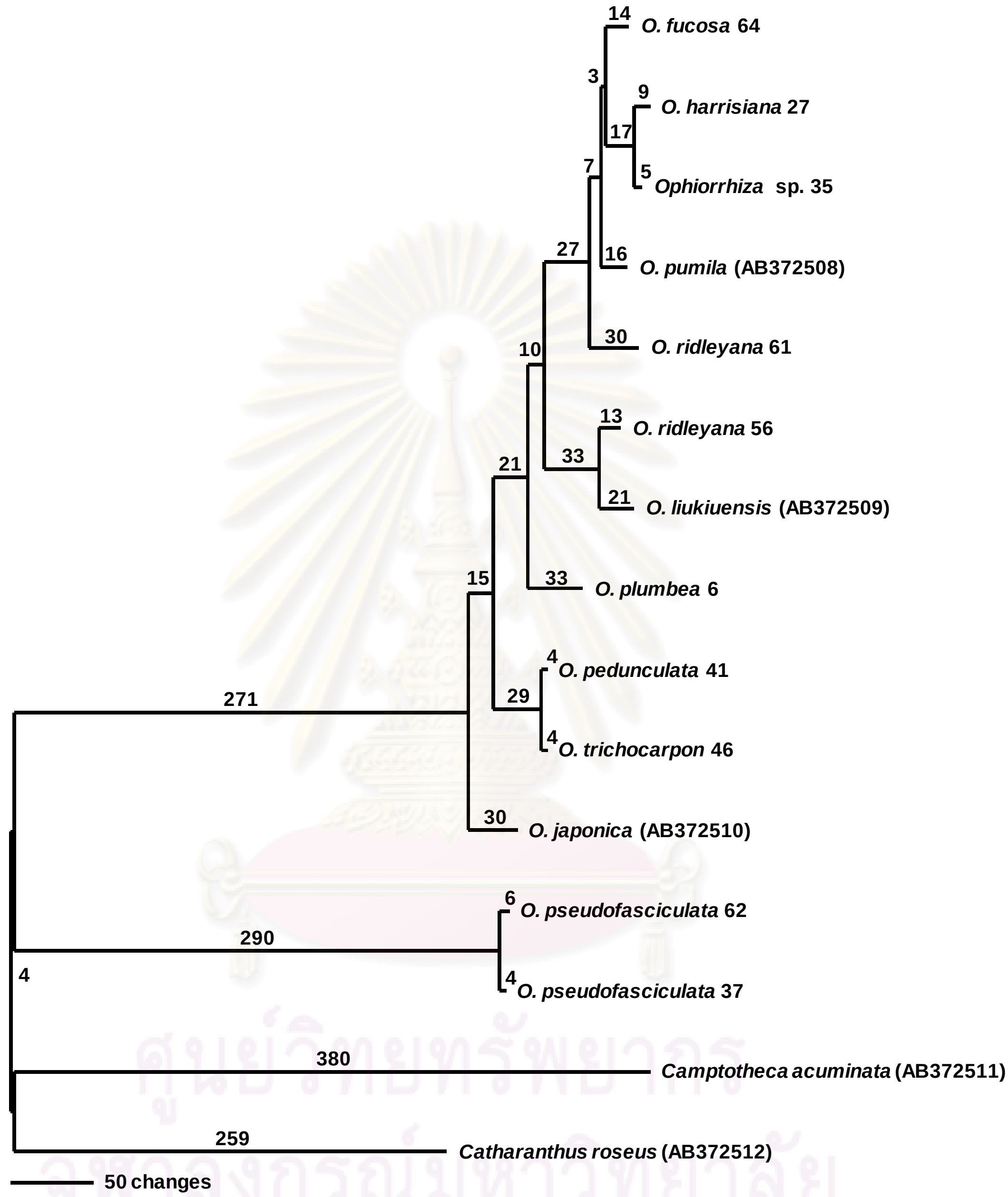


Figure 4.10 The maximum parsimonious phylogram of *Top*I gene. The length of each MPT is 1189; CI = 0.9058, RI = 0.8025 and RC = 0.7269. Numbers above the lines are branch lengths of the MPT. Specimen numbers come after taxa name.

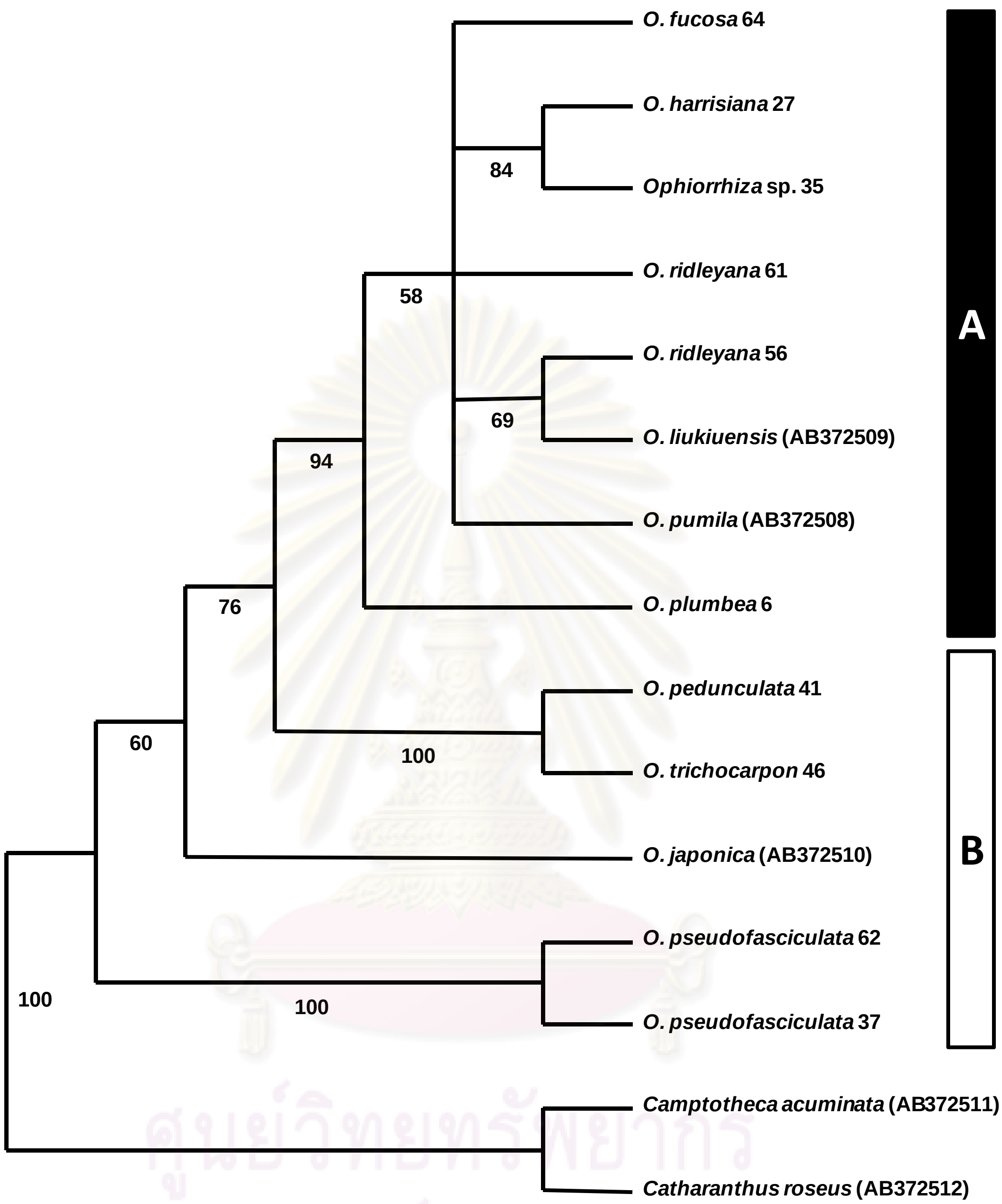
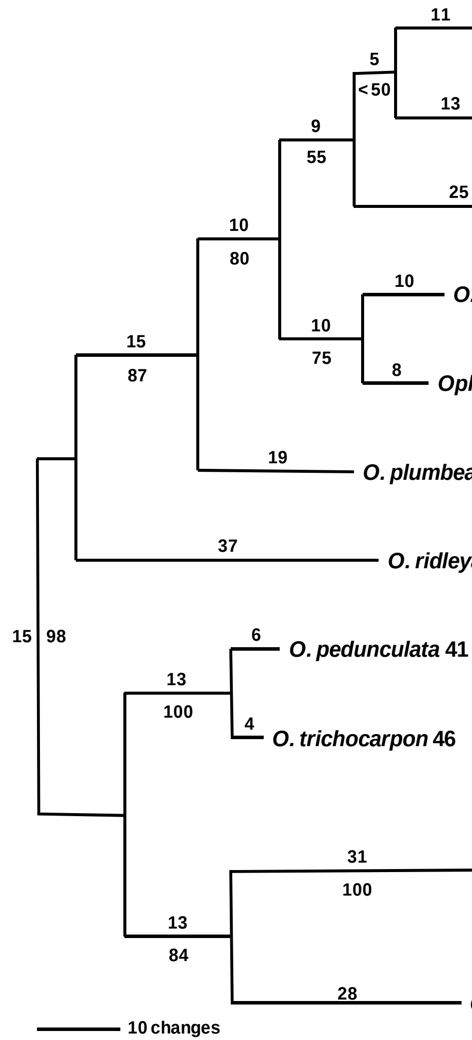


Figure 4.11 The strict consensus tree of two equally MPTs based on the *TopI* gene. The length of each MPT is 1189; CI = 0.9058, RI = 0.8025 and RC = 0.7269. Numbers below the lines are %bootstrap values with 1000 replicates. Specimen numbers come after taxa name. 'A' and 'B' indicates the clade of CPT-producing and -non-producing plants.

4.2.5 Phylogenetic tree of combined data of *mat*K and *topoisomerase* I gene

The *mat*K and *Top*I sequences of *Ophiorrhiza* spp. were combined. The nucleotide sequences of combined *mat*K and *Top*I were aligned using CLC Sequence Viewer program (CLC bio, Aarhus, Denmark) and phylogenetically analyzed using PAUP* 4.0b10 program (Sinauer Assoc Inc, USA).

The obtained combined *mat*K and *Top*I data-matrices were 4381 total characters and numbers of parsimony-informative characters were 119 (2.72%). The single most parsimonious tree was constructed (Figure 4.12). The length of MPT is 295; CI = 0.7966, RI = 0.8137 and RC = 0.6482. The tree was midpoint-rooted without any outgroup added to the analysis. The single most parsimonious tree classified *Ophiorrhiza* spp. into two major clades.



	Leaf extract						Root extract					
	CPT	9-MCPT	9-MCPT analog	10-HCPT	PMI	DPMI	CPT	9-MCPT	9-MCPT analog	10-HCPT	PMI	DPMI
O. fucosa 64	–	–	–	–	–	–	+	+	+	–	+	+
O. pumila	+	+	–	–	+	+	+	–	–	–	+	–
O. ridleyana 61	–	+	–	–	–	–	+	+	–	–	+	+
O. harrisiana 27	+	+	+	–	+	–	+	–	+	–	+	–
Ophiorrhiza sp. 35	–	+	–	–	–	–	–	+	–	–	–	–
O. plumbea 6	–	–	–	–	–	–	+	+	+	–	+	+
O. ridleyana 56	–	+	–	+	–	–	+	+	+	+	+	+
O. pedunculata 41	–	–	–	–	–	–	–	–	–	–	–	–
O. trichocarpon 46	–	–	–	–	–	–	–	–	–	–	–	–
O. pseudofasciculata 62	–	–	–	–	–	–	–	–	–	–	–	–
O. pseudofasciculata 37	–	–	–	–	–	–	–	–	–	–	–	–
O. japonica	–	–	–	–	–	–	–	–	–	–	–	–

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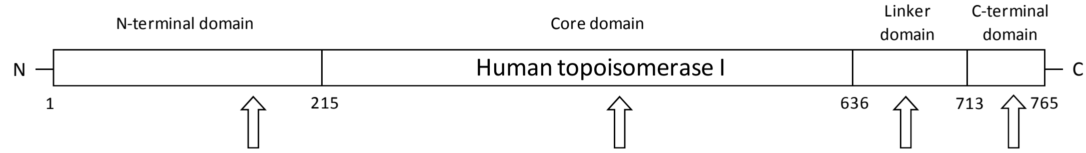
Figure 4.12 The single most parsimonious tree based on a combined *mat*K and *Top*I data matrices. The length of each MPT is 295; CI = 0.7966, RI = 0.8137 and RC = 0.6482. Numbers above the lines are branch lengths of the MPT. Numbers below the lines are %bootstrap values with 1000 replicates. The tree was midpoint-rooted without any outgroup added to the analysis. Specimen numbers come after taxa name. The results from HPLC-MS analysis of compounds detected in the leaf and root extracts of each specimen were indicated by '+' (presence) or '–' (absence) symbols.

4.2.6 The alignment of topoisomerase I amino acid sequences

According to the alignment of *Top*I nucleotide sequences (Appendix D), *O. pseudofasciculata* 62 and *O. ridleyana* 56 had 76-bp nucleotide insertion. This insertion caused numerous gaps in amino acid alignment of TopI. Therefore, Ophi 62 and Ophi 56 were excluded from TopI amino acid alignment (Appendix E). Topoisomerase I amino acid sequences of examined samples of *Ophiorrhiza* spp. (Table 4.1) were aligned with other organisms (Appendix E). The order of taxa in the alignment was arranged in clade A and B of the phylogenetic trees. The 26 positions which showed unique amino acids of each clade were reported in Figure 4.13 (A). The codons translated to these unique amino acids were reported in Figure 4.13 (B).



(A)

TopI amino acid substitution No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Position based on *H. sapiens*	-	67	-	97	-	139	173	-	226	247	331	370	420	421	424	527	530	610	617	679	706	713	717	722	736	743
Homo sapiens	-	T	-	S	-	P	L	-	V	M	N	M	I	Q	I	F	L	E	L	K	T	Q	G	N	W	I
Catharanthus roseus	K	P	S	S	P	P	-	L	I	V	K	M	I	P	F	F	L	D	V	K	E	T	G	N	H	I
O. japonica	K	P	S	F	T	P	T	V	I	I	K	M	I	Q	F	F	L	E	V	K	E	T	G	N	H	I
O. pseudofasciculata 62	K	P	S	F	T	P	T	V	I	I	K	M	I	Q	F	F	L	E	V	K	E	T	G	N	H	I
O. pseudofasciculata 37	K	P	S	F	T	P	T	V	I	I	K	M	I	Q	F	F	L	E	V	K	E	T	G	N	H	I
O. trichocarpon 46	K	P	S	F	T	P	T	V	I	I	K	M	I	Q	F	F	L	E	V	K	E	T	G	N	H	I
O. pedunculata 41	K	P	S	F	T	P	T	V	I	I	K	M	I	Q	F	F	L	E	V	K	E	T	G	N	H	I
O. plumbea 6	E	L	I	F	P	P	A	A	I	I	K	V	I	Q	R	I	I	K	G	E	K	A	S	S	H	M
Ophiorrhiza sp. 35	E	L	I	S	P	A	A	A	I	L	R	V	V	L	C	I	I	K	G	E	K	A	S	S	Q	M
O. harrisiana 27	E	L	I	S	P	A	A	A	M	L	R	V	V	L	C	I	I	K	G	E	K	A	S	S	Q	M
O. liukiuensis	E	L	I	S	P	A	A	A	M	L	R	M	I	Q	C	I	I	K	G	E	K	A	S	S	H	I
O. ridleyana 56	E	L	I	S	P	A	A	A	M	L	R	M	I	Q	F	F	L	E	V	K	E	T	G	N	H	I
O. ridleyana 61	E	L	I	S	P	A	A	A	M	L	R	V	V	I	C	I	I	K	A	E	K	A	S	S	Q	M
O. pumila	E	L	I	S	P	A	A	A	M	L	R	V	V	L	C	I	I	K	G	E	K	A	S	S	Q	M
O. fucosa 64	E	L	I	S	P	A	A	A	M	L	R	V	V	L	C	I	I	K	G	E	K	A	S	S	Q	M
Camptotheca acuminata	K	V	S	F	R	P	-	V	I	V	R	M	I	P	F	F	L	N	V	K	E	T	G	S	H	V
Position based on amino acid alignment	79	125	175	179	227	275	334	345	417	438	522	561	612	614	617	721	724	804	811	877	906	913	917	922	936	943

(B)

Position based on nucleotide alignment	277	374	518	530	673	817	994	1028	1245	1300	1547	1663	1816	1823	1832	2125	2134	2374	2393	2590	2753	2774	2786	2802	2845	2866
Homo sapiens	---	ACC	---	TCT	CCT	CCT	TTG	---	GTA	ATG	AAT	ATG	ATC	CAA	ATT	TTT	CTC	GAG	CTT	AAG	ACA	CAG	GGA	AAT	TGG	ATT
Catharanthus roseus	AAG	CCA	AGC	TCT	CCG	CCC	---	CTT	ATT	GTT	AAA	ATG	ATT	CCA	TTC	TTT	CTT	GAT	GTT	AAA	GAG	ACC	GGC	AAT	CAT	ATA
O. japonica	AAG	CCG	AGC	TTT	ACA	CCT	ACC	GTT	ATT	ATT	AAA	ATG	ATA	CAA	TTC	TTT	CTC	GAG	GTG	AAG	GAG	ACC	GGC	AAT	CAC	ATA
O. pseudofasciculata 62	AAG	CCG	AGC	TTT	ACA	CCT	ACC	GTT	ATT	ATT	AAA	ATG	ATA	CAA	TTC	TTT	CTC	GAG	GTG	AAG	GAG	ACC	GGC	AAT	CAC	ATA
O. pseudofasciculata 37	AAG	CCG	AGC	TTT	ACA	CCT	ACC	GTT	ATT	ATT	AAA	ATG	ATA	CAA	TTC	TTT	CTC	GAG	GTG	AAG	GAG	ACC	GGC	AAT	CAC	ATA
O. trichocarpon 46	AAG	CCG	AGC	TTT	ACA	CCT	ACC	GTT	ATT	ATT	AAA	ATG	ATA	CAA	TTC	TTT	CTC	GAG	GTG	AAG	GAG	ACC	GGC	AAT	CAC	ATA
O. pedunculata 41	AAG	CCG	AGC	TTT	ACA	CCT	ACC	GTT	ATT	ATT	AAA	ATG	ATA	CAA	TTC	TTT	CTC	GAG	GTG	AAG	GAG	ACC	GGC	AAT	CAC	ATA
O. plumbea 6	GAG	CTG	ATC	TTT	CCA	CCT	GCC	GCT	ATT	ATT	AAA	GTG	ATA	CAA	CGC	ATT	ATC	AAG	GGG	GAG	AAG	GCC	AGC	AGT	CAC	ATG
Ophiorrhiza sp. 35	GAG	CTG	ATC	TCT	CCA	GCT	GCC	GCT	ATT	CTT	AGA	GTG	GTA	CTA	TGC	ATT	ATC	AAG	GGG	GAG	AAG	GCC	AGC	AGT	CAA	ATG
O. harrisiana 27	GAG	CTG	ATC	TCT	CCA	GCT	GCC	GCT	ATG	CTT	AGA	GTG	GTA	CTA	TGC	ATT	ATC	AAG	GGG	GAG	AAG	GCC	AGC	AGT	CAA	ATG
O. liukiuensis	GAG	CTG	ATC	TCT	CCA	GCT	GCC	GCT	ATG	CTT	AGA	ATG	ATA	CAA	TGC	ATT	ATC	AAG	GGG	GAG	AAG	GCC	AGC	AGT	CAC	ATA
O. ridleyana 56	GAG	CTG	ATC	TCT	CCA	GCT	GCC	GCT	ATG	CTT	AGA	ATG	ATA	CAA	TTC	TTT	CTC	GAG	GTG	AAG	GAG	ACC	GGC	AAT	CAC	ATA
O. ridleyana 61	GAG	CTG	ATC	TCT	CCA	GCT	GCC	GCT	ATG	CTT	AGA	GTG	GTA	ATA	TGC	ATT	ATC	AAG	GCG	GAG	AAG	GCC	AGC	AGT	CAA	ATG
O. pumila	GAG	CTG	ATC	TCT	CCA	GCT	GCC	GCT	ATG	CTT	AGA	GTG	GTA	CTT	TGC	ATT	ATC	AAG	GGG	GAG	AAG	GCC	AGC	AGT	CAA	ATG
O. fucosa 64	GAG	CTG	ATC	TCT	CCA	GCT	GCC	GCT	ATG	CTT	AGA	GTG	GTA	CTA	TGC	ATT	ATC	AAG	GGG	GAG	AAG	GCC	AGC	AGT	CAA	ATG
Camptotheca acuminata	AAG	GTT	AGT	TTT	CGA	CCT	---	GTT	ATT	GTT	AGA	ATG	ATC	CCA	TTC	TTT	CTT	AAT	GTA	AAG	GAG	ACC	GGT	AGC	CAT	GTA

Figure 4.13 The 26 positions of amino acid substitutions in TopI amino acid alignment. (A) The amino acid substitutions showing two putatively different groups separated by a black line: CPT-producing organisms (below the line) and -non-producing organisms (above the line). The gray boxes indicate amino acid substitutions which have been reported in previous studies. Arrows indicate the locations of below amino acid positions in four domains of a human TopI structure (Champoux, 2001) comprising an N-terminal domain, a core domain, a linker domain, and a C-terminal domain. The numbers beneath the human TopI structure indicate domain boundaries based on amino acid sequences of human TopI. (B) The codons translated into each amino acid substitutions in (A). Hyphens indicate gaps. Red characters represent different amino acid or nucleotide sequences.

4.3 Discussion

Sequence analysis of *mat*K revealed its conserve within the genus *Ophiorrhiza*. The *mat*K genes of all *Ophiorrhiza* species and *Joosia umbellifera* were all 1518 bp in length. Parsimony-informative nucleotides were only 1.45% from 1518 bp. Figure 4.8 showed the number of different nucleotide of each taxon. The *mat*K sequences of *O. fucosa* 64, *O. ridleyana* 61, and *O. ridleyana* 56 were completely 100% identical even if they were different species. Conversely, *mat*K sequences of plant specimens in the same species were different in 1-2 bps, for instance *O. harrisiana* and *O. pseudofasciculata.* Despite the *mat*K tree did not revealed high resolution enough to divide species, the phylogenetic consensus tree of *mat*K (Figure 4.9) revealed two major clades of *Ophiorrhiza* spp. that agree with a *trn*K/*mat*K tree previously published (Nakamura, 2006). Clade A comprised of *O. plumbea, O. harrisiana, O. fucosa, O. ridleyana, Ophiorrhiza* sp. 35, *O. pumila,* and *O. kuroiwae*. Clade B comprised of *O. pseudofasciculata, O. pedunculata, O. trichocarpon, O. japonica,* and *O. hayatana.*

The *mat*K phylogenetic tree showed a correlation of *Ophiorrhiza* spp. with production of CPT and CPT derivatives. All plants in clade A can produce CPT or CPT derivatives. For instance, *O. pumila* produces CPT and 9-MCPT (Yamazaki *et al*., 2003) and *O. kuroiwae* produces CPT and 10-MCPT (Asano *et al.*, 2009). The other *Ophiorrhiza* taxa in clade B are known to be non-CPT-producing plants, e.g. *O. hayatana* can produce only anthraquinones (Chan *et al*., 2005). In clade B, *O. ridleyana* 52 was clustered with *O. pseudofasciculata* and separated from other *O. ridleyana* specimens. Thus, Ophi 52 is clearly not *O. ridleyana* but it is closely related with *O. pseudofasciculata*. Due to the close relationships of genetic and morphological characteristics within species of *O. harrisiana*, Ophi 18 and Ophi 27 were considered as *O. harrisiana*. From these results, Ophi 52 and Ophi 18 were excluded from *Top*I analysis.

The strict consensus tree of nuclear *Top*I gene (Figure 4.11) showed similar topology to the *mat*K tree. *O. liukiuensis* in clade A was previously reported to produce

CPT, 9-MCPT and 10-MCPT (Kitajima *et al*., 2005). Compared with the chloroplast *mat*K tree, the nuclear *Top*I tree gave a much higher number of parsimony informative characters (10.68% of *Top*I and 1.45% of *mat*K) and showed a higher bootstrap percentage supporting the division of clade A and B (94% for *Top*I and 91% for *mat*K). Likewise, the *Top*I tree revealed higher resolution of the phylogenetic relationship between species within the tree and may suggest the evolutionary pattern in the genus *Ophiorrhiza*. The maximum parsimonious phylogram of *Top*I gene (Figure 4.10) suggests that CPT-non-producing *Ophiorrhiza* spp. (clade B) may exist before CPT-producing species (clade A). In fact, TopI enzyme is known to be a target of CPT. Therefore, the *Top*I gene of *Ophiorrhiza* could have evolved responsively to the emerging event of gene mutations for CPT production.

The single phylogenetic tree of combined *mat*K and *Top*I regions (Figure 4.12) also strongly confirmed the separation between the two groups of CPT-producing and CPT-non-producing *Ophiorrhiza* plants with a very high bootstrap value (98%). Currently, it has been no report of subgenus division in the genus *Ophiorrhiza*. In this study, there is obvious correlation between camptothecinoid detection and taxonomic positions of *Ophiorrhiza* spp. based on *mat*K and *Top*I phylogenetic trees. Thus, it is possible to divide *Ophiorrhiza* into two chemotaxonomic groups: camptothecinoid producers and camptothecinoid-non-producers.

The alignments of *mat*K and *Top*I nucleotide sequences showed several polymorphic loci which can be utilized to design molecular markers to differentiate CPT-producing and CPT-non-producing *Ophiorrhiza.* For instance, PCR-RFLP method can be developed using the different enzyme restriction sites between two groups of *Ophiorrhiza.* SCAR marker may be used if there is specific band obtained from RAPD technique.

The alignment of TopI amino acid sequences (Appendix E) showed moderate polymorphisms between *Ophiorrhiza* spp. and other plants; e.g. 68% identity between *O. plumbea* 6 and *Camptotheca acuminata*, and 63% identity between *O. harrisiana* 27 and *Catharanthus roseus*. The amino acid polymorphisms in various residue-positions (Figure 4.13) revealed the division between all examined organisms, which could be separated into two groups of CPT-producing and -non-producing organisms. Previous studies of the structure of human TopI enzyme (Champoux *et al*., 2001; Redinbo *et al*., 1998; Sirikantaramas *et al*., 2008) suggested several mutated amino-acid residues that may contribute to production of a CPT-resistant TopI. The mutations of Leu-530 to Ile which were found only in *O. pumila* and *O. liukiuensis*, could disrupt CPT-binding by shifting the Asp-533 that binds to CPT (numbered according to human TopI) (Sirikantaramas *et al*., 2008) The Asn-722 which lies next to the active-site Tyr-733 has been reported to be mutated to Ser or Asp in CPT-producing plants and mutated cells which are resistant to CPT, such as some human leukemia cell-lines, yeast *Saccharomyces* spp. and viruses (Gupta *et al*., 1995).

In this study, we found that most of CPT-producing *Ophiorrhiza* species had two amino acid mutations of Leu-530 to Ile and Asn-722 to Ser, which were identical to a previous study (Sirikantaramas *et al*., 2008). We also found two amino acid substitutions at Met-370 and Gly-717, which have been previously reported only in yeast and human mutated cells (Wang *et al.*, 1997) but never been reported in plants. Other substituted residues (Figure 4.13) also suggest the amino acid markers in the TopI sequences of the CPT-producing or CPT derivative-producing *Ophiorrhiza* plants were comparable to the amino acid positions in four distinct domains of human TopI. These substituted residues were located near the mutated positions that affect TopI structure (Redinbo *et al*., 1998) but had never been found in CPT-resistant human cancer cells.

The phylogenetic tree and TopI amino acid analysis results confirm that Ophi 37 and Ophi 62 were *O. pseudofasciculata.* Although *Ophiorrhiza* sp. 35 showed only CPT derivatives production but not CPT, it was placed in clade A of both *mat*K and *Top*I phylogenetic trees. Additionally, several mutated TopI residues of this plant were identical to those of CPT-producing plants. Surprisingly, *O. ridleyana* 56 was placed in clade A of both *mat*K and *Top*I phylogenetic trees but had non-mutated amino acid residues in reported critical positions. The full-length *Top*I gene of *O. ridleyana* 56 was closely related with *O. ridleyana* 61 and other CPT-producing *Ophiorrhiza* spp. However, this plant may not have TopI mutation as a self-resistance mechanism, otherwise, there would be new point mutations which cause resistance in TopI. Hence, any further study should focus on the effect of these amino acid substitutions on protein structure, CPT-binding site, and enzyme activity of TopI.

CHAPTER V

CONCLUSION: COEVOLUTION OF *TOPOISOMERASE* I AND CAMPTOTHECIN PRODUCTION

Five species of *Ophiorrhiza*, *O. fucosa, O. harrisiana, O. plumbea, O. ridleyana,* and *Ophiorrhiza* sp. 35, out of eight species collected in this study, are reported the detections of CPT, CPT derivatives, and chemical compounds involved in CPT biosynthesis pathway. The distribution of *Ophiorrhiza* spp. suggests that *Ophiorrhiza* in Thailand had CPT production abilities mainly related to species, not habitat. The sequence analyses of chloroplast *mat*K and nuclear *Top*I genes suggest that genetic factors play an important role in determining CPT and CPT derivatives-producing properties of *Ophiorrhiza* plants. By reason that the molecular phylogenetic trees of both separated and combined *mat*K and *Top*I nucleotide sequences had similar topology and correlated with production of CPT and CPT derivatives, we conclude that *Ophiorrhiza* plants have a coevolution of *mat*K and *Top*I genes with production of CPT and CPT derivatives.

In fact, TopI enzyme is known to be a target of CPT. Therefore, the *Top*I gene of *Ophiorrhiza* could have evolved responsively to the emerging event of gene mutations for CPT production. Several amino acid residues in the *Top*I gene are preserved in CPT-producing *Ophiorrhiza* plants, probably as a self-resistance mechanism to avoid self-toxicity. Despite encoded protein of *mat*K gene is not correlated with TopI enzyme or even CPT, the phylogenetic tree exhibits the coevolution between *mat*K and CPT production. It could be possible that CPT-producing ability is established in ancestor of *Ophiorrhiza* plants in ancient times.

The alignments of *mat*K and *Top*I nucleotide sequences showed several identical positions of CPT-producing *Ophiorrhiza* which can be utilized to design molecular markers for differentiation of anticancer *Ophiorrhiza* species from non-anticancer species. For instance, PCR-RFLP method can be developed using the different enzyme restriction sites between two groups of *Ophiorrhiza.* SCAR marker may be used if there is specific band obtained from RAPD technique.

According to the coevolution of *mat*K, *Top*I genes and production of CPT and CPT derivatives, *mat*K and *Top*I gene sequences could be utilized for prediction of CPT- and CPT derivatives-production ability of any members of *Ophiorrhiza*. Such molecular techniques have greater advantages than chemical techniques to suggest production of CPT and CPT derivatives in *Ophiorrhiza* spp. For instance, we can use this molecular technique for plants that produce trace amounts of CPT at levels below the initial detection point of the equipment. Likewise, some plants may produce only CPT derivatives that are more sensitive than the parental CPT molecule and some of this amount may be lost through the material processing technique. Moreover, our molecular analysis is not affected by seasonal variability, plant elicitor, or stage and part of the plant material. If any plant of the genus is analyzed and placed in clade A of the *mat*K and *Top*I phylogenetic trees, this would indicate a close relationship to CPT-producing plants and thus they may produce some amount of CPT or CPT derivatives.

Additionally, the mutation points in TopI amino acid sequences also supported the nucleotide phylogenetic trees on the prediction of CPT-producing ability in *Ophiorrhiza*. The results in the present study thus strengthen our hypothesis that members of the genus *Ophiorrhiza* producing CPT or CPT derivatives should have specific mutations in the *Top*I gene. CPT-producing *O. ridleyana* 56, which placed in clade A of both *mat*K and *Top*I phylogenetic trees, had non-mutated amino acid residues in reported critical positions. This disagreeable result brings into question that there would be other unreported point mutations in TopI which cause CPT-resistance. This study is fundamental research toward anticancer development from natural resources based on CPT and CPT derivatives. Any further study should focuses on the effect of amino acid substitutions on protein structure, CPT-binding site and enzyme activity of TopI. The anticancer-producing *Ophiorrhiza* species should be used as alternative sources of anticancer research and pharmaceutical industrial production development. Subsequently research should focuses on a quantitative analysis of CPT and CPT derivatives production. Tissue culture technique is also interesting to be utilized for increasing the CPT-producing potential of *Ophiorrhiza* plants in Thailand. Our finding could provide useful information toward recognition of the point mutations in CPT-resistant cancer patients in the future.

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APPENDICES

APPENDIX A

Ophiorrhiza specimens

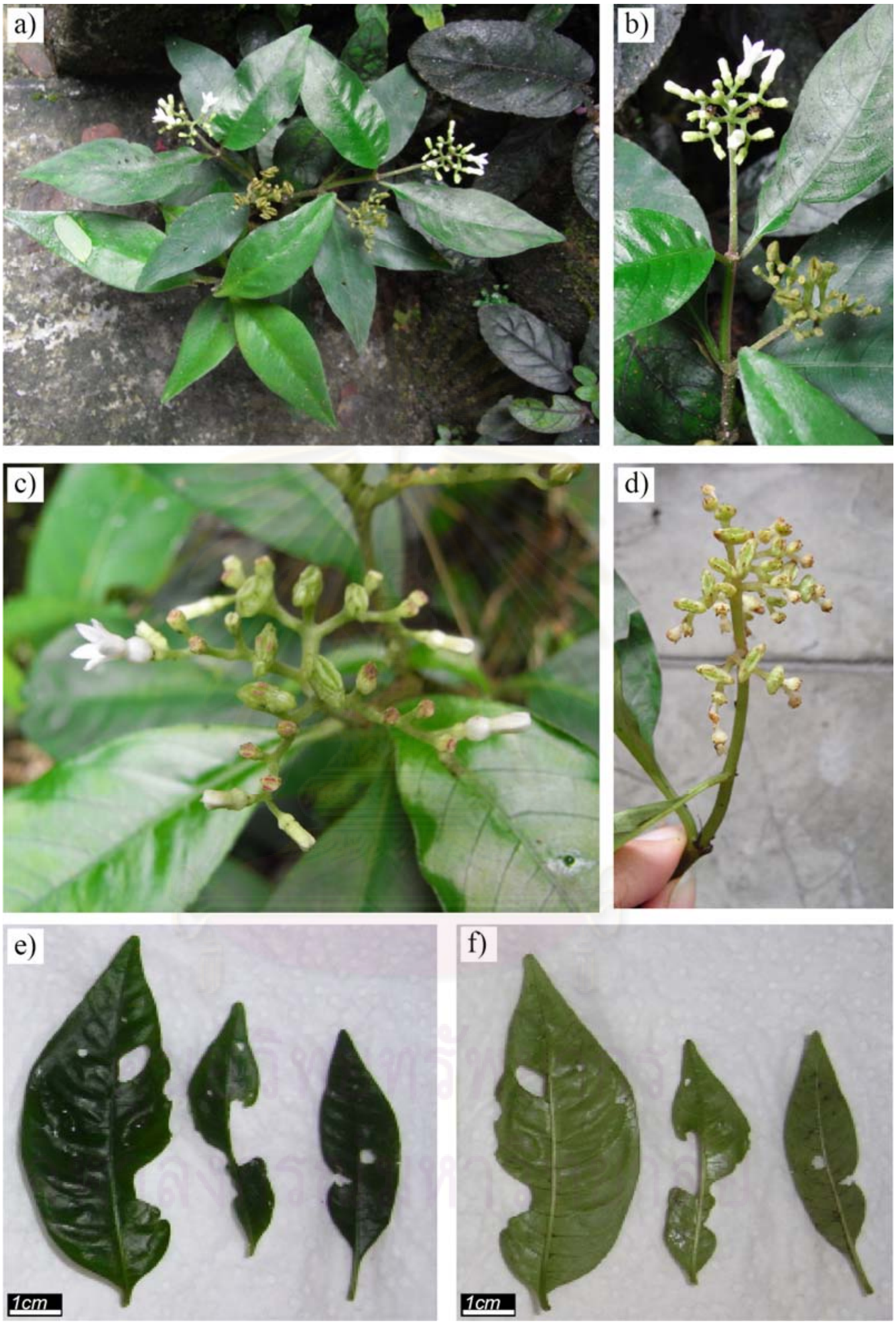


Figure A1 *Ophiorrhiza fucosa* Hance: a) habitat; b) and c) inflorescence; d) peduncle in fruit; e) upper leaf surface; f) lower leaf surface.

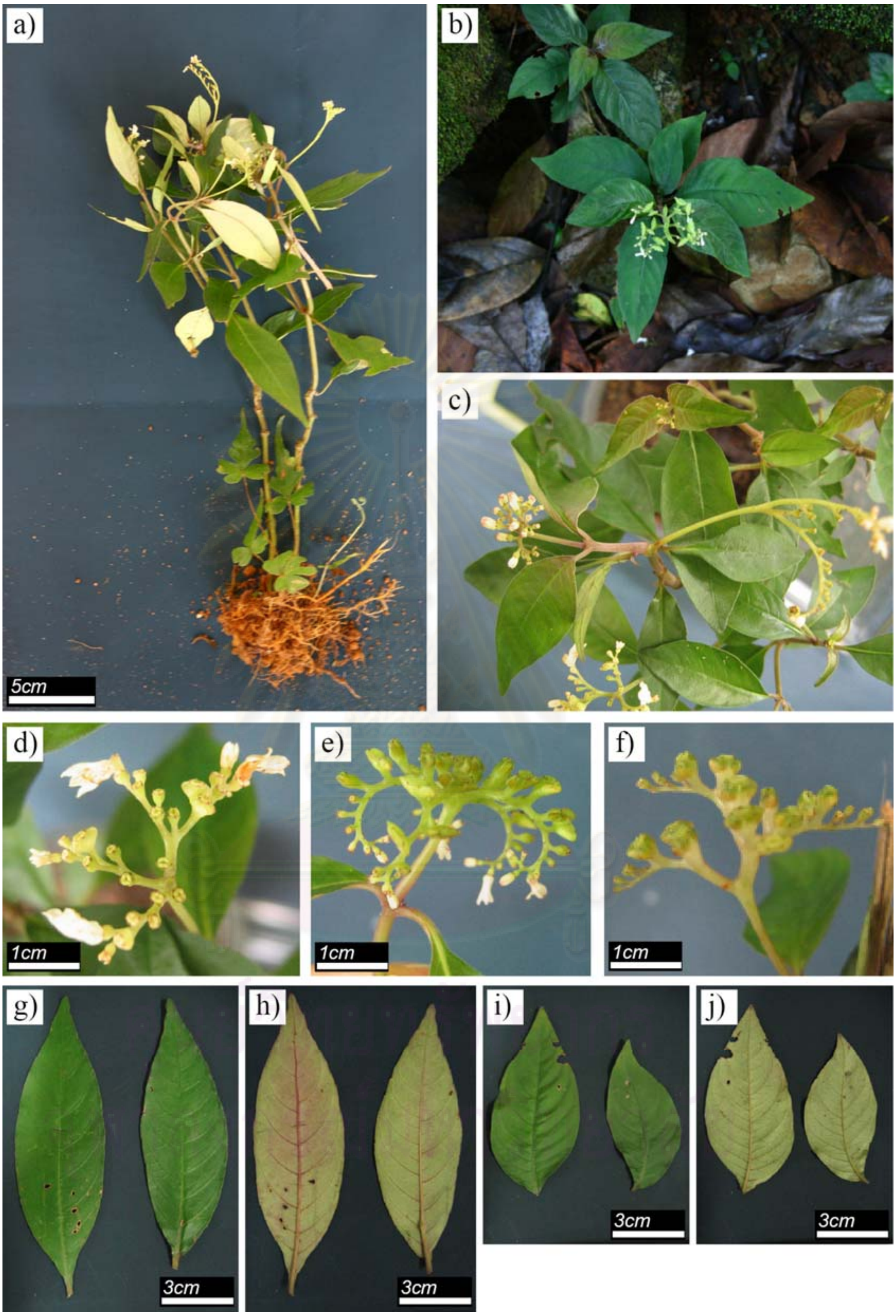


Figure A2 *Ophiorrhiza harrisiana* B. Heyne ex Hook. f.: a) whole plant; b) habitat; c), d), e), and f) inflorescence; g) and i) upper leaf surface; h) and j) lower leaf surface.

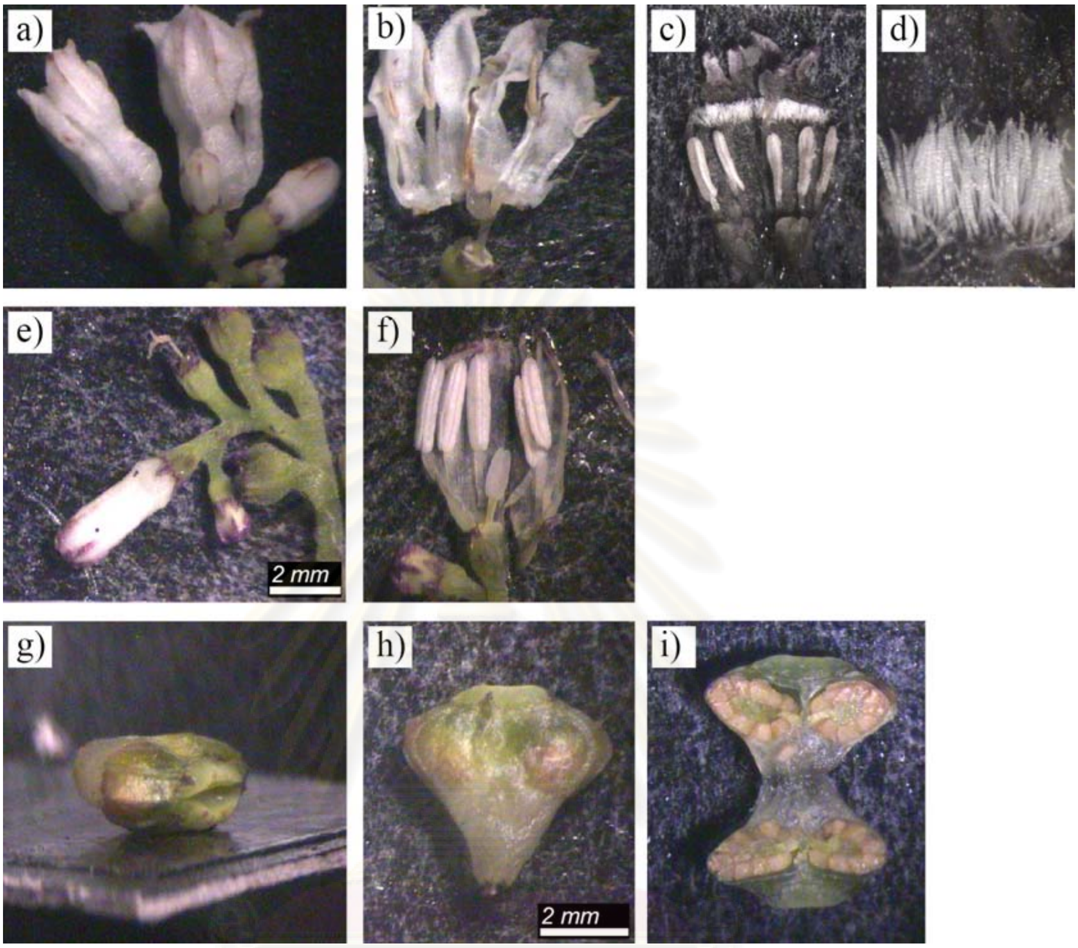


Figure A3 Stereo Microscope images of *Ophiorrhiza harrisiana* B. Heyne ex Hook. f.: a) and e) inflorescence (7X); b) and f) brevistylous flower (7X, 10X); c) longistylous flower (10X); d) hair ring of c) (45X); g), h) and i) fruit (7X). X indicates magnification of image.

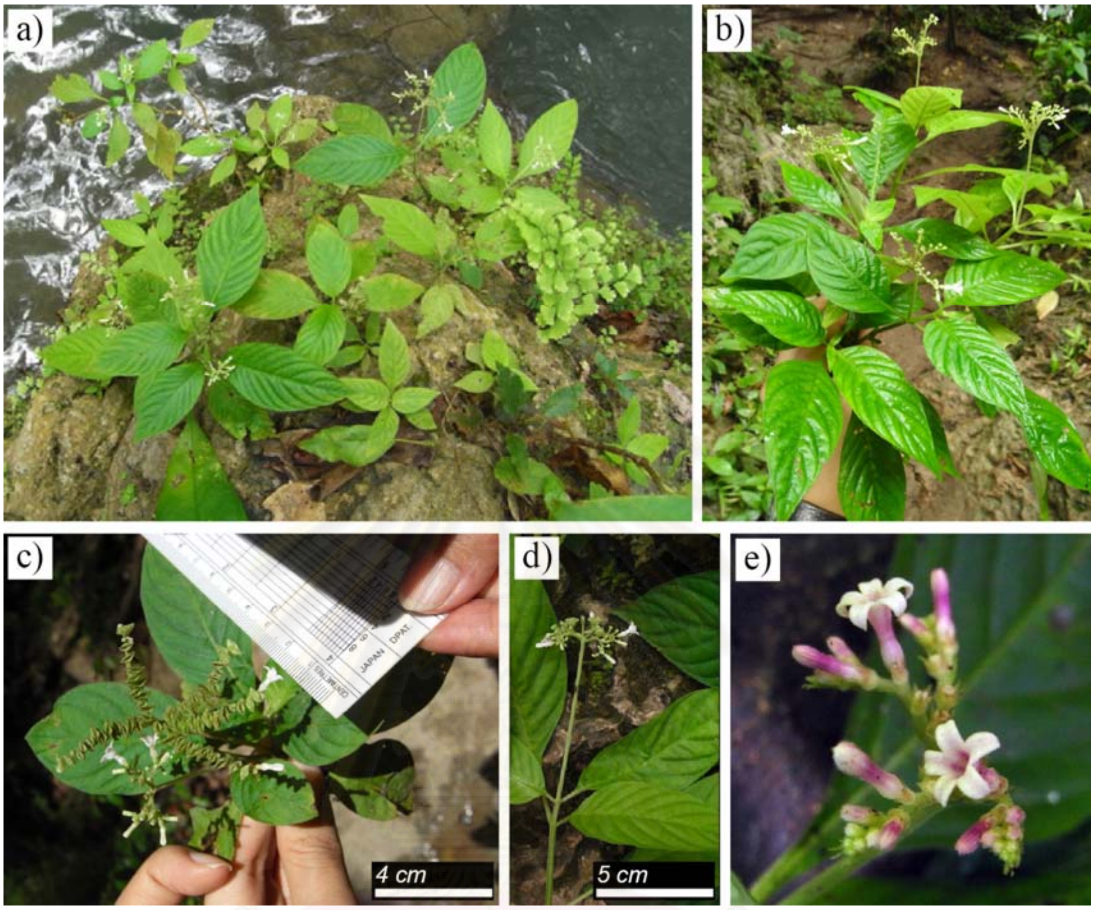


Figure A4 *Ophiorrhiza pedunculata* Schanzer (*O. hispidula* Wall. ex G.Don var. *longipedunculata* Craib): a) habitat; b) habit; c) inflorescence; d) peduncle; e) flowers.

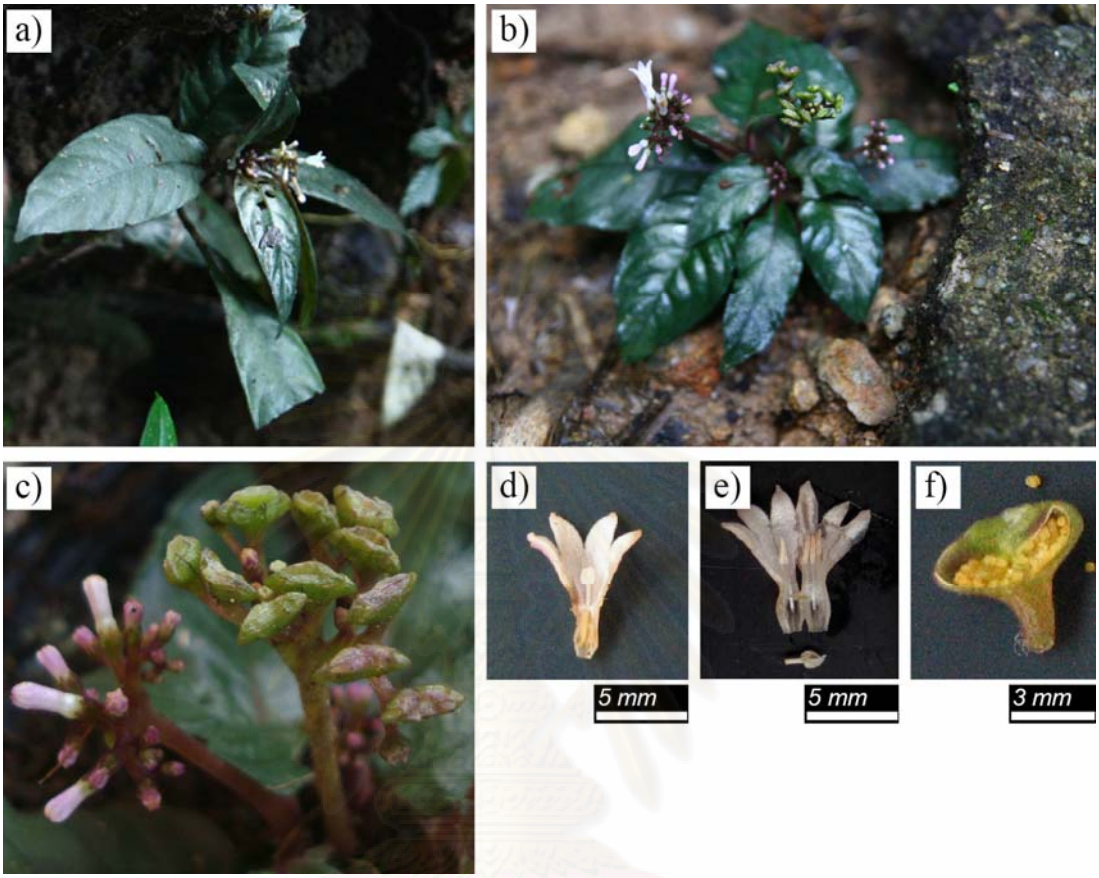


Figure A5 *Ophiorrhiza plumbea* Craib: a) and b) habitat; c) inflorescence; d) longistylous flower; e) brevistylous flower; f) fruit.

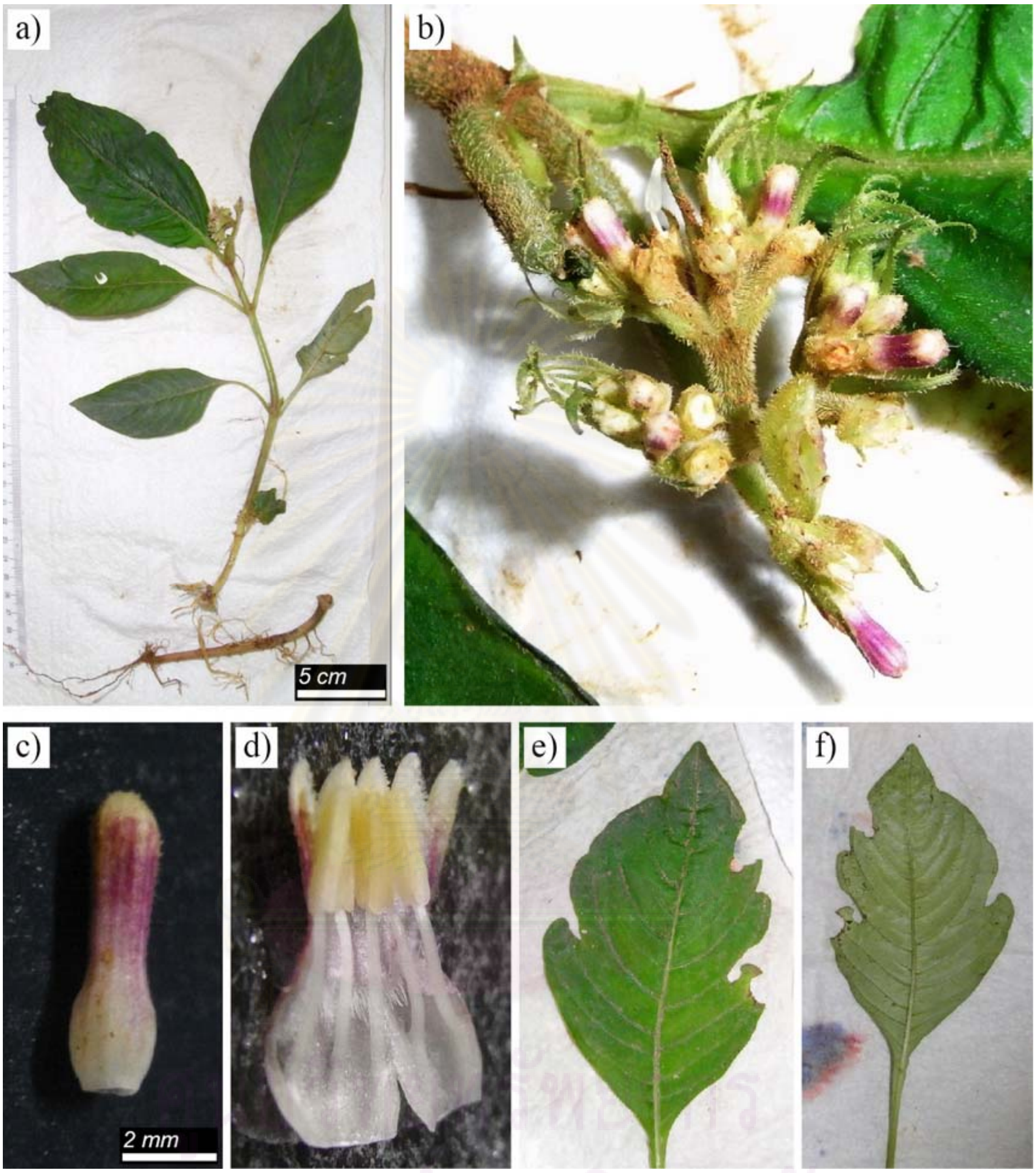


Figure A6 *Ophiorrhiza pseudofasciculata* Schanzer, Ophi 37: a) whole plant; b) inflorescence; c) and d) flower; e) upper leaf surface; f) lower leaf surface.

Figure A7 *Ophiorrhiza pseudofasciculata* Schanzer, Ophi 62: a) habit; b) inflorescence; c) and d) enlarged inflorescence; e) upper leaf surface.

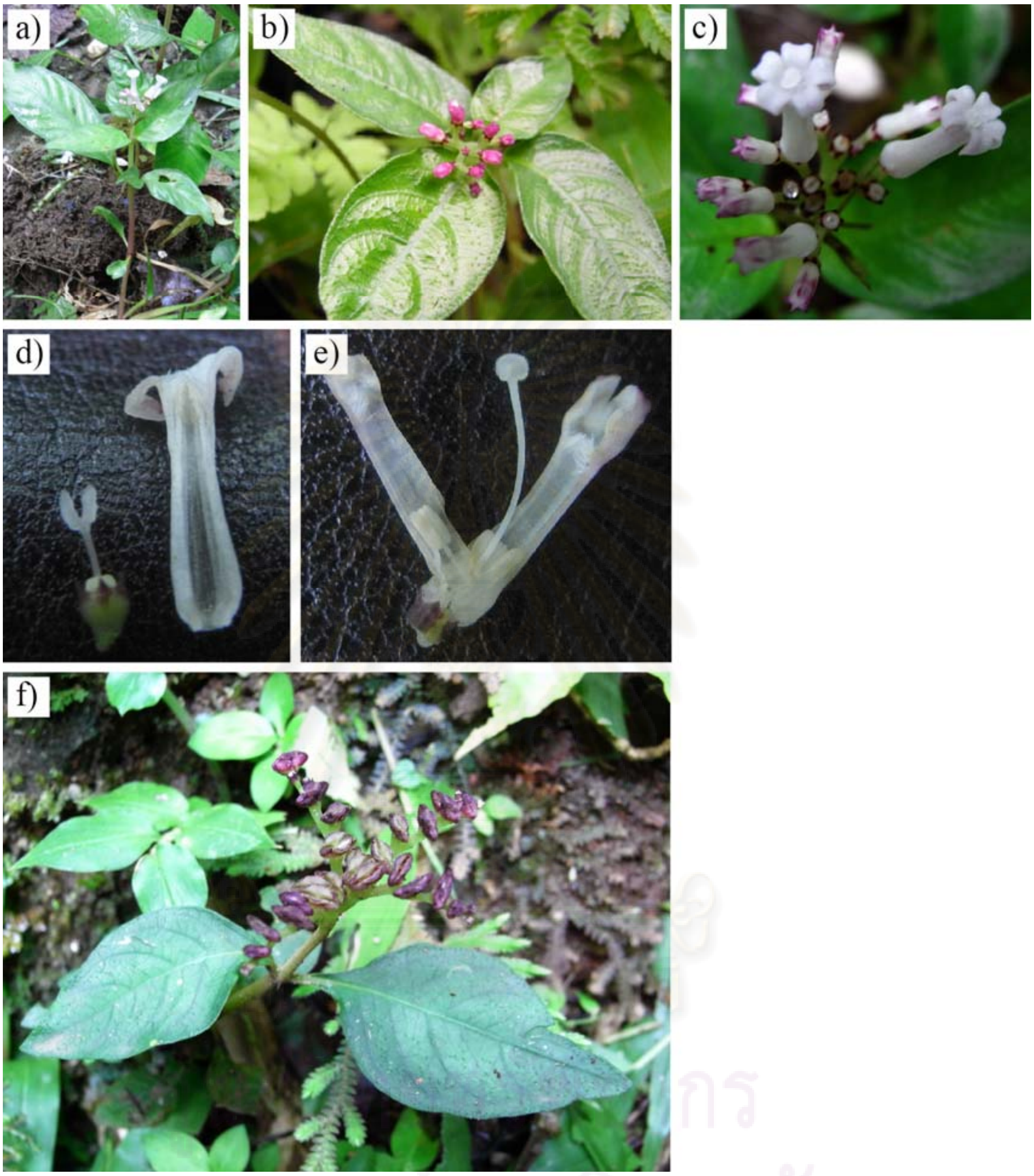


Figure A8 *Ophiorrhiza ridleyana* Craib: a) habit; b) flower buds; c) inflorescence; d) brevistylous flower; e) longistylous flower; f) fruits.

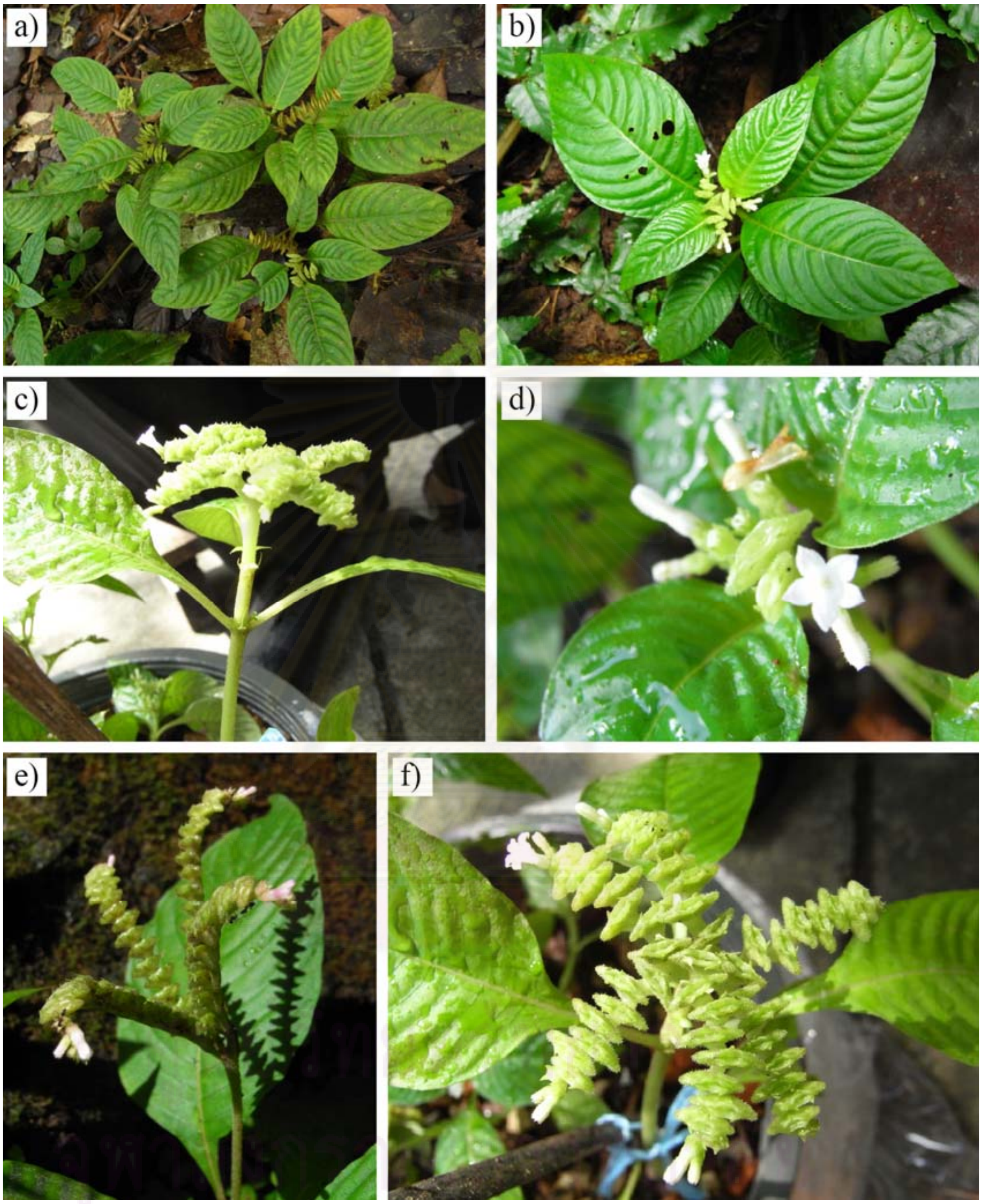


Figure A9 *Ophiorrhiza trichocarpon* Blume var. *glabra* Schanzer: a) habitat; b) habit; c) inflorescence; d) enlarged flower; e) peduncle in fruit; f) enlarged fruits.

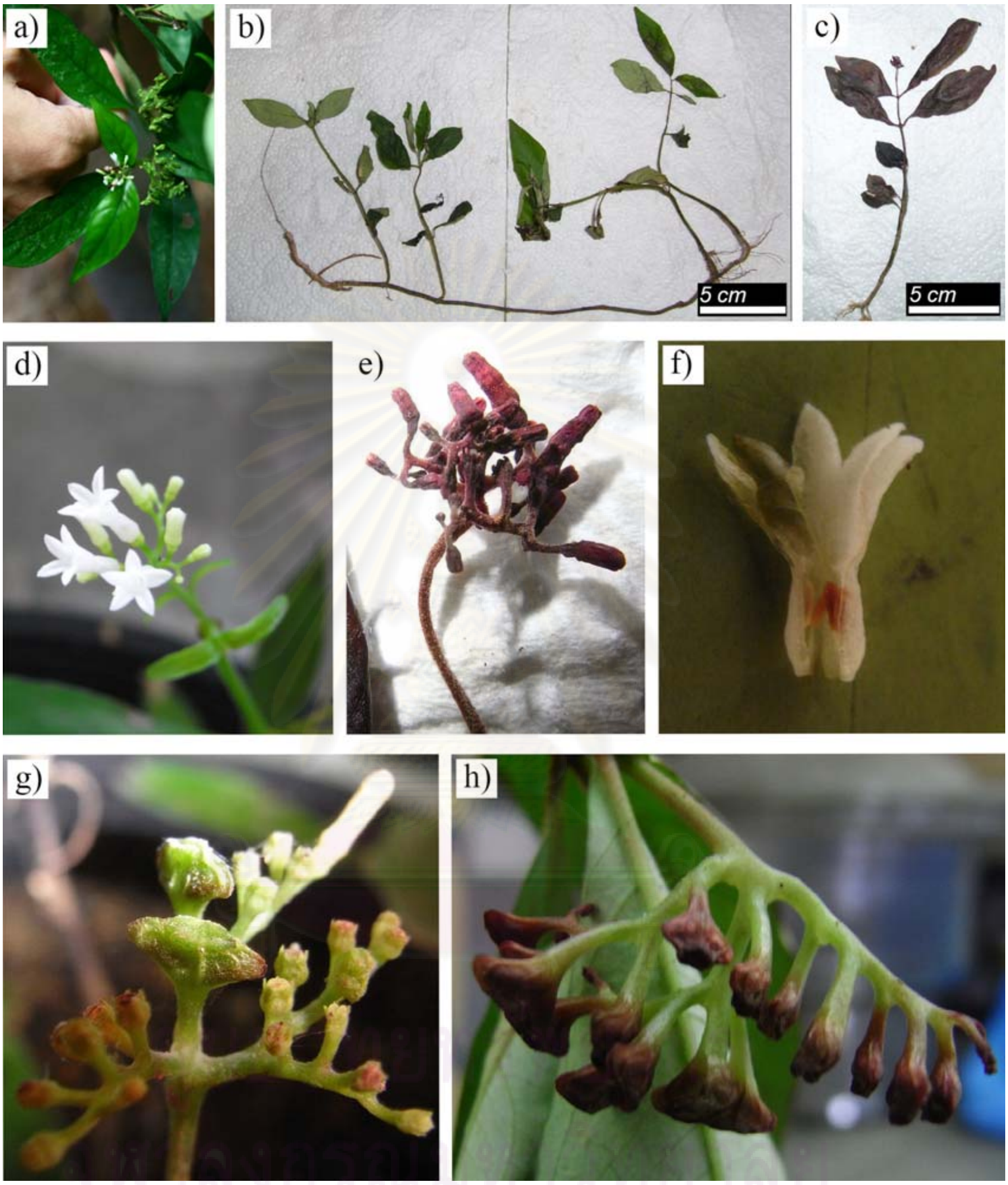


Figure A10 *Ophiorrhiza sp.* 35: a) habit; b) whole plant; c) bruised whole plant; d) inflorescence; e) bruised inflorescence; f) longistylous flower; g) fruits; h) bruised fruits.

APPENDIX B

HPLC-DAD chromatograms, UV spectra and mass spectra of standard compounds

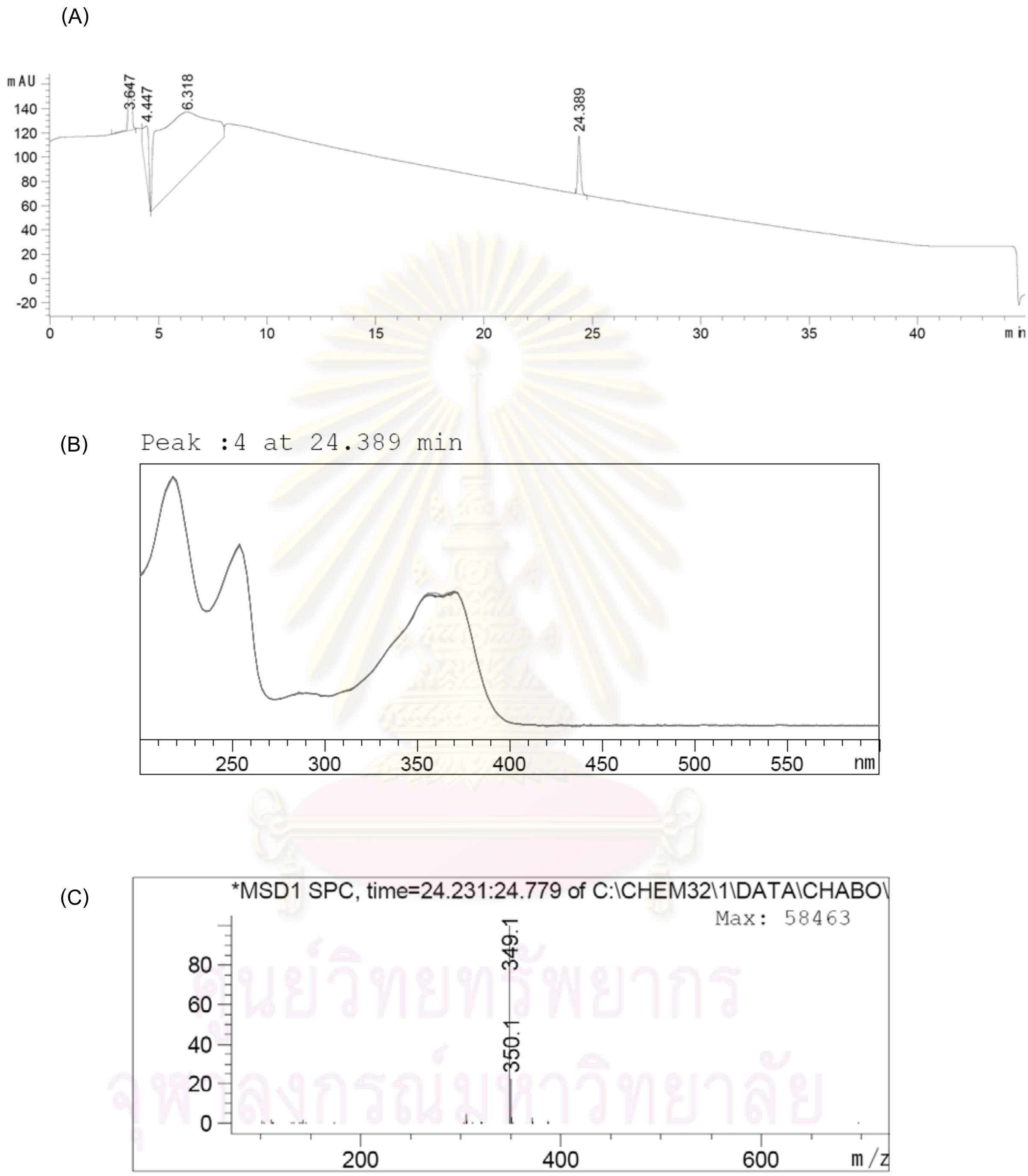


Figure B1 HPLC-DAD chromatograms, UV spectra and mass spectra of 10 ng/10 µL camptothecin standard: (A) HPLC-DAD chromatogram monitored at 254 nm; (B) UV spectrum and (C) mass spectrum.

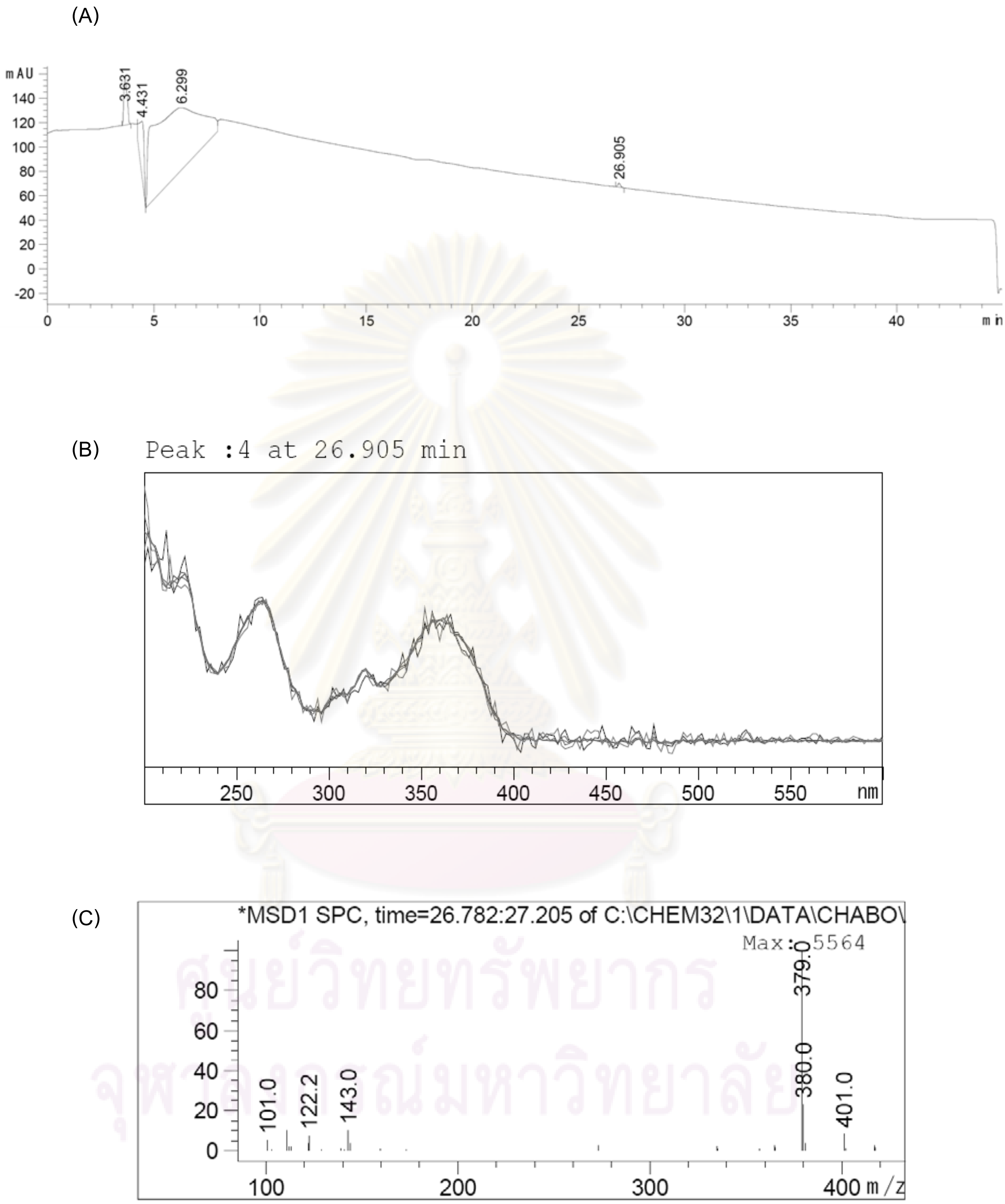


Figure B2 HPLC-DAD chromatograms, UV spectra and mass spectra of 100 ng/10 µL 9-methoxy camptothecin standard: (A) HPLC-DAD chromatogram monitored at 254 nm; (B) UV spectrum and (C) mass spectrum.

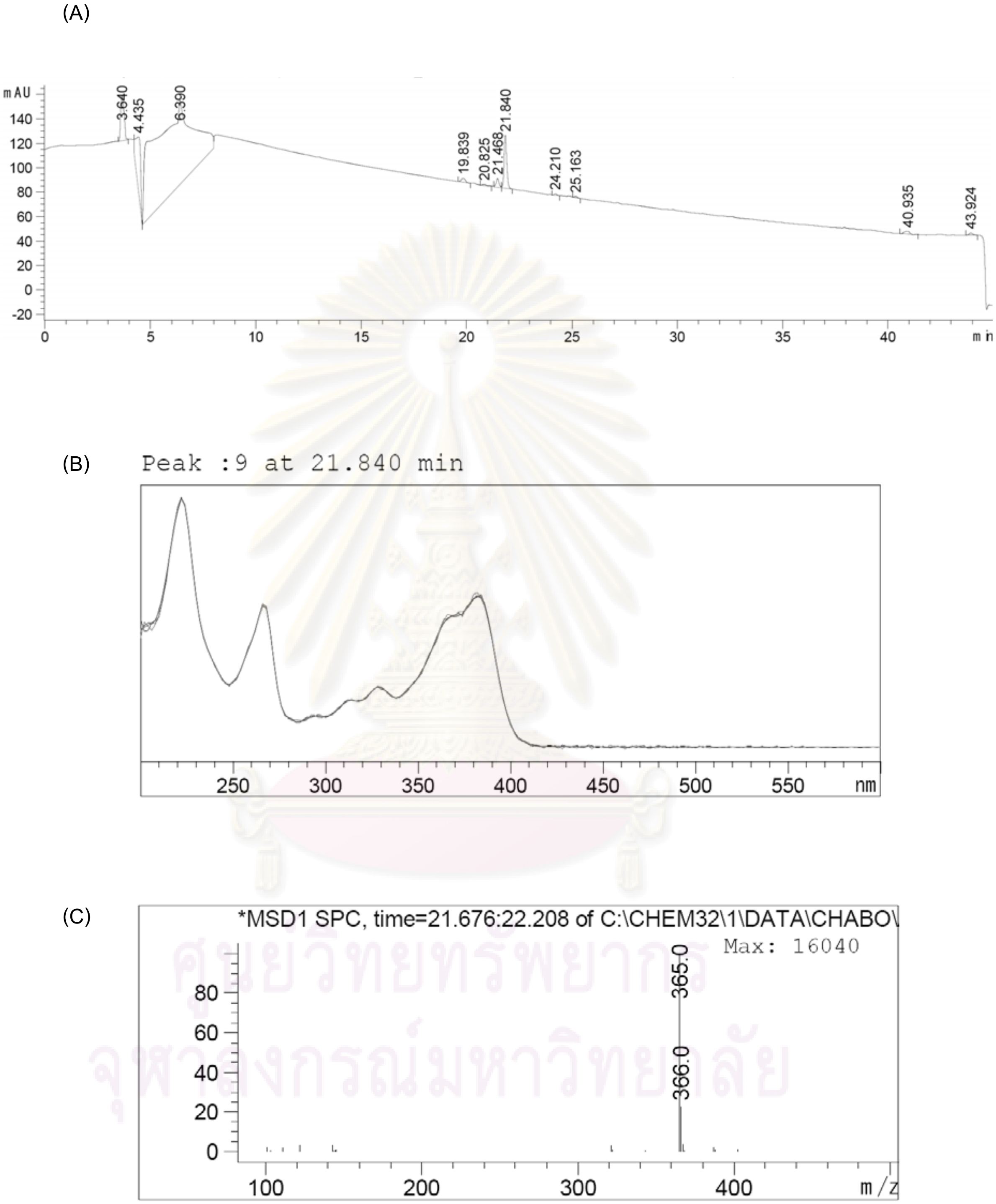
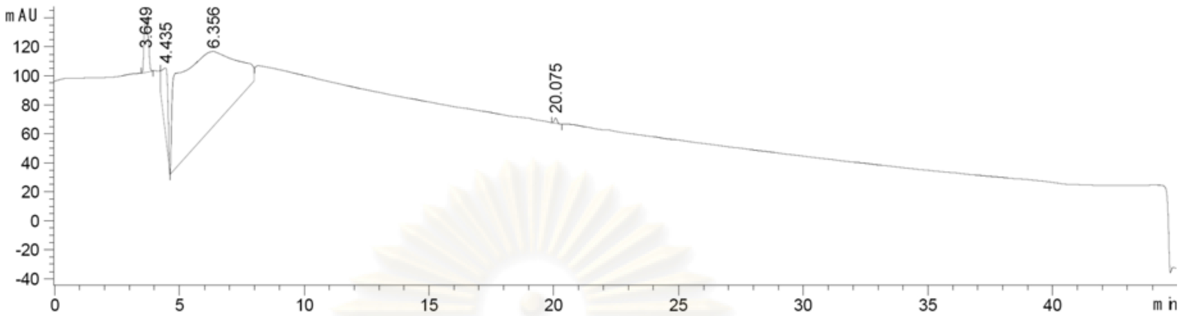
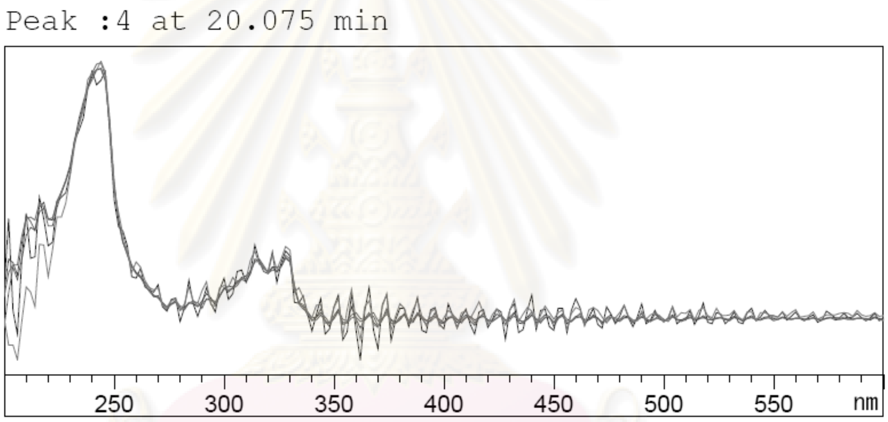


Figure B3 HPLC-DAD chromatograms, UV spectra and mass spectra of 1 µg/µL 10-hydroxy camptothecin standard: (A) HPLC-DAD chromatogram monitored at 254 nm; (B) UV spectrum and (C) mass spectrum.

(A)



(B)



(C)

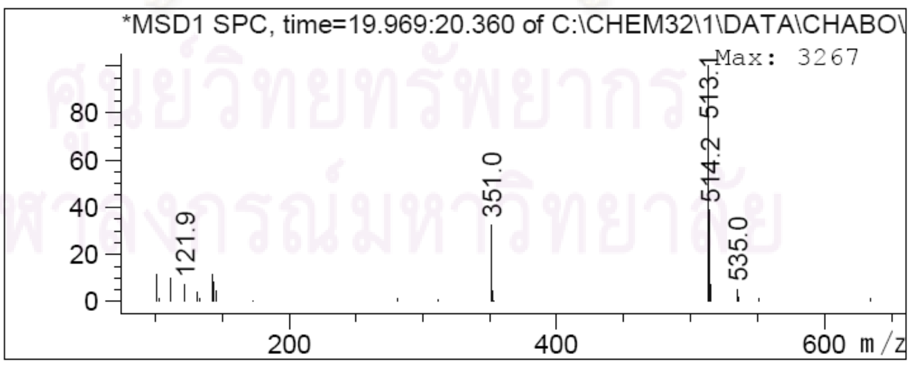
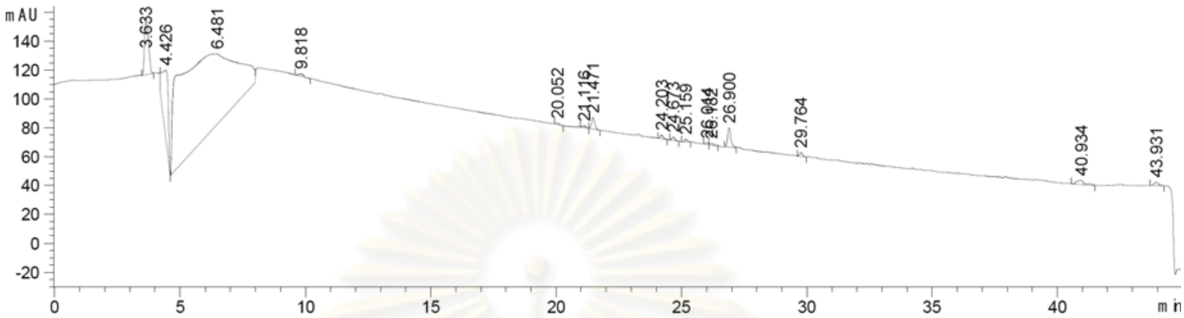
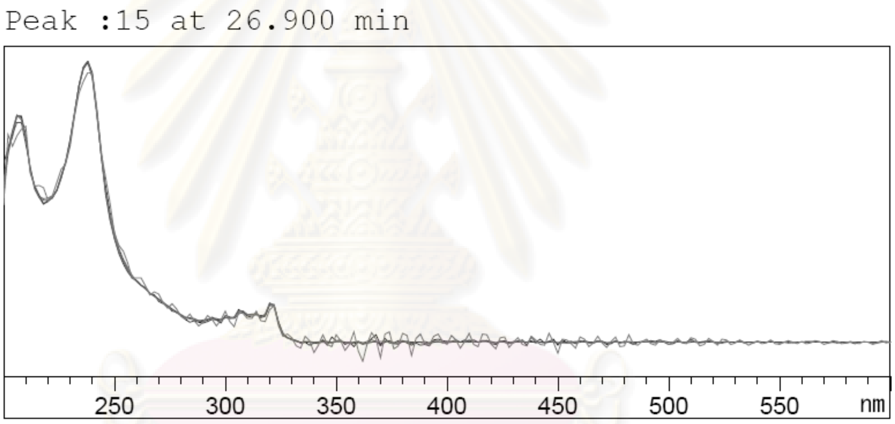


Figure B4 HPLC-DAD chromatograms, UV spectra and mass spectra of 100 ng/10µL pumiloside standard: (A) HPLC-DAD chromatogram monitored at 254 nm; (B) UV spectrum and (C) mass spectrum.

(A)



(B)



(C)

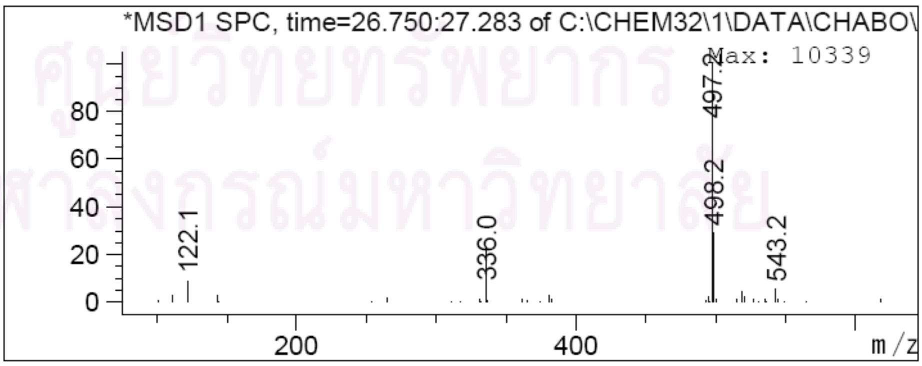
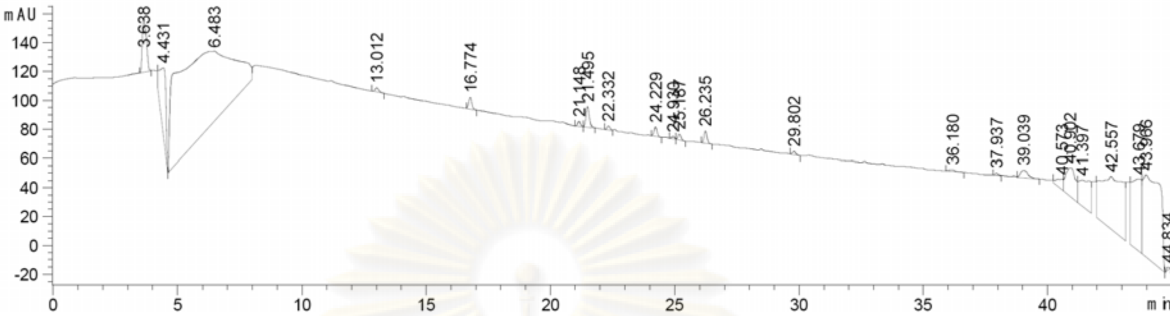


Figure B5 HPLC-DAD chromatograms, UV spectra and mass spectra of 1µg/µL 3(*S*)-deoxy pumiloside standard: (A) HPLC-DAD chromatogram monitored at 254 nm; (B) UV spectrum and (C) mass spectrum.

(A)



(B)

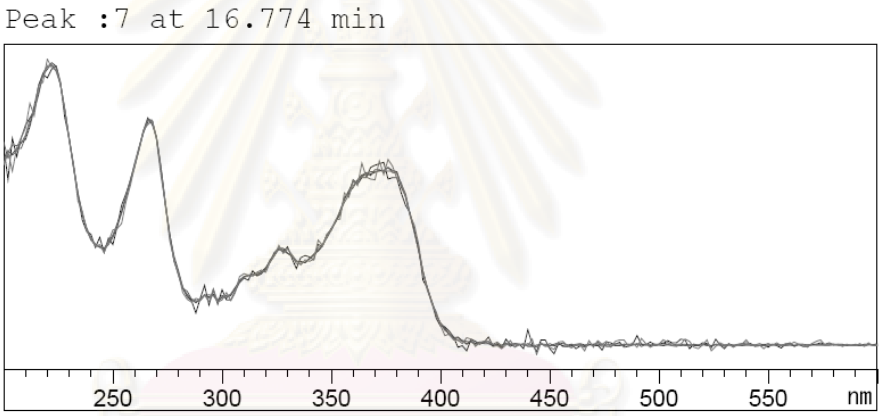
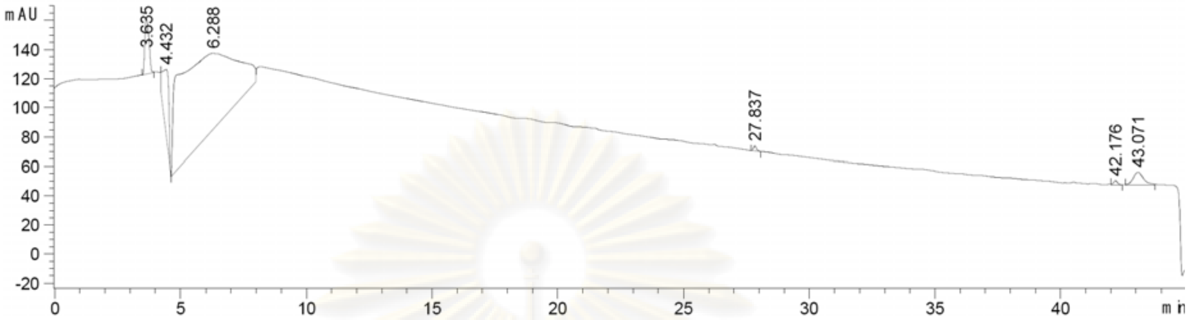
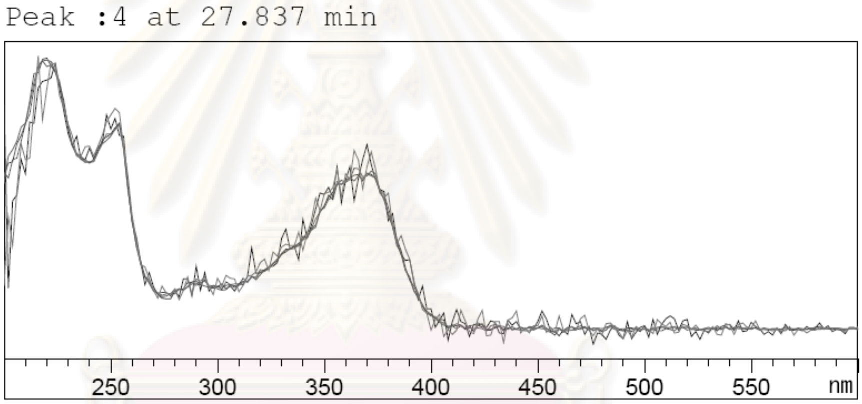


Figure B6 HPLC-DAD chromatograms, UV spectra and mass spectra of 1μg/μL chaboside standard: (A) HPLC-DAD chromatogram monitored at 254 nm; (B) UV spectrum. Mass spectrum cannot be detected.

(A)



(B)



(C)

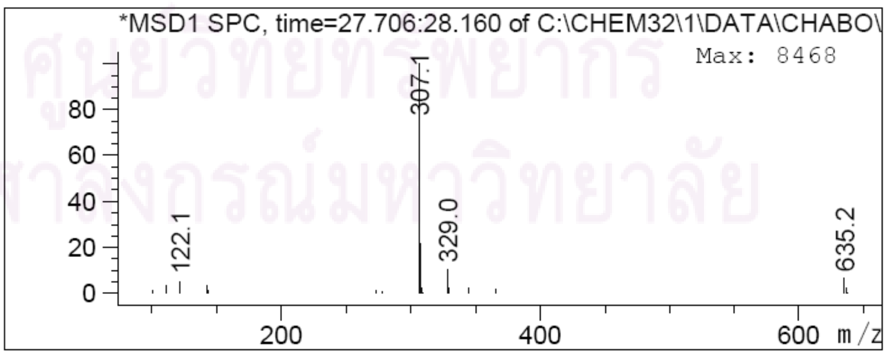


Figure B7 HPLC-DAD chromatograms, UV spectra and mass spectra of 500 ng/µL mappicine standard: (A) HPLC-DAD chromatogram monitored at 254 nm; (B) UV spectrum and (C) mass spectrum.

APPENDIX C

The alignment of *mat*K nucleotide sequences of *Ophiorrhiza* species.

Dots represent identical nucleotides. Red characters represent different nucleotides.

```
                                                     20                                              40                                              60                                              80
O. plumbea 6                 ATGGAGAAAA TCCAAAGATA TTTACAGCTT GATAGATCTC AACAACACGG CTTTTTATAT CCACTTATCT TTCAGGAGTA 80
O. harrisiana 18             .......... .......... .......... .......... .......... .......... .......... .......... 80
O. harrisiana 27             .......... .......... .......... .......... .......... .......... .......... .......... 80
O. fucosa 64                 .......... .......... .......... .......... .......... .......... .......... .......... 80
O. ridleyana 61              .......... .......... .......... .......... .......... .......... .......... .......... 80
O. ridleyana 56              .......... .......... .......... .......... .......... .......... .......... .......... 80
Ophiorrhiza sp. 35           .......... .......... .......... .......... .......... .......... .......... .......... 80
O. pumila                    .......... .......... .......... .......... .......... .......... .......... .......... 80
O. kuroiwae                  .......... .......... .......... .......... .......... .......... .......... .......... 80
O. pseudofasciculata 37      .......... .......... .......... .......... .......... .......... .......... .......... 80
O. ridleyana 52              .......... .......... .......... .......... .......... .......... .......... .......... 80
O. pseudofasciculata 62      .......... .......... .......... .......... .......... .......... .......... .......... 80
O. pedunculata 41            .......... .......... .......... .......... .......... .......... ........T. .......... 80
O. trichocarpon 46           .......... .......... .......... .......... .......... .......... ........T. .......... 80
O. japonica                  .......... .......... .......... .......... .......... .......... .......... .......... 80
O. hayatana                  .......... .......... .......... .......... .......... .......... .......... .......... 80
Joosia umbellifera           ......G..G ..G....... .......... .......... .......... ....C..... .......... .......... 80
                                                    100                                             120                                             140                                             160
O. plumbea 6                 TGTTTATGGA CTTGCTCATG ATCATAGGTT AAACCGATCT AGTTTGTTGG AAAATCCAGG TTATGACAAA AAATCCAGTT 160
O. harrisiana 18             .......... .......... .......... .......... .......... .......... .......... .......... 160
O. harrisiana 27             .......... .......... .......... .......... .......... .......... .......... .......... 160
O. fucosa 64                 .......... .......... .......... .......... .......... .......... .......... .......... 160
O. ridleyana 61              .......... .......... .......... .......... .......... .......... .......... .......... 160
O. ridleyana 56              .......... .......... .......... .......... .......... .......... .......... .......... 160
Ophiorrhiza sp. 35           .......... .......... .......... .....A.... .......... .......... ......G... .......... 160
O. pumila                    .......... .......... .......T.. .......... .......... .......... .......... .......... 160
O. kuroiwae                  .......... .......... .......T.. .......... .......... .......... .......... .......... 160
O. pseudofasciculata 37      .......... .......... .......T.. .......... .......... .......... .......... .......... 160
O. ridleyana 52              .......... .......... .......T.. .......... .......... .......... .......... .......... 160
O. pseudofasciculata 62      .......... .......... .......T.. .......... .......... .......... .......... .......... 160
O. pedunculata 41            .......... .......... .......T.. .......... .......... .......... .......... ....T..... 160
O. trichocarpon 46           .......... .......... .......T.. .......... .......... .......... .......... .......... 160
O. japonica                  .......... .......... .......T.. .......... .......... .......... .......... .......... 160
O. hayatana                  .......... .......... .......T.. .......... .......... .......... .......... .......... 160
Joosia umbellifera           .A......C. .......... .......T.. T......... .T........ .......... ......G..T C...T..... 160
                                                    180                                             200                                             220                                             240
O. plumbea 6                 TCCTAATTGT GAAACGTTTA ATTACTCGAA TGTATCGACA AAATCATTTT ATTATTTTTG CTAATGATTC TAATCAAAAT 240
O. harrisiana 18             .......... .......... .......... ......A... .......... .......... .......... .......... 240
O. harrisiana 27             .......... .......... .......... ......A... .......... .......... .......... .......... 240
O. fucosa 64                 .......... .......... .......... ......A... .......... .......... .......... .......... 240
O. ridleyana 61              .......... .......... .......... ......A... .......... .......... .......... .......... 240
O. ridleyana 56              .......... .......... .......... ......A... .......... .......... .......... .......... 240
Ophiorrhiza sp. 35           .......... .......... .......... ......A... .......... .......... .......... .......... 240
O. pumila                    .......... .......... .......... ......A... .......... .......... .......... .......... 240
O. kuroiwae                  .......... .......... .......... ......A... .......... .......... .......... .......... 240
O. pseudofasciculata 37      .......... .......... .......... ......A... .......... .......... .......... .......... 240
O. ridleyana 52              .......... .......... .......... ......A... .......... .......... .......... .......... 240
O. pseudofasciculata 62      .......... .......... .......... ......A... .......... .......... .......... .......... 240
O. pedunculata 41            .......... .......... .......... ......A... .......... .......... .......... .......... 240
O. trichocarpon 46           .......... .......... .......... ......A... .......... .......... .......... .......... 240
O. japonica                  .......... .......... .......... ......A... .......... .......... .......... .......... 240
O. hayatana                  .......... .......... .......... ......A... .......... .......... .......... .......... 240
Joosia umbellifera           .......... .......... .......... ......A... .......... .......... .......... A..C...... 240
                                                    260                                             280                                             300                                             320
O. plumbea 6                 CGAGTTTTTG GTTGCAACAA GAATTTCTAT CCTCAAACCA TATCAGAAGG GTTTGCATTT ATTGTGGAAA TTCCATTTGA 320
O. harrisiana 18             .......... .......... .......... .......... .......... .......... .......... .......... 320
O. harrisiana 27             .......... .......... .......... .......... .......... .......... .......... .......... 320
O. fucosa 64                 .......... .......... .......... .......... .......... .......... .......... .......... 320
O. ridleyana 61              .......... .......... .......... .......... .......... .......... .......... .......... 320
O. ridleyana 56              .......... .......... .......... .......... .......... .......... .......... .......... 320
Ophiorrhiza sp. 35           .......... .......... .......... .......... .......... .......... .......... .......... 320
O. pumila                    .......... .......... .......... .......... .......... .......... .......... .......... 320
O. kuroiwae                  .......... .......... .......... .......... .......... .......... .......... .......... 320
O. pseudofasciculata 37      .......... .......... .......... .......... .......... .......... .......... .......... 320
O. ridleyana 52              .......... .......... .......... .......... .......... .......... .......... .......... 320
O. pseudofasciculata 62      .......... .......... .......... .......... .......... .......... .......... .......... 320
O. pedunculata 41            .......... .......... .......... .......... .......... .......... .......... .......... 320
O. trichocarpon 46           .......... .......... .......... .......... .......... .......... .......... .......... 320
O. japonica                  .......... .......... .......... .......... .......... .......... .......... .......... 320
O. hayatana                  .......... .......... .......... .......... .......... .......... .......... .......... 320
Joosia umbellifera           .C.T...... ..C.T..... ......AG.. T......TG. .......... A......... .......... ........T. 320
                                                    340                                             360                                             380                                             400
O. plumbea 6                 TATTAGATTA ATATCTTTTC AAGAGGGGAA AAGGGTATTC AAATCTCATA ATTTACGATC AATTCATTCA CTATTTCCTT 400
O. harrisiana 18             .......... .......... .......... ....A..... .......... .......... .......... .......... 400
O. harrisiana 27             .......... .......... .......... ....A..... .......... .......... .......... .......... 400
O. fucosa 64                 .......... .......... .......... ....A..... .......... .......... .......... .......... 400
O. ridleyana 61              .......... .......... .......... ....A..... .......... .......... .......... .......... 400
O. ridleyana 56              .......... .......... .......... ....A..... .......... .......... .......... .......... 400
Ophiorrhiza sp. 35           .......... .......... .......... ....A..... .......... .......... .......... .......... 400
O. pumila                    .......... .......... .......... ....A..... .......... .......... .......... .......... 400
O. kuroiwae                  .......... .......... .......... ....A..... .......... .......... .......... .......... 400
O. pseudofasciculata 37      .......... .......... .......... ....A..... .......... .......... .......... .......... 400
O. ridleyana 52              .......... .......... .......... ....A..... .......... .......... .......... .......... 400
O. pseudofasciculata 62      .......... .......... .......... ....A..... .......... .......... .......... .......... 400
O. pedunculata 41            .......... .......... .......... ....A..... .......... .......... .......... .......... 400
O. trichocarpon 46           .......... .......... .......... ....A..... .......... .......... .......... .......... 400
O. japonica                  .......... .......... .......... ....A..... .......... .......... .......... .......... 400
O. hayatana                  .......... .......... .......... ....A..... .......... .......... .......... .......... 400
Joosia umbellifera           ...AC..... .......C.. ...C...... .G.A...... ......T... .......... .......... .......... 400
                                                    420                                             440                                             460                                             480
O. plumbea 6                 TCTTAGAGAA CCATTTTTCA CATTTGAATT CTGTATTAGA TATACTAATA CCCCACGCCG TCCATCTGGA AATTCTGGTT 480
O. harrisiana 18             .......... .A........ .......... .......... .......... .......... .......... .......... 480
O. harrisiana 27             .......... .A........ .......... .......... .......... .......... .......... .......... 480
O. fucosa 64                 .......... .A........ .......... .......... .......... .......... .......... .......... 480
O. ridleyana 61              .......... .A........ .......... .......... .......... .......... .......... .......... 480
O. ridleyana 56              .......... .A........ .......... .......... .......... .......... .......... .......... 480
Ophiorrhiza sp. 35           .......... .A........ .......... .......... .......... .......... .......... .......... 480
O. pumila                    .......... .A........ .......... .......... .......... .......... .......... .......... 480
O. kuroiwae                  .......... .......... .......... .......... .......... .......... .......... .......... 480
O. pseudofasciculata 37      .......... .......... .......... .......... .........C .......... .......... .......... 480
O. ridleyana 52              .......... .......... .......... .......... .........C .......... .......... ....A..... 480
O. pseudofasciculata 62      .......... .......... .......... .......... .........C .......... .......... .......... 480
O. pedunculata 41            .......... .......... .......... .......... .........C .......... .......... .......... 480
O. trichocarpon 46           .......... .......... .......... .......... .........C .......... .......... .......... 480
O. japonica                  .......... .A........ .......... .......... .......... .......... .......... .......... 480
O. hayatana                  .......... .......... .......... .......... .......... .......... .......... .......... 480
Joosia umbellifera           .......... .A........ .....A.... ..T.G..... .......... ......C..A .....T.... ...C...... 480
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                                                   500                                               520                                               540                                               560
O. plumbea 6               CAAACCCTTC GTTATTGGGT AAAAGATGCC TCTTCCTTGC ATTTATTACG ATTCTTTTTC CACAAGTATT GGAGTTGGAA 560
O. harrisiana 18           .......... .......... .......... ..G....... .......... .......... .......... .......... 560
O. harrisiana 27           .......... .......... .......... .......... .......... .......... .......... .......... 560
O. fucosa 64               .......... .......... .......... .......... .......... .......... .......... .......... 560
O. ridleyana 61            .......... .......... .......... .......... .......... .......... .......... .......... 560
O. ridleyana 56            .......... .......... .......... .......... .......... .......... .......... .......... 560
Ophiorrhiza sp. 35         .......... .......... .......... .......... .......... .......... .......... .......... 560
O. pumila                  .......... .......... .......... .......... .......... .......... .......... .......... 560
O. kuroiwae                .......... .......... .......... .......... .......... .......... .......... .......... 560
O. pseudofasciculata 37    .......... .......... .......... .......... .......... .......... .......... .......... 560
O. ridleyana 52            .......... .......... .......... .......... .......... .......... .......... .......... 560
O. pseudofasciculata 62    .......... .......... .......... .......... .......... .......... .......... .......... 560
O. pedunculata 41          .......C.. .......... .......... .......... .......... .......... .......... .......... 560
O. trichocarpon 46         .......... .......... .......... .......... .......... .......... .......... .......... 560
O. japonica                .......... .......... .......... .......... .......... .......... .......... .......... 560
O. hayatana                .......... .......... .......... .......... .......... .......... .......... .......... 560
Joosia umbellifera         ....A..... .......... ......C... C......... .......... .......... ...G...... ...A...... 560

                                                   580                                               600                                               620                                               640
O. plumbea 6               TACTCTTATT GCTACAAGCA AACCCTGTTT GGATTTTTCA CCAAAAAGAA ATCAAAGATT GTTTTTCTTA TTATATAATT 640
O. harrisiana 18           .......... ........G. .......... .......... .......... .......... .......... .......... 640
O. harrisiana 27           .......... ........G. .......... .......... .......... .......... .......... .......... 640
O. fucosa 64               ........G. ........G. .......... .......... .......... .......... .......... .......... 640
O. ridleyana 61            ........G. ........G. .......... .......... .......... .......... .......... .......... 640
O. ridleyana 56            ........G. ........G. .......... .......... .......... .......... .......... .......... 640
Ophiorrhiza sp. 35         .......... ........G. .......... .......... .......... .......... .......... .......... 640
O. pumila                  .......... ........G. .......... .......... .......... .......... .......... .......... 640
O. kuroiwae                .......... ........G. .......... .......... .......... .......... .......... .......... 640
O. pseudofasciculata 37    .......... .......AG. .......... ..G....... .......... .......... .......... .......... 640
O. ridleyana 52            .......... .......AG. .......... ..G....... .......... .......... .......... .......... 640
O. pseudofasciculata 62    .......... .......AG. .......... ..G....... .......... .......... .......... .......... 640
O. pedunculata 41          .......... .......AG. .......... .......... .......... .......... .......... .......... 640
O. trichocarpon 46         .......... .......AG. .......... .......... .......... .......... .......... .......... 640
O. japonica                .......... .......AG. .......... .......... .......... .......... .......... .......... 640
O. hayatana                .......... .......AG. .......... .......... .......... .......... .......... .......... 640
Joosia umbellifera         .......... ....A..AG. .....A.... T......C.. .......... G.G....... A..A.....C .......... 640

                                                   660                                               680                                               700                                               720
O. plumbea 6               CACATGTATA TGAATACGAA TCCATTTTTG CCTTTCTCCG TAAGCAATCT TCTCATTTGG GATCAACATC TTTTGGAGTC 720
O. harrisiana 18           .......... .......... .......... .......... .......... .......... .......... .......... 720
O. harrisiana 27           .......... .......... .......... .......... .......... .......... .......... .......... 720
O. fucosa 64               .......... .......... .......... .......... .......... .......... .......... .......... 720
O. ridleyana 61            .......... .......... .......... .......... .......... .......... .......... .......... 720
O. ridleyana 56            .......... .......... .......... .......... .......... .......... .......... .......... 720
Ophiorrhiza sp. 35         .......... .......... .......... .......... .......... .......... .......... .......... 720
O. pumila                  .......... .......... .......... .......... C......... .......... .......... .......... 720
O. kuroiwae                .......... .......... .......... .......... .......... .......... .......... .......... 720
O. pseudofasciculata 37    .......... .......... ........G. .......... .......... .......... .......... .......... 720
O. ridleyana 52            .......... .......... ........G. .......... .......... .......... .......... .......... 720
O. pseudofasciculata 62    .......... .......... ........G. .......... .......... .......... .......... .......... 720
O. pedunculata 41          .......... .......... ........G. .......... .......... .......... .......... .......... 720
O. trichocarpon 46         .......... .......... ........G. .......... .......... .......... .......... .......... 720
O. japonica                .......... .......... ........G. .......... .......... .......... .......... .......... 720
O. hayatana                .......... .......... ..T.....G. .......... .......... .......... .......... .......... 720
Joosia umbellifera         .T.......G .......... ........C. T........T ...C...... .........C .......... .......... 720

                                                   740                                               760                                               780                                               800
O. plumbea 6               TTTCTTGAAC GAATATATTT CTACGGAAAA AAAGAAAGGC TTGTAGAAGT CGTTGCGGAG GATTTTCAGG TTAGTTTATG 800
O. harrisiana 18           .......... .......... .......... .......... .......... .......... .......... .......... 800
O. harrisiana 27           .......... .......... .......... .......... .......... .......... .......... .......... 800
O. fucosa 64               .......... .......... .......... .......... .......... .......... .......... .......... 800
O. ridleyana 61            .......... .......... .......... .......... .......... .......... .......... .......... 800
O. ridleyana 56            .......... .......... .......... .......... .......... .......... .......... .......... 800
Ophiorrhiza sp. 35         .......... .......... .......... ..G....... .......... .......... .......... .......... 800
O. pumila                  .......... .......... .......... .......... .......... .......... .......... .......... 800
O. kuroiwae                .......... .......... .......... .......... .......... .......... .......... .......... 800
O. pseudofasciculata 37    .......... .......... .......... .......... .......... .......... .......... .......... 800
O. ridleyana 52            .......... .......... .......... .......... .......... .......... .......... .......... 800
O. pseudofasciculata 62    .......... .......... .......... .......... .......... .......... .......... .......... 800
O. pedunculata 41          .......... .......... .......... .......... .......... .......... .......... .......... 800
O. trichocarpon 46         .......... .......... .......... ..N....... .......... .......... .......... .......... 800
O. japonica                .......... .......... .......... ......C... .......... .......... .......... .......... 800
O. hayatana                .......... .......... .......... ......C... .......... .......... .......... .......... 800
Joosia umbellifera         .......... .......... ...T...... .T....CTT. .......... TT....TA.. .......... .....C.... 800

                                                   820                                               840                                               860                                               880
O. plumbea 6               GTTATTTACA GACCCTTTCA TGCATTATGT TAGGTATCAA GGAAAATCAA TTCTGGTTTC AAAGGATACG CCTCTTTTGA 880
O. harrisiana 18           .......... .........G .......... .......... .......... .......... .......... .......... 880
O. harrisiana 27           .......... .........G .......... .......... .......... .......... .......... .......... 880
O. fucosa 64               .......... .........G .......... .......... .......... .......... .......... .......... 880
O. ridleyana 61            .......... .........G .......... .......... .......... .......... .......... .......... 880
O. ridleyana 56            .......... .........G .......... .......... .......... .......... .......... .......... 880
Ophiorrhiza sp. 35         .......... .........G .......... .......... .......... .......... .......... .......... 880
O. pumila                  .......... .........G .......... .......... .......... .......... .......... .......... 880
O. kuroiwae                .......... .......... .......... .......... .......... .......... .......... .......... 880
O. pseudofasciculata 37    .......... .......... .......... .......... .......... .......... .......... .......... 880
O. ridleyana 52            .......... .......... .......... .......... .......... .......... .......... .......... 880
O. pseudofasciculata 62    .......... .......... .......... .......... .......... .......... .......... .......... 880
O. pedunculata 41          .......... .......... .......... .......... .......... .......... .......... .......... 880
O. trichocarpon 46         .......... .......... .......... .......... .......... .......... .......... .......... 880
O. japonica                .......... .......... .......... .......... .......... .......... .......... .......... 880
O. hayatana                .......... .......... .......... .......... .......... .......... .......... .......... 880
Joosia umbellifera         ...G..C.A. ..T....... .......... .......... ........T. ..T....... ...A.GG... ..C....... 880

                                                   900                                               920                                               940                                               960
O. plumbea 6               TGAATAAATG GAAATCTTAT CTTGTCCATT TTTGGCAATG TCATTTTGAT CTGTGGTTTC ACTCGGGAAG GTTCTATATA 960
O. harrisiana 18           .......... .......... .......... .......... .......... .......... ....A..... .......... 960
O. harrisiana 27           .......... .......... .......... .......... .......... .......... ....A..... .......... 960
O. fucosa 64               .......... .......... .......... .......... .......... .......... ....A..... .......... 960
O. ridleyana 61            .......... .......... .......... .......... .......... .......... ....A..... .......... 960
O. ridleyana 56            .......... .......... .......... .......... .......... .......... ....A..... .......... 960
Ophiorrhiza sp. 35         .......... .......... .......... .......... .......... .......... ....A..... .......... 960
O. pumila                  .......... .......... .......... .......... .......... .......... ....A..... .......... 960
O. kuroiwae                .......... .......... .......... .......... .......... .........G .......... .......... 960
O. pseudofasciculata 37    .......... .......... .......... .......... .......... .......... .......... .......... 960
O. ridleyana 52            .......... .......... .......... .......... .......... .......... .......... .......... 960
O. pseudofasciculata 62    .......... .......... .......... .......... .......... .......... .......... .......... 960
O. pedunculata 41          .......... .......... .......... .......... .......... .......... .......... .......... 960
O. trichocarpon 46         .......... .......... .......... .......... .......... .......... .......... .......... 960
O. japonica                .......... .......... .......... .......... .......... .......... .......... .......... 960
O. hayatana                .......... .......... .......... .......... .......... .......... .......... .......... 960
Joosia umbellifera         ....G..... .....A...C ......A... .......... .T........ ..T....... ....C..... .G........ 960
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                             980                    1,000                   1,020                   1,040
O. plumbea 6              AATGAATTAT CCAATCATTC CTTTGACTTT ATGGGCTATC TTTCAAGTGT GCAACTAAGC CCGTCAATGG TACGGAGCCA 1040
O. harrisiana 18          .......... .......... .......... .......... .......... ........A. .......... .......... 1040
O. harrisiana 27          .......... .......... .......... .......... .......... ........A. .......... .......... 1040
O. fucosa 64              .......... .......... .......... .......... .......... ........A. .......... .......... 1040
O. ridleyana 61           .......... .......... .......... .......... .......... ........A. .......... .......... 1040
O. ridleyana 56           .......... .......... .......... .......... .......... ........A. .......... .......... 1040
Ophiorrhiza sp. 35        .......... .......... .......... .......... .......... ........A. .......... .......... 1040
O. pumila                 .......... .......... .......... .......... .......... ........A. .......... .......... 1040
O. kuroiwae               .......... .......... .......... .......... .......... ........A. .......... .......... 1040
O. pseudofasciculata 37   .......... .......... .......... .......... .......... ........A. .......... .......... 1040
O. ridleyana 52           .......... .......... .......... .......... ...T...... ........A. .......... .......... 1040
O. pseudofasciculata 62   .......... .......... .......... .......... .......... ........A. .......... .......... 1040
O. pedunculata 41         .......... .......... .......... .......... .......... ........A. .......... .......... 1040
O. trichocarpon 46        .......... .......... .......... .......... .......... ........A. .......... .......... 1040
O. japonica               .......... .......... .......... .......... .......... ........A. .T........ .......... 1040
O. hayatana               .......... .......... .......... .......... .......... ........A. .......... .......... 1040
Joosia umbellifera        ..CC.....C ....C..... .C..A..... ..A..T...T .......... ..G.....A. ..T....... ....A..T.. 1040

                             1,060                  1,080                   1,100                   1,120
O. plumbea 6              AATGCTAGAA AATTCATTTC TAATCAATAA TGCTATTAAG AAATTGGATA CCCTTGTTCC AATTATTCCT CTTATTGGAT 1120
O. harrisiana 18          .......... .......... .......... .......... .......... .......... .......... .......... 1120
O. harrisiana 27          .......... .......... .......... .......... .......... .......... .......... .......... 1120
O. fucosa 64              .......... .......... .......... .......... .......... .......... .......... .......... 1120
O. ridleyana 61           .......... .......... .......... .......... .......... .......... .......... .......... 1120
O. ridleyana 56           .......... .......... .......... .......... .......... .......... .......... .......... 1120
Ophiorrhiza sp. 35        .......... .......... .......... .......... .......... .......... .......... .......... 1120
O. pumila                 .......... .......... .......... .......... .......... .......... .......... .......... 1120
O. kuroiwae               .......... .......... .......... .......... .......... .......... .......... .......... 1120
O. pseudofasciculata 37   .......... ...G...... .......... .......... .....T.C.. .......... .......... .......... 1120
O. ridleyana 52           .......... ...G...... .......... .......... .....T.C.. .......... .......... .......... 1120
O. pseudofasciculata 62   .......... ...G...... .......... .......... .....T.C.. .......... .......... .......... 1120
O. pedunculata 41         .......... ...G...... .......... .......... .....T.... .......... .......... .......... 1120
O. trichocarpon 46        .......... ...G...... .......... .......... .....T.... .......... .......... .......... 1120
O. japonica               .......... .......... .......... .......... .....T.... .......... .......... .......... 1120
O. hayatana               .......... .......... .......... .......... .....T.... .......... .......... .......... 1120
Joosia umbellifera        .......... ...G...... .......... .......... .....T.... .......... .......... ......C... 1120

                             1,140                  1,160                   1,180                   1,200
O. plumbea 6              CATTGGCTAA AGCGCAATTT TGTAACCTAT TAGGACATCC CGTTAGTAAG CCGGTTTGGG CTGATTTATC AGATTCTGAT 1200
O. harrisiana 18          .......... .......... .......... .......... .......... ..A....... .......... .......... 1200
O. harrisiana 27          .......... .......... .......... .......... .......... ..A....... .......... .......... 1200
O. fucosa 64              .......... .......... .......... .......... .......... ..A....... .......... .......... 1200
O. ridleyana 61           .......... .......... .......... .......... .......... ..A....... .......... .......... 1200
O. ridleyana 56           .......... .......... .......... .......... .......... ..A....... .......... .......... 1200
Ophiorrhiza sp. 35        .......... .......... .......... .......... .......... ..A....... .......... .......... 1200
O. pumila                 .......... .......... .......... .......... .......... ..A....... .......... .......... 1200
O. kuroiwae               .......... .......... .......... .......... .......... .......... .......... .......... 1200
O. pseudofasciculata 37   .......... .......... .......... .......... G......... .......... .......... .......... 1200
O. ridleyana 52           .......... .......... .......... .......... G......... .......... .......... .......... 1200
O. pseudofasciculata 62   .......... .......... .......... .......... G......... .......... .......... .......... 1200
O. pedunculata 41         .......... .......... .......... .......... .......... .......... .......... .......... 1200
O. trichocarpon 46        .......... .......... .......... .......... .......... .......... .......... .......... 1200
O. japonica               .......... .......... .......... .......... .......... .......... .C........ .......... 1200
O. hayatana               .......... .......... .......... .......... .......... .......... .......... .......... 1200
Joosia umbellifera        .......... ...CA..... .......... .......... .......... .........A .......... .......... 1200

                             1,220                  1,240                   1,260                   1,280
O. plumbea 6              ATTATTGACC GATTTGGGTA TATATGCAGA AACCTTTCTC ATTATCATAG CGGTTCTTCC AAAAAAAAGA GTTTGTATCG 1280
O. harrisiana 18          .C........ .......... .......... .......... .......... .......... .......... .......... 1280
O. harrisiana 27          .......... .......... .......... .......... .......... .......... .......... .......... 1280
O. fucosa 64              .......... .......... .......... .......... .......... .......... .......... .......... 1280
O. ridleyana 61           .......... .......... .......... .......... .......... .......... .......... .......... 1280
O. ridleyana 56           .......... .......... .......... .......... .......... .......... .......... .......... 1280
Ophiorrhiza sp. 35        .......... .......... .......... .......... .......... .......... .......... .......... 1280
O. pumila                 .......... .......... .......... .......... .......... .......... .......... .......... 1280
O. kuroiwae               .......... .......... .......... .......... .......... .......... .......... .......... 1280
O. pseudofasciculata 37   .......... .......... .......... .......... .......... .......... .......... .......... 1280
O. ridleyana 52           .......... .......... .......... .......... .......... .......... .......... .......... 1280
O. pseudofasciculata 62   .......... .......... .......... .......... .......... .......... .......... .......... 1280
O. pedunculata 41         .......... .......... .......... .......... .......... .......... .......... .......... 1280
O. trichocarpon 46        .......... .......... .......... .......... .......... .......... .......... .......... 1280
O. japonica               .......... .......... .......... .......... .......... .......... .........G .......... 1280
O. hayatana               .......... .......... .......... .......... .......... .......... .......... .......... 1280
Joosia umbellifera        ........A. ........C. ........A. ..G....... .......... T..A...... .......... .......... 1280

                             1,300                  1,320                   1,340                   1,360
O. plumbea 6              AATAAAGTAT ATACTTCGAC TTTCTTGTGT TAAAACTTTG GCTCGGAAAC ACAAAAGTAC TGTACGTGTT TTTTTGAAAA 1360
O. harrisiana 18          .......... .......... .......... .......... .......... .......... .......... .......... 1360
O. harrisiana 27          .......... .......... .......... .......... .......... .......... .......... .......... 1360
O. fucosa 64              .......... .......... .......... .......... .......... .......... .......... .......... 1360
O. ridleyana 61           .......... .......... .......... .......... .......... .......... .......... .......... 1360
O. ridleyana 56           .......... .......... .......... .......... .......... .......... .......... .......... 1360
Ophiorrhiza sp. 35        .......... .......... .......... .......... .......... .......... .......... .......G.. 1360
O. pumila                 .......... .......... .......... .......... .......... .......... .......... .......... 1360
O. kuroiwae               .......... .......... .......... .......... .......... .......G.. .......... .......... 1360
O. pseudofasciculata 37   .......... .......... .......... .......... .......... .......... G......... .......... 1360
O. ridleyana 52           .......... .......... .......... .......... .......... .......... G......... .......... 1360
O. pseudofasciculata 62   .......... .......... .......... .......... .......... .......... G......... .......... 1360
O. pedunculata 41         .......... .......... .......... .......... .......... .......... .......... .......... 1360
O. trichocarpon 46        .......... .......... .......... .......... .......... .......... .......... .......... 1360
O. japonica               .......... .......... .......... .......... .......... .......... .......... .......... 1360
O. hayatana               .......... .........T .......... .......... .......... .......... .......... .......... 1360
Joosia umbellifera        .......... .......... .........C .......... .T........ .......... ........C. .......... 1360

                             1,380                  1,400                   1,420                   1,440
O. plumbea 6              GATTAGGCTC GTATTTTTTG GACGAATTAC TCCTGTCGGA AGAAGAAGTC CTTTCTTTGA ACTTCCCAAG AGCTTCTTCG 1440
O. harrisiana 18          .......... .......... .......... .......... .......... .......... .......... .......... 1440
O. harrisiana 27          .......... .......... .......... .......... .......... .......... .......... .......... 1440
O. fucosa 64              .......... .......... .......... .......... .......... .......... .......... .......... 1440
O. ridleyana 61           .......... .......... .......... .......... .......... .......... .......... .......... 1440
O. ridleyana 56           .......... .......... .......... .......... .......... .......... .......... .......... 1440
Ophiorrhiza sp. 35        .......... .......... .......... .......... .......... .......... .......... .......... 1440
O. pumila                 .......... .......... .......... .......... .......... .......... .......... .......... 1440
O. kuroiwae               .......... .......... .......... .......... .......... .......... .......... .......... 1440
O. pseudofasciculata 37   .......... .......... .......... .......... .......... .......... .......... .......... 1440
O. ridleyana 52           .......... .......... .......... .......... .......... .......... .......... .......... 1440
O. pseudofasciculata 62   .......... .......... ......C... .......... .......... .......... .......... .......... 1440
O. pedunculata 41         .......... .......... .......... .......... .......... .......... .......... .......... 1440
O. trichocarpon 46        .......... .......... .......... .......... .......... .......... .......... .......... 1440
O. japonica               .......... .......... .......... .......... .......... .......... .......... .......... 1440
O. hayatana               .......... .......... .......... .......... .......... .......... .......... .......... 1440
Joosia umbellifera        .......... .G.A...... ..A.....C. ..AC...... .......... .......... C....T.... .........T 1440
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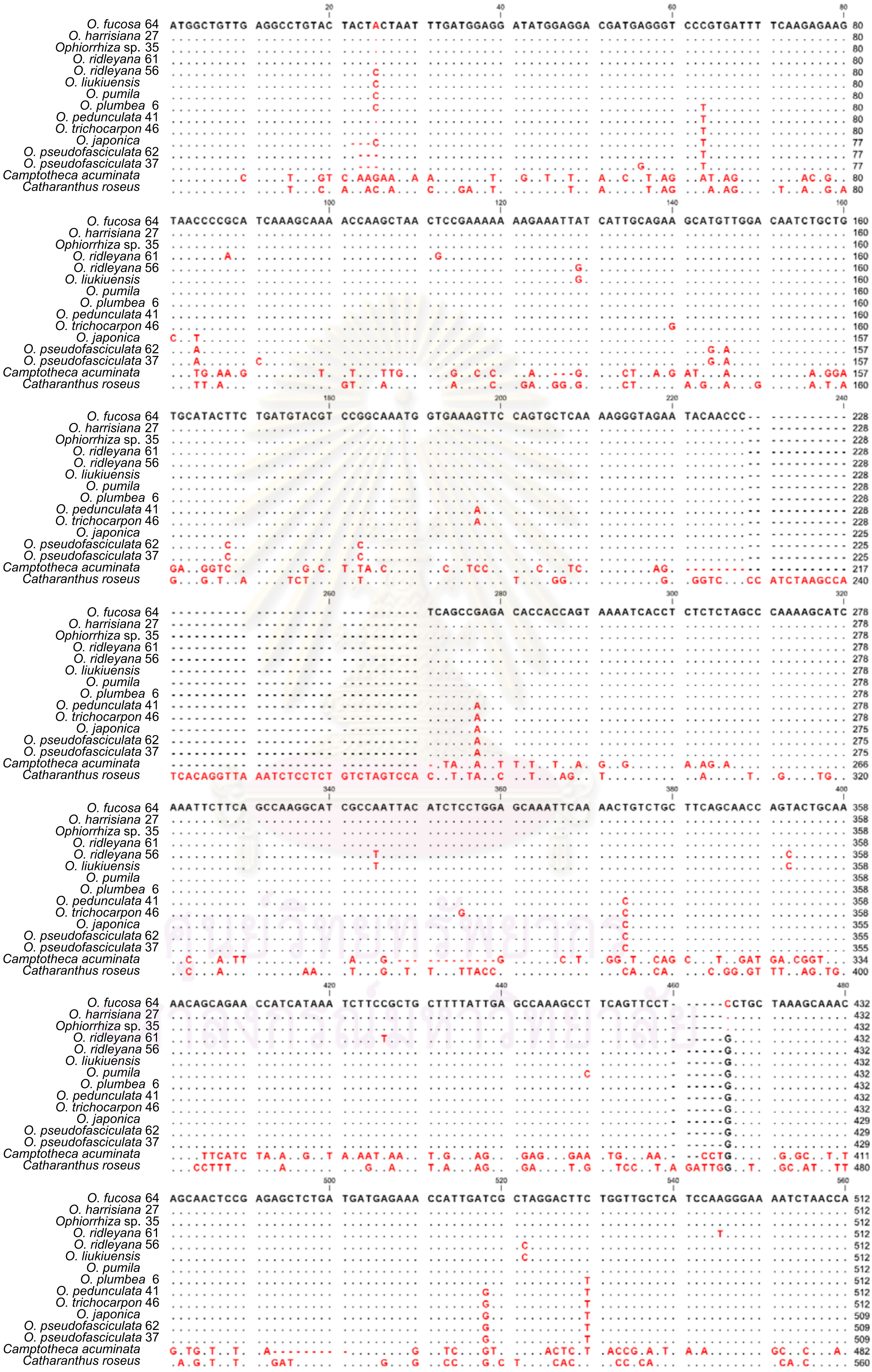
		1,460		1,480		1,500			
O. plumbea 6	ACTTTTCGGG	GGGTATGTAG	AAATCGAATT	TGGTATTTGG	AAATTACTTA	TATCAACGAT	CTGATCAATC	ATCAATGA	1518
O. harrisiana 18	....	......A...	....	....	....	....	....	....	1518
O. harrisiana 27	....	......A...	....	....	....	....	....	....	1518
O. fucosa 64	....	......A...	....	....	....	....	....	....	1518
O. ridleyana 61	....	......A...	....	....	....	....	....	....	1518
O. ridleyana 56	....	......A...	....	....	....	....	....	....	1518
Ophiorrhiza sp. 35	....	......A...	....	....	....	....	....	....	1518
O. pumila	....	......A...	....	....	....	....	....	....	1518
O. kuroiwae	....	......A...	....	....	....	....	....	....	1518
O. pseudofasciculata 37	....	......A...	......G...	....	....	....	....	....	1518
O. ridleyana 52	....	......A...	......G...	....	....	....	....	....	1518
O. pseudofasciculata 62	....	......A...	......G...	....	....	....	....	....	1518
O. pedunculata 41	....	......A...	......G...	....	....	....	....	....	1518
O. trichocarpon 46	....	......A...	......G...	....	....	....	....	....	1518
O. japonica	....	......A...	....A.G...	....	....	....	.....T....	....	1518
O. hayatana	....	......A...	......G...	....	....	....	.....T....	....	1518
Joosia umbellifera	....	...A..A...	..G...G..G	....	.T....T...	......T...	....C....T	....	1518

APPENDIX D

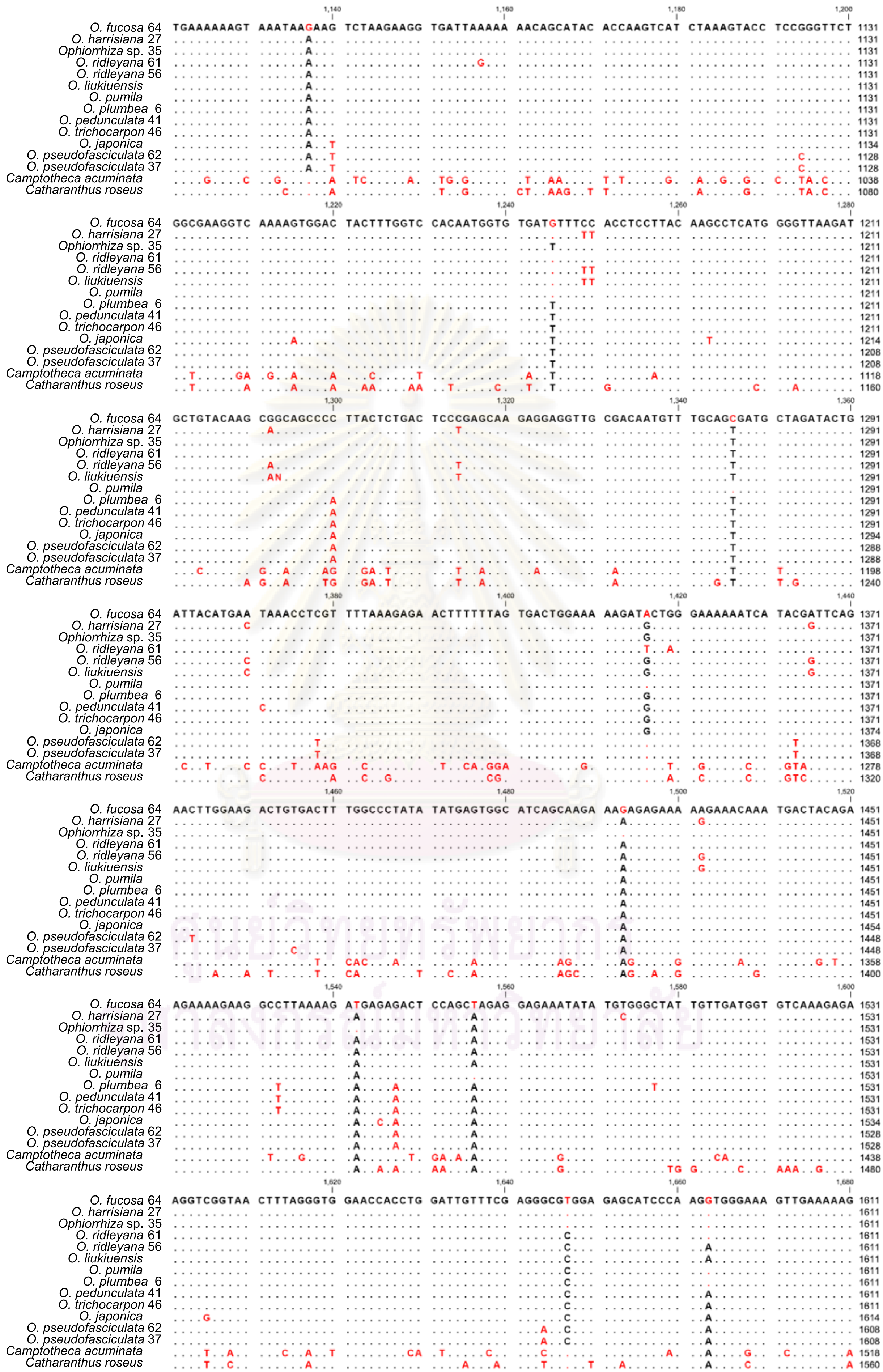
The alignment of *Top*I nucleotide sequences of *Ophiorrhiza* species.

Hyphens indicate gaps and dots represent identical nucleotides.

Red characters represent different nucleotides.


O. fucosa 64
O. harrisiana 27
Ophiorrhiza sp. 35
O. ridleyana 61
O. ridleyana 56
O. liukiuensis
O. pumila
O. plumbea 6
O. pedunculata 41
O. trichocarpon 46
O. japonica
O. pseudofasciculata 62
O. pseudofasciculata 37
Camptotheca acuminata
Catharanthus roseus


O. fucosa 64 TGCTAATAAG GAGGGTGGTG ATTCTTGTTC AATTCGAAGG CCAGTTGTCG AAGAAAG--- ---------- --TGAAGATT 577
O. fucosa 64 CAGAGGATGA AAAACCTCTG TCACGGAGGT TCCCATCAAA GTCGAGTTTA GGGGAATCTA CTAGCAAGTC TTACGACTCG 657
O. fucosa 64 GATGAAAAGA AGCCATTAGC AGCAAAAGTT CAACAAAATG GTTCTGCTTC GCGTGACGGT CCAATGAACA AATCTGCTTC 737
O. fucosa 64 TTTGTCAAAT AAAAGAGCTC CAGGGGAGGT TAAATCCTTG AATCAGTCTT CACTTAAGAA ACCTAAGCTT TCTAGTCCAA 817
O. fucosa 64 TCACATCCAT CAGCAACAAG CAGGCAGCTG TAAAACCAGA ACCA------ AAGGCTGAGG ATGATGATGA TAATATTCCT 891
O. fucosa 64 TTATCTCAAC GAGGGAAGAA GTCACAAACT GCAGCCAGTA AACCAAAGGA TGAGGATGAT GATAATGCTC CAATATCCCA 971
O. fucosa 64 AAGAATCAAG AAGTCACCAG CTTCTGTTAG CAAATCATCA GCACCTGTAA AAAAAGCATC TACAGTTGTA TCACCTTCTC 1051
O. harrisiana 27
Ophiorrhiza sp. 35
O. ridleyana 61
O. ridleyana 56
O. liukiuensis
O. pumila
O. plumbea 6
O. pedunculata 41
O. trichocarpon 46
O. japonica
O. pseudofasciculata 62
O. pseudofasciculata 37
Camptotheca acuminata
Catharanthus roseus


O. fucosa 64
O. harrisiana 27
Ophiorrhiza sp. 35
O. ridleyana 61
O. ridleyana 56
O. liukiuensis
O. pumila
O. plumbea 6
O. pedunculata 41
O. trichocarpon 46
O. japonica
O. pseudofasciculata 62
O. pseudofasciculata 37
Camptotheca acuminata
Catharanthus roseus
1,140 1,160 1,180 1,200
TGAAAAAAGT AAATAAGAAG TCTAAGAAGG TGATTAAAAA AACAGCATAC ACCAAGTCAT CTAAAGTACC TCCGGGTTCT 1131
1,220 1,240 1,260 1,280
GGCGAAGGTC AAAAGTGGAC TACTTTGGTC CACAATGGTG TGATGTTTCC ACCTCCTTAC AAGCCTCATG GGGTTAAGAT 1211
1,300 1,320 1,340 1,360
GCTGTACAAG CGGCAGCCCC TTACTCTGAC TCCCGAGCAA GAGGAGGTTG CGACAATGTT TGCAGCGATG CTAGATACTG 1291
1,380 1,400 1,420 1,440
ATTACATGAA TAAACCTCGT TTTAAAGAGA ACTTTTTTAG TGACTGGAAA AAGATACTGG GAAAAAATCA TACGATTCAG 1371
1,460 1,480 1,500 1,520
AACTTGGAAG ACTGTGACTT TGGCCCTATA TATGAGTGGC ATCAGCAAGA AAGAGAGAAA AAGAAACAAA TGACTACAGA 1451
1,540 1,560 1,580 1,600
AGAAAAGAAG GCCTTAAAAG ATGAGAGACT CCAGCTAGAG GAGAAATATA TGTGGGCTAT TGTTGATGGT GTCAAAGAGA 1531
1,620 1,640 1,660 1,680
AGGTCGGTAA CTTTAGGGTG GAACCACCTG GATTGTTTCG AGGGCGTGGA GAGCATCCCA AGGTGGGAAA GTTGAAAAAG 1611

1,700 1,720 1,740 1,760

O. fucosa 64	CGCATCCATC CAAGGGATAT TACCATAAAT ATTGGAAAGG ATGCTCCAAT TCCTGAATGT CCCATCCCCG GTGAAAGATG	1691
O. harrisiana 27	....	1691
Ophiorrhiza sp. 35	....	1691
O. ridleyana 61	.......G..	1691
O. ridleyana 56	.......G..	1691
O. liukiuensis	.......G..	1691
O. pumila	.......G.. ....T.	1691
O. plumbea 6	.......G..	1691
O. pedunculata 41	.......G..	1691
O. trichocarpon 46	.......G..	1691
O. japonica	.......G..	1694
O. pseudofasciculata 62	.......G..	1688
O. pseudofasciculata 37	.......G..	1688
Camptotheca acuminata	T.T....GC. .G..T..C.. ...G...... ....A. ....C..T.. ...A...... ..A.....T. ....C..	1598
Catharanthus roseus	..T....G.. .TT.C..... ...A...... ....T.. ...A.....C ..TG.T..T.	1640

1,780 1,800 1,820 1,840

O. fucosa 64	GAAGGAAGTA AGGAATGACA ACACTGTGAC ATGGTTAGCA TATTGGAATG ATCCAGTAAA TCTAAAGGAA TGCAAATATG	1771
O. harrisiana 27	....	1771
Ophiorrhiza sp. 35	....	1771
O. ridleyana 61	...A...... .... .A........	1771
O. ridleyana 56	...A...... ....A..... ....A.... ..A....... .T........	1771
O. liukiuensis	...A...... .... .T........ .....A.... ..A.......	1771
O. pumila	...A...... ....T......	1771
O. plumbea 6	...A...... ....A.... ..A....... C.......C.	1771
O. pedunculata 41	...A...... ....A..... ....A.... ..A....... .T........	1771
O. trichocarpon 46	...A...... ....A..... ....A.... ..A....... .T........	1771
O. japonica	...A...... ....A..... .... .T.....T.. .....A.... ..A....... .T........	1774
O. pseudofasciculata 62	...A...... ....NN..... ....A.... ..A....... .T........	1768
O. pseudofasciculata 37	...A...... ....G.. ....A..... ....A.... ..A....... .T........	1768
Camptotheca acuminata	...A..A... ...C.C.... .T..G..T.. ....C .T........ .....A.C.. G.C..GA... .T...G..C.	1678
Catharanthus roseus	...A...... C.CC...... .T..G..... ......G... .T........ .....A.T.. ..C....... .T........	1720

1,860 1,880 1,900 1,920

O. fucosa 64	TTTTTCTAGC ACCCAGCAGT ACCTTAAAGG GGCAAAGTGA CAAGGAGAAA TATGAGAAAG CAAGGCTCTT AAAGGATTAC	1851
O. harrisiana 27	....	1851
Ophiorrhiza sp. 35	....	1851
O. ridleyana 61	.... .G........ ....T ....G....	1851
O. ridleyana 56	.... .G........ ....T..	1851
O. liukiuensis	.... .G........	1851
O. pumila	.... .G........	1851
O. plumbea 6	........G. .G........ ....T..	1851
O. pedunculata 41	.... .G........ ....T..	1851
O. trichocarpon 46	.... .G........ ....T..	1851
O. japonica	.... .G........	1854
O. pseudofasciculata 62	.... .G........ ....G..	1848
O. pseudofasciculata 37	.... .G........	1848
Camptotheca acuminata	.G..CT.G.. .G.T...... T.T..G.... ....C....... ....G.. G.....C.TT	1758
Catharanthus roseus	.......G.. TG........ .... .A.T...... ...A...... .....A.... ....T.. G.....C...	1800

1,940 1,960 1,980 2,000

O. fucosa 64	ATACATGGCA TCAGAGCTGC TTATACTAAG GATTTTACTA ATAATAAAGA TCCCATGAAG AAGCAAATAG CAGTTGCAAC	1931
O. harrisiana 27	....	1931
Ophiorrhiza sp. 35	....	1931
O. ridleyana 61	.... .G........	1931
O. ridleyana 56	....	1931
O. liukiuensis	....	1931
O. pumila	....G...	1931
O. plumbea 6	....	1931
O. pedunculata 41	....	1931
O. trichocarpon 46	....	1931
O. japonica	....	1934
O. pseudofasciculata 62	....	1928
O. pseudofasciculata 37	....	1928
Camptotheca acuminata	.....A.... ....A.. A.....A... ......G.A. GC..---G.. .AT..C.... CG........ ....T..	1835
Catharanthus roseus	.....A..A. .T.....G.. ....G... GC..---... .....C....	1877

2,020 2,040 2,060 2,080

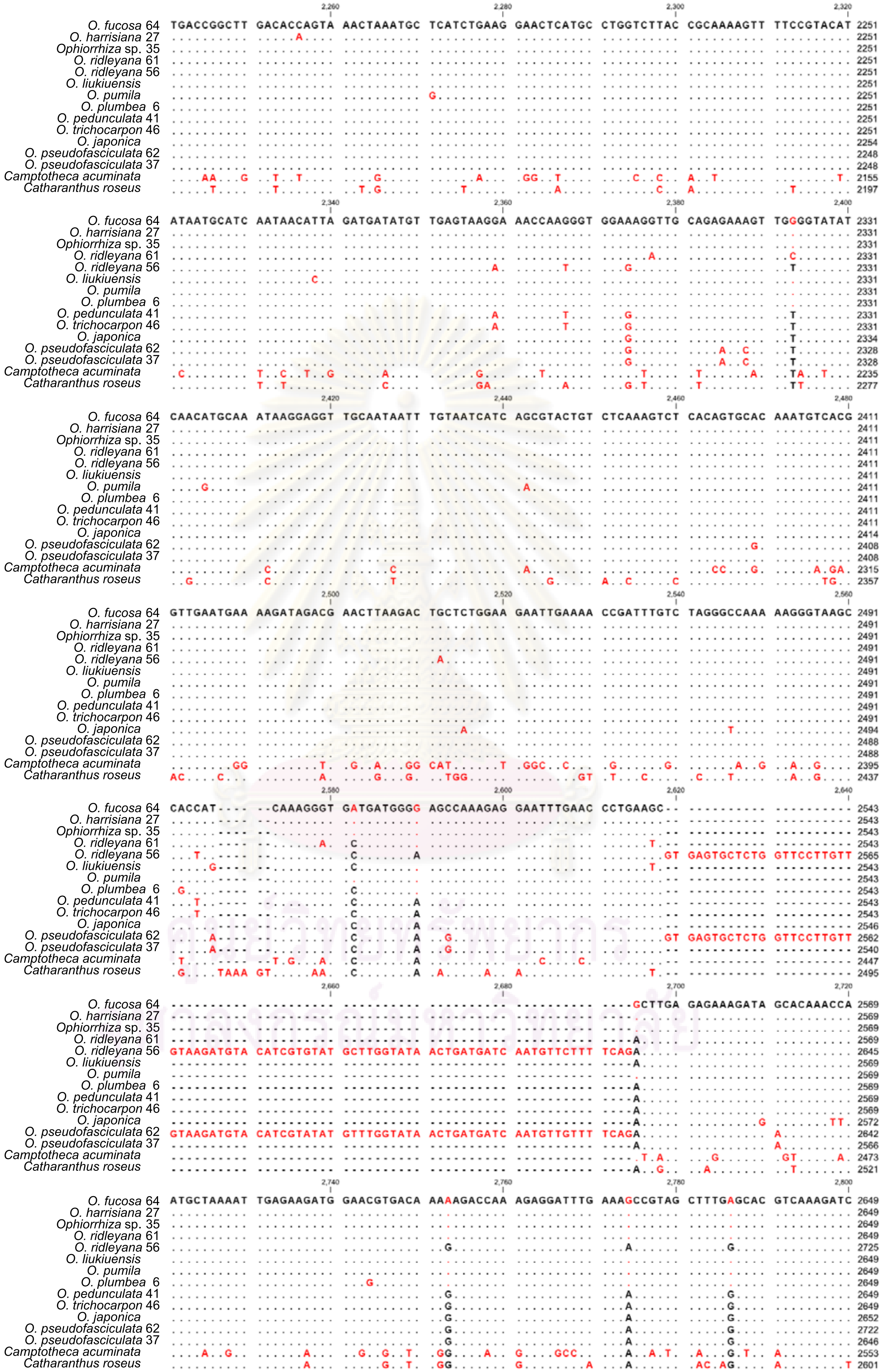
O. fucosa 64	TTATCTTATT GACAAACTAG CTCTGAGGGC AGGCAATGAG AAGGATGATG ATGAAGCTGA TACAGTTGGT TGCTGCACAC	2011
O. harrisiana 27	....	2011
Ophiorrhiza sp. 35	....	2011
O. ridleyana 61	....	2011
O. ridleyana 56	....C.....	2011
O. liukiuensis	....	2011
O. pumila	....	2011
O. plumbea 6	....	2011
O. pedunculata 41	....C.....	2011
O. trichocarpon 46	....C.....	2011
O. japonica	....C.....	2014
O. pseudofasciculata 62	....C.....	2008
O. pseudofasciculata 37	....C.....	2008
Camptotheca acuminata	....T...T... ....C..... ....GT	1915
Catharanthus roseus	.....C..C. ..T....G.. ....C..... C..A....A. ....G... ..T......T	1957

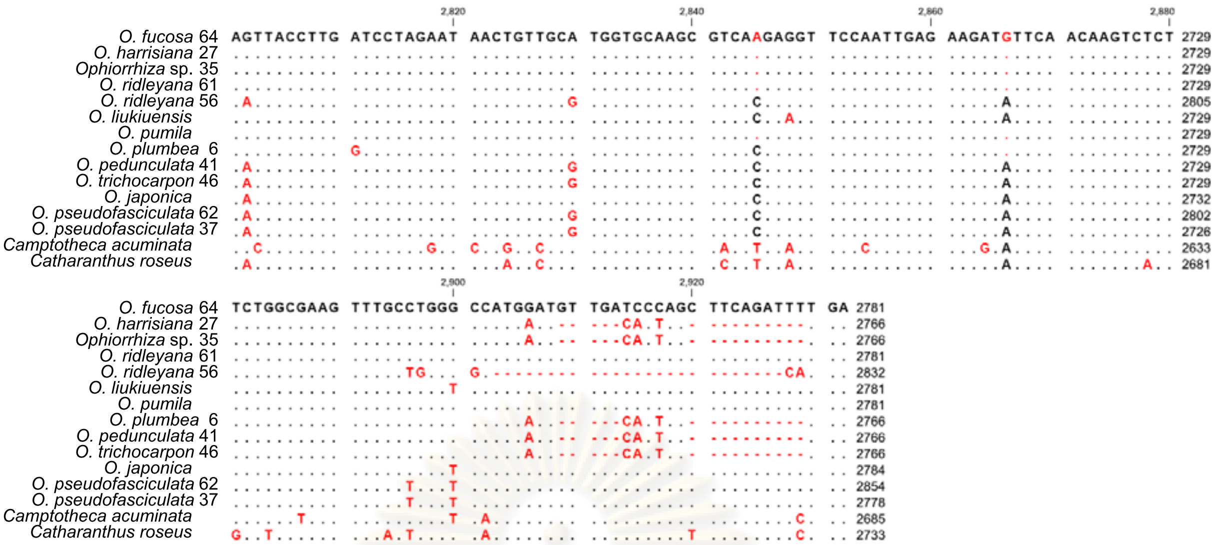
2,100 2,120 2,140 2,160

O. fucosa 64	TGAAAGTAGA AAATGTAGAA CCTGTGCCTC CAAATATCTT AAAGATTGAC TTTATCGGTA AGGATTCCAT TAGATATCAA	2091
O. harrisiana 27	....	2091
Ophiorrhiza sp. 35	...G......	2091
O. ridleyana 61	....C.	2091
O. ridleyana 56	....T.. ....T..... ...C......	2091
O. liukiuensis	....	2091
O. pumila	....	2091
O. plumbea 6	....	2091
O. pedunculata 41	....T.. ....T..... ...C......	2091
O. trichocarpon 46	....T.. ....T..... ...C......	2091
O. japonica	....T..... ...C......	2094
O. pseudofasciculata 62	....T..... ...C......	2088
O. pseudofasciculata 37	....T..... ...C......	2088
Camptotheca acuminata	....AAA..... ...GC..T.. G...T..... ..CC.T.... .A.....A..	1995
Catharanthus roseus	....T.... ....T.. ....T....T ...C.T.... .A.....T.. C.....C...	2037

2,180 2,200 2,220 2,240

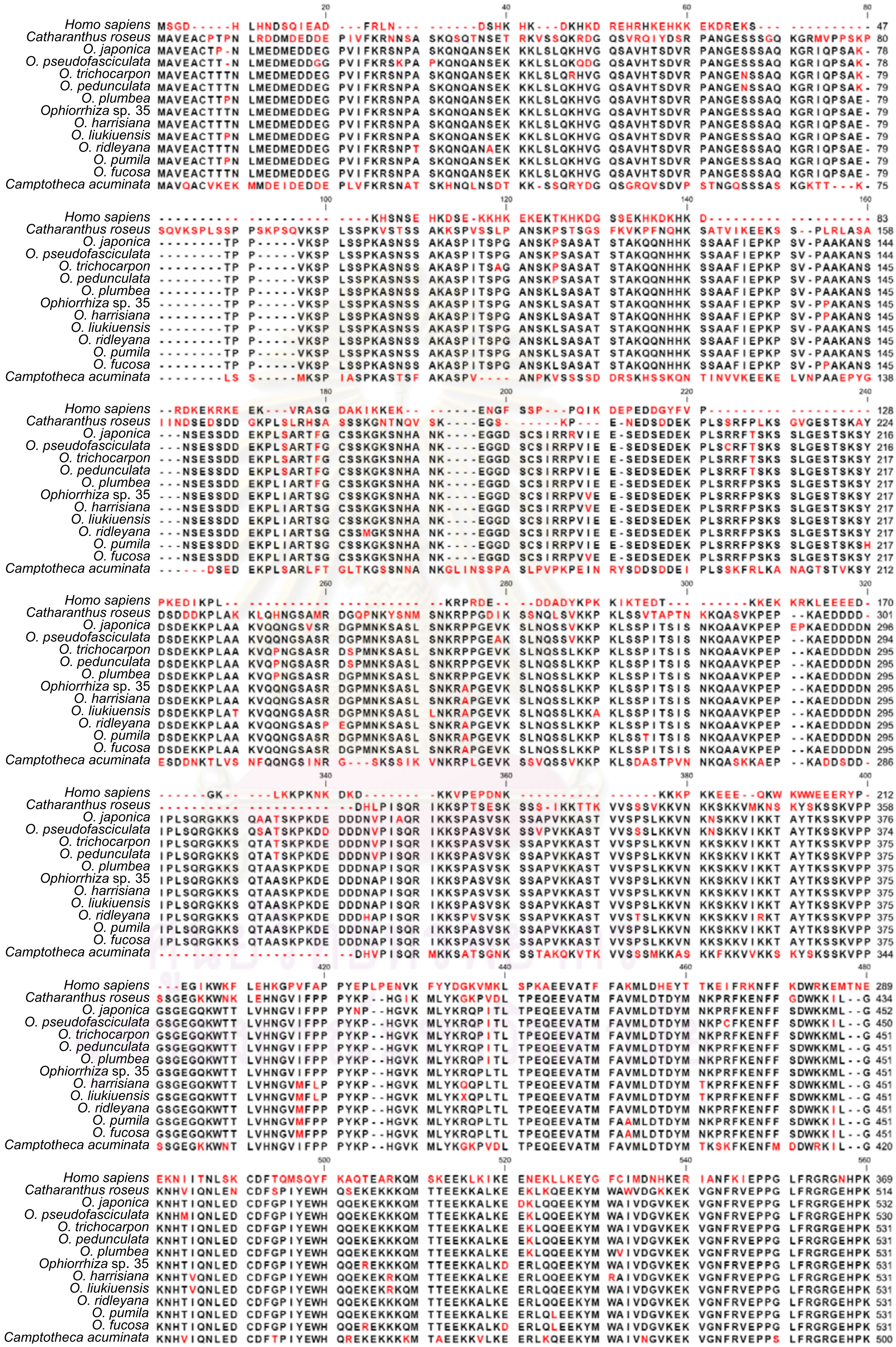
O. fucosa 64	AATGAGGTCC AGGTTGAACC TGCTGTTTTC AAGGCAATTC AACAGTTCCG AAGTGGGAAA GAGGGTAGTG AAGACCTTTT	2171
O. harrisiana 27	....	2171
Ophiorrhiza sp. 35	....	2171
O. ridleyana 61	.... C.........	2171
O. ridleyana 56	....	2171
O. liukiuensis	....T... C.........	2171
O. pumila	....	2171
O. plumbea 6	.... C.........	2171
O. pedunculata 41	.... .C........	2171
O. trichocarpon 46	....	2171
O. japonica	.... .G........	2174
O. pseudofasciculata 62	.... .T........	2168
O. pseudofasciculata 37	....	2168
Camptotheca acuminata	........TG .A.......T .C....G.A. .... .G........ ..C...A... AGA...G... .T..T.....	2075
Catharanthus roseus	........TG ....G. .... .G.....T.. ....C..CA GT..T.....	2117


O. fucosa 64
O. harrisiana 27
Ophiorrhiza sp. 35
O. ridleyana 61
O. ridleyana 56
O. liukiuensis
O. pumila
O. plumbea 6
O. pedunculata 41
O. trichocarpon 46
O. japonica
O. pseudofasciculata 62
O. pseudofasciculata 37
Camptotheca acuminata
Catharanthus roseus
TGACCGGCTT GACACCAGTA AACTAAATGC TCATCTGAAG GAACTCATGC CTGGTCTTAC CGCAAAAGTT TTCCGTACAT
ATAATGCATC AATAACATTA GATGATATGT TGAGTAAGGA AACCAAGGGT GGAAAGGTTG CAGAGAAAGT TGGGGTATAT
CAACATGCAA ATAAGGAGGT TGCAATAATT TGTAATCATC AGCGTACTGT CTCAAAGTCT CACAGTGCAC AAATGTCACG
GTTGAATGAA AAGATAGACG AACTTAAGAC TGCTCTGGAA GAATTGAAAA CCGATTTGTC TAGGGCCAAA AAGGGTAAGC
CACCAT---- --CAAAGGGT GATGATGGGG AGCCAAAGAG GAATTTGAAC CCTGAAGC--
GCTTGA GAGAAAGATA GCACAAACCA
ATGCTAAAAT TGAGAAGATG GAACGTGACA AAAAGACCAA AGAGGATTTG AAAGCCGTAG CTTTGAGCAC GTCAAAGATC


2,820
2,840
2,860
2,880
O. fucosa 64
O. harrisiana 27
Ophiorrhiza sp. 35
O. ridleyana 61
O. ridleyana 56
O. liukiuensis
O. pumila
O. plumbea 6
O. pedunculata 41
O. trichocarpon 46
O. japonica
O. pseudofasciculata 62
O. pseudofasciculata 37
Camptotheca acuminata
Catharanthus roseus
AGTTACCTTG ATCCTAGAAT AACTGTTGCA TGGTGCAAGC GTCAAGAGGT TCCAATTGAG AAGATGTTCA ACAAGTCTCT 2729
2,900
2,920
TCTGGCGAAG TTTGCCTGGG CCATGGATGT TGATCCCAGC TTCAGATTTT GA 2781

APPENDIX E

The alignment of TopI amino acid sequences of *Ophiorrhiza* species and other organisms. Hyphens indicate gaps. Red characters represent different residues.



		580		600		620		640	
Homo sapiens	MGMLKRRIMP	EDIIINCSKD	AKVPS-PPPG	HKWKEVRHDN	KVTWLVSWTE	NI-QGSIKYI	MLNPSSRIKG	EKDWQKYETA	447
Catharanthus roseus	MGKLKKRIRP	CDITINIGKD	APIPECPVPG	ERWKEVRHDN	TVTWLAFWND	PINPKEFKYV	FLAASSTLKG	LSDKEKYEKA	594
O. japonica	MGKLKKRIRP	RDITINIGKD	APIPECPIPG	ERWKEVRNDN	TVTWLAFWID	PINQKEFKYV	FLAASSTLKG	QSDKEKYEKA	612
O. pseudofasciculata	MGKLKKRIRP	RDITINIGKD	APIPECPIPG	ERWKEVRNGN	TVTWLAYWND	PINQKEFKYV	FLAASSTLKG	QSDKEKYEKA	610
O. trichocarpon	MGKLKKRIRP	RDITINIGKD	APIPECPIPG	ERWKEVRNDN	TVTWLAYWND	PINQKEFKYV	FLAASSTLKG	QSDKEKYEKA	611
O. pedunculata	MGKLKKRIRP	RDITINIGKD	APIPECPIPG	ERWKEVRNDN	TVTWLAYWND	PINQKEFKYV	FLAASSTLKG	QSDKEKYEKA	611
O. plumbea	VGKLKKRIRP	RDITINIGKD	APIPECPIPG	ERWKEVRNDN	TVTWLAYWND	PINQKERKYV	FLGASSTLKG	QSDKEKYEKA	611
Ophiorrhiza sp. 35	VGKLKKRIHP	RDITINIGKD	APIPECPIPG	ERWKEVRNDN	TVTWLAYWND	PVNLKECKYV	FLAPSSTLKG	QSDKEKYEKA	611
O. harrisiana	VGKLKKRIHP	RDITINIGKD	APIPECPIPG	ERWKEVRNDN	TVTWLAYWND	PVNLKECKYV	FLAPSSTLKG	QSDKEKYEKA	611
O. liukiuensis	MGKLKKRIRP	RDITINIGKD	APIPECPIPG	ERWKEVRNDN	TVTWLAFWND	PINQKECKYV	FLAASSTLKG	QSDKEKYEKA	611
O. ridleyana	VGKLKKRIRP	RDITINIGKD	APIPECPIPG	ERWKEVRNDN	TVTWLAYWND	PVNIKECKYV	FLAASSTLKG	QSVKEKYEKA	611
O. pumila	VGKLKKRIRP	RDITINIGKD	APIPECPIPG	ERWKEVRNDN	TVTWLAYWND	PVNLKECKYV	FLAASSTLKG	QSDKEKYEKA	611
O. fucosa	VGKLKKRIHP	RDITINIGKD	APIPECPIPG	ERWKEVRNDN	TVTWLAYWND	PVNLKECKYV	FLAPSSTLKG	QSDKEKYEKA	611
Camptotheca acuminata	MGKLKKCIRP	SDITINIGKD	APIPECPIPG	ESWKEIRHDN	TVTWLAFWND	PIKPREFKYV	FLAASSSLKG	QSDKEKYEKA	580

		660		680		700		720	
Homo sapiens	RRLKKCVDKI	RNQYREDW-K	SKEMKVRQRA	VALYFIDKLA	LRAGNEKEEG	ETADTVGCCS	LRVEHINLHP	ELDGQEYVVE	526
Catharanthus roseus	RLLKDYIQGI	RAAYTKDFAS	-KDPTKKQIA	VATYLIDKLA	LRAGNEKDDD	E-ADTVGCCT	LKVENVEPVP	P-----NILK	667
O. japonica	RLLKDYIHGI	RAAYTKDFTN	NKDPMKKQIA	VATYLIDKLA	LRAGNEKDDD	E-ADTVGCCT	LKVENVEPVP	P-----NILK	686
O. pseudofasciculata	RLLKDYIHGI	RAAYTKDFTN	NKDPMKKQIA	VATYLIDKLA	LRAGNEKDDD	E-ADTVGCCT	LKVENVEPVP	P-----NILK	684
O. trichocarpon	RLLKDYIHGI	RAAYTKDFTN	NKDPMKKQIA	VATYLIDKLA	LRAGNEKDDD	E-ADTVGCCT	LKVENVEPVP	P-----NILK	685
O. pedunculata	RLLKDYIHGI	RAAYTKDFTN	NKDPMKKQIA	VATYLIDKLA	LRAGNEKDDD	E-ADTVGCCT	LKVENVEPVP	P-----NILK	685
O. plumbea	RLLKDYIHGI	RAAYTKDFTN	NKDPMKKQIA	VATYLIDKLA	LRAGNEKDDD	E-ADTVGCCT	LKVENVEPVP	P-----NILK	685
Ophiorrhiza sp. 35	RLLKDYIHGI	RAAYTKDFTN	NKDPMKKQIA	VATYLIDKLA	LRAGNEKDDD	E-ADTVGCCT	LRVENVEPVP	P-----NILK	685
O. harrisiana	RLLKDYIHGI	RAAYTKDFTN	NKDPMKKQIA	VATYLIDKLA	LRAGNEKDDD	E-ADTVGCCT	LKVENVEPVP	P-----NILK	685
O. liukiuensis	RLLKDYIHGI	RAAYTKDFTN	NKDPMKKQIA	VATYLIDKLA	LRAGNEKDDD	E-ADTVGCCT	LKVENVEPVP	P-----NILK	685
O. ridleyana	RVLKDYIHGI	RAAYTKGFTN	NKDPMKKQIA	VATYLIDKLA	LRAGNEKDDD	E-ADTVGCCT	LKVENVEPVP	P-----NILK	685
O. pumila	RLLKDYIHGI	RAAYTKDFTN	NKDPMKKQVA	VATYLIDKLA	LRAGNEKDDD	E-ADTVGCCT	LKVENVEPVP	P-----NILK	685
O. fucosa	RLLKDYIHGI	RAAYTKDFTN	NKDPMKKQIA	VATYLIDKLA	LRAGNEKDDD	E-ADTVGCCT	LKVENVEPVP	P-----NILK	685
Camptotheca acuminata	RLLKDFIQGI	RAAYTKDFAS	-KDITKRQIA	VATYLIDKLA	LRAGNEKDDD	E-ADTVGCCT	LKVENVEPKP	P-----SILK	653

		740		760		780		800	
Homo sapiens	FDFLGKDSIR	YYNKVPVEKR	VFKNLQLFME	NKQPEDDLFD	RLNTGILNKH	LQDLMEGLTA	KVFRTYNASI	TLQQQLKELT	606
Catharanthus roseus	FDFLGKDSIR	YQNEVEVEAA	VFKAIQQFRS	GKEGSSDLFD	LLDTSKLNAH	LKELMPGLTA	KVFRTYNASI	TLDDMLSRET	747
O. japonica	FDFLGKDSIR	YQNEVQVEPA	VFKAIQQFRS	GKEGSEDLFD	RLDTSKLNAH	LKELMPGLTA	KVFRTYNASI	TLDDMLSKET	766
O. pseudofasciculata	FDFLGKDSIR	YQNEVQVEPA	VFKAIQQFRS	GKEGSEDLFD	RLDTSKLNAH	LKELMPGLTA	KVFRTYNASI	TLDDMLSKET	764
O. trichocarpon	FDFLGKDSIR	YQNEVQVEPA	VFKAIQQFRS	GKEGSEDLFD	RLDTSKLNAH	LKELMPGLTA	KVFRTYNASI	TLDDMLSKKT	765
O. pedunculata	FDFLGKDSIR	YQNEVQVEPA	VFKAIHQFRS	GKEGSEDLFD	RLDTSKLNAH	LKELMPGLTA	KVFRTYNASI	TLDDMLSKKT	765
O. plumbea	IDFIGKDSIR	YQNEVQVEPA	VFKAIQQFRS	GKEGSADLFD	RLDTSKLNAH	LKELMPGLTA	KVFRTYNASI	TLDDMLSKET	765
Ophiorrhiza sp. 35	IDFIGKDSIR	YQNEVQVEPA	VFKAIQQFRS	GKEGSEDLFD	RLDTSKLNAH	LKELMPGLTA	KVFRTYNASI	TLDDMLSKET	765
O. harrisiana	IDFIGKDSIR	YQNEVQVEPA	VFKAIQQFRS	GKEGSEDLFD	RLDTSKLNAH	LKELMPGLTA	KVFRTYNASI	TLDDMLSKET	765
O. liukiuensis	IDFIGKDSIR	YQNEVQVEPA	VFKAIQQFRS	GKEGCADLFD	RLDTSKLNAH	LKELMPGLTA	KVFRTYNASI	TLDDMLSKET	765
O. ridleyana	IDFIGKDSIR	YQNEVQVEPA	VFKAIQQFRS	GKEGSEDLFD	RLDTSKLNAH	LKELMPGLTA	KVFRTYNASI	TLDDMLSKET	765
O. pumila	IDFIGKDSIR	YQNEVQVEPA	VFKAIQQFRS	GKEGSEDLFD	RLDTSKLNAH	LKELMPGLTA	KVFRTYNASI	TLDDMLSKET	765
O. fucosa	IDFIGKDSIR	YQNEVQVEPA	VFKAIQQFRS	GKEGSEDLFD	RLDTSKLNAH	LKELMPGLTA	KVFRTYNASI	TLDDMLSKET	765
Camptotheca acuminata	FDFLGKDSIR	YQNEVEVELP	VYKAIQQFRT	GKRGGDDLFD	KLDTSKLNAH	LKGLMPGLTA	KVFRTYNASI	TLDEMLSRET	733

		820		840		860		880	
Homo sapiens	APDENIPAKI	LSYNRANRAV	AILCNHQRAP	PKTFEKSMMN	LQTKIDAKKE	QLADARRDLK	SAKADAKVMK	----DAKTKK	682
Catharanthus roseus	KGGD-VAEKV	VVYQHANKEV	AIICNHQRTV	SKSHSAQMLR	LNEKIEELKA	VVEELKSDLS	RVKKGKPPLK	SKNADGKPKR	826
O. japonica	KGGE-VAEKV	VVYQHANKEV	AIICNHQRTV	SKSHSAQMSR	LNEKIDELKT	AMEELKTDLS	RVKKGKPP--	SKGADGKPKR	843
O. pseudofasciculata	KGGE-VAENV	VVYQHANKEV	AIICNHQRTV	SKSHSAQMSR	LNEKIDELKT	ALEELKTDLS	RAKKGKPP--	TKGADGKAKR	841
O. trichocarpon	NGGE-VAEKV	VVYQHANKEV	AIICNHQRTV	SKSHSAQMSR	LNEKIDELKT	ALEELKTDLS	RAKKGKPL--	SKGADGKPKR	842
O. pedunculata	NGGE-VAEKV	VVYQHANKEV	AIICNHQRTV	SKSHSAQMSR	LNEKIDELKT	ALEELKTDLS	RAKKGKPL--	SKGADGKPKR	842
O. plumbea	KGGK-VAEKV	GVYQHANKEV	AIICNHQRTV	SKSHSAQMSR	LNEKIDELKT	ALEELKTDLS	RAKKGKPP--	SKGADGEPKR	842
Ophiorrhiza sp. 35	KGGK-VAEKV	GVYQHANKEV	AIICNHQRTV	SKSHSAQMSR	LNEKIDELKT	ALEELKTDLS	RAKKGKPP--	SKGDDGEPKR	842
O. harrisiana	KGGK-VAEKV	GVYQHANKEV	AIICNHQRTV	SKSHSAQMSR	LNEKIDELKT	ALEELKTDLS	RAKKGKPP--	SKGDDGEPKR	842
O. liukiuensis	KGGK-VAEKV	GVYQHANKEV	AIICNHQRTV	SKSHSAQMSR	LNEKIDELKT	ALEELKTDLS	RAKKGKPP--	AKGADGEPKR	842
O. ridleyana	KGGK-IAEKV	AVYQHANKEV	AIICNHQRTV	SKSHSAQMSR	LNEKIDELKT	ALEELKTDLS	RAKKGKPP--	SKDADGEPKR	842
O. pumila	KGGK-VAEKV	GVYQRANKEV	AIICNHQRTV	SKSHSAQMSR	LNEKIDELKT	ALEELKTDLS	RAKKGKPP--	SKGDDGEPKR	842
O. fucosa	KGGK-VAEKV	GVYQHANKEV	AIICNHQRTV	SKSHSAQMSR	LNEKIDELKT	ALEELKTDLS	RAKKGKPP--	SKGDDGEPKR	842
Camptotheca acuminata	KGGN-VAEKI	VVYQHANKEV	AIICNHQRTV	SKSHPAQMTR	LNGKIDELKG	ILDGLQTDLA	RAKKGKPPL-	-EDADGKPKR	810

		900		920		940		960	
Homo sapiens	VV--ESKKKA	VQRLEEQLMK	LEVQATDREE	NKQIALGTSK	LNYLDPRITV	AWCKKWGVPI	EKIYNKTQRE	KFAWAIDMAD	760
Catharanthus roseus	NLNPESLEKK	IAQTNAKIEK	MERDKETKED	LKTVALGTSK	INYLDPRITV	AWCKRHEVPI	EKIFNKSLLA	KFTWAMDV-D	905
O. japonica	NLNPEALERK	MAQINAKIEK	MERDKETKED	LKTVALGTSK	INYLDPRITV	AWCKRHEVPI	EKIFNKSLLA	KFAWSMDV-D	922
O. pseudofasciculata	NLNPEALERK	ITQTNAKIEK	MERDKETKED	LKTVALGTSK	INYLDPRITV	AWCKRHEVPI	EKIFNKSLLA	KFAWSMDV-D	920
O. trichocarpon	NLNPEALERK	IAQTNAKIEK	MERDKETKED	LKTVALGTSK	INYLDPRITV	AWCKRHEVPI	EKIFNKSLLA	KFAWAMN---	919
O. pedunculata	NLNPEALERK	IAQTNAKIEK	MERDKETKED	LKTVALGTSK	INYLDPRITV	AWCKRHEVPI	EKIFNKSLLA	KFAWAMN---	919
O. plumbea	NLNPEALERK	IAQTNAKIEK	MEGDKKTKED	LKAVALSTSK	ISYLGPRITV	AWCKRHEVPI	EKMFNKSLLA	KFAWAMN---	919
Ophiorrhiza sp. 35	NLNPEALERK	IAQTNAKIEK	MERDKKTKED	LKAVALSTSK	ISYLDPRITV	AWCKRQEVPI	EKMFNKSLLA	KFAWAMN---	919
O. harrisiana	NLNPEALERK	IAQTNAKIEK	MERDKKTKED	LKAVALSTSK	ISYLDPRITV	AWCKRQEVPI	EKMFNKSLLA	KFAWAMN---	919
O. liukiuensis	NLNPESLERK	IAQTNAKIEK	MERDKKTKED	LKAVALSTSK	ISYLDPRITV	AWCKRHEVPI	EKIFNKSLLA	KFAWSMDV-D	921
O. ridleyana	NLNPESLERK	IAQTNAKIEK	MERDKKTKED	LKAVALSTSK	ISYLDPRITV	AWCKRQEVPI	EKMFNKSLLA	KFAWAMDV-D	921
O. pumila	NLNPEALERK	IAQTNAKIEK	MERDKKTKED	LKAVALSTSK	ISYLDPRITV	AWCKRQEVPI	EKMFNKSLLA	KFAWAMDV-D	921
O. fucosa	NLNPEALERK	IAQTNAKIEK	MERDKKTKED	LKAVALSTSK	ISYLDPRITV	AWCKRQEVPI	EKMFNKSLLA	KFAWAMDV-D	921
Camptotheca acuminata	NLTPEALERK	IGQTNAKIEK	MERDKETKEG	LKTIALGTSK	ISYLDPRITV	AWCKRHEVPI	EKVFNKSLLA	KFAWSMDV-D	889

Homo sapiens	EDYEF	765
Catharanthus roseus	PSFRF	910
O. japonica	PSFRF	927
O. pseudofasciculata	PSFRF	925
O. trichocarpon	---H-	920
O. pedunculata	---H-	920
O. plumbea	---H-	920
Ophiorrhiza sp. 35	---H-	920
O. harrisiana	---H-	920
O. liukiuensis	PSFRF	926
O. ridleyana	PSFRF	926
O. pumila	PSFRF	926
O. fucosa	PSFRF	926
Camptotheca acuminata	PSFRF	894

VITA

Miss Varalee Viraporn was born on January 12, 1983 in Bangkok, Thailand. She received her Bachelor's degree of Pharmacy in 2005 from Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

Publication

Viraporn, V., Yamazaki, M., Saito, K., Denduangboripant, J., Chayamarit, K., Chuanasa, T., and Sukrong, S. 2011. Correlation of camptothecin-producing ability and phylogenetic relationship in the genus *Ophiorrhiza* (Rubiaceae). Planta medica (accepted).