Dissertation

The Quality Control of Thai Triphala Formulations

タイの生薬製剤「Triphala（トリファラ）」の品質に関する研究

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CONTENT

ABBREVIATION

accD	acetyl-coA carboxylase beta subunit
ARMS	amplification refractory mutation system
*atp*F-*atp*H	ATPase type F - ATPase type H
bp	base pair
BLAST	Basic Local Alignment Search Tool
CBOL	the Consortium for the Barcode of Life
cm	centimetre
CO1	cytochrome c oxidase 1
DDBJ	DNA Data Bank of Japan
EMBL	the European Molecular Biology Laboratory
h	hour
InDels	insertion/deletion polymorphisms
in.	inch
ITS	internal transcribed spacer
*ndh*J	H-quinone oxidoreductase subunit J
NJ	neighbor-joining method
kb	kilobase
K2P	Kimura 2-Parameter
*mat*K	maturase K
min	minute
PCR	polymerase chain reaction
PCR-RFLP	polymerase chain reaction-restriction fragment length polymorphism
RAPD	randomly amplified polymorphic DNA
*rbc*L	riburose bisphosphate carboxylase large chain
*rpo*B	RNA polymerase beta-subunit
*rpo*C1	RNA polymerase gamma-subunit
s	second
SCAR	sequence characterized amplified region
SNPs	single nucleotide polymorphisms
syn.	synonym
TAE	Tris-acetate-EDTA
*trn*L-F	Leu tRNA - Phe tRNA
*psb*A-*trn*H	photosystem II protein A - His tRNA
*psb*K-*psb*I	photosystem II protein K - photosystem II protein I
var.	variety
YCF5	cytochrome c biogenesis protein
μL	microlitre
^{o}C	degree centigrade
μM	micromolar

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CHAPTER 1

INTRODUCTION

1.1 Background information

The World Health Organization (WHO) has defined traditional medicine (TM) as "the summary of the knowledge, skill, and practices relied upon the theories, beliefs, and native experiences to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness".[1)] On the other hand, complementary medicine or alternative medicine refers to "a broad set of health care practice that are not part of that country's own tradition and are not integrated into the dominant health care system.[1)] The philosophy and practices of the traditional medicine systems are affected by the prevailing conditions, environment, and geographic area within which it first evolved.[2)] However, a holistic approach to living, equilibrium of the mind, body, and the environment, and a focus on health rather than on disease are a key philosophy that found in many systems of traditional medicine around the world.[2)]

The recently available data of traditional and complementary medicine product showed that the market is increasing continuously. For example, The Chinese herbal medicine was evaluated to about US$ 83.1 billion in 2012 (more than 20% from 2011). Besides in the South Korea herbal market, annual herbal commidities rised from US$ 4.4 billion in 2009 to US$ 7.4 billion in 2014.[3)] In addition, the global herbal crude drug and formulations market is prophysied to reach US$ 11.5 billion by 2020, which Asia-Pacific region is the fastest growing market at 9.1% CAGR (Compound Annual Growth Rate), according to Global Industry Analysts.[4)] The National Medicine Plants Board, Government of India expected that because of the recurrence of global traditional and alternative healthcare system, the demand of worldwide herbal market which stood at $120 billion in 2013 is estimated to reach $7 trillion by 2050.[5)] The reasons of these occurrences are the high cost of modern health systems, the awareness about the side effect of modern medicine, and the low cost of herbal remedies.[6)]

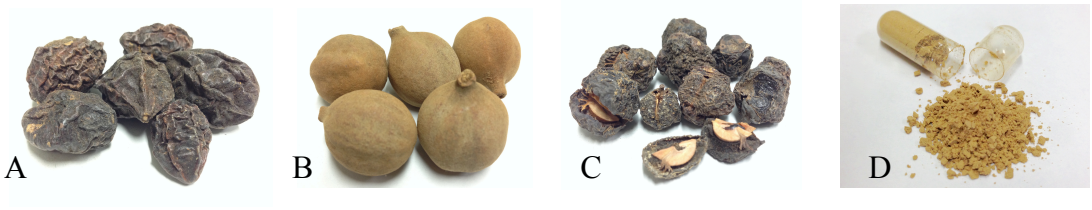


Fig. 1 Triphala formulation and its ingredients
A. *Terminalia chebula* B. *T. bellirica* C. *Phyllanthus emblica* D. Triphala formulation

1.2 Triphala

According to the Ayurvedic system of medicine, the body is composed of *tridosha* meaning three humours, *vata*, translated into the wind, corresponds to mind and nervous system, the *pitta* translated into fire or bile and is responsible for all metabolic transformations including digestion and assimilation of the food, while *kepha* is translated as water or mucous and it is responsible for the anabolic functions such as development of muscle and bone tissue.[7] Triphala is the one of the most important and well-known polyherbal formulations in Ayurvedic system for protecting gastrointestinal organ as well as several organ.[8] The Triphala is the herbal formulation consisting of the powderized fruit of three plants, *Phyllanthus emblica* (Euphorbiaceae), *Terminalia chebula* and *T. bellirica* (Combretaceae) in ratio 1:1:1. It is an important medicine of the "Rasayana" (Rasa: plasma, Ayana: path) group and is believed to promote health, immunity and longevity.[9-10] The Triphala is believed to have balancing and rejuvenating effects on three constitution elements (*vata*, *pitta* and *kepha*) that effect to human life.[7], [11] Traditionally, this formulation has been prescribed as first line treatment for many ailments such as laxative in chronic constipation, detoxifying agent for colon, food digestive problem, rejuvenator of the body[9], high blood pressure disease, large intestine inflammation and ulcerative colitis[12]. Pharmacological studies have shown that Triphala extract posseses anticancer, immunomodulatory activity[12], radioprotective activity[7], antioxidant activity[12-13], free radical scavenging activity[14], anti-inflammatory activity[15] and hypolipidemic activity[16] and so on. The Triphala was demonstrated to have several chemical compounds in the megaext mixture, namely, alkaloids, carbohydrates, glycosides, terpenoids, tannins, phenolic compounds, flavonoids, and proteins[17].

1.3 Thai Triphala

Thai traditional medicine (TTM), the indigenous medicinal practices in Thailand is a mixture of Indian, Ayurvedic and Thai beliefs.[18] Therefore, TTM shares some formulations with Ayurvedic medicine, but TTM uses a native material for ingredients. For example, Triphala in Ayurvedic medicine consists of three dried fruits in equal proportion, but the ingredients of Thai Triphala are contained in different proportion relied on body types or elements of the body, as listed in Table 1.[19] The elements are earth, water, air, and fire that shared concept with Chinese, Greek and Indian Philosophers.[20] Thai Triphala has been traditionally used for a adapting the balance of body elements to climate change for strength and healthiness.[21] Moreover, it is used for detoxifying the body system, particularly gastrointestinal system, blood, and lymph system.[22] Changing in these 4 elements is able to influence discomfort and sickness.[21] However, commercial Triphala in Thai market appears only equal proportion of dosage form.

Table 1 Composition of Thai Triphala

Elements	Ratios		
	T. chebula	*T. bellirica*	*P. emblica*
Pitta or bile (fire + water)	8	12	4
Vata or wind (air + space)	12	4	8
Kapha or mucous (water + earth)	4	8	12
Malas or waste product (faces)	8	8	8

1.4 The Quality Control of Triphala

The quality assurance of botanicals and herbal preparations is required to confirm scientific proof and clinical validation via using chemical standardization, biological assays, animal models and clinical trials.[23] It is an accept fact that qualitative and quantitative analysis of major bioactive chemical component (marker component) of crude drug constituent an important and reliable part of quality control protocol as any change in the quality of the drug directly affects the constituents.[24]

1.4.1 The Quality Control of Triphala by chemical marker constituents

The quality control of herbal medicine that is, the profile of the constituents in the final product has implications in efficacy and safety.[25] Triphala has been reported that it contains enormous amount of tannins such as ellagic acid and gallic acid because these chemical substances are the major tannin-related ingredients.[24-26] High-performance Liquid Chromatography (HPLC) has been utilized for estimating the chemical constituents of Triphala. Singh DP *et al*. developed HPLC condition for separation and quantitative determination of the major polyphenols from Triphala.[27] The reversed phase C_{18} column run by an acidic mobile phase achieved in the efficient separation of gallic acid, tannic acid, syringic acid and epicatechin including ascorbic acid within 20 minute from Triphala.[24] Moreover, the other studies also utilized the RP-HPLC with different chromatographic conditions.[24), 28] The fruits of *T. chebula*, the one of Triphala ingredients was determined by reversed-phase HPLC and capillary electrophoresis (CE).[29] The result revealed that HPLC and CE were success to apply the assay of tannin. Furthermore, Juang *et al*. compared the chemical components of commercially dried *T. chebula* fruits (two varieties: *T. chebula* and *T. chebula* var. *parviflora*) from local herbal markets in Taiwan by using HPLC.[30]

Ascorbic acid was found in Triphala preparation and its ingredients. There are two isomers of ascorbic acid: L-ascorbic acid (reduced form) and dehydroascorbic acid (oxidized form). Oxidized L-ascorbic to dehydroascorbic acid is changeable and a key step of antioxidant activity but dehydroascorbic acid is unstable and occurs irreversible hydrolytic ring cleavage to 2,3-diketogulonic acid, which has no biological effects.[31] Because dehydroascorbic acid is able to absorb ultraviolet, in the determination of total ascorbic acid content, homocysteine was used for reducing dehydroascorbic acid to L-ascorbic acid.[32] Therefore, the amount of dehydroascorbic acid can calculate by subtracting the reduced ascorbic acid content from the total ascorbic acid content.

1.4.2 The Quality Control of Triphala by DNA technology

Analyzed by using traditional morphological and chemical methods is very difficult to authenticate Triphala (a mixture of fruit powders). Because of that, a molecular technique is introduced to identify the ingredients. Previously, the randomly amplified polymorphic DNA (RAPD) based sequence characterized amplified region (SCAR) marker technique was developed and applied to authenticate only *P. emblica*.[33] But, all three ingredients in Triphala should be identified.

1.5 Molecular Marker Method Based on Single-Nucleotide Polymorphism

The tragedy cases from Chinese-herb nephropathy (CHN) were reported around the world about weight-reducing pills (the Chinese traditional medicine) of *Stephania tetradra* S. Moore substituted by *Aristolochia fangchi* Y.C. Wu ex L.D. Chow et S.M. Hwang (rich in aristolochic acid), causing progressive renal failure in women that intake of these pills.[34-35] From this evidence, the cause of the problem is an incorrect identification of the ingredients of medicinal materials, so the accurate identification is important for the safety of patients. Therefore, the ability of herbal differentiation from close related species, inferior substitutes, adulterants and artificial materials affects with patient safety and herbal efficacy.[36] Traditionally, herbal medicines are identified and authenticated by a morphological and histological characteristic including organoleptic markers such as morphological characteristics, appearance, smell, taste, texture, size, and color which rely heavily on the experienced collectors, botanists or experts.[37] Chemical components are utilized as identification marker as well, however, the chemical contents usually vary by physiological and environmental conditions, harvesting period, post-harvesting processing and storage condition.[38] Recently, DNA method has been played roles for identification of medicinal materials because the genetic composition is quite not influenced by other factors like traditional methods.[39] Nonetheless, the advantages of this method are expensive or in limited supply. DNA markers used in plant genome analysis are able to divide to three types: Hybridization-based methods, PCR-based method, and Sequencing-based markers.[40]

Single nucleotide polymorphisms (SNPs), the genetic variation in plant and animal kingdom, have become to utilize increasingly as a molecular marker system for practically distinguishing single base differences within the genome since the late 1990s.[41-43] In medical field, the SNPs from human genome can explain the cause of genetic disorders (such as galactosemia, an inborn disease caused by mutations in *GALT* gene encoding for galactose metabolism enzyme, galactose-1-phosphate uridylyltransferase) and conceive the structure, expression and function of regular genes as well as and presymptomatic and prenatal diagnosis.[44-45] Moreover, in pharmacogenetic analysis and in population genetics and evolutionary studies, the SNPs play a substantial role by means of using hybridization, primer extension, oligonucleotide ligation, allele-specific polymerase chain reaction (PCR), and endonuclease cleavage for SNP genotyping.[46] The attraction of identification and analysis of SNPs in plant species has augmented.[43, 47-48] For example, Hayashi *et al*. used SNPs and small insertion/deletion polymorphism (InDels) as DNA markers for genetic analysis and bleeding of rice. Moreover, they suggested that SNP genotyping with allele-

specific PCR plays a priceless role for genetic developing in crops, especially rice, i.e. gene mapping, map-based cloning, and marker-assisted selection.[49] In sunflower (Helianthus), the important crop in food and confectionary industries, Lai Z *et al.* identified 605 expressed sequence tags (ESTs) exhibited SNPs variation, tissue-specific expression pattern, and were candidate function namely cell transport, metabolism, and plant defense.[50] Therefore, there are several advantages of SNPs such as simple to utilize and automate, and most plentiful polymorphisms in genome.[51-53]

ARMS (amplification refractory mutation system), an easy and cost-effective method for authenticating herbs and their adulterants, is an allele-specific polymerase chain reaction (PCR) that exhibits differentiation of alleles at specific loci differing by as little as 1 bp.[53] The ARMS technique has been successfully performed to diagnosis the genetic disorders[54-55] and to authenticate many medicinally commercial commodities especially herbal medicines and medicinal raw material from animals such as *Alisma orientale* (Sam.) Juzep.[56], *Panax ginseng* L.[57], *Dendrobium officinale* Kimura et Migo[53, 58], *Anemarrhena asphodeloides* Bunge.[59], *Tribulus terrestris* L.[60], *Croton caudatus* Geisel.[61], *Swertia mussotii* Franch.[62], *Cucumis melo* L.[63], last but not least *Pantheran tigris* (tiger's bone used in traditional Chinese medicines).[64]

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) is a simple and reliable method that uses a restriction endonuclease that has a high affinity for unique restriction sites for species authentication. Restriction enzymes are specific endonucleases enzyme produced by many types of prokaryotes to destroy invading, foreign DNA molecules by recognizing and cleaving specific DNA sequence, generally consisting of about four to six bases.[65] Recently, the PCR-RFLP has been used as one of the methods for authenticating medicinal plants and their adulterants. For example, *Glehnia littoralis* Fr. Schmidt ex Miquel[66], *Atractylodes lancea* De Candolle[67], seven *Epimedium* species[68], *Panax ginseng* L.[69-70], narcotic *Mitragyna* plants collected from Thailand[71], *Poria cocos* Wolf[72], *Fritillaria cirrhosa* D.Don[73], *Akebia* plants[74], three medical *Stemona* species[75], and *Phyllanthus amarus*[76].

1.6 DNA Barcoding

DNA barcoding is a rapid and accurate species identification and classification method by using a standardized DNA region.[77] Recently, the term 'DNA barcoding', first used in 2003 by scientists from the University of Guelph in Ontario, Canada, has been utilized in the literature.[78-82] The DNA barcoding workflow is similar to a barcode in goods packaging. From an identified specimen under investigation (i.e. holotype) and a standard part of the genome, these barcode sequences will be as the reference barcode sequences for comparing with each unknown specimen.[83] The two main purposes are (1) an assignment of unknown species, and (2) improvement of the discovery of new species and simplification of identification.[79, 82, 84-85] However, the DNA barcoding expects to promise in providing a practical, standardized genetic variation discrimination method with fast and accurate identification. It shows promise in providing a practical, standardized genetic variation of discrimination method with fast and accurate identification. The advantages of DNA barcoding are the way to support many scientific fields (e.g. biogeography[80], biodiversity assessment[87-88], forensic analysis[89-90], ecological studies[91-92], evolutionary

biology[91] and epidemiology[93-94]) and in bio-industry[82]. There are many published scientific literatures about the role of DNA barcoding in authentication of herbal products.[95-99]

Several gene regions have been used for species-level identification that should be universally appropriate to a great number of eukaryote.[100] However, the CBOL (the Consortium for the Barcode of Life) plant working group (http://barcoding.si.edu), an international initiative supporting the development of DNA barcoding, intends to promote worldwide standards as well as coordinate research in DNA barcoding.[100] Furthermore, the CBOL has been founded to stimulate the idea of a database of documented and vouchered reference sequences to serve as a huge library for comparing with unidentified taxa.[100] The gene encoding the mitochondrial cytochrome c oxidase 1 (CO1), a protein-coding region contained 658 bp of high-copy numbers per cell was deliberately selected as the standard barcode for animal identification.[79-80), 101] Then, the CO1 was suggested as the locus that could perform recognition tags for all organisms[81] However, even the CO1 has been recommended to use as DNA barcode, it is not commonly use in plant and fungi.[91] Since, the mitochondrial genes in plant are very low substitution rate and slow evolution, so they were not proper for barcoding.[81] The CBOL group searched for alternative barcoding regions for the plant kingdom. Seven leading candidate plastid DNA regions (four coding genes: *mat*K, *rbc*L, *rpo*B, and *rpo*C1 and three noncoding spacer: *atp*F-*atp*H, *trn*H-*psb*A, and *psb*K-*psb*I) were compared the performance to be as a standard plant barcode.[102] Finally, *mat*K + *rbc*L combination was recommend as the plant barcode because *mat*K provide high specie resolution but low universality. On the other hands, *rbc*L gives high universality but less resolution. Nonetheless, Singh *et al.* said that these two recommended loci were not able to discriminate among closely related species (such as *Dendrobium* species[103] and Indian *Berberis* species[104]). Therefore, the China Plant BOL group suggested that ribosomal DNA ITS should be supplement to the *mat*K + *rbc*L combination as plant barcode because the nrITS showed highest differentiate efficiency among four candidate markers (plastid *rbc*L, *mat*K, *psb*A-*trn*H and nuclear ribosomal ITS).[105]

Recently, the DNA barcoding has played a role to authenticate herbal medicinal material including food and beverage from adulterants derived from closely related species or from species from other families.[96), 106-107]

1.7 Genus Terminalia (Combretaceae family)

The family Combretaceae, a pantropical family that placed in order Myrtales, comprises approximately 525 species in 17 genera of trees, shrubs, and lianas distributing mainly in tropical and subtropical Africa, Central and South America, Southern Asia and northern Australia.[108] Within the family, the Combretaceae was divided to two subfamilies, Strephonematoideae, and Combretoideae.[109] The subfamily Strephonematoideae contains a single genus Strephonema with three species distributed in western tropical Africa. Their common morphological characteristics are a semi-inferior ovary, cotyledons conduplicate and the appearance of tangential bands of apotracheal parenchyma in wood. Whereas, the subfamily Combretoideae, containing 19 genera, is characterized by the inferior ovary, cotyledons flattened with variously folded or rarely conduplicate, and wood without tangential bands of apotracheal

parenchyma.[110] Genus Combretum, placed in the largest genera in the family, occurs in all continents, but it is reported that Africa is the area that contains the greatest genetic diversity of Combretum.[110] On the other hands, the second largest genus is Terminalia.[111] In Asia, Combretaceae is found six genera: Anogeissus (DC.) Wall, Calycopteris Lam., Combretum Loefl., Lumnitzera Wild., Quisqualis L., and Terminalia L. The species in Combretaceae family have many economic values not only in dying and tanning but also in the traditional pharmaceutical industries. The study of phylogenetic relationship of family Combretaceae has generated since in 1984 when Dahlgren *et al*. released the first comprehensive cladistic analysis framework of order Myrtales. The study considered that Combretaceae was placed in one of the core families of Mytales, namely Myrtaceae, Heteropyxidaceae, Psiloxylaceae, Melastomataceae, Memecylaceae, Rhynchocalacaceae, Combretaceae, Crypteroniaceae, Penaeaceae, Oliniaceae, Lythraceae, Trapaceae, Alzateaceae, and Onagraceae.[112] In the same year, Johnson *et al.* analyzed the phylogenetic study of 19 families of the Myrtales on the basis of morphological and anatomical characters. The Combretaceae contained in the same clades of seven core families: Panaeceae, Alzateaceae, Oliniaceae, Rhynchocalyceae, Crypteroniaceae, Memecylaceae, and Melastamotaceae.[113] Then, Conti *et al.* (1996) performed the phylogenetic relationship based on 80 nucleotide sequences of plastid *rbc*L gene representing 36 species from Myrtales and 44 species from other Rosidae. The firstly reanalyzed comprehensive phylogenetic framework revealed that Combretaceae included in the same clade with Onagraceae and Lythraceae.[114] The intraordinal relationships, analyzed in 50 *rbc*L sequences among the species of Myrtales using parsimony and maximum likelihood method, confirmed the former studies that the Combretaceae combined in the sister group with Onagraceae and Lythraceae.[115] Tan *et al*. (2002) analyzed the first molecular phylogeny on the Combretaceae focused on subfamily Combretoideae based on nuclear ITS region and the plastid *rbc*L, and the intergenic spacer between the *psa*A and *ycf*3 gene (PY-IGS). As the summarized results of study, the ingroup taxa separated into two clades: Laguncularieae and Combreteae which five *Terminalia* species include in Combreteae clade (subtribe Terminaliinae).[109] The most recent molecular phylogenetic study on Combretaceae with massive specimens was revealed by Maurin *et al.*[108] The DNA sequence data of nuclear ITS and plastid *rbc*L, *psa*A-*ycf*3 spacer and *psb*A-*trn*H spacer was collected and analyzed by maximum parsimony method. Genus Terminalia was unidentified as monophyletic which it was split into two groups: one bearing mainly African species and another containing cheifly Asian species.

Table 2 DNA Barcoding and DNA Sequence-based Markers were utilized for Identifying Species of Several Herbal Medicines.

Specie	Common name	Family	Genomic region	Reference
Rheum officinale	Chinese rhubarb	Polygonaceae	nrITS *rbc*L *trn*H-*psb*A *ndh*J *rpo*C1 *rpo*B *acc*D YCF5	Song J *et al.*[97)]
R. tanguticum	Turkish rhubarb			
R. palmatum	Chinese rhubarb			
Fagopyrum dibotrys	Buckwheat			
Polygonum bisturta	Bistort			
P. aviculare	Common knotgrass			
Persicaria orientalis	Prince's feather			
P. tinctoria	Chinese indigo			
Fallopia multiflora	Chinese knotweed			
F. japonica	Japanese knotweed			
Astragalus membrananeus	Huang qi	Fabaceae	ITS2	Gao T *et al.*[116)]
A. mongolicus	Milk vetch			
Pueraria tuberosa	Indian kudzu			
Salvia miltiorrhiza	Red sage	Lamiaceae	*rbc*L *mat*K *trn*L-F *trn*H-*psb*A ITS	Wang M *et al.*[117)]
Mentha piperita	Peppermint	Lamiaceae	*rbc*L *rpo*B *mat*K *trn*H-*psb*A	Mattia FD *et al.*[107)]
M. aquatica	Water mint			
M. spicata	Spearmint			
Ocimum basilicum	Basil			
O. gratissimum	Clove basil			
O. tenuiflorum	Holy basil			
Origanum majorana	Marjoram			
O. vulgare	Oregano			
O. pseudodictamnus	-			
O. heracleoticum	Greek oregano			
Salvia officinalis	Common sage			
S. rutilans	Pineapple sage			
S. sclarea	Clary			
S. uliginosa	bog sage			
Thymus vulgaris	Common thyme			
Rosmarinus officinalis	Rosemary			
Panax ginseng	Ginseng	Araliaceae	*rbc*L *mat*K *trn*H-*psb*A ITS2	Liu Z *et al*[118)].
P. notoginseng	Notoginseng			
Acanthopanax gracilistylus	-			
Sida cordifolia	Indian Ephedra	Malvaceae	*mat*K *rbc*L *trn*H-*psb*A ITS2	Vassou SL *et al.*[119)]
Bupleurum chinense	Chaihu	Apiaceae	*rbc*L *mat*K *trn*H-*psb*A ITS2	Chao Z *et al.*[120)]
B. scorzonerifolium	Shannon Chaihu			
Dendrobium nobile	Noble Dendribium	Orchidaceae	*mat*K *rbc*L *rpo*B *rpo*C1 *trn*H-*psb*A ITS	Singh HK *et al.*[103)]
Ophiocordyceps sinensis	Chinese caterpillar fungus	Ophiocordycipitaceae	ITS	Xiang L *et al.*[99)]

Table 3 The Characteristic of 17 Genera in Combretaceae [110), 121)]

Subfamily	Tribe	Sub-tribe	Genus	Geographical distribution	Characteristic
Strephonematoideae Engl. & Diels (1899)			*Strephonema* Hook.f (1867)	Tropical West Africa	Semi-inferior of ovary, the calyx-tube arising from its side; seeds with big hemispherical cotyledons
Combretoideae Engl. & Diels (1899)	Laguncularieae Engl. & Diels (1899)		*Laguncularia* C.F.Gaertn (1807)	Tropical East & West America, tropical West Africa	Mangrove, leaves opposite with two petiolar grands but without margin glands
			Lumnitzera Willd. (1803)	Tropical East Africa to Australia	Mangrove; leaves spiral with no petiolar glands but presence glands on margin.
			Macropteranthes F. Muell.	Australia	Leaves spiral or opposite with no gland on petiole and margin; its prophylls accress to form winged friut.
			Densiea Byrnes	Australia	Leaves no petiolar glands but presence glands on margin near base; hypanthium adnate to ovary on ventral side.
	Combreteae DC. (1828)	Terminaliinae (DC.) Exell & Stace (1996)	*Anogeissus* (DC.) Wall (1831)	Tropical West Africa to Southeast Asia	Leaves sometimes with pocket-shaped domantia, with no petiolar glands and stalked glands; upper hypanthium deciduous before fruiting; fruit 2-winged, flattened, dry and achene like.
			Buchenavia Eichler (1866)	Tropical America	Leave spiral with pocket-shaped and glands on petiole; fruit 5 ridged.
			Conocarpus L. (1753)	Tropical West and East America, and Northeast Africa and Southern Yemen	Mangrove-like shrubs or trees; leaves spiral with bowl-shaped domatia and petiolar glands and small stalked glands; fruit 2 winged, flattened, dry.
			Pteleopsis Engl. (1894)	Africa	Leaves with no domatia and glands; andronoecious; petal usually present; fruit 2-winged.
			Terminalia L. (1767)	Tropics and subtropics	Leaves spiral with domatia and petiolar glands; petal absent; fruit 2- to 5-winged or ridged or ± terete.
			Finetia Gangnep. (1917)	Thailand and Laos	One species: *F. rivularis* (Gagnep.) Lecomte Leaves opposite or subopposite without domatia and glands; flowers bisexual; fruits 4-libbed.
		Combretinae Exell & Stace (1966)	*Calycopteria* Lam. (1793)	Southeast Asia	One species: *C. floribunda* (Roxb.) Lam. Scrambling shrub, leaves opposite or sub-opposite with scale; flowers 5-merous with accrescent calyx forming 5 wings.
			Combretum Loefl. (1758)	Tropics and subtropics	Shrubs or lianas, rarely trees; leaves with stalked glands and/or scales; petal present; fruits 2- to 5-winged or ridged or ± terete, dry or succulent, not achene-like.
			Guiera Adans (1789)	Tropical West Africa	Shrub; leaves opposite or sub-opposite with scale-like glandular trichomes; flowers 5-numerous; petal present; fruit dry, achene-like.
			Meiostemon Exell & Stace (1966)[122)]	Zambia, Zimbabwe and Madagascar	Scandent shrubs or a small trees; leaves opposite with scales; petal 4 inserted near the margin of the disk; fruit 4-winged
			Quisqualis L. (1762)[123)]	Tropical old world	Leaves with sub-epidermal crystalliferous idioblasts and stalked glands (no scales); persistent petiole; petal present; hypanthium tubular or cylindrical.
			Thiloa Echler (1866)[124)]	Tropical South America	Small trees, shrubs or lianas; leaves opposite; flowers 4-merous, petal absent; fruits pseudocarp, dry, indehiscent, 1-seed with 4 broad papery wing

Terminalia, the name obtained from "terminus", refer to the leaves of the plant in this genus mostly appear at the tips of shoots. The estimated number of the genus is about 190 species that distributed to tropics of Africa, America, and Asia, covering to South Africa, Australia, and Pacific islands.[110), 125-126)] Approximately 70 species of *Terminalia* plants were recorded in Southeast Asia that it appeared the most genetic diversity.[104), 127-128)] The characteristics of genus *Terminalia* are as follows:[125), 127-128)]

- Habit: deciduous trees, usually buttressed, rarely shrub; branching often sympodial and pagoda-like
- Leaves: usually spiraled and crowded into pseudowhorls at tips of branchlets, often hairy when young and become glabrescent, frequently with two or more domatia or glands at or near the base of the lamina or on the petiole.
- Inflorescence: axillary or terminal spikes, racemes or sometimes panicles with bisexual flowers toward the base and male flowers toward the apex of the inflorescence.
- Calyx: calyx lobes 4 or 5 deltoid, ovate or triangular
- Petal: absent
- Stamen: 8 to 10, versatile
- Fruit: variable in shape and size, often fleshy and drupelike, sometimes dry and leathery or corky, often longitudinally 2-, 3-, or 5-winged nut, or -ridge; endocarp usually with at least partially sclerenchymatous.

Thailand found sixteen indigenous species with two exotic species[129)]: *T. arjuna* (Roxburgh ex Candolle) Wight & Arnott (the native species from India)[125)] and *T. ivorensis* A. Chev. (the native species from West Africa[130)] used for garden decoration) as show in Table 4-6.

T. chebula Retz. is native specie in South Asia from India to Nepal, South East Asia extending to the south of China (West Yunnan).[125)] *T. chebula* are not only utilized in dye industry but also as medicines in Thai traditional medicine and Ayurvedic medicine. In Flora of China Vol. 13 (2007), two varieties of *T. chebula* considered on the basis of indumentum on branchlets and both surfaces of leaf blade. *T. chebula* Retz. var. *chebula* appears tawny tomentose only when young or glabrous on both surfaces of leaf blade and branchlets, while *T. chebula* Retz. var. *tomentella* (Kurz) C.B. Clarke appressed tawny villous or densely attached silvery tomentose at least when young.[125)] Moreover, *T. chebula* var. *parviflora* was reported in Taiwan as a commercial Fructus Chebulae, the dried ripe fruit of *T. chebula* used in Chinese traditional medicine.[30)] Thailand reported that there are two varieties of *T. chebula*: *T. chebula* Retz. var. *chebula* and *T. chebula* Retz. var. *nana* Gagnep.[128), 129)] Their difference is habit which *T. chebula* var. *chebula* is large tree (10-20 m) but *T. chebula* var. *nana* is shrub (0.7 - 2 m). In addition, Krachai *et al.* reported that they share the character of morphology, palynology and anatomy but the habit and the appearance of tannin in a bundle sheath near the lower epidermis of leaves can separate both species to two varieties.[131)] As Flora of British India (1896), six varieties of *T. chebula* were mentioned: [132-133)]

1. Variety chebula: fruit 1-1.5 inches, ellipsoid or obovoid, five-ribbed
2. Variety typica: young ovary are rough without calyx teeth
3. Variety citrina: young ovary are quite glabrous; fruit ovate, round base
4. Variety tomentella: young ovary glabrous, fruit ovoid
5. Variety gangetica: fruits covered with brown silky hair
6. Variety parviflora: fruits more acute ribbed

Moreover, there are variations of characteristic of *T. chebula* var. *chebula* as follows in Table 7.

A. The Important Characteristics of *Terminalia* Leaves

Two domatia on the lamina of *T. elliptica*

Glands on the petiole of *T. citrina*

Golden hairs in young leaves of *T. chebula*

B. The Variations of Some *Terminalia* Inflorescences

T. catappa

T. elliptica

T. chebula

C. The Fruit Variations of Some *Terminalia* Species

T. arjuna

T. glaucifolia

T. elliptica

T. bellirica

T. citrina

T. chebula

Fig. 2 The Main Characteristic of *Terminalia* spp.

Table 4 Characteristic of Bark and Ecology of *Terminalia* Plants in Thailand[128)]

Species	Thai name[129)]	Bark	Ecology				
			Habitat	Altitude (m)	Defoliating	Flowering	Fruiting
T. bellirica (Gaertn.) Roxb.	สมอพิเภก Samo phiphek	Blackish, brittle, longitudinally fissured & cracked, thick, cut yellow	Wide spread, though out mixed deciduous forests, dry deciduous dipterocarp forests and dry evergreen forest.	Low alt. up to 500	Nov.-Jan	Mar.-Apr	Sep.-Nov.
T. catappa L.	หูกวาง Huu kwaang	Smooth	Indigenous on sandy or rocky beaches	0-10	Jan.-Feb. Jul.-Aug.	Mar.-Apr.	Aug.-Sep.
T. foetidissima Griff.	อู่ชด Uu chot	Light brown, slightly fissured	By streams in evergreen forests	Low alt. up to 500	Feb.-Mar.	Apr.-May	Nov.-Mar.
T. citrina (Gaertn.) Roxb. ex Fleming	สมอดีงู Samo dee nguu	Smooth, grayish-brown with slightly shallow patches	Scrattered in lowland forests and frequent seashores	Up to ca. 200	-	Jun.-Jul.	Sep.-Nov.
T. chebula Retz. var. *chebula*	สมอไทย Samo thai	Rough, scaly	Scrattered in teak forests in Northern Thailand and in mixed deciduous and dry evergreen forests, normally in clayey-sandy soil.	0-800 (-1000)	Feb.-Mar.	Apr.-May	Nov.-Mar.
T. chebula Retz var. *nana* Gagnep.	สมอเตี้ย Samo tia	Rough, scaly, grayish-black, with longitudinally fissured and cracked	In poor soil, or on sandy and rocky ground, scrattered in open deciduous forests.	200-450	Mar.-Apr.	Jun.-Aug	-
T. myriocarpa Heurck & Muell.-Arg var. *hirsuta* Craib	ซ่าง Saang	Brown, scaly	Along streams in hill evergreen forests.	700-1200	-	-	-
T. mucronata Craib et Hutchinson	ตะแบกเลือด Tabaek lueat	Smooth with dimple marks, light grayish-brown	Widely distributed in mixed deciduous and dry dipterocarp forests.	Up to 700	Nov.-Feb.	Mar.-Apr.	June
T. glaucifolia Craib	แหนนา Haen naa	Grayish-black, slightly cracked with shallow, longitudinal fissured	Scattered in mixed deciduous forests on ridges and slopes and in savannas.	250-400	-	-	-

Table 4 Characteristic of Bark and Ecology of *Terminalia* Plants in Thailand (cont.)

Specie	Thai name	Bark	Ecology			
			Habitat	Altitude (m)	Defoliating	Flowering	Fruiting
T. calamansanai (Blanco) Rolfe	สกุณี Sakunee	Grayish-brown, shallowly fissured	Widely distributed in deciduous forests and lowland forests, by road-sides, savannas and rice-fields	0-170	Feb.-Apr.	Aug.-Dec.	Dec.-Apr.
T. harmandii Gagnep.	แหนกลัก Haen klak	-	Deciduous tree, in open deciduous forest near swamps.	-	-	-	-
T. nigrovenulosa Pierre. (syn *T. triptera* Stapf)	ขี้อ้าย Khee aai	Smooth, brownish with shallow, longitudinal streaks, cut with bright orange-red colour	Common in low land mixed deciduous forests and dry evergreen forests, on sandy soil and limestone formation	-	Feb.-Mar.	Sep.-Oct.	-
T. franchetii Gagnep. var. *tomentosa* Nanakorn	สมอใบขน Samo bai khon	Grayish-black	Only know from the type locality, the tree is scattered among limestone hills in deciduous forest	1100-1200	-	Feb.-Mar.	Mar.-May
T. pedicellata Nanakorn	เปื๋อย Puei	-	Scattered in dry deciduous forest, mainly in savannas, sandstone and rocky soil	300-360	-	Mar.-Apr	Apr.-May
T. cambodiana Gagnep.	เปื๋อยน้ำ Puei nam	-	By streams in evergreen forest	-	-	-	-
T. pierrei Gagnep.	ตะแบกกราย Tabaek kraai	Smooth, light grayish-brown with shallow patches	Scattered in dry deciduous forest on sandy soil and rocky ground in North-eastern region in mixed deciduous and dry green forests on poor soil.	-	Feb.-Mar	Apr.-Jul	Aug.-Oct
T. elliptica Willd. (syn *T. alata* Heyne ex Roth., *T. tomentosa* (Roxb.) Wight & Arn.)	รกฟ้า Rok faa	Rough, grayish-black, deeply cracked, inner bark reddish	Common in mixed deciduous forests and dry dipterocarps forest	100-1000	Jan.-Apr.	Jun	Feb.-Mar.

Table 5 Characteristic of *Terminalia* Leaf in Thailand[128)]

Species	Characteristic	Length (cm)	Surface	Apex	Base	Nerve	Petiole	Gland
T. bellirica	Coriaceous, obovate	4-16 by 2-10	Glabrous	-	-	Widely spaced, 6-8 pairs	Glabrous, 3-9 cm	2, at about the middle or near leaf-base
T. catappa	Chartaceous, obovate	12-25 by 8-15	Shiny and glabrous	-	Very narrow cordate base	6-9 pairs	Stout, 0.5-1.2 cm long	Nodular, obscure on the lower surface near leaf-base, 0.1-0.3 cm in diameter
T. foetidissima	Membranous to chartaceous, obovate	7-12 by 3-6	Glabrescent	Slightly acuminate or obtuse	cuneate	Obscure above, slightly raised beneath, widely spaced, 8-10 pairs	Slender, glabrous, 1.5-2.2 cm	2, near leaf-base or at about the middle
T. citrina	Coriaceous, oblong-elliptic	3-14 by 2-6	Glabrous	Shortly acuminate	Rounded or broadly cuneate	9-12 pairs, slightly raised with reticulate venetion beneath	1-2.5 cm, glabrous	2, near leaf-base
T. chebula var. *chebula*	Coriaceous, broadly ovate to ovate-elliptic	8-15 by 6-10	Glabrescent	Acute or abruptly acuminate	Cuneate, slightly cordate or rounded	Obscure above, slightly raised and usually brownish puvescent beneath	1-3 cm, glabrous or sparsely pubescent	2, near leaf-base
T. chebula var. *nana*	Alternate, subopposite or opposite, coriaceous, ovate to ovate-elliptic	3.5-7 by 2-6.3	Margin ciliate, sparsely tomentellous on both surface particularly on nerve beneath	Acuminate	Rounded or obtuse	-	Apressed pubescent, 0.5-1.2 cm	2, on margin near the base of lamina, 2 nodular glands on petiole
T. myriocarpa var. *hirsuta*	Subopposite or opposite, chartaceous, oblong to broadly elliptic	10-20 by 6-9	Glabrescent or pubescent on nerve beneath	Pointed or acuminate	Rounded or subcordate	Parallel, 16-26 pairs, prominent beneath	Stout, 0.4-0.7 cm, densly tomentose to glabrous	2 stalked glands ca 1 mm in diameter prominent at the leaf-base
T. mucronata	Chartaceous, oblong to oblong-elliptic	8-15 by 5-8	Tomentose with hyaline margin when young	Mucronate or acuminate	Slightly attenuate or obtuse	Slightly raised with scalariform venation beneath	Tomentose, 1-2 cm	2 nodular glands at the jount of leaf-base
T. harmandii	Opposite or subopposite, thin coriaceous, ovate-elliptic to suborbicular	5-6.5 by 3-3.5	Glabrous or only slightly pubescent along midrib on upper surface, obscured on upper surface, slightly raised on under surface	Acute or sharply acuminate	Obtuse or slightly cuneate	Sparsely pubescent on nerves on under surface, 6-8 pairs	5-8 mm, sparsely appressed pubescent to glabrous, usually with axillary dormant bud	2 glands on margin near leaf-base
T. nigrovenulosa	Alternate or subopposite, chartaceous, ovate or ovate-elliptic	6-10 by 3-6	Glabrescent	Usually acuminate	Slightly cuneate or rounded	-	0.5-1.2 cm, slender, glabrous	2 basal glands on margin near leaf-base

Table 5 Characteristic of *Terminalia* Leaf in Thailand (cont.)

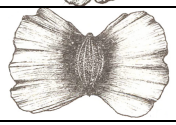
Species	Characteristic	Length (cm)	Surface	Apex	Base	Nerve	Petiole	Gland
T. franchetii var. *tomentosa*	Yellowish-green, alternate, subcoriaceous, ovate to ovate-elliptic	4-6 by 1.5-4 cm	Densely tomentose on both surface	Shortly mucronate to subacuminate	Obtuse or slightly cordate	Ascending, obscure on upper surface	Tomentellous, 0.4-1.5 cm	2 nodular glands on margin near the base of lamina
T. pedicallata	Brownish, subcoriaceous, broadly ovate to oblong-elliptic	5-8 by 3-6 cm	Rufous tomentose on both surface	Mucronate	Rounded or slightly attenuate	Slightly ascending, obscure above, prominently raised beneath	Tomentose, 0.8-1.4 cm	2 prominent nodular glands at the leaf-base
T. cambodiana	Opposite or verticillate on short shoots, membranous, obovate to ovate-elliptic	5-7 by 2.5-4 am	Glabescent or sparsely pubescent particularly on nerve beneath	Rounded or obtuse	Attenuate or cuneate	Slightly ascending, 7-9 pairs	Tomentellous to glabrous, 0.4-1.2 cm	2 nodular glands and 2 basal glands on margin near laef-base
T. pierrei	Chartaceous, ovate-elliptic to ovate-oblong	3-7.5 by 1-3 cm	Rusty pubescent particularly on nerves beneath	Shortly acute or acuminate	Rounded, obtuse or slightly cordate, sparsely tomentellous to glabrescent	8-10 pairs, obscure above, raised beneath	Appressed rufous pubescent, 0.3-0.6 cm	2 glands on margin near the base of lamina
T. elliptica	Oblong to ovate-oblong	10-15 by 5-10 cm	Tomentose to glabrous, usually with 2 stalked glands (domatia) prominent on midrib near base beneath	Acute or subacute	Obtuse, frequently oblique	Parallel, 10-16 pairs	Glabrous, 1-2 cm	1-3 mm in diameter

Table 6 Comparison of *Terminalia* Fruits in Thailand[128)]

- Fruit drupaceous

Species	Description				Illustration
	Characteristic	Lenght	Ridge	Other	
T. bellirica	Subglobose to broadly ellipsoid	2-3 by 1.5-2 cm	Slightly 5 longitudinal	Exocarp densely & finely velvety pubescent, endocarp densely sclerenchymatous, very hard when dry	
T. catappa	Ellipsoid, glabrous	3-7 by 2-5 cm	-	Pericarp fibrous, laterally compressed with keel all round	
T. foetidissima	Ovoid to ellipsoid	4-8 by 3-6 cm	-	Slightly compressed at one side, drying wrinkled	
T. citrina	Ellipsoid to subglobose, glabous	2-3 by 0.8-2 cm	5-angular	Smooth, slightly laterally compressed, wrinkled and blackish when dry	
T. chebula var. *chebula*	Subglobose to ellipsoid, glabrous	2.5-4 by 1.5-2.5 cm	Smooth or 5-angular	Wrinkled, turning blackish when dry	
T. chebula var. *nana*	Globose or subglobose	2.5-3 by 1.5-1.8 cm	-	Greenish yellow, often reddish-purple tinted and sparsely lucid dotted, wrinkled when dry	

- Fruit 2-, 3-, or 5-winged nut
    - Nuts 2-winged

Species	Description				Illustration
	Characteristic	Length of Nut	Wing	Other	
T. myriocarpa var. *hirsuta*	Obscurely trigonal or compressed ellipsoid	0.3-0.4 by 0.1-0.2 cm	2	Occationally rudimentary development of a 3rd wing	
T. mucronata	Suborbicular in outline	2-4 by 2.5-3 cm	2	Wings coriaceous densely, finely rusty pubescent	
T. glaucifolia	2 broad winged, body velvety pubescent	0.5-1.2 by 1.5-2.6 cm	2	Upper surface with prominently 2 longitudinal ridges, lower surface with 2 longitudinal grooved 1-2 mm depth, winged striate 2.5-5 cm by 3.5-5 cm, yellow hay to dark brown color	
T. calamansanai	2 coriaceous straite winged	4-8 by 1.5-4 cm	2	Fruit body trigonal, 2.5-6 by 1.5-3 cm velutinous pubescent, wings longer than broad 2-4 by 1.5-3 cm	
T. harmandii	Subellipsoid to trigonal in outline	1.5-1.7 by 1.3-1.5 cm	2	Rounded or spherical at centre, proximal part abruptly narrow, distal part expaned into 2 narrow wing, wings 2-3 mm broad, pericarp hard brownish-black, glabrous	

- Nuts 3- or 5- winged

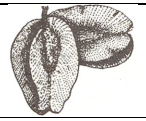
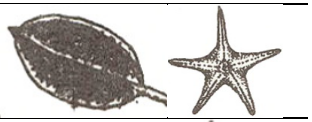
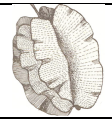
Species	Description				Illustration
	Characteristic	Length of Nut	Wing	Other	
T. nigrovenulosa	Oblong (rarely oblique)	1.5-3.3 by 1-1.8 cm	3	Wings coriaceous, glabrous	
T. franchetii var. *tomentosa*	Densely reddish tomentose, laterally compressed	0.7-0.9 by 0.5-0.7 cm	3	Oblique at base, orbicular in outline, stalk 0.4-0.6 cm, wing membranous, 2-3 mm broad	
T. pedicellata	Densely pubescent	1.4-1.8 by 0.7-1.0 cm	3	Oblong to ellipsoid in outline, stalk 0.7-1.8 cm, wings subcoriaceous, 2-3 mm broad	
T. cambodiana	Oblong	1.4-1.7 by 0.7-0.8 cm	5	Wings oblong, more or less equal, glabrous, 0.2 mm broad.	
T. pierrei	Ovoid-oblong	0.9-1.2 by 0.6-0.8 cm	5	Wings densely brownish-red pubescent, 1-3 mm broad	
T. elliptica		4-6 by 2.5-5 cm	5	Wings coriaceous, glabrous, oblong, 1-1.5 by 3-4 cm broad	

Table 7 The Variation of Characteristic of *T. chebula*

Characteristic		India[132-134)]	Thailand[128)]	Myanmar[135)]	China[125)]
Leave	Young parts	-	rusty villous	dense rusty-coloured tomentum	tomentose or silvery villous
	Full-grown leaf	-	coriaceous	coriaceous, glabrous above, or altogether glabescent	both surface glabrous
	Shape	ovate or elliptic	broadly ovate or ovate-elliptic	oblong	elliptic
	Apex	acute not acuminate	acute or abruptly acuminate	bluntish acuminate or apiculate	mucronate
	Base	rounded	cuneate, slightly cordate or rounded		obtused-rounded or cuneate, oblique
	Length	-	8-15 x 6-10 cm	6-8 in.	7-18 x 4.5-10 cm
	Petiole	1 in.	1-3 cm	1.5-2 in.	1-3 cm
	Gland	2 glands	2 nodular glands	with or without	2(-4) glands 1-5 mm
Flower	Inflorescence	raceme	axillary or terminal panicles		simple spike, smt. panicle
	Calyx	calyx-teech hairy within	outside glabrous, inside densely villous, calyx segment triangular	very villous all over (especially inside)	tube distally cupular, abaxial glabrous, adaxial tomentose
	Stamen	-	3-4 mm.	-	10, 3-4 mm
	Ovary	-	glabrous, ovoid, 1 cm long	-	-
	Style	-	glabrous, 2.5-3 mm.	-	-
	Disc	-	lobed, densely villous	oval or oblong-oval, villous	-
Fruit	Shape	ellipsoid or oboviod from a broad base	subglobose to ellipsoid	oval	ovoid/ ellipsoid/ cylindric ovoid
	Length	3/4-1 by 1/4 in	1.5-2.5 cm	1-1.5 in.	2-4.5 x 1.2-2.5 cm
	Rib	5-ribbed	5-angular	5-angular	5-ridge
	Color when dry	yellowish grey	wrinkled, turning black	yellow	deeply wrinkled blackish brown
Note		divided to 6 varieties	Habit var. chebula: Tree var. nana: Shrub	-	Leave blade var. chebula: both glabrous var. tomentosa: both tawny villous

1.8 Objectives of the study

This project aims to evaluate the quality of powdered Triphala preparation and related *Terminalia* plants collected from Thailand using chemical constituents and molecular technology. The particular objectives of the study were:

1. To authenticate *Terminalia* crude drugs, "Samo" and the ingredients of Triphala collected from Thai herbal markets using molecular technology.
2. To clarify the phylogenetic relationships of Thai medicinal *Terminalia* species.
3. To enquire for suitable DNA barcoding markers to differentiate *Terminalia* plants and their crude drugs collected from Thailand.
4. To determine the chemical markers (gallic acid, ellagic acid, and ascorbic acid) in Triphala and its ingredients including related medicinal *Terminalia* crude drugs.

CHAPTER 2

MATERIALS AND METHODS

2.1 Molecular Analysis of *Terminalia* spp. Distributed in Thailand and Authentication of Crude Drugs from *Terminalia* Plants

2.1.1. Sampling

Leaf samples of nine *Terminalia* species, including one alien species (*T. ivorensis* H. Perrier), were collected from Thailand, and some specimens obtained from Queen Sirikit Botanic Garden, Chiang Mai, Thailand. Five of which namely *T. bellirica* (Gaertn.) Roxb., *T. chebula* Retz. var. *chebula*, *T. chebula* Retz. var. *nana* Gagnep., *T. catappa* L., and *T. citrina* (Gaertn.) Roxb. ex Fleming are used as medicine. Moreover, *P. emblica* L. and *Combretum indicum* (L.) DeFilipes were collected for nucleotide sequence reference and phylogenetic tree construction, respectively. Voucher samples were deposited in Queen Sirikit Botanic Garden Herbarium (QBG) and the Herbarium of the Laboratory of Molecular Pharmacognosy of Graduate School of Medical Science, Kanazawa University, Japan. Three crude drug samples (Samo Thai, Samo Phiphek, and Makampom) and nine commercial Triphala formulations were obtained from local Thai market.

2.1.2 Isolation of Total DNA

Total DNA was extracted by DNeasy Plant Mini Kit (Qiagen, German) followed the manufacturer's instructions with minor modifications.

2.1.3 Polymerase Chain Reaction (PCR) Amplification

Nuclear internal transcribed spacer (ITS) were amplified using 100-120 ng of total DNA as the template in 25 μL of a reaction mixture that contain 12.5 μL of 2x PCR Buffer for KOD FX Neo, 0.4 mM of each dNTP, 0.15 μM of each primer (Table 10), and 0.5 unit of KOD FX Neo DNA polymerase (Toyobo, Japan). PCR amplification was done under the following cycling parameters: initial denaturation at 94 ^{o}C for 2 min, followed by 30 cycles of denaturation at 94 ^{o}C for 15 s, 30 s annealing at the annealing temperature of each primer used, and elongation at 68 ^{o}C for 45 s; and final elongation at 68 ^{o}C for 5 min. The amplified products were electrophoresed on a 2.0% agarose gel and purified by Fast Gene^{TM} Gel/PCR Extraction Kit (Nippon Genetics Co.Ltd, Japan).

2.1.4 Sequence Analysis

The purified PCR fragment was subjected to direct sequencing with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) using an ABI PRISM 310 sequencer (Applied Biosystems). The obtained DNA information was aligned using 'DNASIS' version 3 (Hitachi, Japan).

2.1.5 Phylogenetic Analysis

The aligned DNA sequences of the ITS1-5.8S-ITS2 region were analyzed using Molecular Evolutionary Genetic Analysis (MEGA) version 5.2.2 Software.[136] Maximum likelihood calculation was carried out using the Kimura 2-parameter model with 1000 bootstrap replications. Apart from the nucleotide sequences of the nine *Terminalia* species distributed in Thailand as determined in our present study, 26 *Terminalia* samples from DDBJ/EMBL/GenBank were used for phylogenetic tree reconstruction. *C. indicum* (L.) DeFilipps of the same tribe Combreteae in Family Combretaceae was used as outgroup species for phylogenetic tree rooting.[103]

2.1.6 PCR-RFLP Analysis

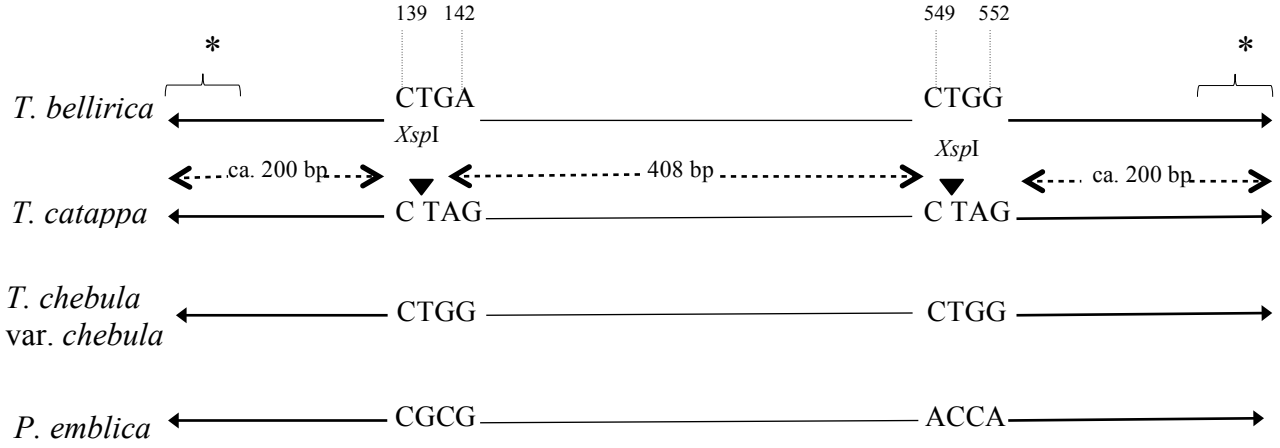
Ter.Af (forward primer) and Ake-26SR (reverse primer) were used for PCR amplification of approximately 800 bp in the ITS1-5.8S-ITS2 region. The purified PCR products from *T. catappa, T. chebula* var. *chebula* and *T. bellirica* were digested with 10 units of restriction enzyme *Xsp*I (Takara Bio, Inc., Japan) at 37 ^{o}C for 2 h to distinguish *T. catappa* from the other species. For the identification of *T. chebula* var. *chebula* and *T. bellirica,* 10 units of restriction enzyme *Aor*13HI (Takara Bio, Inc., Japan) were added, and digestion was carried out at 55 ^{o}C for 2 h. For the authentication of the Triphala formulation, PCR-RFLP analysis of *P. emblica* L. (one of the three ingredients of Triphala) was conducted with both restriction enzymes (Fig. 3A). The digested fragments were separated by 3.0% agarose gel electrophoresis and visualized by staining with GelRed™ Nucleic Acid Gel Stain (Wako Chemicals, Japan).

2.1.7ARMS Analysis

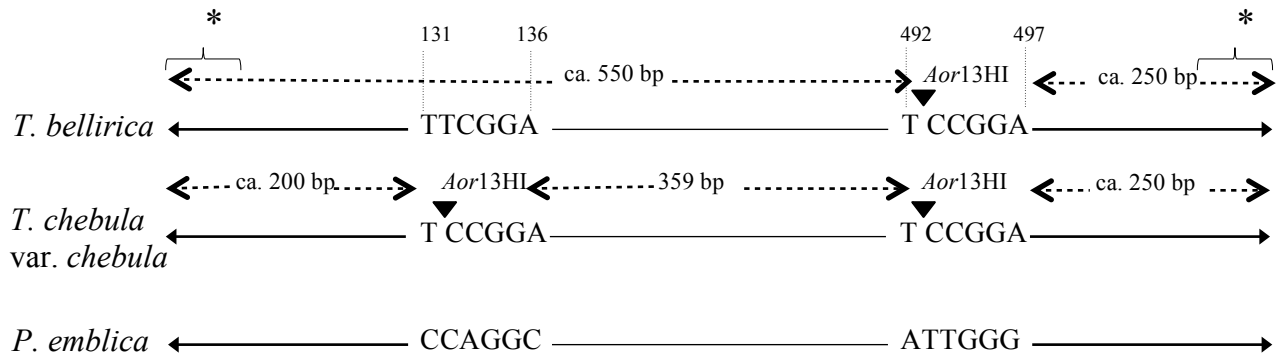
From the nucleotide substitutions at different positions in the ITS region (Fig. 3B), six species-specific primers were designed as follows: TCA.Af and Ter.Br for the identification of *T. catappa*; TCH.Af and Ter.Br for the identification of *T. chebula* var. *chebula*; TBE.Cf and Ter.Br for the identification of *T. bellirica*, and PHE.Bf and PHE.Br for the identification of *P. emblica*, and were used in the authentication of Triphala (Table 10). Multiplex-ARMS-PCR amplification was performed by using the DNA templates of all the four species, as well as crude drugs and Triphala with multiple species-specific primers. Twenty-five microliters of the reaction mixture consisted of 12.5 μL of GoTaq® Green Master Mix (Promega, USA), 0.2 μM species-specific primer, and 100-120 ng of DNA templates of leaf samples. Crude drug and Triphala samples were mixed with 12.5 μL of 2x PCR Buffer for KOD FX Neo, 0.4 mM of each dNTP, 0.15 μM of each three sets of species-specific primers, and 0.5 unit of KOD FX Neo DNA Polymerase (Toyobo, Japan). Amplification was carried out under the following conditions: pre-heating at 94 ^{o}C for 2 min, followed by 30 cycles at 94 ^{o}C for 15 s, 60 ^{o}C for 30 s, and 68 ^{o}C for 45 s, and a final extension at 68 ^{o}C for 5 min. The amplified PCR products were detected by 2.0% agarose gel electrophoresis and visualized by staining with GelRed™ Nucleic Acid Gel Stain under UV light irradiation.

A PCR-RFLP

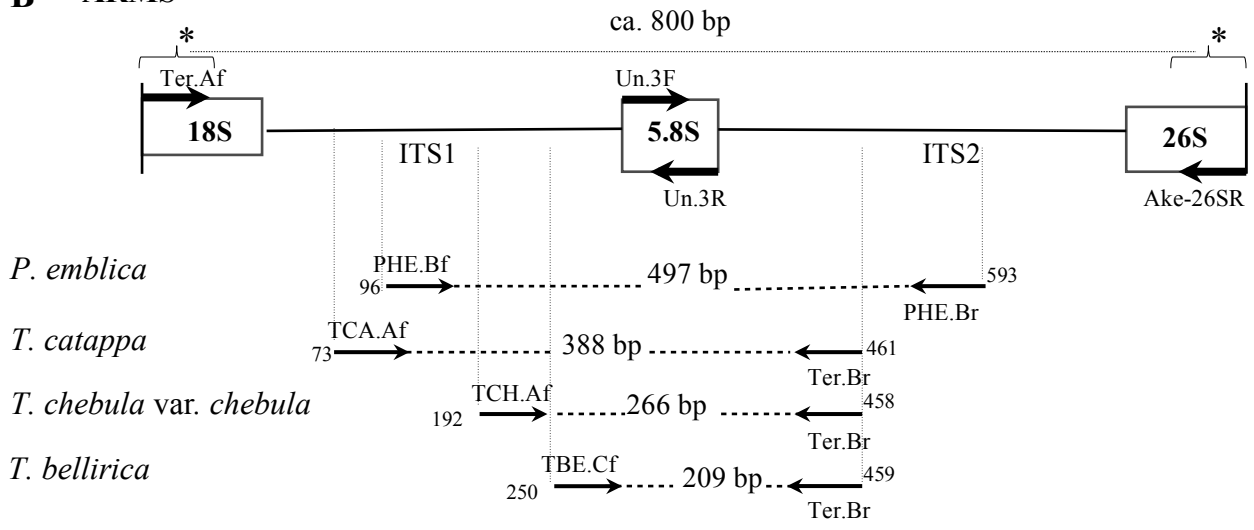
Restriction Enzyme *Xsp*I (C^TAG)



Restriction Enzyme *Aor*13HI (T^CCGGA)



B ARMS



*not analyzed correctly

Fig. 3 PCR-RFLP (A) and ARMS (B) Analysis of *Terminalia* Plants and Triphala Formulations
A. Restriction Sites of *Xsp*I and *Aor*13HI in the rDNA-ITS region
B. Amplification Fragment Sites of Species-specific Primers for *P. emblica*, *T. catappa*, *T. chebula* var. *chebula*, and *T. bellirica*

Table 8. List of Samples Collected from Thailand in This Study

Species (accession no.)	Vernacular name[129)]	Number of samples	Voucher no.	Collection site
T. bellirica (Gaertn.) Roxb. (LC050567)	Samo Phiphek สมอภิเพก	10	B13090711-2	Sanam Chai Khet, Chacheongsao
			B130917221	Maerim, Chiang Mai
			B130910241	Muang, Chiang Mai
			B130912611-3	Ban Tak, Tak
			B130917221	Muang, Chiang Mai
			B130917271-2	Sansai, Chiang Mai
T. chebula Retz. var. *chebula* (LC050565)	Samo Thai สมอไทย	17	C130908311-2	Muang, Lamphun
			C130914311-2	
			CN130908321	
			CN130914311	
			C130909221	Maerim, Chiang Mai
			C130910231-2, C130910241	Muang, Chiang Mai
			C130911511	Muang, Udon Thani
			C130912611-3	Ban Tak, Tak
			C130915271-2	Sansai, Chiang Mai
			C130917281	Muang, Lampang
T. chebula Retz. var. *nana* Gagnep. (LC050566)	Samo Nang สมอนั่ง	1	CN130916221	Maerim, Chiang Mai
T. catappa L. (LC050568)	Huu Kwaang หูกวาง	7	D130910241-5	Muang, Chiang Mai
			D130911511	Muang, Udon Thani
			D130913261	Maerim, Chiang Mai
T. citrina (Gaertn.) Roxb. ex Fleming (LC050564)	Samo Deenguu สมอดีงู	3	R130918811	Phra Nakhon, Bangkok
			R140630811	
			R140630821	
T. ivorensis A. Chev. (LC050569)	Huu Krajong หูกระจง	7	I130908211	Sankampaeng, Chiang Mai
			I130908331-2	Muang, Chiang Mai
			I130910241	Muang, Chiang Mai
			I130912621	Ban Tak, Tak
			I130913261-2	Maerim, Chiang Mai
T. glaucifolia Craib (LC050562)	Hean แหน	1	K130909221	Maerim, Chiang Mai
T. elliptica Willd. (LC050570)	Rok Faa รกฟ้า	5	A130909411-2	Mae Sariang, Mae Hong Son
			A130911511	Muang, Udon Thani
			A130912611-2	Ban Tak, Tak
T. mucronata Craib & Hutch (LC050563)	Ma Kluea Lueat มะเกลือเลือด	7	M130912611-3	Ban Tak, Tak
			G130912611-3	Ban Tak, Tak
			M130917711	Muang, Lampang
Combretum indicum (L.) DeFilipps (Combretaceae) (LC050571)	Leb mue nang เล็บมือนาง	2	L130917711	Muang, Lampang
			L130918241	Muang, Chiang Mai
Phyllanthus emblica L. (Euphorbiaceae) (LC089029)	Makhampom มะขามป้อม	11	P140605211-2	Maerim, Chiang Mai
			P140605221-2	Muang, Chiang Mai
			P140605661-2	Ban Tak, Tak
			P140605231-2	Mae Chaem, Chiang Mai
			P140605241-2	Omkoi, Chiang Mai
			P140605251	Hot, Chiang Mai

Table 9 List of *Terminalia* Crude Drugs and Triphala Formulations

Crude drug name	Expected drug origin	Sample ID	Collection Date	Collection Site
Samo Thai	*T. chebula*	DF 2, 6	11 Sep 2013	Muang, Udon Thani
		DF 12	13 Sep 2013	Muang, Tak
		DF 17, 22, 24	7 Sep 2013	Samphanthawong, Bangkok
		DF 28	14 Sep 2013	Muang, Chiang Mai
		DF 32-33	17 Sep 2013	Muang, Lampang
		DF 38	10 Oct 2013	Hat Yai, Songkhla
Samo Phiphek	*T. bellirica*	DF 3, 5, 10	11 Sep 2013	Muang, Udon Thani
		DF 13	13 Sep 2013	Muang, Tak
		DF 18, 23, 25	7 Sep 2013	Samphanthawong, Bangkok
		DF 30	14 Sep 2013	Muang, Chiang Mai
		DF 34-35	17 Sep 2013	Muang, Lampang
		DF 40	10 Oct 2013	Hat Yai, Songkhla
Makampom	*P. emblica*	DF 4, 7-8	11 Sep 2013	Muang, Udon Thani
		DF 15	13 Sep 2013	Muang, Tak
		DF 20, 26	7 Sep 2013	Samphanthawong, Bangkok
		DF 31	14 Sep 2013	Muang, Chiang Mai
		DF 36-37	17 Sep 2013	Muang, Lampang
		DF 42	10 Oct 2013	Hat Yai, Songkhla
Triphala formulation	*T. chebula* : *T. bellirica* : *P. emblica* (1 : 1 : 1)	TP 1	17 Sep 2013	Muang, Lampang
		TP 2	7 Sep 2013	Bang Krathum, Phitsanulok
		TP 3	7 Sep 2013	Sampran, Nakornpathom
		TP 4	7 Sep 2013	Muang, Prachin Buri
		TP 5-7	7 Sep 2013	Samphanthawong, Bangkok
		TP 8	11 Sep 2013	Muang, Udon Thani
		TP 9	14 Sep 2013	Muang, Chiang Mai

Table 10 Primers Used in This Study

Primer name	Sequence (5' → 3')	Length (bp)	Tm (^{o}C)
Ter.Af	CGA GAA GTC CAC TGA ACC TT	20	60
Ake-26SR	GTA AGT TTC TTC TCC TCC GC	20	60
Un.3F	CGA CTC TCG GCA AGG GAT AT	20	65
Un.3R	AAC TTG CGT TCA AAG ACT CG	20	60
PHE.Bf	CCT TGT GCA CCT GAA GCC A	19	58
PHE.Br	TTC GGC CAA ATG AAC GAG G	19	60
TCA.Af	CGT TTT TTA AAT GCC CGG GAT A	22	62
TCH.Af	AGC GCC AAG GTA CTC CAA CAA	22	68
TBE.Cf	GGG CTG CTG TTC AAC GTC ATA AT	23	68
Ter.Br	GAT CTG GAG GCA ACG CGA	18	58

2.2 **A Comparison of Different DNA Barcoding Markers for Identification of *Terminalia* Plants and Their Crude Drugs Collected from Thailand.**

2.2.1. Sampling

Leaf samples of nine *Terminalia* species, including one exotic species (*T. mantaly* H. Perrier), were collected from Thailand. Voucher samples were deposited in Queen Sirikit Botanic Garden Herbarium (QBG) and the Herbarium of the Laboratory of Molecular Pharmacognosy of Graduate School of Medical Science, Kanazawa University, Japan.

2.2.2 Isolation of Total DNA

Total DNA was extracted by DNeasy Plant Mini Kit (Qiagen, German) followed the manufacturer's instructions with minor modifications.

2.2.3 Polymerase Chain Reaction (PCR) Amplification

100-120 ng of Genomic DNA template was amplified in 25 μL of a reaction mixture that contain 12.5 μL of 2x PCR Buffer for KOD FX Neo, 0.4 mM of each dNTP, 0.15 μM of each primer, and 0.5 unit of KOD FX Neo DNA polymerase (Toyobo, Japan). The primers used for PCR amplification of the DNA barcode markers include ITS1, ITS2, *rbc*L, *psb*A-*trn*H, and *mat*K PCR amplification (Table 11) was done under the following cycling parameters: initial denaturation at 94 ^{o}C for 2 min, followed by 30 cycles of denaturation at 94 ^{o}C for 15 s, 30 s annealing at the annealing temperature of each primer used, and elongation at 68 ^{o}C for 45 s; and final elongation at 68 ^{o}C for 5 min. The amplified products were electrophoresed on a 2.0% agarose gel and purified by Fast Gene^{TM} Gel/PCR Extraction Kit (Nippon Genetics Co.Ltd, Japan).

Table 11 Primers Used in This Study

Locus	Primer name	Sequence (5'-3')	Annealing temp. (^{o}C)
ITS1	AkeF	CGAGAAGTCCACTGAACCTT	62
	Un.3R	AACTTGCGTTCAAAGACTCG	
ITS2	Un.3F	CGACTCTCGGCAAGGGATAT	62
	Ake-26SR	GTAAGTTTCTTCTCCTCCGC	
*rbc*L	*rbc*L 1F	ATGTCACCACAAACAGAAAC	60
	*rbc*L 724R	TCGCATGTACCTGCAGTAGC	
*psb*A-*trn*H	*trn*H-*psb*AF	ACTGCCTTGATCCACTTGGC	60
	*trn*H-*psb*AR	CGAAGCTCCATCTACAAATGG	
*mat*K	*mat*K-1RKIM-f	ACCCAGTCCATCTGGAAATCTTGGTTC	62
	*mat*K-3FKIM-r	CGTACAGTACTTTTGTGTTTACGAG	

2.2.4 Data Analysis

The obtained DNA information of plant samples was aligned with those from DDBJ database (Table 5) by ClustalX software. The comparative levels of variability and discrimination power for six markers were performed by using MEGA 5.2.2 software.[136] Particularly, Kimura 2-Parameter (K2P) distance matrices were undertaken for each locus using as a reference. Three methods of species identification are (1) BLAST method was done using BLAST1 analysis searched by nucleotide database at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi)[80]; (2) Distance method was performed by using Kimura 2-parameter model of evolution[136]; (3) the neighbor-joining (NJ) tree based on different loci was performed using MEGA 5.2.2.[137]

Table 12 List of Samples Collected from Thailand and Nucleotide Data from DDBJ/EMBL/GenBank

Species	Voucher no.	Accession no.					Locality	Type of origin
		Chloroplast			Nuclear			
					ITS			
		*psb*A-*trn*H	*mat*K	*rbc*L	ITS1	ITS2		
T. chebula var. *chebula*	C130908311	LC102825	LC107046	LC107019	LC110354	LC110354	Lamphun	Plant specimen
	CN130908321	LC102826	LC107047	LC107020	LC110355	LC110355	Lamphun	
	C130909221	LC102827	LC107048	LC107021	LC110356	LC110356	Chiang Mai	
	C130911511	LC102828	LC107049	LC107022	LC110359	LC110359	Udon Thani	
	C130912613	LC102829	LC107050	LC107023	LC110357	LC110357	Tak	
	C130917281	LC102830	LC107051	LC107024	LC110358	LC110358	Lampang	
T. chebula	HBG810107	FJ381883	-	FJ381812	FJ381775	FJ381775	Philippines	GenBank Data
	KYUM<JPN>:186	-	AB924845	-	-	-	Cambodia	
	TC3	-	KT274004	-	-	-	India	
	TC4	-	KT274005	-	-	-	India	
	TC6	-	KT279719	-	-	-	India	
	TB64	-	-	JX856795	-	-	India	
	2	-	-	KT203920	-	-	India	
	3	-	-	KT203921	-	-	India	
	4	-	-	KT203922	-	-	India	
	JNVU/BD/H/C/2010/4	-	-	JF747602	-	-	India	
	-	-	-	-	HM236857	HM236857	India	
	-	-	-	-	KC984654	KC984654	India	
	SYS442	-	-	AF425710	AF334769	AF334769	China	
T. chebula var. *nana*	CN130916221	LC102831	LC107052	LC107025	LC110360	LC110360	Chiang Mai	Plant specimen
T. bellirica	B130910241	LC102818	LC107053	LC107026	LC110361	LC110361	Chiang Mai	Plant specimen
	B130912611	LC102819	LC107054	LC107027	LC110362	LC110362	Tak	
	B130917221	LC102820	LC107055	LC107028	LC110363	LC110363	Chiang Mai	
	B130917271	LC102821	LC107056	LC107029	LC110364	LC110364	Chiang Mai	
	OM1673	FJ381879	-	FJ381808	FJ381773	FJ381773	Tropical Africa	GenBank Data
	TB1	-	KT274002	-	KT235565	KT235565	India	
	TB2	-	KT271003	KT274009	-	-	India	
	TB3	-	-	KT274010	-	-	India	
	TB4	-	KT279718	KT279740	KT279734	KT279734	India	
	TB5	-	-	-	KT279735	KT279735	India	
	TB1	-	-	-	-	-	India	
	JNVU/BD/H/C/2010/2	-	-	JF747600	-	-	India	
	-	-	-	-	HM236856	HM236856	India	
	SYS364	-	-	AF425714	AF334768	AF334768	China	
	BB0333	KR533036	-	-	-	KR532654	China	
	BB1061	KR533033	-	-	-	-	China	
	J037	KR533035	-	KR530130	-	-	China	
	J053	-	-	KR530131	-	-	China	
	J155	-	-	-	-	KR532655	China	
	J348	KR533034	-	KR530132	-	KR532656	China	
T. citrina	R130918811	LC102832	LC107057	LC107030	LC110365	LC110365	Bangkok	Plant specimen
	R140630811	LC102833	LC107058	LC107031	LC110366	LC110366	Bangkok	
	R140630821	LC102834	LC107059	LC107032	LC110367	LC110367	Bangkok	
	R130907111	LC102835	LC107060	LC107033	LC110368	LC110368	Chacheongsao	
T. catappa	D130910241	LC102822	LC107061	LC107034	LC110369	LC110369	Chiang Mai	Plant specimen
	D130911511	LC102823	LC107062	LC107035	LC110370	LC110370	Udon Thani	
	D130913261	LC102824	LC107063	LC107036	LC110371	LC110371	Chiang Mai	
	J.R.Abbott25019 (FLAS)	GU135388	GU135057	-	-	-	America	GenBank Data
	Conti1003WIS	-	-	U263338	-	-	America	
	TCA2	-	-	KT274012	KT235566	KT235566	India	
	TCA3	-	-	KT279741	KT279736	KT279736	India	
	TCA4	-	-	-	KT279737	KT279737	India	
	JNVU/BD/H/C/2010/3	-	-	JF747601	-	-	India	
	JX518026	JX518026	-	-	-	-	Africa	
	RA2941	FJ381882	-	FJ381811	-	-	Madagascar	
T. glaucifolia	K130909221	LC102836	LC107061	-	LC110372	LC110372	Chiang Mai	Plant specimen
T. elliptica (*syn T. alata T. tomentosa*)	A130909411	LC102816	O	LC107037	LC110373	LC110373	Mae Hong Son	Plant specimen
	A130911511	LC102817	O	LC107038	LC110374	LC110374	Udon Thani	
	A130912611	LC110382	O	LC107039	LC110375	LC110375	Tak	
	OM1667	FJ381891	-	FJ381819	FJ381781	FJ381781	India	GenBank Data
T. mucronata	M130912611	LC102841	LC107065	LC107040	LC110376	LC110376	Tak	Plant specimen
	M130917711	LC102842	LC107066	LC107041	LC110377	LC110377	Lampang	
T. mantaly	I130908211	LC102837	LC107067	LC107042	LC110378	LC110378	Chiang Mai	Plant Specimen
	I130908331	LC102838	LC107068	LC107043	LC110379	LC110379	Chiang Mai	
	I130912621	LC102839	LC107069	LC107044	LC110380	LC110380	Chiang Mai	
	I130913261	LC102840	LC107070	LC107045	LC110381	LC110381	Tak	
	OM1088	FJ381887	-	FJ381815	FJ381778	FJ381778	Madagascar	GenBank Data

2.3 **A Comparative Chemical Markers of *Terminalia* Crude Drugs and Triphala Formulations Collected from Thailand.**

2.3.1 Herb Materials

Fourty-two samples, identified from their external appearance were derived from Triphala formulations with their ingredients (Samo Thai, Samo Phiphek, and Makampom) as well as Samo thed and Samo deengu, as Table 13.

2.3.2 Equipment

UV detector:	L-2400 Hitachi High-Technologies Corporation Detector
Pump:	Pump L-1230, Hitachi High-Technologies
Chromatograph:	Chromato-PRO runtime Instruments Inc.
Column oven:	SSC-2230 Senshu Scientific co., Ltd.

- Determinalion of Gallic Acid and Ellagic Acid followed Juang *et al*.[30)]

2.3.3 Sample Preparation of Gallic Acid and Ellagic Acid Determinalion

1. 0.3 g of pulverized samples was extracted with 70% MeOH (25 ml) by ultrasonication at room temperature for 15 min
2. Centrifuged at 1500 *g* for 5 min
3. The extraction was repeated three times
4. The resulting extracts combined, filtered through a 0.45 μm filter
5. The test solution was obtained by dilution with 70% MeOH to a final volume of 100 mL.
6. Aliquots (10 μL) were injected for HPLC

2.3.4 Analytical Conditions of Gallic Acid and Ellagic Acid Determinalion

Pre-column:	Nova-Pak™ silica (Millipore)
Column:	Nacalai Tesque Cosmosil™ $5C_{18}$-AR reversed-phase column (250x4.6 mm i.d.; 5 μm)
Mobile phase:	(A) aqueous phosphoric acid (pH 2.75) (B) 80:20 (v/v) mixture of acetonitrile and mobile phase A
Flow-rate:	0.8 mL/min
Wavelength:	216 nm for tannin, 254 nm for ellagic acid

Gradient elution program:

0-10 min	95:5 - 92:8
10-20 min	92:8 - 88:12
20-30 min	88:12 - 86:14
30-40 min	86:14 - 81:19
40-50 min	81:19 - 80:20
50-65 min	80:20 - 70:30
65-70 min	70:30 - 0:100
70-80 min	0:100 - 95:5

- Determinalion of Ascorbic Acid followed Hashimaoto[138)]

2.3.5 Sample Preparation of Ascorbic Acid Determination

(i) Sample's reduced ascorbic acid

1. Triphala and pounded crude drugs 0.15 g are extracted with 2% meta phosphoric acid by ultrasonic extraction for 30 min in cooling temperature. Then, sample tubes are centrifuged 3000 rpm for 10 min.
2. Supernatant is added with acetonitrile 2 times of supernatant volume, filtered through a membrane filter (0.45 μm).

(ii) Sample's total ascorbic acid (oxidized + reduced form)

1. Sample solutions of reduced ascorbic acid 2 ml are added with 0.1% homocystein and 10% Na_2PO_4 each 1 ml, then, the solutions are heated in water bath at 40 ^oC for 20 min.
2. The solution are analyzed the amount of total ascorbic acid

2.3.6 Analytical Conditions of Ascorbic Acid Determination

Column:	TOSOH TSK-gel silica-60
Mobile phase:	CH_3CN : 100 mM NH_4COOH (72 : 28)
Injection volume:	15 μL
Flow-rate:	0.5 mL/min
Wavelength:	278 nm
Column temperature:	35 ^oC

Table 13 List of Crude Drug Samples and Triphala Formulation

Crude drug name	Expected drug origin	Sample ID	Collection Date	Collection Site
Samo Thai	*T. chebula*	DF 2, 6	11 Sep 2013	Muang, Udon Thani
		DF 12	13 Sep 2013	Muang, Tak
		DF 17, 22, 24	7 Sep 2013	Samphanthawong, Bangkok
		DF 28	14 Sep 2013	Muang, Chiang Mai
		DF 32-33	17 Sep 2013	Muang, Lampang
		DF 38	10 Oct 2013	Hat Yai, Songkhla
Samo Thed	*T. chebula*	DF 1	11 Sep 2013	Muang, Udon Thani
		DF 11	13 Sep 2013	Muang, Tak
		DF 16, 21	7 Sep 2013	Samphanthawong, Bangkok
		DF 29	14 Sep 2013	Muang, Chiang Mai
		DF 39	10 Oct 2013	Hat Yai, Songkhla
Samo Phiphek	*T. bellirica*	DF 3, 5, 10	11 Sep 2013	Muang, Udon Thani
		DF 13	13 Sep 2013	Muang, Tak
		DF 18, 23, 25	7 Sep 2013	Samphanthawong, Bangkok
		DF 30	14 Sep 2013	Muang, Chiang Mai
		DF 34-35	17 Sep 2013	Muang, Lampang
		DF 40	10 Oct 2013	Hat Yai, Songkhla
Samo Deengu	*T. citrina*	DF 14	13 Sep 2013	Muang, Tak
		DF 19	7 Sep 2013	Samphanthawong, Bangkok
		DF 27	14 Sep 2013	Muang, Chiang Mai
		DF 41	10 Oct 2013	Hat Yai, Songkhla
Makampom	*P. emblica*	DF 4, 7-8	11 Sep 2013	Muang, Udon Thani
		DF 15	13 Sep 2013	Muang, Tak
		DF 20, 26	7 Sep 2013	Samphanthawong, Bangkok
		DF 31	14 Sep 2013	Muang, Chiang Mai
		DF 36-37	17 Sep 2013	Muang, Lampang
		DF 42	10 Oct 2013	Hat Yai, Songkhla
Triphala formulation	*T. chebula* : *T. bellirica* : *P. emblica* (1 : 1 : 1)	TP 1	17 Sep 2013	Muang, Lampang
		TP 2	7 Sep 2013	Bang Krathum, Phitsanulok
		TP 3	7 Sep 2013	Sampran, Nakornpathom
		TP 4	7 Sep 2013	Muang, Prachin Buri
		TP 5-7	7 Sep 2013	Samphanthawong, Bangkok
		TP 8	11 Sep 2013	Muang, Udon Thani
		TP 9	14 Sep 2013	Muang, Chiang Mai

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Molecular Analysis of *Terminalia* spp. Distributed in Thailand and Authentication of Crude Drugs from *Terminalia* Plants

3.1.1 Identification of Terminalia Samples Collected from Thailand by BLAST

The inquisitive nuclear ITS sequences of *Terminalia* species were identified by using BLAST (Basic Local Alignment Search Tool) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Four species (*T. chebula* var. *nana*, *T. citrina*, *T. glaucifolia*, and *T. mucronata*) were not appeared in GenBank database. On the other hands, the BLAST result was able to identify *T. chebula* var. *chebula*, *T. bellirica*, *T. catappa*, and *T. elliptica* (syn. *T. alata* and *T. tomentosa*) from the reference library. The best BLAST result of Huu Krajong or *T. ivorensis* in Thai Plant Names[129)], the alien species used as decoration in garden and roadside in Thailand, was the most identical with *T. mantaly* FJ381778 in identities score 99%, whereas *T. ivorensis* FJ381776 was 96%.

Table 14 The Result of the Best BLAST Hit Using ITS Sequence

Query	Score (bits)	E value	Identities	Gaps	Strand	BLAST result
T. chebula var. *chebula*	1074	0.0	640/677 (95%)	2/677 (0%)	Plus/Plus	*T. chebula* FJ381775
T. chebula var. *nana*	1190	0.0	664/676 (98%)	1/676 (0%)	Plus/Plus	*T. chebula* FJ381775
T. citrina	1188	0.0	664/676 (98%)	2/676 (0%)	Plus/Plus	*T. chebula* FJ381775
T. bellirica	1223	0.0	670/677 (99%)	0/676 (0%)	Plus/Plus	*T. bellirica* KC602394
T. catappa	1214	0.0	667/677 (99%)	0/676 (0%)	Plus/Plus	*T. catappa* KT235566
T. elliptica	1177	0.0	667/680 (98%)	9/680 (1%)	Plus/Plus	*T. tomentosa* FJ381781
T. gluacifolia	1074	0.0	640/677 (95%)	1/677 (0%)	Plus/Plus	*T. bellirica* KC602394
T. mucronata	1125	0.0	652/676 (96%)	0/676 (0%)	Plus/Plus	*T. chebula* FJ381775
T. ivorensis	1234	0.0	674/677 (99%)	1/677 (0%)	Plus/Plus	*T. mantaly* FJ381778
	1083	0.0	646/676 (96%)	1/676 (0%)	Plus/Plus	*T. ivorensis* FJ381776

Table 15 Comparison of the Nucleotide Sequences Between Huu Krajong Sample Obtained in This Study and the Nucleotide Sequences of *T. mantaly* (FJ381778) and *T. ivorensis* (FJ381776) Retrieved from GenBank Database.

Species	Nucleotide number																												
	ITS1													5.8S					ITS2										
	42	76	78	83-84	92	103	107-109	130-131	163	169	173-174	209	217	265	292	303	329	409	451	518	547	551	616	618	627	629	647		
Huu Krajong I130908211	C	T	T	CG	T	-	TGA	GA	G	G	CA	C	G	A	G	C	T	C	A	A	G	A	T	T	A	C	A		
T. mantaly FJ381778	*	*	Y	**	*	C	**G	**	*	*	**	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*		
T. ivorensis FJ381776	A	C	T	TT	C	-	CAG	TC	A	T	TG	T	C	G	C	M	C	T	G	G	A	G	C	C	C	G	-		

Huu Krajong (I130908211) collected from Chiang Mai, Thailand
Photograph: Intharuksa A.

Type of *T. mantaly* H. Perrier
(http://www.efloras.org/florataxon.aspx?flora_id=12&taxon_id=250074095)

T. ivorensis
(http://www.prota4u.org/plantphotos/Terminalia%20ivorensis.full.7.jpg)

Fig. 4 The Morphology of Inquisitive Specimen and The Reference of *T. mantaly* and *T. ivorensis*

From the comparison of ITS1-5.8S-ITS2 sequences (Table 13), there are three different bases between inquisitive sequence and *T. mantaly* sequence as nucleotide sequence reference at base no. 78, 103, and 109 in ITS1 region. On the other hands, the other bases are the different bases between the query and *T. ivorensis*. From the analysis of pairwise distance, the result showed that 0.15% in Huu Krajong and *T. mantaly* (FJ381778), while between Huu Krajong and *T. ivorensis* (FJ381776) exhibited 4.29%. Furthermore, in morphology, the characteristic of Huu Krajong

(I130908211) (Fig. 4A) is similar to *T. mantaly* (Fig. 4B) more than *T. ivorensis* (Fig. 4C), as Table 16. Therefore, Huu Krajong deserves to name *T. mantaly*.

Table 16 Comparison of *T. mantaly* and *T. ivorensis* Morphology

	T. mantaly[139)]	*T. ivorensis*[140)]
Local names	Umbrella tree	Black afara
Height (m)	10-12	15-46
Leaves	Smooth; in terminal rosettes of 4-9 unequal leaves; thickened stems; length up to 7 cm; apex broadly rounded; base very taped; margin wavy	6.4-12.7 x 2.5-6 cm, whorled, simple, oval, blunt tipped with orange-brown hairs below and veins above
Flowers	Small, greenish, in erect spikes to 5 cm long.	Axillary spikes 7.6-10.2 cm with bisexual flowers nearly the apex. The lower receptacle is densely tomentose, the upper receptacle less so.
Fruit	Small oval; ca. 1.5 cm long with no obvious wing	Winged and quite variable in size, especially in the width of the wing
Native location	Madagascar	Cameroon, Ghana, Guina, Liberia, Nigeria

3.1.2 Sequence Analysis of Nuclear ITS Region

The assembled nucleotide sequences of all samples have been deposited in the DDBJ Nucleotide Sequence Database. Nucleotide differences in the ITS1-5.8S-ITS2 regions, where selected nucleotides were compared with each other and the involved nucleotides in five medicinal *Terminalia* species, are summarized in Table 17. However, All *Terminalia* species were collected from different locations, but the result revealed that no diversities were observed. Direct sequencing of PCR product of the ITS1-5.8S-ITS2 regions revealed that the lengths were 677 bp in *T. bellirica* and *T. catappa,* and 675 bp in *T. chebula* var. *nana* whereas it was 674 bp in *T. chebula* var. *chebula* and *T. citrina*. Among the five species, there were 90 variable sites: 81 sites were SNPs, and nine sites were indels. On the basis of the characteristics of the overlapping peaks in the electropherogram, in the starting 5.8S coding region of *T. chebula* var. *chebula* and *T. citrina*, one A base shift deletion was noted at nucleotide no. 279, whereas that in *T. chebula* var. *nana* occurred at nucleotide no. 278. Moreover, *T. citrina* had one G base shift deletion at nucleotide no. 604 in the ITS2 region. Therefore, we were able to identify these three species roughly. DNA sequence analysis of *T. chebula* var. *chebula*, *T. chebula* var. *nana*, and *T. citrina* revealed that they had few differences at nucleotide nos. 277-486. For example, *T. chebula* var. *nana* had two A bases, one Y (T or C) overlapping nucleotide signal, as well as G and A at nucleotide nos. 277-278, 466, and 485-486, respectively, whereas *T. chebula* var. *chebula* had two deletions, C, and two R (A or G) overlapping nucleotide signals, respectively. *T. citrina* had two overlapping nucleotide signals (R and Y) instead of A and T in *T. chebula* var. *chebula* at nucleotide nos. 408 and 417, respectively. *T. chebula* var. *chebula* had three overlapping nucleotide signals (Y and RR) at nucleotide nos. 451 and 485-6, respectively, whereas *T. citrina* had T and GA at the same nucleotide numbers.

Table 17 Variation in ITS Regions of Medicinal *Terminalia* Species

Species	Nucleotide number 1)																																
	ITS 1																												5.8S 2)			
	44-5	53	60	76	83-5	93-4	105	108-10	112	115	119	125	128	132	140-2	150	157	160-1	167	169	174	179	194	209-11	215-7	224	250	267	269-72	277-9	408	413	417
T. chebula var. *chebula*	AT	G	A	T	-CA	CA	T	AGC	-	T	T	-	G	C	CGA	G	T	G-	T	G	T	C	R	TCC	AAC	Y	C	G	TGCG	--A ---	A	C	T
T. chebula var. *nana*	**	*	*	*	_**	**	*	***	_	*	*	_	*	*	***	*	*	*_	*	*	*	*	*	***	***	*	*	*	****	-A* --*	*	*	*
T. citrina	**	*	*	*	_**	**	*	***	_	*	*	_	*	*	***	*	*	*_	*	*	*	*	*	***	***	*	*	*	****	--* ---	R	*	Y
T. bellirica	G*	A	*	C	-TG	TG	*	***	-	*	Y	G	-	T	T**	A	A	R-	A	*	C	Y	G	MYG	G*T	T	*	A	C*TC	TA*	*	*	*
T. catappa	*C	A	G	*	ATG	T*	-	TAT	T	C	*	G	A	*	TAG	A	C	CA	C	A	C	*	G	***	GGT	T	T	A	CATC	--*	*	T	*

Species	Nucleotide number																																					Length (bp)
	ITS 2																																					
	443	451	460	462	466	472	476-7	480	483	485-6	489	506	517	519	522	525	527	529	539	543	547	551	555	560	583	588-9	600	604	608	610	613	620	622-3	628-9	634	638	654	
T. chebula var. *chebula*	G	Y	T	C	C	C	AG	A	G	RR	Y	C	T	A	A	A	C	A	C	T	C	G	-	A	C	CG	A	G	C	T	C	A	CM	CC	G	R	R	674
T. chebula var. *nana*	*	*	*	*	Y	*	**	*	*	GA	*	*	*	*	*	*	*	*	*	*	*	*	_	*	*	**	*	*	*	*	*	*	**	**	*	*	*	675
T. citrina 3)	*	T	*	*	*	*	**	*	*	GA	*	*	*	*	*	*	*	*	*	*	*	*	_	*	*	**	*	* _	*	*	*	*	**	**	*	*	*	674
T. bellirica	A	T	G	T	T	*	*M	T	C	GT	T	*	*	*	G	C	T	G	A	C	T	*	G	G	*	TA	G	*	G	C	T	*	AT	**	C	A	G	677
T. catappa	*	T	A	*	T	T	TT	T	C	GT	T	T	C	G	*	*	*	*	*	C	T	A	-	G	T	*A	G	*	G	C	T	G	*T	TT	T	A	A	677

An asterisk (*) indicates the same nucleotide as the top sequence; hyphen (-) indicates nucleotide deletion; Y denotes C and T; R denotes A and G; and M denotes A and C.

1) The number 1 at the nucleotide position is the first nucleotide of ITS1
2) *T. chebula* var. *chebula* and *T. citrina* have two overlapping alignments from nucleotide number 279 in the same individual, whereas *T. chebula* var. *nana* also has two overlapping alignments from nucleotide number 278 in 5.8S coding region.
3) *T. citrina* has two overlapping alignments from nucleotide number 604 (ITS2 region) in the same individual.

From the results of sequence analysis, it appeared that the nucleotide sequences in the ITS regions of medicinal *Terminalia* species were species-specific. *T. chebula* var. *chebula*, *T. chebula* var. *nana*, and *T. citrina* contained few different nucleotides each others, but they have many dissimilar nucleotides with *T. bellirica* and *T. catappa*. Thereby, the other nucleotide regions will be performed to search the better region for discrimination of these three species in the future. From morphological characteristic, *T. chebula* var. *chebula* could be distinguished from *T. citrina* on the basis of fruit shape: *T. citrina* had an ellipsoid and ambiguously 5-angled drupe with 5-angled seeds as well as, the size *T. citrina* fruit is a smaller size than *T. chebula* var. *chebula*. Whereas *T. chebula* var. *chebula* carried a subglobose drupe with irregular seeds.[128] In Thai traditional medicine and Ayurvedic medicine, the fruit of *T. citrina* was utilized to treat gastricintestinal disorders, identical with *T. chebula*.[128, 141] In Thailand, there are two varieties of *T. chebula*: *T. chebula* var. *chebula* and *T. chebula* var. *nana*. Krachai *et al.* reported that the morphological, palynological, and anatomical characteristics of those varieties are similar. *T. chebula* var. *nana* is shrub with height 0.6-1.5 m and tannin in bundle sheath near the leaf lower epidermis is nonexistence. On the conversely, the tannin was found in *T. chebula* var. *chebula*. Therefore, the habit and the presence of tannin in bundle sheath near the leaf lower epidermis are the key characteristics for distinguishing those two varieties.[131]

3.1.3 Phylogenetic Analysis of Terminalia Species on nrITS Region

Combretaceae is ubiquitously distributed in the tropics and some warm temperate zones. It is reported that outcrossing is the primary mode of reproduction in *Terminalia*.[142-143] In many tropical tree species, outcrossing is predominant, leading to high genetic diversity within population.[144-146] According to Stace,[110] the greatest genetic diversity of *Terminalia* species is found in Southeast Asia. Exell recorded in Flora Malesiana that fifty *Terminalia* species were discovered form Malay peninsular to Oceania islands.[128] Seventeen *Terminalia* species were found in Thailand.[128] In the present study, we collected nine *Terminalia* species from Thailand, including two species (*T. chebula* var. *nana* and *T. mucronata*) endemic to Southeast Asia and one exotic species in Asia (*T. mantaly*).

The molecular phylogenetic relationship among *Terminalia* species distributed in Thailand was reconstructed on the basis of the nucleotide sequences in the ITS regions obtained in the present study along with those retrieved from DDBJ/EMBL/GenBank DNA database. Maurin *et al.*[121] analyzed the ITS sequences of *Terminalia* species collected mainly in Africa as well in Asia, Australia, and the Pacific islands. As shown in Fig. 5, *Terminalia* is divided into two groups: an African group with a few taxa from Asia and the Pacific islands and an Asian group.

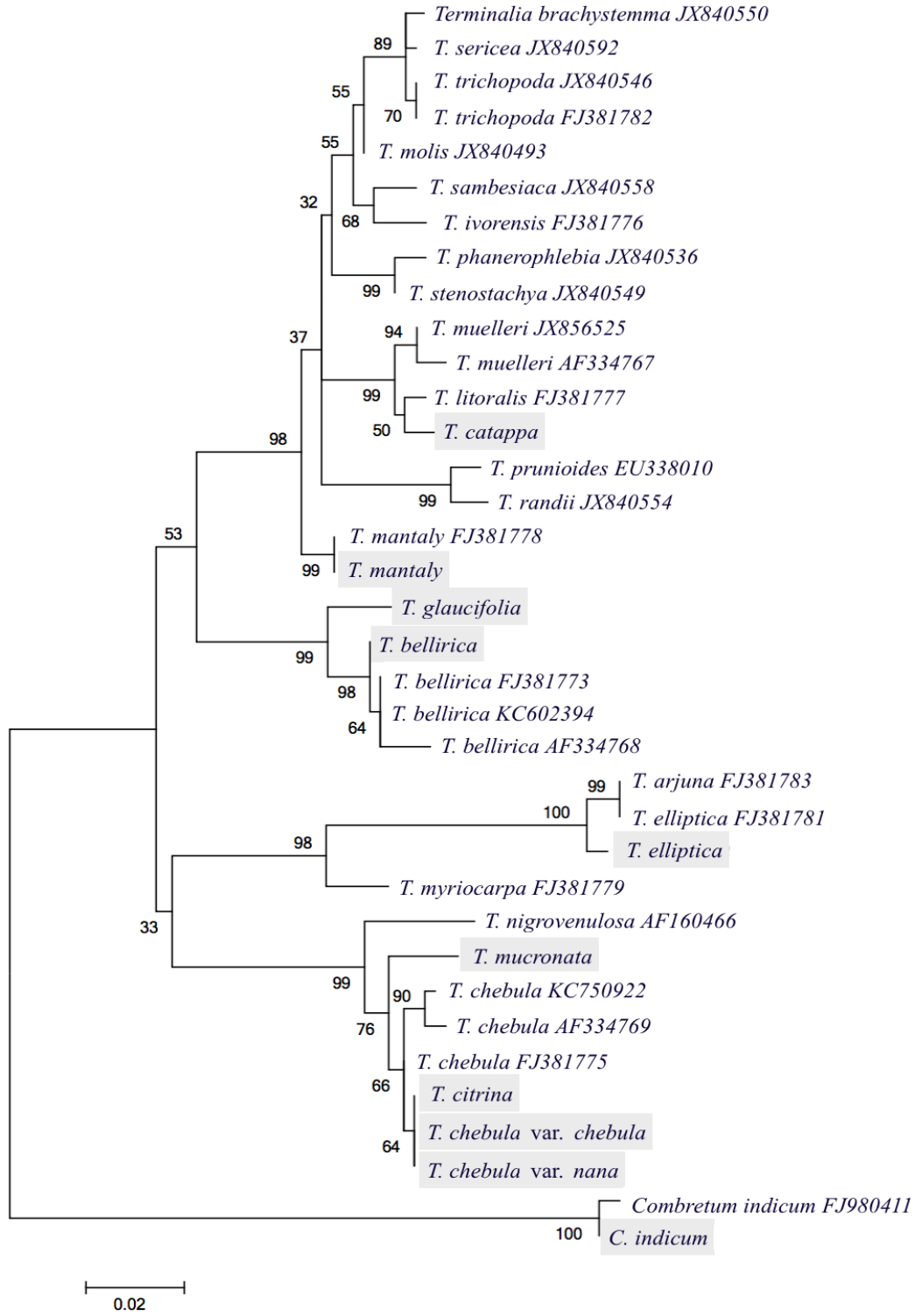


Fig. 5 Dendrogram of Maximum Likelihood Tree for Genus Terminalia and Genus Combretum Outgroup.

Based on approximately 0.6 kb aligned nucleotide sequence of nuclear ITS1-5.8S-ITS2 region. Numbers at nodes indicate bootstrap values with 1000 replications. Branch lengths are proportional to the number of substitutions per site (refer to scale bar). Sequence data of species in gray brackets were obtained in the present study and those of other species were retrieved from the DNA database.

3.1.4 *Authentication of Medicinal Terminalia Species, Their Crude Drugs and Triphala Formulations*

Vast improvements in the identification and analysis of SNPs in plants were noted in last ten years. Such methods as PCR-RFLP and ARMS, which use PCR markers based on SNPs, have been adopted for the authentication of herbs.

Of the *Terminalia* samples collected in Thailand, five had medicinal value, including *T. chebula* var. *chebula*, *T. chebula* var. *nana*, *T. citrina*, *T. bellirica*, and *T. catappa*. We subjected *T. chebula* var. *chebula*, *T. bellirica*, and *T. catappa* to PCR-RFLP analysis. Because, examination of the aligned nucleotide sequences in the ITS1-5.8S-ITS2 regions revealed almost identical nucleotide sequences in *T. chebula* var. *chebula*, *T. chebula* var. *nana,* and *T. citrina*. Therefore, the PCR-RFLP method could not differentiate them. The amplified fragments of these three *Terminalia* species and *P. emblica* were approximately 800 bp long. The nucleotide sequences showed species-specific sequences at sites subjected to the restriction enzyme analysis. To exclude *T. catappa*, the *Xsp*I restriction enzyme was used for diagnosis. Three fragments distinct to *T. catappa* were found. *Xsp*I recognized 5' CTAG 3', which was found in the ITS region of *T. catappa* only at two sites, whereas *T. chebula* var. *chebula*, *T. bellirica*, and *P. emblica* could not to be digested at the same sites. *Xsp*I digestion cleaved the nucleotide sequence of *T. catappa* was cleaved into three amplicons that were approximately 200, 400, and 200 bp long (Fig. 3A). The cleaved products of *T. catappa* appeared as two bands in 3.0% TAE agarose gel electrophoretogram (Fig. 6A). Then, to discriminate *T. chebula* var. *chebula*, *T. bellirica*, and *P. emblica*, the *Aor*13HI restriction enzyme was used to recognize the specific nucleotides in the three species. It was found that *Aor*13HI recognized 5' TCCGGA 3'. As shown in Fig. 6B, the amplified sequence of *T. bellirica* was cleaved into two fragments that were approximately 550 and 250 bp long, and that of *T. chebula* var. *chebula* was cleaved into three fragments that were approximately 200, 350, and 250 bp long, *P. emblica* could not to be digested by the same restriction enzyme.

SNPs were noted in the nucleotide sequences of the ITS regions of medicinal *Terminalia* species (Table 16). Different nucleotides at position nos. 93-94, 215-217, and 267-277 were used to design species-specific primers for the authentication of *T. catappa*, *T. chebula* var. *chebula*, and *T. bellirica*, respectively. The reverse primers of the three diagnostic primer pairs had the same sequences (Table 10). Therefore, three specific primers TCA.Af/Ter.Br, TCH.Af/Ter.Br, and TBE.Cf/Ter.Br, were fabricated for discriminating *T. catappa*, *T. chebula* var. *chebula*, and *T. bellirica*, respectively. The amplified products from those primer sets had different sizes: *T. catappa*, 388 bp; *T. chebula* var. *chebula*, 266 bp; and *T. bellirica*, 209 bp (Fig. 7). As a consequence, the three pairs of diagnostic primers could be used to authenticate the medicinal *Terminalia* plants.

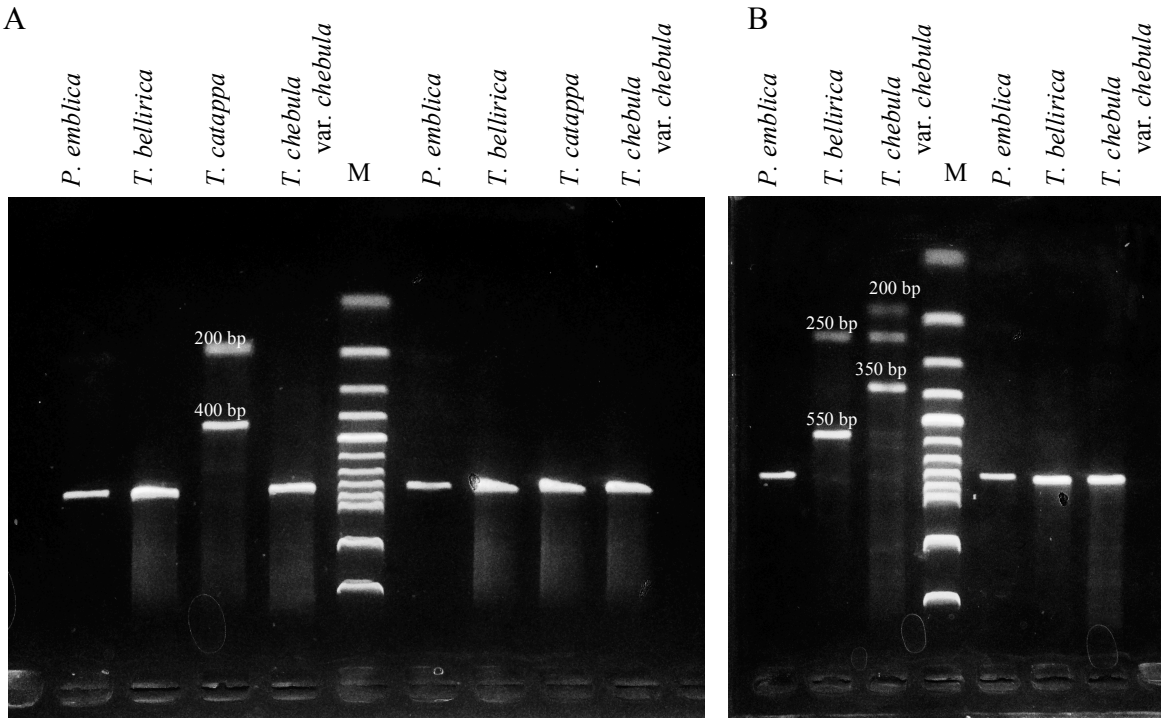


Fig. 6 PCR-RFLP Analysis Using Restriction Enzymes *Xsp*I and *Aor*13HI

3.0% TAE agarose gel electrophoregram of PCR product of *P. emblica*, *T. bellirica*, *T. catappa*, and *T. chebula* var. *chebula* generated by primers Ter.Af and Ake-26SR, and then digested with restriction enzyme.

A. *Xsp*I (C^TAG)

The ITS1-5.8S-ITS2 fragments of *P. emblica, T. bellirica*, *T. catappa*, and *T. chebula* var. *chebula* were amplified, and digested fragments (left 4 lanes) and non-digested fragments (right 4 lanes) were separated by agarose gel electrophoresis, Lane M, 1 kb DNA ladder (BioTools Inc., Japan).

B. *Aor*13HI (T^CCGGA)

The amplified fragments of *P. emblica, T. bellirica,* and *T. chebula* var. *chebula* in the ITS1-5.8S-ITS2 region were separated into the cleaved amplicons (left 3 lanes), Lane M, and uncleaved amplicons (right 3 lanes).

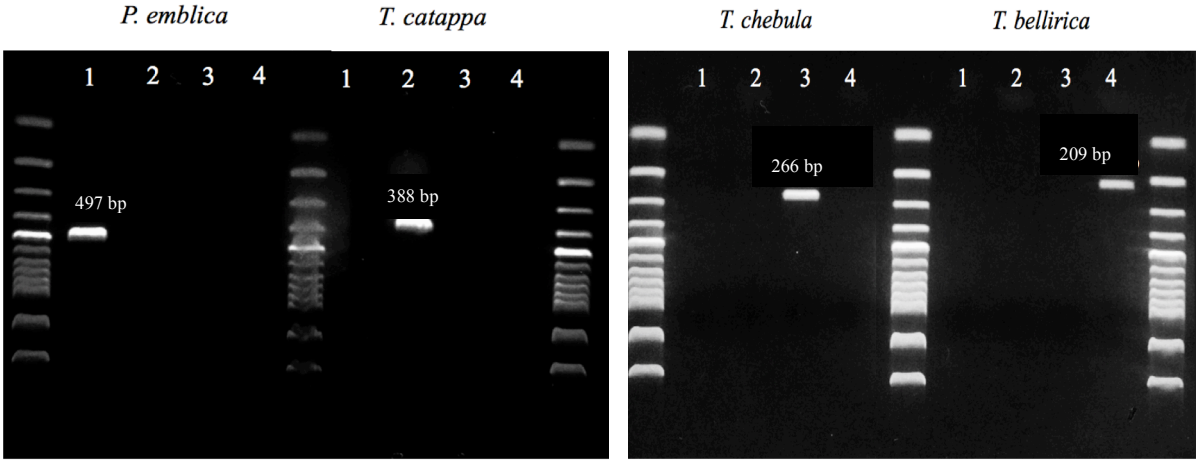


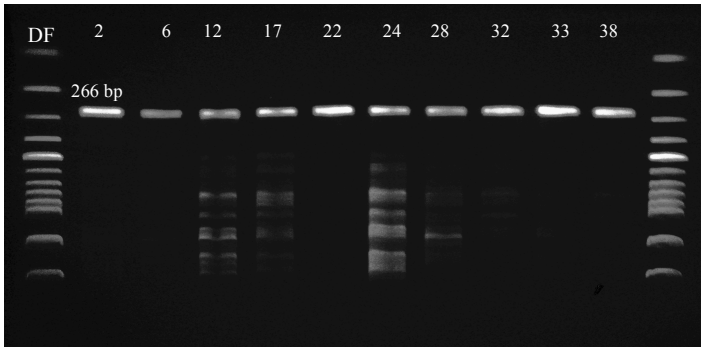
Fig. 7 Authentication of *P. emblica*, *T. catappa*, *T. chebula* var. *chebula*, and *T. bellirica* by ARMS

Lanes 1-4 are the same as species-specific primer pairs: 1. PHE.Bf/PHE.Br, 2. TCA.Af/Ter.Br, 3. TCH.Af/Ter.Br, and 4. TBE.Cf/Ter.Br

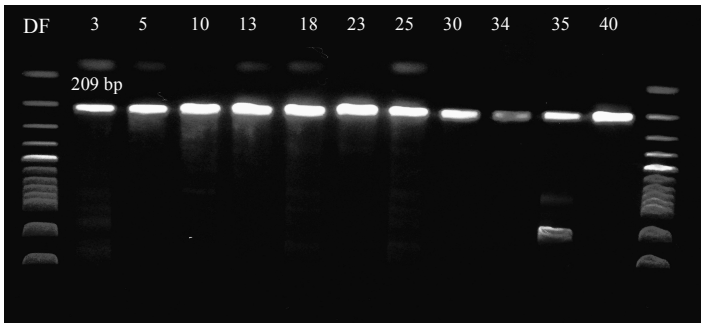
lane M is 1 kb DNA ladder

The two methods for separating the three medicinal *Terminalia* species and *P. emblica*, PCR-RFLP and ARMS, were used to authenticate *Terminalia* crude drugs including *P. emblica* crude drugs and the ingredients of Triphala, which consisted of *T. chebula*, *T. bellirica*, and *P. emblica* (or Samo Thai, Samo Phiphek, and Makampom, respectively, the Thai names of their crude drugs) in the ratio of 1:1:1. However, the PCR-RFLP method could not authenticate *P. emblica* with two restriction enzymes. On the other hands, the ARMS method was preferred for its simplicity and efficiency.[53] The nucleotide sequence of the ITS region of *P. emblica* was aligned and searched for different sites for designing the specific primer. The result showed that amplicon of 497 bp was amplified by PHE.Bf/PHE.Br only for *P. emblica* (Fig. 7), which can authenticate its crude drug. The amplification was conducted with the combination of these species-specific primers under identical concentration and temperature conditions. The multiplex-ARMS-PCR amplification efficiently produced three apparently amplified PCR products as shown in Fig. 8. Therefore, this technique enabled the authentication of *Terminalia* crude drugs and the ingredients of Triphala. For instance, the 209 bp fragment was found only in the genomic DNA sample of Samo Phiphek. Three similar fragments that were 209, 266, and 497 bp long were found in the genomic DNA samples of Triphala. These results clearly showed that ARMS is effective for the identification of *Terminalia* crude drugs and Triphala formulations.

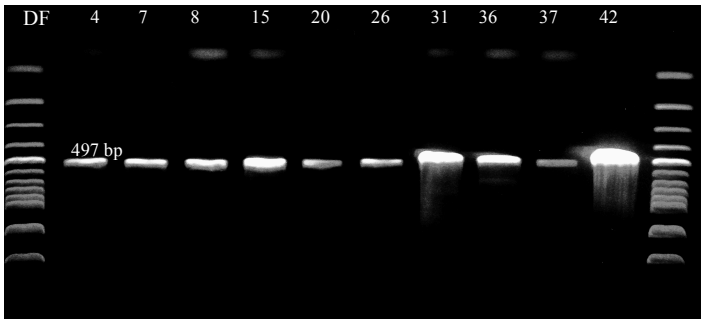
A. Samo thai (crude drug whose botanical origin was predicted as *T. chebula*)



B. Samo phiphek (*T. bellirica* crude drug)



C. Makampom (*P. emblica* crude drug)



D. Triphala formulation

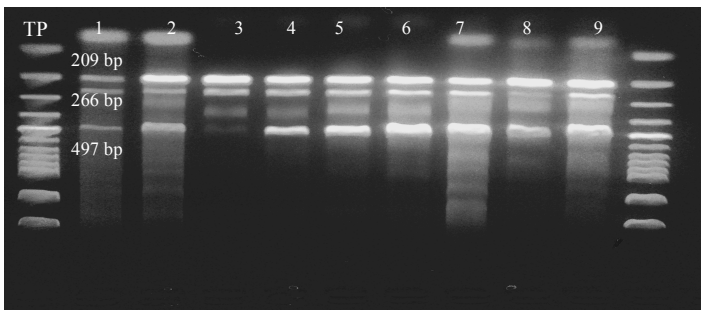


Fig. 8. Authentication of Crude Drugs and Triphala Formulations Obtained Commercially by Using Specific Primer Pairs of ARMS

3.2 A Comparison of Different DNA Barcoding Markers for Identification of *Terminalia* Plants and Their Crude Drugs Collected from Thailand.

3.2.1 Molecular Analysis of Different DNA Barcoding Markers in Terminalia Species Collected from Thailand.

The *Terminalia* specimens collected from Thailand were amplified and aligned direct sequence by five primer sets on six different DNA barcoding markers: coding chloroplast *rbc*L and *mat*K, non-coding chloroplast *psb*A-*trn*H, non-coding nuclear ITS1-5.8S-ITS2, ITS1, and ITS2.

Table 18 Variation in Chloroplast *rbc*L Regions of Thai *Terminalia* Species

Species	Nucleotide number										Lenght (bp)
	155	272	356	379	390	546	612	635	637-638	663-664	
T. chebula var. *chebula*	G	C	C	G	G	T	C	G	CT	TG	673
T. chebula var. *nana*	*	*	*	*	*	*	*	*	**	**	673
T. citrina	*	*	*	*	*	*	*	*	**	**	673
T. bellirica	A	*	*	*	*	*	*	*	A*	**	673
T. catappa	A	T	*	*	T	G	*	C	**	**	673
T. elliptica	*	*	T	*	*	*	T	*	A*	**	673
T. mucronata	*	*	*	*	*	*	*	*	AC	**	673
T. mantaly	*	*	T	A	*	*	T	*	A*	AA	673

An asterisk (*) indicates the same nucleotide as top sequence; hyphen (-) indicates nucleotide deletion.

Table 19 Variation in Chloroplast *mat*K Regions of Thai *Terminalia* Species

Species	Nucleotide number																		Lenght (bp)
	42	45	83	88	158	164	241	267	299	304	347-348	365	378	393	410	427	444	463	
T. chebula var. *chebula*	C	G	A	C	A	C	A	G	A	C	T	A	C	A	G	C	G	C	466
T. chebula var. *nana*	*	*	*	*	*	*	G	*	*	*	*	*	T	*	*	*	*	*	466
T. citrina	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	466
T. bellirica	*	*	*	*	*	*	*	*	G	*	C	C	*	G	A	A	T	*	466
T. catappa	*	A	G	T	*	T	*	T	G	G	C	C	*	*	*	A	T	*	466
T. glaucifolia	*	*	*	*	*	*	*	*	G	*	C	C	*	G	A	A	T	*	466
T. elliptica	*	A	G	T	*	*	*	*	G	G	C	C	*	*	*	A	T	*	466
T. mucronata	*	*	*	*	G	*	*	*	G	*	*	*	*	*	*	A	*	*	466
T. mantaly	T	A	*	*	*	*	*	*	G	*	C	*	*	*	*	A	T	T	466

An asterisk (*) indicates the same nucleotide as top sequence; hyphen (-) indicates nucleotide deletion.

Table 20 Variation in Nuclear ITS1 and 5.8S Regions of Thai *Terminalia* Species

Species	Nucleotide number 1)																																		
	ITS1																																		
	44-46	51-53	60	76-85	87	93-94	104-105	107-111	114	118-119	124	126-128	130-131	139-141	148-149	155-156	159-160	164	166	168	173	175	178	182	187	191	194	209-211	215-219	225	243	251	259	265-266	268
T. chebula var. *chebula*	ATA	AGA	A	TTTCC-AACA	C	CA	GT	CAGCC	T	TA	A	G-G	TC	CGA	AG	TC	-G	G	T	G	T	A	C	-	R	C	R	TCC	AA-CA	Y	T	C	C	TT	G
T. chebula var. *nana*	***	***	*	*****_****	*	**	**	*****	*	**	*	*_*	**	***	**	**	_*	*	*	*	*	*	*	_	*	*	*	***	**_**	*	*	*	*	**	*
T. citrina	***	***	*	*****_****	*	**	**	*****	*	**	*	*_*	**	***	**	**	_*	*	*	*	*	*	*	_	*	*	*	***	**_**	*	*	*	*	**	*
T. bellirica	G**	*A*	*	*C***-**TG	*	TG	**	*****	*	Y*	G	A-*	*T	T**	*A	A*	-R	*	A	*	C	*	Y	-	G	*	G	MYG	G*-T*	T	*	*	*	**	A
T. catappa	*C*	*A*	*	***TTA**TG	*	T*	*C	T*T*T	C	**	G	AA*	**	TAG	*A	C*	CA	*	C	A	C	*	*	-	G	*	G	***	GG-T*	T	*	T	*	**	A
T. glaucifolia	R*R	*A*	*	C****-***K	*	TG	R*	*****	*	**	G	A-*	**	*RY	*A	AY	-*	R	C	*	C	*	*	-	G	T	G	***	G*-T*	T	*	*	*	**	A
T. elliptica	**G	G**	*	*CCT*-GG**	T	T*	A*	*G*T*	C	*G	G	A-T	**	**G	GA	**	-*	*	C	*	C	*	*	-	G	*	G	***	GGATG	T	A	*	T	CC	A
T. mucronata	***	***	*	*****-**TG	Y	**	**	*****	*	**	*	*_*	**	***	**	C*	-*	*	C	*	*	G	*	A	G	T	G	***	**_**	T	*	*	*	**	*
T. mantaly	*C*	*A*	G	***T*-***G	*	T*	CG	TGA**	C	**	G	AA*	GA	T*G	*A	C*	-A	*	C	*	C	*	*	-	G	*	G	***	G*-T*	T	*	T	*	**	A

Species	ITS1	Length (bp)	Nucleotide number						Length (bp)
			5.8S 2)						
	270-274		277-279	284	286	410	415	419	
T. chebula var. *chebula*	TGCGA	271	--A ---	T	A	A	C	T	159
T. chebula var. *nana*	*****	271	-A* --*	*	*	*	*	*	160
T. citrina	*****	271	--* ---	*	*	*	*	C	159
T. bellirica	C*TC*	271	TA* ---	*	*	*	*	*	161
T. catappa	CATC*	274	--*	*	*	*	T	*	159
T. glaucifolia	C*TT*	271	AA*	G	T	*	*	*	161
T. elliptica	C*TCG	273	CA*	*	*	G	*	C	161
T. mucronata	*****	271	-T*	*	*	*	*	*	160
T. mantaly	CATC*	272	--*	*	*	*	*	*	159

An asterisk (*) indicates the same nucleotide as top sequence; hyphen (-) indicates nucleotide deletion; Y denotes C and T; R denotes A and G; and M denotes A and C.

1) The number 1 at the nucleotide position is the first nucleotide of ITS1

2) *T. chebula* var. *chebula* and *T. citrina* have two overlapping alignments from nucleotide number 279 in the same individual, whereas *T. chebula* var. *nana* also has two overlapping alignments from nucleotide number 278 in 5.8S coding region.

Table 21 Variation in Nuclear ITS2 Regions of Thai *Terminalia* Species

Species	Nucleotide number[1)]																																						
	ITS2																																							
	445-446	448-449	451-454	463	465	469-471	475	479-480	483	486	488-489	492-493	496-498	502	510	521	523	526-527	529	531	533	543	547-548	550-551	555-556	599	564-565	576-577	580	587	592-593	604	606-608	611-614	617					
T. chebula var. *chebula*	G-	AGA	GCGY	T	C	CCA	C	AG	A	G	RR	Y-	TCC	A	C	T	A	AC	A	C	A	C	TA	GC	GG	-	AA	AT	C	C	CG	A	AGT	CCGT	C					
T. chebula var. *nana*	*-	***	***C	*	*	Y**	*	**	*	*	AG	T-	***	*	*	*	*	**	*	*	*	*	**	**	**	-	**	**	*	*	**	*	***	****	*					
T. citrina[2)]	*-	***	***T	*	*	***	*	**	*	*	GG	C-	***	*	*	*	*	**	*	*	*	*	**	**	**	-	**	**	*	*	**	*	*** *-*	****	*					
T. bellirica	A-	*A*	***T	G	T	T**	*	*M	T	C	GT	T-	***	T	*	*	*	G*	C	T	G	A	C*	*T	**	G	G*	**	*	*	TA	G	***	*G*C	T					
T. catappa	*-	*A*	***T	A	*	T**	T	TT	T	C	GT	T-	**T	T	T	C	G	**	*	*	*	*	C*	*T	A*	-	G*	**	*	T	*A	G	***	*G*C	T					
T. glaucifolia	A-	*A*	***T	G	*	TY*	*	*A	T	C	GT	W-	***	T	*	*	*	GY	C	*	*	*	C*	RT	**	-	G*	*Y	*	*	TA	G	***	*GRC	Y					
T. elliptica	AA	G**	ATCG	G	T	**G	*	*A	T	C	GC	TT	GTT	T	*	*	*	GA	*	*	*	*	**	*T	AA	-	GG	G*	*	*	GA	*	GAC	*G**	*					
T. mucronata	*-	***	***T	*	*	T**	*	**	*	*	GA	T-	**T	*	*	*	*	**	*	*	*	*	*G	**	**	-	**	**	A	*	**	*	***	T***	*					
T. mantaly	*-	*A*	***T	A	*	TT*	T	GA	T	C	GT	T-	**T	T	*	C	G	**	*	*	*	*	C*	*T	**	-	G*	**	*	T	*A	G	***	*G*C	T					

	Nucleotide number								Length (bp)	Total length (bp)
	ITS2									
	624	626-627	632-633	633	638	642	655	658		
T. chebula var. *chebula*	A	CM	CC	C	G	R	-	R	244	674
T. chebula var. *nana*	*	*C	**	*	*	A	-	G	244	675
T. citrina	*	*C	**	*	*	A	-	A	245	674
T. bellirica	*	AT	**	*	C	A	-	G	244	677
T. catappa	G	*T	TT	*	T	A	-	A	244	677
T. glaucifolia	*	AC	**	*	Y	A	C	G	244	676
T. elliptica	*	*C	*T	*	T	A	-	A	246	679
T. mucronata	*	*C	**	*	*	A	-	A	244	676
T. mantaly	*	*T	*T	A	A	A	A	A	245	676

An asterisk (*) indicates the same nucleotide as top sequence; hyphen (-) indicates nucleotide deletion; Y denotes C and T; R denotes A and G; and M denotes A and C.

1) The number 1 at the nucleotide position is the first nucleotide of ITS1 and continues to ITS2

2) *T. citrina* has two overlapping alignments from nucleotide number 607 (ITS2 region) in the same individual.

Table 22 Variation in *psb*A-*trn*H Regions of Thai *Terminalia* Species

Species	Nucleotide number *psb*A-*trn*H																																		
	1-2	4-8	10	12-13	16	18	23	27	34	47	74	89	93-94	103	117	123	125-150	154-155	157	160	162	164	167	170-180	182	186-190	201-209	239	245	247	255-256	262	277	281
T. chebula var. *chebula*	CG	-TTTT	G	AT	G	-	G	G	T	G	C	G	T-	-	-	C	--------------------------	TT	A	C	T	T	T	AAAAAATTCT	T	TTAAA	-------	T	T	G	AA	T	C	T
T. chebula var. *nana*	**	C-CC*	*	*C	C	-	C	*	*	*	*	*	*-	-	-	*	--------------------------	**	*	*	*	*	*	******A***	*	*****	-------	*	*	*	**	*	T	*
T. citrina	*A	C-C**	*	*C	C	-	C	*	C	*	*	*	*-	-	-	*	--------------------------	**	*	*	*	*	*	**********	*	-----	-------	*	*	*	**	*	T	*
T. bellirica	*A	C*C**	*	G*	C	-	*	T	*	*	*	*	C-	-	-	*	--------------------------	*A	*	A	A	*	*	T****G*ACAC	A	-----	ATAAGAA	G	A	A	**	*	T	C
T. catappa	*A	C*C**	*	G*	C	-	*	T	*	*	*	A	*-	A	-	A	--------------------------	*A	*	-	A	A	-	-**---TC***	*	-----	ATAAGAA	G	C	C	--	G	T	C
T. glaucifolia	TA	AA-**	*	GC	C	-	*	T	*	*	*	*	*-	-	-	*	--------------------------	*A	*	A	A	*	*	T****G*ACAC	A	-----	ATAAGAA	G	A	A	**	*	T	C
T. elliptica	*A	-*-**	*	G*	C	G	*	T	*	*	*	*	*C	-	-	*	--------------------------	*A	T	A	A	*	*	T*********	*	-----	ATAAGAA	G	*	*	**	*	T	*
T. mucronata	*A	C-C**	*	G*	C	-	*	T	*	A	*	*	*-	-	A	*	--------------------------	**	*	C	*	*	*	**********	*	-----	-------	G	*	*	**	*	T	*
T. mantaly	*A	C-CCC	C	GC	C	-	C	T	*	*	T	A	*-	-	-	A	TTTAATGTGTTATTTTAATAATATTA	*A	*	A	*	*	*	**T*******	*	-----	ATAAGAA	G	C	A	--	*	T	C

Species	Nucleotide number *psb*A-*trn*H																				Length (bp)
	283-284	286-300	310-326	345	360	372-380	390-392	394-395	397-415	437	455-459	461-465	498	502-516	518-519	523	528	532	534	541	
T. chebula var. *chebula*	TT	TTT------TAATCT	CTGTAAAATATTAAATA	T	-	TTTATCTAA	TTT	AT	TTTATAAAAAAA-----TT	T	-----	-----	T	T-----------AAA	CA	T	T	T	A	G	551
T. chebula var. *nana*	**	***------******	*****************	*	-	*********	***	**	************-----**	*	-----	-----	*	*-----------***	**	*	*	*	*	*	551
T. citrina	**	***------******	*****************	*	-	*********	***	**	************-----**	*	-----	-----	*	*----TTTATAA***	A*	*	*	*	*	*	553
T. bellirica	AC	**GAAGTTA*TT***	*----------------	*	-	*********	A*A	**	************TGAAA**	*	-----	TACAA	A	*------TATAA***	A*	G	C	G	*	*	533
T. catappa	AC	**GAAGTTA*TT***	*----------------	*	A	*********	A*A	**	************TTAAA**	G	TTACC	TACAA	*	A--TATTTATAA***	**	G	C	*	*	*	544
T. glaucifolia	CA	**GAAGTTA*TT***	*----------------	*	-	*********	A*A	**	************TGAAA**	*	-----	TACAA	C	*------TATAA***	A*	G	C	G	*	*	531
T. elliptica	**	--------------A	T-------------C*C	*	-	---------	*GA	**	--------------AAA--	*	-----	TACAA	*	*--------------	AT	*	*	*	*	T	491
T. mucronata	**	***------******	*----------------	*	-	*********	***	--	************-----**	*	-----	-----	*	*--------TAA***	**	*	*	*	*	*	517
T. mantaly	A*	**GAAGTTA*TT***	*----------------	C	-	*********	GGA	**	************TGAAA**	G	TTACC	TACAA	*	*TATATTTATAA***	**	G	C	*	T	*	559

An asterisk (*) indicates the same nucleotide as top sequence; hyphen (-) indicates nucleotide deletion.

Direct sequencing of *Terminalia* specimens collected from Thailand was compared in each DNA barcoding markers. Among all analyzed DNA barcodes, the region that contains the lowest variable sites is *rbc*L. The 673 bp of coding chloroplast *rbc*L showed twelve variable sites among nine *Terminalia* species (Table 18). Moreover, there is the identical nucleotide sequence amplified on *rbc*L with *T. chebula* var. *chebula*, *T. chebula* var. *nana*, and *T. citrina*, so, the narrow inter-specific divergence *rbc*L restricted to discriminate on the closing species.[126] High universality but fewer species discrimination is afforded by *rbc*L, while *mat*K provided high species resolution but low universality.[81] Our study showed that there are no different base between *T. chebula* var. *chebula* and *T. citrina* on the nucleotide sequence alignment on the *mat*K region in length 467 bp. Because ITS region provides high discrimination capacity among species, it was ubiquitously utilized for identifying herbal medicinal materials.[114] The ITS of nuclear ribosomal DNA sequence contains three parts: noncoding ITS1 and ITS2, and a coding 5.8S region. 5.8S, coding region located at the center of ITS1 and ITS2 exhibits low diversity, which there are some different nucleotide base found among nine species in this study.[147] The ITS1 region provides high identification ability but poor PCR amplification. On the other hands, ITS2 sequences contain the high capacity of phylogenetic reconstruction at genus and species level, high success rate of PCR amplification.[148] Therefore, the ITS2 not only was selected as a barcoding marker but also succeeded in authenticating medicinal material. The Chinese medicinal *Bupleurum* plants[120], plants species in the family Araliaceae[149], the Indian *Sida* medicinal plant[119] are the example of studies used the ITS2 region as the standard DNA barcode for authentication. However, in this study, the ITS2 was unusable to discriminate *T. chebula* var. *chebula*, *T. chebula* var. *nana*, and *T. citrina* because they have identical ITS2 sequence as well as, there are some published studies reported that the discrimination of some species by using ITS2 sequence was ambiguous such as between *Caragana tibetica* and *C. ordosica* (Fabaceae), and between *Hedera nepalensis* and *H. nepalensis* var. *sinensis* (Araliaceae).[116, 149] The non-coding *psb*A-*trn*H intergenic spacer presents high numbers of substitutions as shown in Table 22, so many previous researches suggest that the *psb*A-*trn*H is a suitable marker for DNA barcoding of plant.[117, 148] In 491-559 bp, the nucleotide sequences of Thai *Terminalia* species were differentiated by the *psb*A-*trn*H region, in particular, *T. chebula* var. *chebula*, *T. chebula* var. *nana*, and *T. citrina,* which they could not resolve by ITS region.

3.2.2 Sequencing Amplification and Base Composition of Terminalia Species Collected from Thailand

Table 22 Sequence Data Analysis of Single Marker DNA Barcodes of *Terminalia* spp.

Marker		*rbc*L	*mat*K	*psb*A-*trn*H	ITS	ITS1	ITS2
Length range		673	467	491-559	674-679	271-274	244-246
Aligned length		673	467	628	685	279	248
Average G+C		42.71	34.10	19.09	61.39	62.59	64.41
C (%)		675 (97.40)	445 (95.29)	514 (79.44)	538 (78.08)	207 (59.48)	181 (72.98)
V (%)		15 (2.16)	22 (4.71)	130 (20.09)	150 (21.77)	75 (21.55)	67 (27.02)
Pi (%)		12 (1.73)	20 (4.31)	107 (16.54)	142 (20.61)	72 (20.69)	64 (25.81)
S (%)		3 (0.43)	2 (0.43)	23 (3.55)	8 (1.16)	3 (0.86)	3 (1.21)
Species identification (%) BLAST1	Genus level	85.19	100	100	100	100	100
	Specie level	35	23.08	85	100	85	85

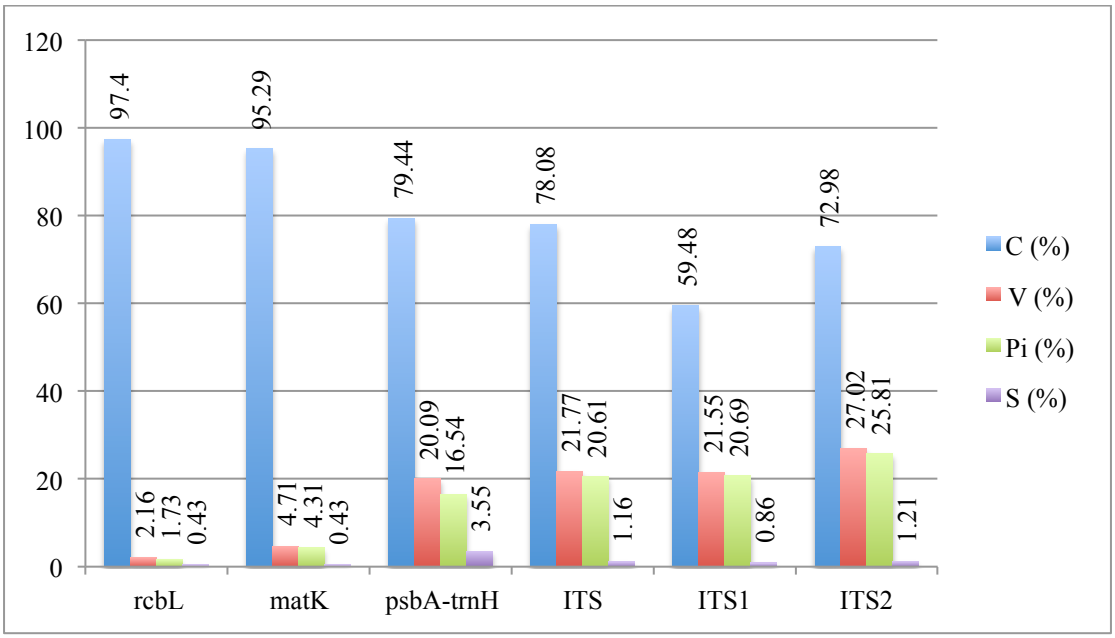


Fig. 9 Sequence Data Analysis of Single Marker DNA Barcode of Thai *Terminalia* Plant

C: conserve sites; V: variable sites; Pi: Parsimony-Informative sites; S: singleton sites

The three universal chloroplast DNA primers followed CBOL Plant working group[102] and one primer set of nuclear ITS region used in *Akebia* sp. and *Uncaria* sp. were amplified and sequenced. The amplified PCR from *rbc*L, *mat*K, *psb*A-*trn*H, and ITS carried out with almost potential barcodes, except an *rbc*L nucleotide of *T. glaucifolia*). A total of 116 closely related sequences belonging to nine *Terminalia* species collected from Thailand and 98 sequences obtained from GenBank database were analyzed. All alignments required the addition of gap, especially in *psb*A-*trn*H. The aligned sequences calculated total lengths of 674, 467, 628, 685, 279, and 248 nucleotides and the average percentage of G + C contents were 42.71, 34.10, 19.01, 61.39, 62.59, and 64.41 in *rbc*L, *mat*K, *psb*A-*trn*H, ITS1-5.8S-ITS2, ITS1, and ITS2, respectively. The parsimony criterion showed that of the respective variable sites, 14

of the 18, 20 of the 22, 107 of the 130, 142 of the 150, 72 of the 75, and 64 of the 67 were parsimony-informative sites (Table 22).

3.2.3 Discrimination Performances of the Six Candidate DNA Markers

The different molecular genetic techniques for species identification have evaluated the reliability of species authentication by trying to use them to authenticate each of inquisitive nucleotide sequences in corresponding reference alignment. There are three major methods of identification for appraisement: the methods based on sequence comparison (BLAST and genetic method) and tree topology.[137)]

1. BLAST1

BLAST1 method determined the identity of a query based on the best hit of the query sequence and the E-value for the match must be fewer than a cutoff value. The species identities of the query sequences were determined using BLAST1 method in genus level and species level. The results indicated that, as the result of the BLAST1 method, ITS precisely identified 100% of the samples at species and genus level. Except only *rbc*L marker, all loci reached 100% of BLAST1 result in genus level. At the species level, ITS (100%) ranked first in percentage of identification success rates, while the lowest result is *mat*K (23.08%) (Table 23).

2. Distance method[80)]

Table 23 Comparative Performances and Variability of Different DNA Barcoding Markers

Marker		Mean % variation (S.E%)	Range%
Single marker	*rbc*L	0.49 (1.51)	0 - 1.54
	*mat*K	1.14 (0.30)	0 - 2.83
	*psb*A-*trn*H	5.53 (0.69)	0 - 10.57
	ITS	6.70 (0.67)	0 - 13.63
	ITS1	8.81 (1.31)	0 - 20.00
	ITS2	9.29 (1.40)	0 - 18.59
Marker combination	*rbc*L + *mat*K	0.66 (0.14)	0 - 1.55
	*rbc*L + *psb*A-*trn*H	2.38 (0.30)	0 - 4.10
	ITS + *psb*A-*trn*H	5.90 (0.47)	0 - 10.27
	ITS1 + *psb*A-*trn*H	6.25 (0.62)	0 - 10.29
	ITS2 + *psb*A-*trn*H	6.56 (0.62)	0 - 11.21

All pairwise genetic distances were analyzed amongst the reference sequences, and between each inquisitive sequence and each of the reference sequences, using Kimura 2-parameter model of evolution. In single marker, the highest mean % variation is the ITS2 (9.29%; S.E. 1.40), followed by ITS1 (8.81%; S.E. 1.31), ITS (6.70%; S.E. 0.67), *psb*A-*trn*H (5.53%; S.E. 0.69), *mat*K (1.14%; S.E. 0.30), and *rbc*L (0.49%; S.E. 1.51). To evaluate potential benefits of multi-locus barcodes over the single marker followed the suggestion of the Consortium for the Barcode of Life (CBOL) Plant Working Group.[102)] Comparable K2P variability consequences for the analyzed combinations are showed in Table 23. The marker

combination of *rbc*L and *mat*K was frequently used for species discrimination.[150] Kress and Ericson have suggested the *rbc*L + *psb*A-*trn*H as an efficient two-locus DNA barcoding for land plants.[147] Therefore, we analyzed multiple combinations of four markers that exhibited good results of genetic diversity level in the previous analyzes: *psb*A-*trn*H, ITS, ITS1, and ITS2. Moreover, the *rbc*L + *mat*K followed CBOL and the *rbc*L + *psb*A-*trn*H followed were evaluated for species level identification. It is found to be the highest with the combination of ITS2 + *psb*A-*trn*H (6.56%; S.E. 0.62) followed by ITS1 + *psb*A-*trn*H, ITS + *psb*A-*trn*H, *rbc*L + *psb*A-*trn*H, and *rbc*L + *mat*K.

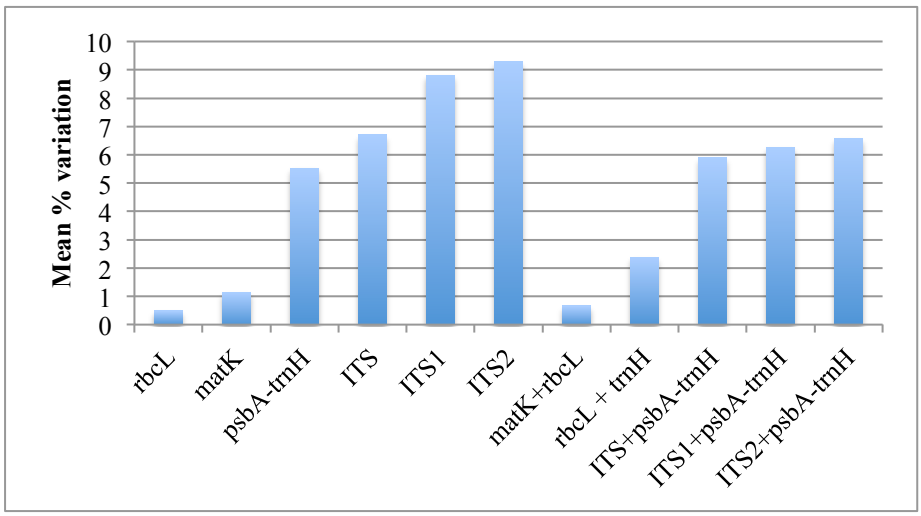


Fig. 10 The Average K2P Distance (transformed into percent) of Different DNA Barcoding Markers

3. Tree method

The data from individual markers and two-locus combination were performed phylogenetic tree by using the neighbor-joining (NJ) method. In phylogenic trees that were constructed with single markers, the four non-coding markers performed better species resolution than the two coding markers. However, no single markers showed clear resolution on nine *Terminalia* species. As the result shown in Fig 11C, the NJ tree based on *psb*A-*trn*H could not resolve *T. bellirica* and *T. glaucifolia* because *T. glaucifolia* appeared in the same clade with *T. bellirica*. Whereas, the samples of *T. chebula* var. *chebula*, *T. chebula* var. *nana* and *T. citrina* existed in the same clade of phylogenetic tree used ITS, ITS1, or ITS2 region. In two-marker combination, *rbc*L + *mat*K combination, the core markers recommended to utilize in plants by CBOL Plant working group, disabled to resolve the species. As the Fig 11, the species that were not resolved by *rbc*L were resolved by *psb*A-*trn*H, so that the combination of *rbc*L and *psb*A-*trn*H suggested by Kress and Erickson could differentiate all *Terminalia* species.[147] As the result of K2P variability, the ITS, ITS1, ITS2 and *psb*A-*trn*H were showed high result, so C, ITS1, and ITS2 markers were complemented by the *psb*A-*trn*H markers. Only the two-locus DNA markers of ITS + *psb*A-*trn*H clearly resolved all the species of genus *Terminalia*.

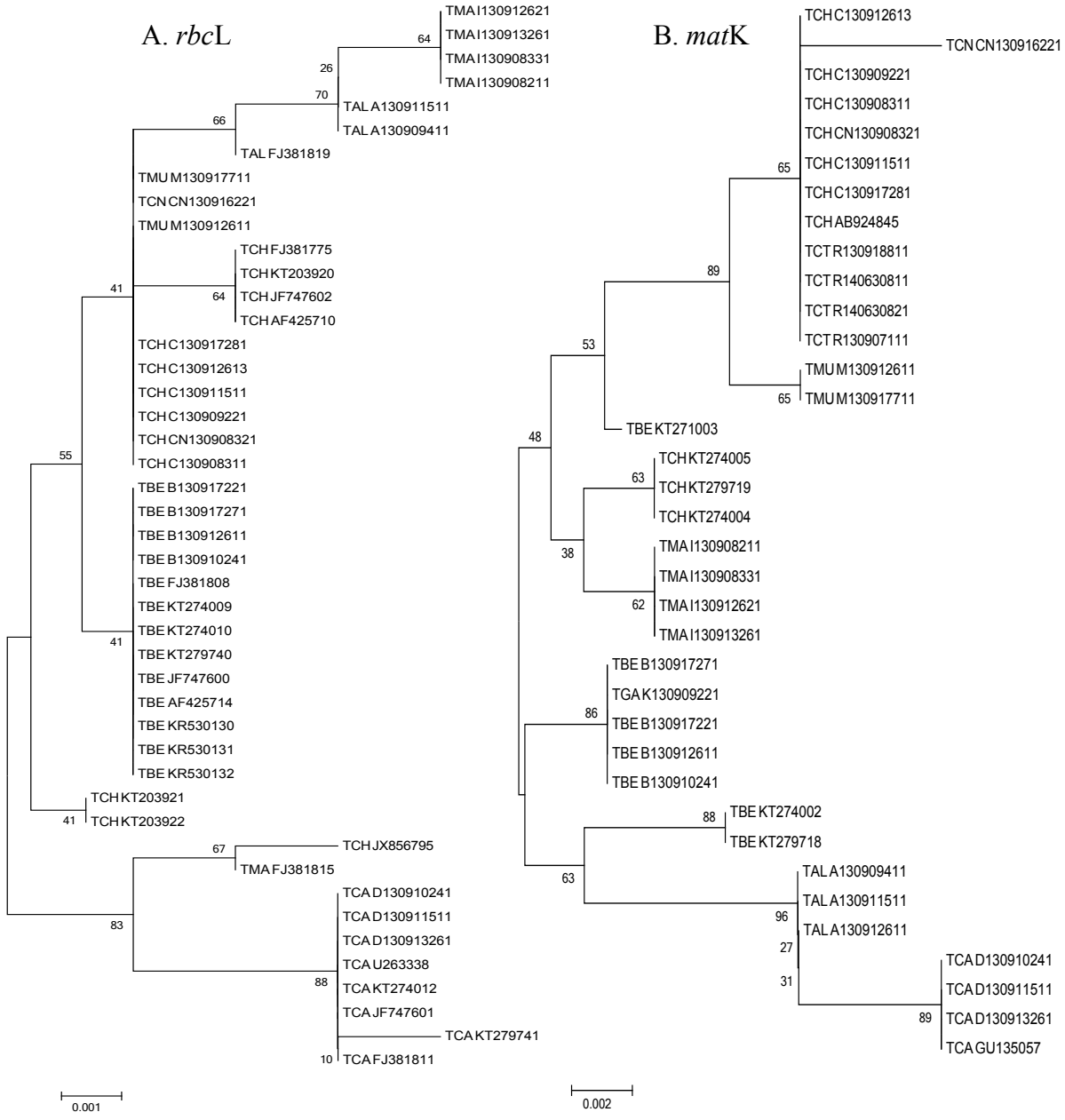
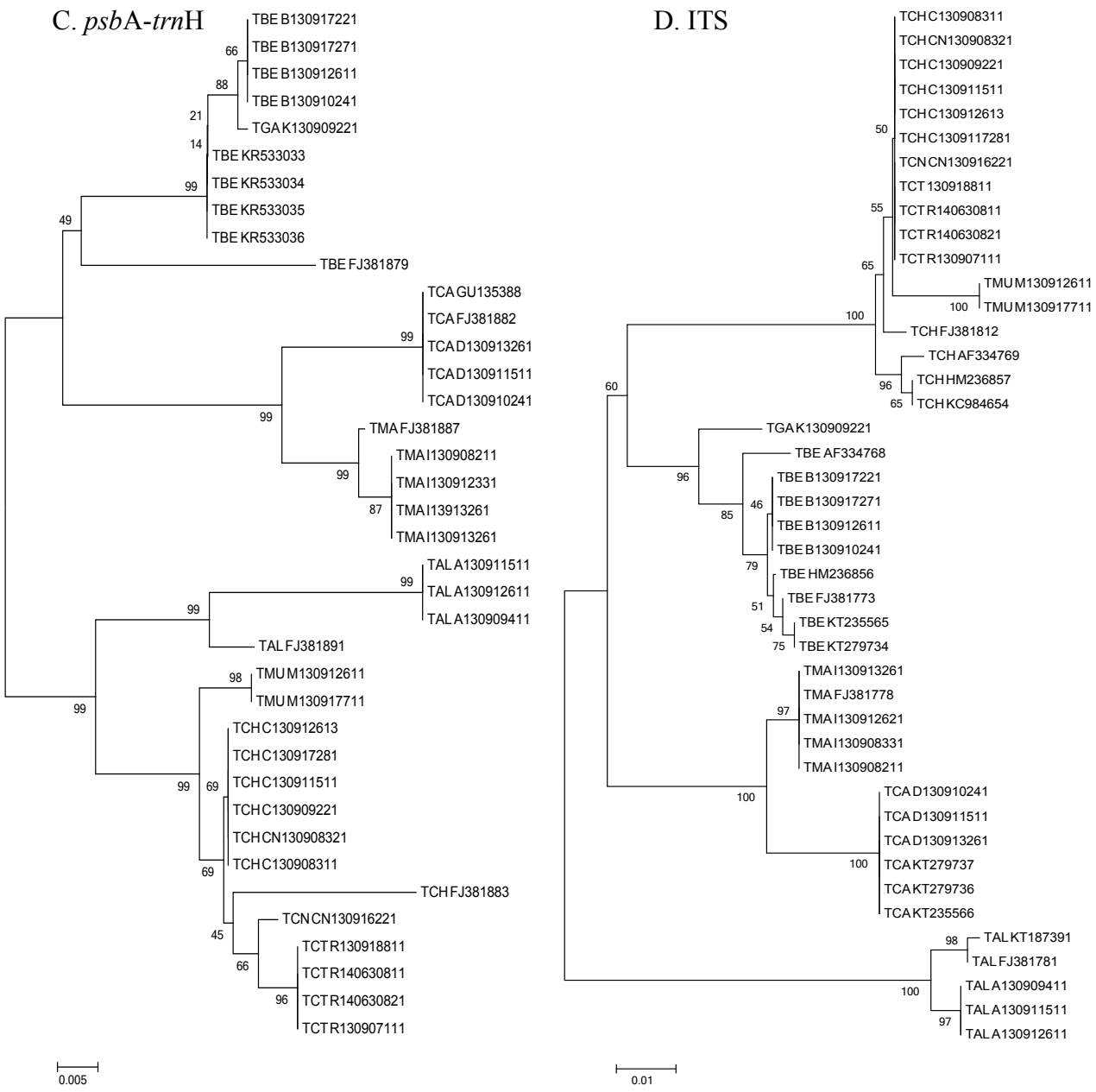


Figure 11 Neighbor-joining Reconstructions Analyzed by MEGA 5.2.2 Software for Six Markers: A) *rcb*L, B) *mat*K, C) *psb*A-*trn*H, D) ITS, E) ITS1, and F) ITS2 and five multiple-locus combinations: G) *mat*K + *rbc*L, H) *rbc*L + *psb*A-*trn*H, I) ITS + *psb*A-*trn*H, J) ITS1 + *psb*A-*trn*H, and K) ITS2 + *psb*A-*trn*H. Details of sample, voucher number and accession numbers for each marker can be retrieve from Table 12. The specimens analyzed in this study were shown in voucher number, while the others from GenBank database were shown in accession number. TCH denotes *T. chebula* var. *chebula*, TCN denotes *T. chebula* var. *nana*, TCT denotes *T. citrina*, TBE denotes *T. bellirica*, TCA denotes *T. catappa*, TGA denotes *T. glaucifolia*, TMU denotes *T. mucronata*, TAL denotes *T. alata*, TMA denotes *T. mantaly*.


C. psbA-trnH
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TBE B130912611
TBE B130910241
TGA K130909221
TBE KR533033
TBE KR533034
TBE KR533035
TBE KR533036
TBE FJ381879
TCA GU135388
TCA FJ381882
TCA D130913261
TCA D130911511
TCA D130910241
TMA FJ381887
TMA I130908211
TMA I130912331
TMA I13913261
TMA I130913261
TAL A130911511
TAL A130912611
TAL A130909411
TAL FJ381891
TMU M130912611
TMU M130917711
TCH C130912613
TCH C130917281
TCH C130911511
TCH C130909221
TCH CN130908321
TCH C130908311
TCH FJ381883
TCN CN130916221
TCT R130918811
TCT R140630811
TCT R140630821
TCT R130907111
0.005
D. ITS
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TCH CN130908321
TCH C130909221
TCH C130911511
TCH C130912613
TCH C1309117281
TCN CN130916221
TCT 130918811
TCT R140630811
TCT R140630821
TCT R130907111
TMU M130912611
TMU M130917711
TCH FJ381812
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TCH HM236857
TCH KC984654
TGA K130909221
TBE AF334768
TBE B130917221
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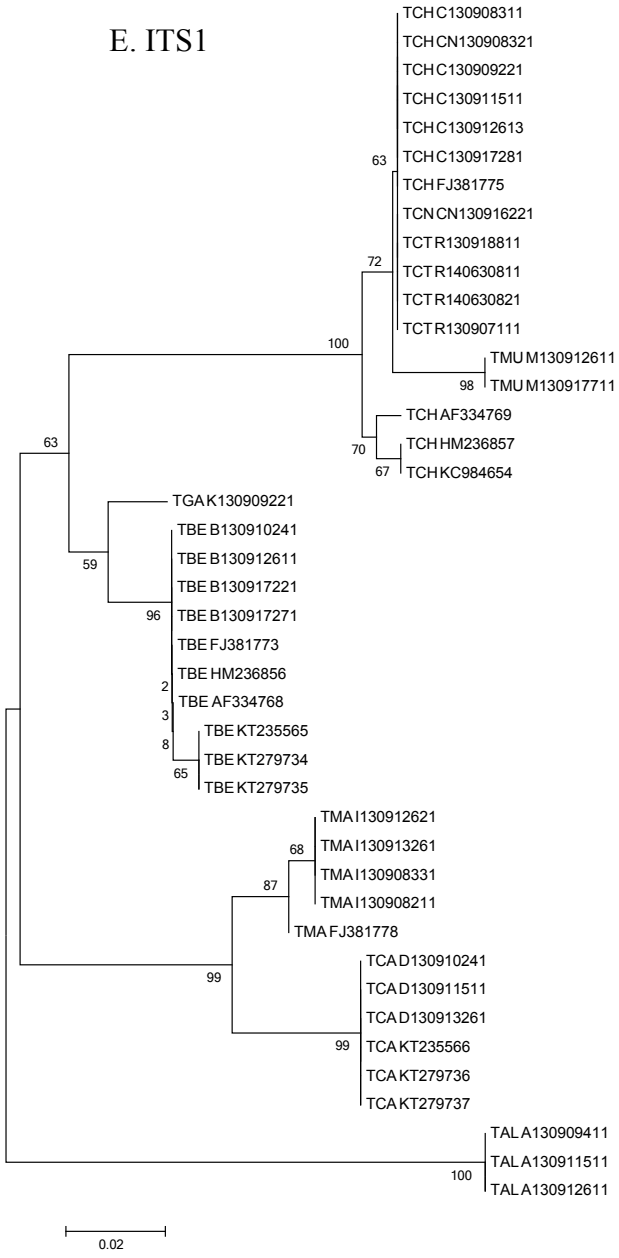
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TCNCN130916221
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TCTR140630811
TCTR140630821
TCTR130907111
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TMUM130917711
TCHAF334769
TCHHM236857
TCHKC984654
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TBEB130917271
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TBEHM236856
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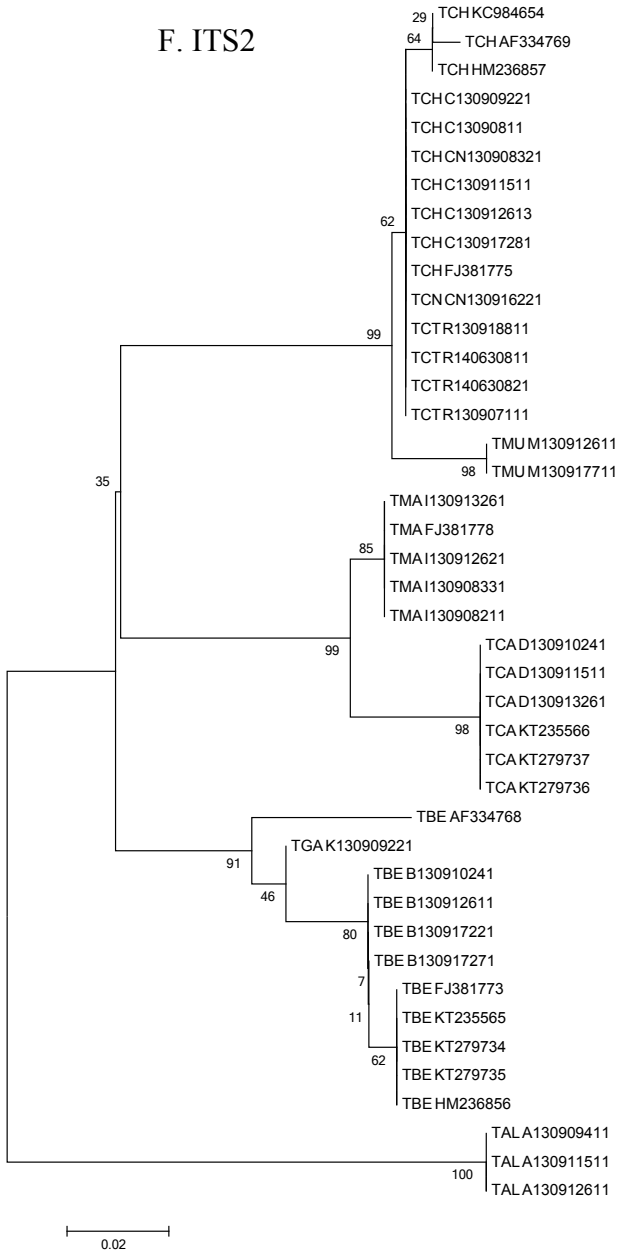
F. ITS2
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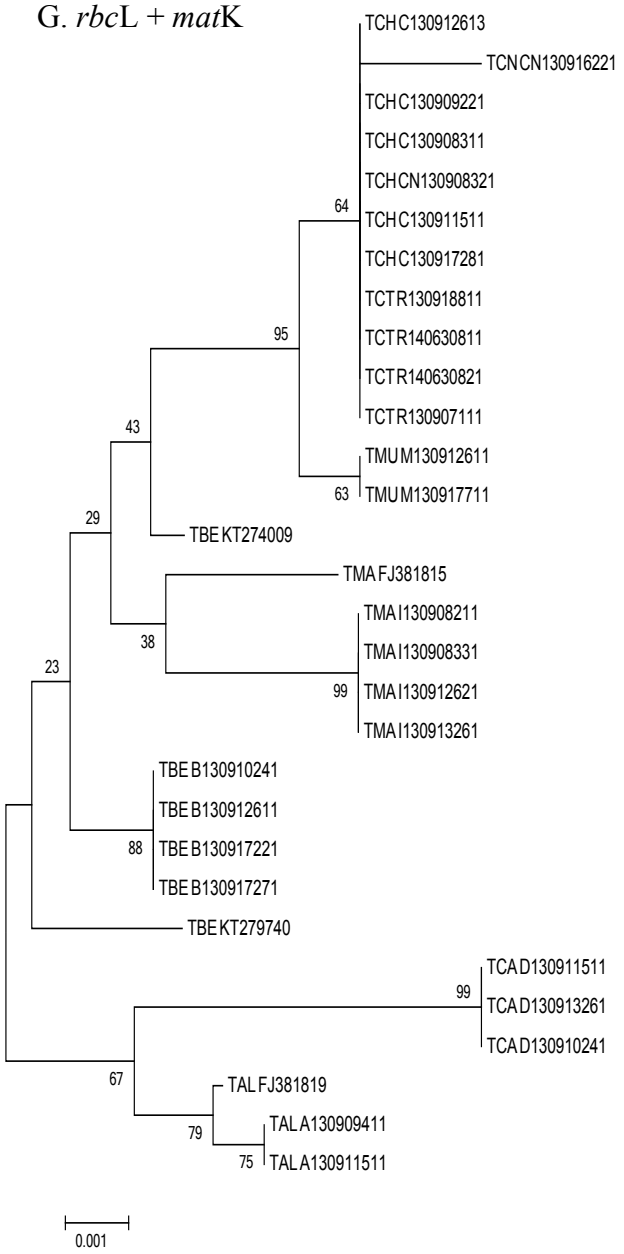
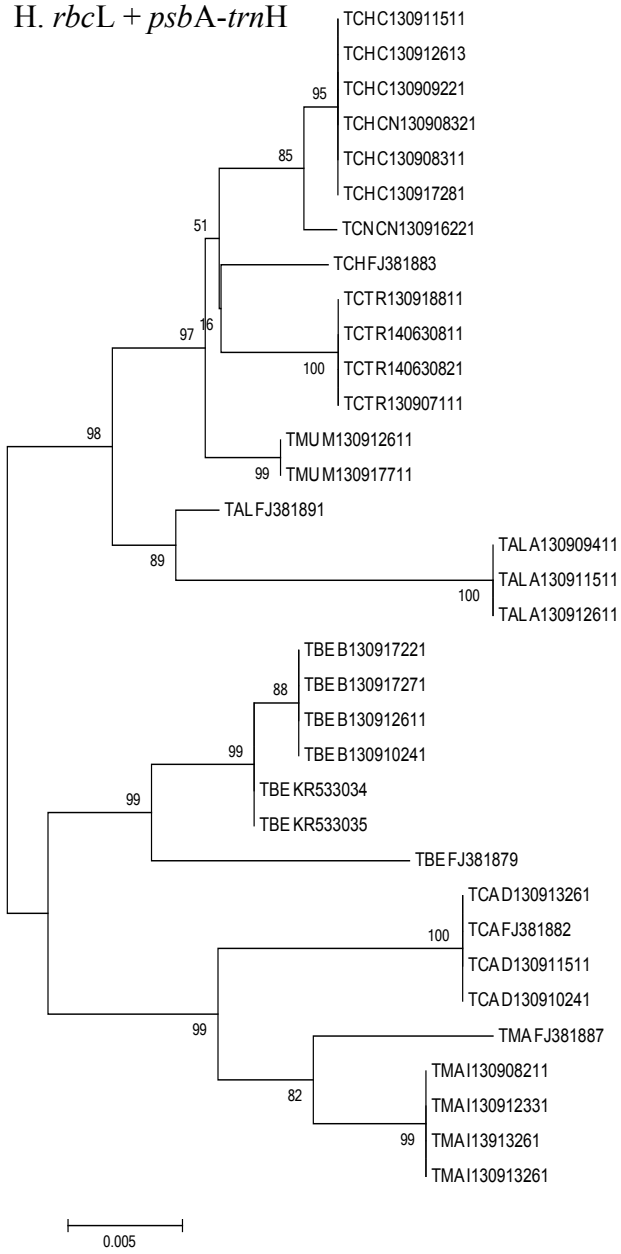
0.02

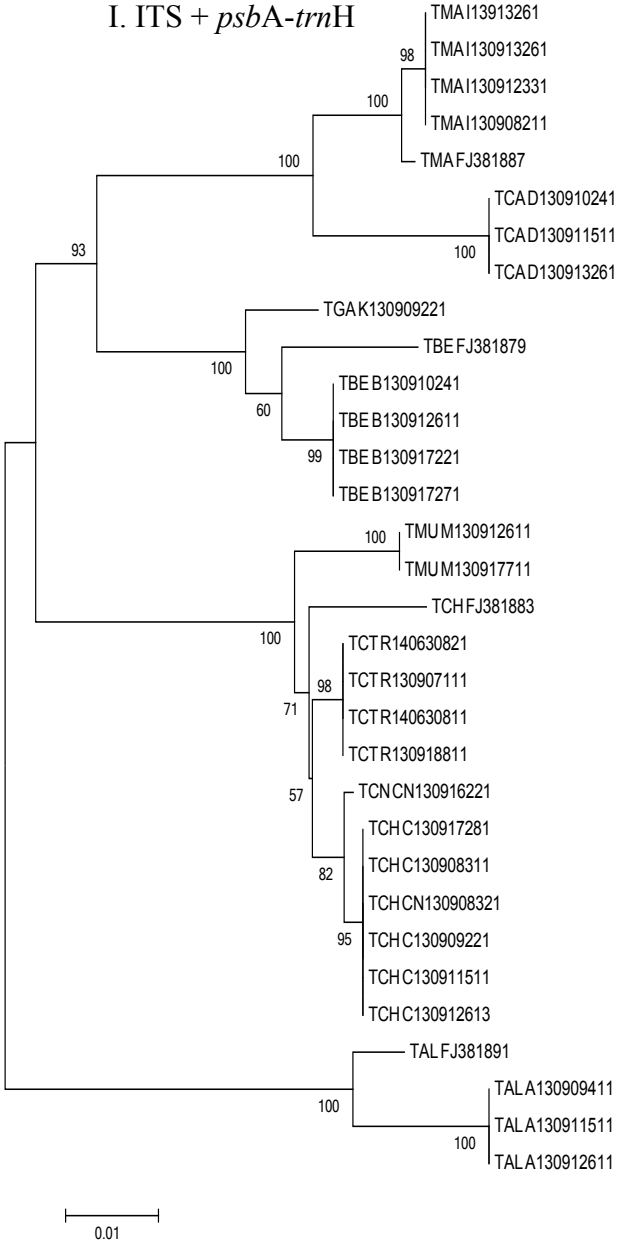
G. *rbc*L + *mat*K



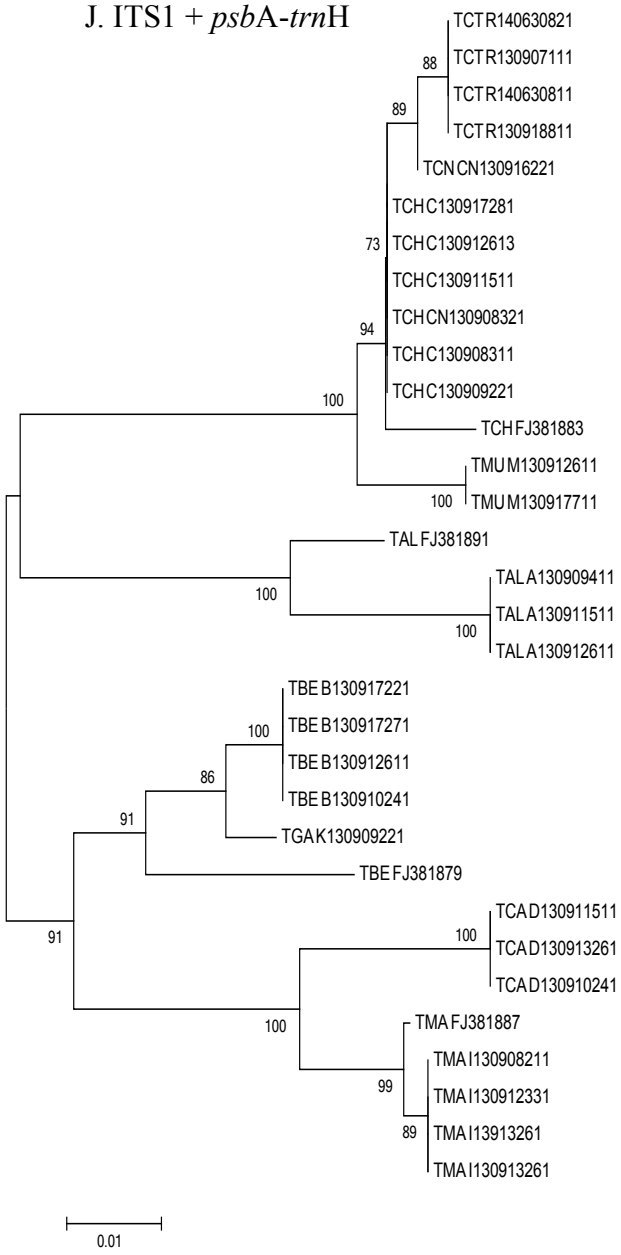
H. *rbc*L + *psb*A-*trn*H



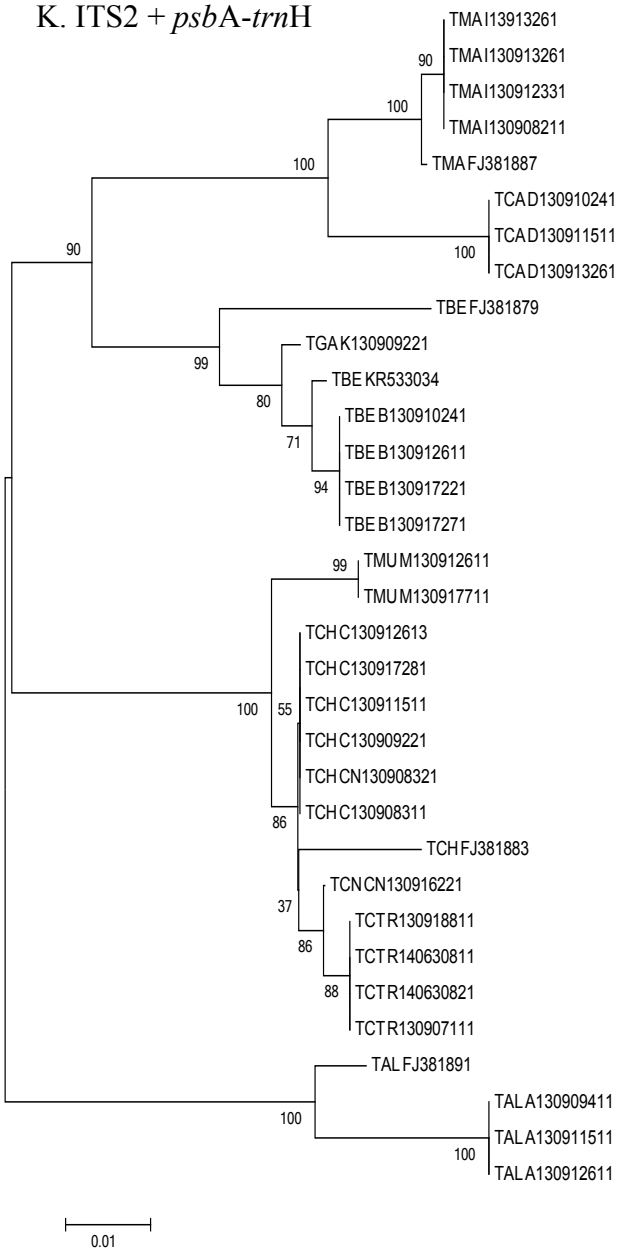
I. ITS + *psb*A-*trn*H



J. ITS1 + *psb*A-*trn*H



K. ITS2 + *psb*A-*trn*H



CHAPTER 4

SUMMARY

In this study, nine *Terminalia* species (Family Combretaceae) collected from Thailand including one of the exotic species: *T. ivorensis*. Five of which namely *T. chebula* var. *chebula*, *T. chebula* var. *nana*, *T. citrina*, *T. bellirica*, and *T. catappa* are utilized as indigenous medicine. Triphala, the famous formulation in Thai traditional medicine and Ayurvedic medicine composes of *T. chebula* var. *chebula*, *T. bellirica* and *Phyllanthus emblica* (Family Euphorbiaceae). These three crude drugs and nine commercial Triphala formulations were obtained from local Thai market.

The commercial Triphala and its crude drugs usually found in powder form that it is very tough to identify the botanical origin by morphological and chemical methods. DNA technology plays an important role to solve these limitations. Eighty-one single nucleotide polymorphisms (SNPs) and nine insertion-deletions (indels) were observed, and the nucleotide sequences of this region showed species-specific sequences. Based on these differences, the PCR-RFLP method, and the ARMS method were applied to identify medicinal *Terminalia* species and compare the authenticating capacity of Triphala. *T. chebula* var. *chebula*, *T. bellirica*, *T. catappa*, and *P. emblica* were analyzed for discrimination. Two steps of PCR-RFLP method were performed: (1) the restriction enzyme *Xsp*I was utilized to recognize *T. catappa* from the other species, and then, (2) the remained species were authenticated by *Aor*13HI enzyme. From 3.0% TAE agarose gel electrophoregram of PCR product, *T. chebula* var. *chebula* and *T. bellirica* were able to cleave by the *Aor*13HI restriction enzyme into three fragments and two fragments respectively, while, *P. emblica* could not to be digested by the same restriction enzyme. The species-specific primers based on SNPs of the nucleotide sequences of the ITS regions were searched for separating the three medicinal *Terminalia* species and *P. emblica*. The amplification was conducted with the combination of these species-specific primers under resemble concentration and temperature conditions. The multiplex-ARMS-PCR amplification efficiency produced four apparently amplified PCR products that the four pairs of diagnostic primers could be utilized to authenticate all of these four species. Both methods were able to authenticate the three medicinal *Terminalia* species and *P. emblica*. Nonetheless, the ARMS method had better select to conduct for differentiating *Terminalia* crude drugs including *P. emblica* crude drugs and the ingredients of Triphala because it provides the simplicity and efficiency. This technique achieved to utilize the authenticating by using the different length of amplified amplicons: 209 bp for *T. bellirica*; 266 bp for *T. chebula*: 497 bp for *P. emblica*. Consequently, these results clearly exhibited that the ARMS is effective for identification of *Terminalia* crude drugs and Triphala formulations.

All *Terminalia* specimens were amplified and performed direct sequencing on the ITS1-5.8S-ITS2 region. The aligned sequences obtained from samples with secure identity authentication were used to search the database. The query sequences were identified using BLAST database. As a consequence, the BLAST output of Huu Krajong (หูกระจง) samples exhibited the highest identities score 99% with *T. mantaly*. From the result of BLAST output, sequencing analysis, and comparison of morphology, Huu Krajong deserved to designate as *T. mantaly*. The aligned ITS1-5.8S-ITS2 nucleotide sequences of five medicinal *Terminalia* species were correlated

each other and analyzed. The result revealed that no diversities were observed, even though all specimens were collected from different locations. In the ITS region, the nucleotide sequences of *T. chebula* var. *chebula*, *T. chebula* var. *nana*, and *T. citrina* are identical, but they have many dissimilar nucleotides with *T. bellirica* and *T. catappa*. Based on the nucleotides obtained in the present study along with those retrieved from DDBJ/EMBL/GenBank DNA database, the phylogenetic tree was reconstructed using maximum likelihood method. The tree revealed that *Terminalia* is divided into two groups: an African group with a few taxa from Asia and the Pacific islands and an Asian group.

There are four *Terminalia* medicinal fruits merchandised in local raw drug markets in Thailand: Samo Thai, Samo Thed, Samo Deengu, and Samo Phiphek. For the safety of patients, the authentication of botanical origin is prerequisite before utilization. Recently, DNA barcode was utilized for species identification and classification method by using a standardized DNA region. In preliminary research, six candidate markers (*rbc*L, *mat*K, *psb*A-*trn*H, ITS, ITS1, and ITS2) were evaluated in nine *Terminalia* species to find a best marker or combination of markers for species level identification. Direct sequencing of *Terminalia* specimens was compared in each DNA barcoding markers. Among all analyzed DNA barcodes, the region that contains the lowest variable sites is *rbc*L (2.59%), whereas, the highest one is ITS2 (27.02%). A total of 116 closely related sequences belonging to nine Terminalia nucleotide sequences obtained in this study and 98 sequences retrieved from DDBJ/EMBL/GenBank DNA database were analyzed the discrimination performances of the six candidate DNA markers. Three major methods of identification: the methods based on sequence comparison ((1) BLAST and (2) genetic method) and (3) tree topology. In BLAST1 method, the result revealed that ITS correctly identified 100% at the species and genus level. ITS2 ranked first comparable K2P variability consequences in distance method. The data from single markers and two-locus combination were conducted phylogenetic tree by using the neighbor-joining (NJ) method. No individual markers exhibited clear resolution among nine *Terminalia* species. On the other hands, the two-locus combination of ITS + *psb*A-*trn*H clearly discriminated all the species of *Terminalia*. As a consequence of three methods, in the author opinion, ITS2 is recommended to identifying *Terminalia* species, which may supplement with *psb*A-*trn*H. In future, the author will utilize ITS2 to identify four *Terminalia* medicinal fruits especially Samo thai and Samo thed that the botanical origin still ambiguous.

The chemical marker constituents in the final product specify in efficacy and safety of medicine. The triphala has been reported that it contains enormous of major tannin-related ingredients namely ellagic acid and gallic acid. Moreover, ascorbic acid was found in all of the Triphala ingredients. HPLC has been performed for estimating the chemical constituents of Triphala. Thai Triphala and its ingredients including related *Terminalia* crude drugs sold in local Thai herbal markets will be determined the chemical markers (gallic acid, ellagic acid, and ascorbic acid).

CHAPTER 5

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