การวิเคราะห์ลักษณะทางจุลทรรศน์และอณูพันธุศาสตร์ของพืชสกุลโมรินดาบางชนิดในประเทศไทย

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์สาธารณสุข
วิทยาลัยวิทยาศาสตร์สาธารณสุข จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2553

MICROSCOPIC AND MOLECULAR ANALYSES OF SELECTED *MORINDA* SPECIES IN THAILAND

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Public Health Sciences
College of Public Health Sciences
Chulalongkorn University
Academic Year 2010

Thesis Title	MICROSCOPIC AND MOLECULAR ANALYSES OF SELECTED *MORINDA* SPECIES IN THAILAND
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Field of Study	Public Health Sciences
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วรลักษณ์ รุญญะมาลัย : การวิเคราะห์ลักษณะทางจุลทรรศน์และอณูพันธุศาสตร์ของพืชสกุลโมรินดาบางชนิดในประเทศไทย (MICROSCOPIC AND MOLECULAR ANALYSES OF SELECTED *MORINDA* SPECIES IN THAILAND) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : อ. ดร. กาญจนา รังษีหิรัญรัตน์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ. ดร. นิจศิริ เรืองรังษี, 92 หน้า.

พืชสกุลโมรินดาที่มักพบในประเทศไทยมี 4 สายพันธุ์ได้แก่ *Morinda citrifolia* L., *M. elliptica* Ridl., *M. umbellata* L. และ *M. coreia* Ham. สายพันธุ์ *M. citrifolia* ถูกใช้เป็นยาสมุนไพรในหลายประเทศ รวมถึงประเทศไทย ได้รับการบรรจุเป็นสมุนไพรในงานสาธารณสุขมูลฐานเพื่อแก้อาการคลื่นเหียน อาเจียน พืชสกุลโมรินดามีชื่อไทยเรียกพ้องกันว่า "ยอ" เนื่องจากความแตกต่างทางสัณฐานวิทยา และการศึกษาพืชสกุลโมรินดามีค่อนข้างจำกัด ดังนั้นการตรวจหาเอกลักษณ์ประจำสายพันธุ์ มีความจำเป็นต่อการนำสมุนไพรมาใช้เป็นยา วัตถุประสงค์ของการศึกษาครั้งนี้เพื่อศึกษาทางจุลทรรศน์ ด้วยการตัดขวางของเส้นกลางใบ และการหาค่าคงที่ของใบ รวมถึงการศึกษาทางอณูพันธุศาสตร์ โดยใช้เทคนิค PCR-RFLP บริเวณตำแหน่ง ITS ผลการศึกษาพบว่าปากใบเป็นเซลล์ชนิด Paracytic ทั้ง 4 สายพันธุ์ ซึ่งสัมพันธ์กันตามลักษณะเซลล์ปากใบของพืชประจำวงศ์ Rubiaceae และการหาค่าคงที่ของใบเป็นค่าที่จำเพาะในแต่ละสายพันธุ์แต่ละชนิด และผลจากการตัดขวางเส้นกลางใบพบว่าลักษณะของเซลล์เนื้อเยื่อ สามารถสร้างเป็นรูปวิธานในการแยกเอกลักษณ์ของแต่ละสายพันธุ์ได้ด้วย นอกจากนี้ ผลการศึกษาทางอณูพันธุศาสตร์พบว่าใช้เอนไซม์ตัดจำเพาะจำนวน 3 ชนิด คือ *MseI*, *EcoRI* และ *MspI* กับบริเวณ ITS ซึ่งผลการศึกษานั้นให้รูปแบบการตัดด้วยเอนไซม์จำเพาะที่แสดงเป็นลายพิมพ์ดีเอ็นเอที่แตกต่างกัน จึงสามารถใช้แยกพืชสกุลโมรินดาทั้ง 4 สายพันธุ์ออกจากกันได้ ซึ่งสามารถสร้างเป็นเครื่องหมายโมเลกุล โดยสรุป การศึกษาทางจุลทรรศน์และทางอณูพันธุศาสตร์ สามารถใช้ในการจำแนกพืชสกุลโมรินดาทั้ง 4 ชนิดได้

สาขาวิชา วิทยาศาสตร์สาธารณสุข ลายมือชื่อนิสิต วรลักษณ์ รุญญะมาลัย

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5279411053 : MAJOR PUBLIC HEALTH SCIENCES
KEYWORDS : MICROSCOPIC ANALYSIS / MOLECULAR ANALYSIS / *MORINDA* SPECIES / LEAF MEASUREMENT / POLYMERASE CHAIN REACTION - RESTRICTION FRAGMENT LENGTH POLYMORPHISM

WORALAK ROONYAMARAI : MICROSCOPIC AND MOLECULAR ANALYSES OF SELECTED *MORINDA* SPECIES IN THAILAND. ADVISOR : KANCHANA RUNGSIHIRUNRAT, Ph.D., CO-ADVISOR : ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., 92 pp.

There are four species of plant in the Genus *Morinda* commonly found in Thailand; *Morinda citrifolia* L., *M. elliptica* Ridl., *M. umbellata* L., *and M. coreia* Ham. *M. citrifolia* is used in folk medicine of many countries, including Thailand. *M. citrifolia* or Yor has been notified as a medicinal plant in Primary Health Care for anti-nauseate and anti-emetic. Due to their relatively high degree of morphological variability and inadequate study of *Morinda* species, an accurate investigation on their identity is essential. The purpose of this study was to examine the microscopic characteristics including of cross section of midrib, leaf measurement, and the molecular analysis by using PCR-RFLP technique on the ITS regions. The results of leaf measurement of four species followed the previous reported of rubiaceous plants which could be categorized the stomatal cells in Paracytic type and the leaf measurement revealed specific comparative data of which display in the term of constant number. Dichotomous key can be generated from cross section of midrib structure for identification of each species. Along with the molecular analysis, three restriction enzymes (*MseI*, *EcoRI* and *MspI*) have shown distinct and polymorphic of banding pattern from digestion on ITS regions. Based on the differentiation among the digestion pattern *Morinda* species, it could be applied as molecular marker for discrimination or contamination of samples. In conclusion, both microscopic and molecular analyses were able to authenticate 4 *Morinda* species.

Field of Study : Public Health Sciences.. Student's Signature Woralak Roonyamarai....
Academic Year: 2010..... Advisor's Signature Kanchana Rungsihirunrat
Co-advisor's Signature Nijsiri Ruangrungsi

ACKNOWLEDGEMENTS

The author would like to express her deepest gratitude and appreciation to her thesis advisor, Dr. Kanchana Rungsihirunrat and her thesis co-advisor, Assoc. Prof. Dr. Nijsiri Ruangrungsi, for their advice, guidance, valuable suggestion, encouragement, and support throughout this study. Without their helps, this investigation has been impossible.

Her deepest gratitude goes to Mr. Niran Vipunngeun, College of Public Health Sciences, Chulalongkorn University, for his kind assistance in microscopic and drawing techniques throughout this study.

The author would like to thank and deep appreciation to Prof. Surasak Taneepanichskul, M.D., Dean of College of Public Health Sciences, Chulalongkorn University.

The author also wishes to express her special thanks to Dr. Chanida Palanuvej, as thesis committee and Assist. Prof. Dr. Worapan Sitthithaworn, as external examiner for their critical perusal, valuable suggestion and advices for improve the thesis.

The author thanks to Dr. Ladawan Lerslerwong and her team at Department of Plant Sciences, The Faculty of Natural Resources, Prince of Songkla University, for their kind supports in collecting plant specimens.

This research is supported by a grant from the College of Public Health Sciences, Chulalongkorn University and thanks are also due to all staff members, friends and others persons whose names have not been mentioned here for helping the author through the difficult times, kindness, hopeful and friendships.

Finally and the most importantly, the author would like to express her heart-felt gratitude to Roonyamarai family for their supporting, encouragement and their trying understanding. The author cannot start or finish this work without them. She would like to express her deep gratitude and dedicate this degree to them.

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5.8s rDNA	=	5.8s ribosomal DNA
18s rDNA	=	18s ribosomal DNA
26s rDNA	=	26s ribosomal DNA
A, T, C, G	=	Nucleotide containing the base adenine, thymine, cytosine, and guanine, respectively
AFLP	=	Amplified fragment length polymorphism
bp	=	Base pair
$^{\circ}C$	=	Degree celsius
CTAB	=	Cetyltrimethyl ammonium bromide
cm	=	Centimeter
DNA	=	Deoxyribonucleic acid
dNTPs	=	Deoxynucleotide triphosphate (dATP, dTTP, dGTP, dCTP)
g	=	Gram
ITS	=	Internal transcribed spacer
kb	=	Kilobase
M	=	Molar
m	=	Meter
mm	=	Millimeter
mm^2	=	Square millimeter
mM	=	Millimolar
min	=	Minute
ml	=	Milliliter
nDNA	=	Nuclear DNA
PCR	=	Polymerase chain reaction
pH	=	Potential of Hydrogen ion
rDNA	=	Ribosomal deoxyribonucleic acid
RAPD	=	Random amplification polymorphic DNA
RFLP	=	Restriction fragment length polymorphism
rpm	=	Revolution per minute
SD	=	Standard deviation

spp.	=	Species
U	=	Unit
UV	=	Ultraviolet
µg	=	Microgram
µl	=	Microliter
µM	=	Micromolar
µm	=	Micrometer

CHAPTER I

INTRODUCTION

Rubiaceae is a family of flowering plants comprising about 110 genus and 600 species. *Morinda*, a genus belonging to the Rubiaceae family contains approximately 80 spp. in tropical zone of the world [1]. More than nine species with five varieties of *Morinda* are found in Thailand. *Morinda* species have been used as folk medicine and alternative medicine in many countries in India [2], Malaysia [3], Indonesia [4], and other countries in South-East Asia [5] including Thailand. One of the most commonly found is *M. citrifolia*, commercially known as Indian Mulberry or Noni. It's native range from tropical Asia to Australia, and the species is now cultivated throughout the tropics and widely naturalized near the sea coast in many islands of the West Indies and also in El Salvador and Surinam to the Hawaiian Islands [6]. In Thailand, *M. citrifolia* is one of the most frequently found and cultivated not only in the rural village but also in the urban area. The others *M.* spp. which have been found in the open habitats and secondary forest in the lowland areas of the Southern part of the country are *M. elliptica* and *M. umbellata* [1]. And another species that found in mixed deciduous forest and in the Northern, Southwestern, or Northeastern part of Thailand is *M. coreia* [7]. *M. citrifolia*; also known as "Yor", has been recommended for Primary Health Care System as Thai medicinal plants for anti-nauseate, anti-emetic, treatment of diseases causing cachexia and wasting disease, or other properties [8]. Many parts of this plant such as root, bark, leaf and fruit have been used in both traditionally and modernly for health care. This plant is high in carbohydrates and dietary fiber [9]. In addition, both fruits and leaves have been taken as food and the leaves are cooked as vegetables [10]. Previous studies showed that traditional uses were possible effective on human health [11]. The vernacular name "Yor" belongs to several *Morinda* species as above-mentioned, for example *M. citrifolia* is called "Yor" or "Yor-Ban"; *M. elliptica* is called "Yor-Thuean" or "Yor-Pa"; *M. umbellata* is called "Yor-Yan"; and *M. coreia* is called "Yor-Pa" [12]. Thus, misidentification caused by similarity in their authentic counterparts may lead to some adverse effects. In addition, the processing of medicinal plants in to powder for

encapsulation or pills resulting in morphological and anatomical changed [13]. The methods employed for authentication of herbal materials are necessary.

Macroscopic and microscopic examinations are major methods in pharmacognostic study and used for medicinal plants identification. Macroscopic involves the comparison of morphological characters such as size, shape, color, flowers or fruit that are visible with naked eye. Microscopic examination focuses on anatomical structures of plant materials such as trichomes (hair), the arrangement of stomata in epidermis or the presence/absence of compounds by using microscope. It consists of cross section of midrib, stomatal measurement, and leaf measurements [14] that possible to identify and confirm plants species on their specific characters by certain physical constants [15]. Macroscopic and microscopic examinations can be used as rapid and inexpensive identification techniques. An only investigation of microscopic analysis may not always provide the complete identification but when use in association with other analytical methods, it can support any additional useful information for invaluable evidence [16].

In the few decades, DNA fingerprinting assay has been applied and also introduced to identify herbal medicine. Analyzing of DNA that is present in all organisms is suitable for identifying plant materials because genetic composition is unique for each individual [17]. DNA extracted from leaves, stems or roots of plant carry the same genetic information which is not affected by physiological conditions, environmental factors and/or developmental factors during plant growth. It may become an alternative method to identify herbal medicine by using polymerase chain reaction (PCR) technique. PCR in combination with restriction fragment length polymorphism (PCR-RFLP) has been widely used for DNA fingerprinting [18]. Species-specific region in nuclear DNA, mitochondrial DNA and chloroplast DNA have been used for species-level identification. The internal transcribed spacer (ITS) region, found on either side of transcribed region of the 18S, 5.8S and 26S nuclear DNA, is one of the most popular sequence for phylogenetic analysis in plants for species level identification [13]. The ITS region is highly repeated in the plant nuclear genome which present in up to many thousands of copies arranged in tandem repeats. Previous studies of *M. citrifolia* were found incompletely classified the characteristic of each cells type by morphology method, but have a distinct relationship with *M. elliptica* by using amplified fragment length polymorphism (AFLP) technique [19]. Moreover, *M. citrifolia* in Thailand have been closed relation with the exotic *M.*

citrifolia form Hawaiian by using random amplified polymorphic DNA (RAPD). Nevertheless, based on using RAPD as a marker to identify the genetic discrimination between different locations of *M. citrifolia*, but also confirmed the distinct relationship with *M. elliptica* [20].

Authentication of medicinal plant can be achieved through the application of a variety of different methodologies. This study aimed to use various aspects of method such as microscopic and molecular analyses for authentication of *Morinda* species.

CHAPTER II

LITERATURE REVIEWS

Morinda species

Morinda, a genus belongs to family Rubiaceae, subfamily Rubioideae [10] compose of approximately 80 species distributed in the tropical area. These species may be trees, shrubs or climbers. Leaves are opposite which the stipules are interpetiolar, free, or united with bases of petioles and undivided. Inflorescences are terminal (although sometimes looking axillary) and white flower 4-6-merous. The corolla is infundibular or hypocrateriform, which the throat of tube is glabrous or pilose. Stamens are inserted in the tube or may be near the throat with short to long filaments and included or exserted of anthers. The two-lobed of stigma are either included or distinctly exserted. Capitula when fruiting stage is infructescences or called "syncarps" consisting of fused drupes that is 1-seeded pyrenes [1]. In the past years, chemical analysis revealed that the plants belonging to family Rubiaceae are known to contain large amounts of anthraquinones. *In vitro* biological activities assay revealed some biological properties such as antimicrobial, antifungal, hypotensive, analgesic, antimalarial, antioxidant, antileukemic, mutagenic functions. Moreover, one of the extensively reviewed on anthraquinones biosynthetic studies used in the family Rubiaceae is *Morinda* species [21].

The most frequently seen is *M. citrifolia* known commercially as Indian Mulberry or Noni. It is native from tropical Asia to Australia, cultivated, and naturalized near the sea coast in many islands of the West Indies, and also in El Salvador and Surinam to the Hawaiian Islands [6]. In Thailand, more than nine species, five varieties of plants are recorded [12]. *M. citrifolia* is found and cultivated not only in the rural village but also in the urban area. The others *M.* spp. which have been seen in the open habitats and secondary forest in the lowland areas of the Southern part of the country are *M. elliptica* and *M. umbellata* [1]. And another species that found in mixed deciduous forest and in the Northern, Southwestern, or Northeastern part of Thailand is *M. coreia* [7]. In addition, each *Morinda* species mentioned above was called in Thai vernacular name under the name "Yor" which details as shown in **Table 1** [12, 22].

Table 1 List of *Morinda* species Thai vernacular name

No.	Scientific name	Thai vernacular name
1	*M. citrifolia* L.	Yor (ยอ), Yor-ban (ยอบ้าน)
2	*M. elliptica* Ridl.	Yor-pa (ยอป่า), Yor-thuean (ยอเถื่อน)
3	*M. umbellata* L.	Yor-yan (ยอย่าน)
4	*M. coreia* Ham.	Yor-pa (ยอป่า)

a) *Morinda citrifolia* L.

The common name is "Indian mulberry" or commercially name called "Noni". It is a small to medium-sized tree which is grows in the tropics and Pacific regions. In Thailand, both fruits and leaves have been cooked in many kinds of food and eaten with rice [10]. This plant has been used as a folk medicine in Philippines, India, Australia, Vietnam, Polynesia, and throughout Eastern and South-Eastern of Asia [23]. In folk medicine of Philippines, *M. citrifolia* fresh leaves are used to cure rapidly on ulcers, and the fruits are recorded to use as an emmenagogue [24]. In Malay Peninsula, the leaves are used to treat by heating for coughs, nausea, enlarged spleen, and colic. An infusion of the root bark is applied as an antiseptic and using as cleanse of wounds [5]. In Bombay, the leaves are used as a healing application to wounds and ulcers as well as internal administration as a tonic and febrifuge [2]. While in Tonga, it is commonly used for diarrhea in infants and the crushed leaves moistened in water are applied to aching joints or massaged into aching muscle [23]. In addition, charred leaves made into a decoction with a little mustard are a remedy for infantile diarrhea. The decoction with aromatics is also given for treatment of dysentery [25]. Moreover, many properties of *M. citrifolia* have been increasingly studied and investigated. Chemical investigations showed that the root-bark contained a crystal glucoside, morindine ($C_{27}H_{10}O_{15}$), coloring-matter, and the fruit contained volatile oil (Morinda oil) [24]. In addition, morindicone and morinthone were reported as two new compounds of the chemical constituents from stems by NMR techniques. The methanolic extracts of stems were reported in many properties: antimicrobial, antioxidant, anti-HIV, and hypotensive activities [26]. Drying leaves extract could be applied to relief for inflammation and pain from poison fish and insect stings. Its occurrence of the volatile compounds was also confirmed of no toxicological significance [27]. Furthermore, the extracts of leaves were reported to mitigate the

UVB-induced skin injury, for example, UVB-induced erythema in human skin [28]. *M. citrifolia* fruit juice has been investigated for modulate immune system. The results elucidated possible mechanisms on stimulation of the immune cytokines.

b) *Morinda elliptica* Ridl.

It is a small tree usually about 4-5 m. tall with white bark. It's the most medicinally used in Malay Peninsula which the common name called "Mengkudu jantan" [3]. The leaves are eaten to sharpen the appetite, to treat fever, headache, cholera, and diarrhea or dysentery. A decoction is used as a lotion in case of hemorrhoids and for bathing after childbirth. They are also made into a poultice or smeared with oil and heated [5]. In addition, the barks are used as antipyretic and anti-inflammatory agent for the field of Thai traditional medicine. Phytochemical from previous studied on roots of *M. elliptica* was established 2-formyl-1-hydroxyanthrquinone, a new naturally occurring anthraquinone derivative based on spectral studies [29]. Moreover, isolation from the leaves and branches was reported a new plumieride type iridoid glycloside called morinipticoside [30]. The results from anthraquinones extracted from roots of *M. elliptica* have reported antibacterial, antifungal, and antileukemic properties [31]. Furthermore, it was found that anthraquinones as well as antioxidant vitamins contents were produced in *M. elliptica* cell suspension cultures [32].

c) *Morinda umbellata* L.

It is a scrambling shrub or climbing and divaricated branches which have reported in India through Southern China and Malaya. According to Indian reported, Tamil folk medicine used the leaves in conjunction with certain aromatics and administered to treat diarrhea and dysentery in doses of half a tea-cupful twice daily [2]. Also in Indo China and Vietnam, the root and leaves have been used as antidysenteric [5]. Besides anthraquinones that found in *M. umbellata*, the root-bark contains a glucoside, morindine, and the coloring-matter, morindone. *Morinda* root is used for medicine and dyestuff [33]. The yellow dye given form *Morinda* roots was employed in America as a violent purgative, and a decoction of the leaves and roots is used as a vermifuge for children [24]. Previous studied showed the first reported on the pharmacognostic parameters of *M. umbellata*, the common name called "Nuna", to identify any adulterants which composed of microscopic characters, fluorescence

analysis, physic-chemical characters, and preliminary phytochemical screening to thin layer and paper chromatographic studies [34].

d) *Morinda coreia* Ham.

Synonym: *M. tinctoria* Roxb. [12]

It is a small or may be middle-sized and usually pubescent or tomentose tree. The root and stem were reported to use as a styptic, astringent to the bowels. It was dried and boiled as an infusion and used as tonic [2]. In Malay Peninsula, leaves and roots were prescribed for ague in decoction. The root crushed in alcohol could be provided to stop vomiting in cholera. Moreover, the bark was administered in case of astringent [5]. The methanolic extract of leaves and brunches were studied and five new iridoid glucosides and a new phenolic glycoside were isolated [35]. The chloroform extract of fruits has been investigated for wound healing properties in the treated rats and revealed the potential as a therapeutic drug for wound healing activity [36]. Moreover, it showed liver-protective properties against D-galactosamine ($_{D}$-GalN)-induced liver damage of rats by histopathogical studies [37]. Any previous biological studies showed the antioxidant property of *M. coreia* that could help the immune-stimulating property and maintained blood stasis which might have a valuable plant potential to anti-carcinogenic activities and help to reduce the risk of chronic disease [38]. The beverage of fruits produced by fermenting was examined for ability to inhibit some organisms, and the results showed the highest inhibition against bacterial and also presented a high amount of potassium [39]. Furthermore, fermenting of ripe fruit extracts plus molasses were produced as a liquid fertilizer showing the possibility of microbiological succession potential and supplementary plant nutrition [40].

Medicinal plants have long been widely used for disease prevention and therapy and becoming popular in many countries. The history of herbal medicine or Thai traditional medicine in Thailand has presented from the Sukhothai period basically in utilization to Thai people in a primary health care system. It is from a holistic perspective to people's health [41]. Usage of medicinal plants and other materials still rely of people for their everyday health care is needed. People learn how to use the plants as traditional remedies from their experiences that some plants have highly effectiveness, even though they may not understand about scientific evidence [42]. There is natural origin which has few adverse effects associated with

herbal remedies. Although modern medicine may be available in these countries, herbal medicines have often maintained popularity for historical and cultural reasons. During the past decades, in developing world, people increasingly use medical plants, herbal medicines and the products derived from them for their primary health care because of the understanding that how plants function in the body for healing [43]. Nowadays, the unceasingly growth of the pharmaceutical technologies of biological activities or chemical synthesis from medicinal products have not diminished importance in many countries. As many products form plants have greatly developed have more popularity. Because of less people have no understanding on the scientific insight to explain the properties of plants; so, regulations of the assurance of the quality, safety and efficacy of medicinal plants have been evidenced [4]. Due to similarity of the local name of *Morinda* species and their pharmacological action, misidentification caused by similarity in their authentic counterparts may lead to some adverse effects. Thus, identification is also a great importance to ensure the highest effectiveness and to ensure against from the adulteration. On the contrary, there is no supporting the preparation of model guidelines to identify *Morinda* species, have been helpful in strengthening of their role in health care. However, inadequate study in *Morinda* species is generally considered to be investigated [44]. There are several example ways for examination of medicinal plants authentication and, if possible, the ways of microscopic and molecular derived from a standardized have been discussed.

Microscopic analysis

Pharmacognostic study is consisting of various methods used to characterize and identify the medicinal plants such as macroscopic, microscopic characterization, and phytochemical screening which can revealed for quality control as described in Pharmacopoeia [45]. Under the word of Pharmacognosy, microscopic analysis has been recording used for medicinal plants examination. Pharmacognosy was regarded coincident with the increasingly used of alternative medicine or complementary medicine which has been generally requirement in order to quality control in medicinal plant products in many countries [14]. In addition, the microscopic evaluation is an observation under the microscope. The remarkable of each species can be compared with the description from a known sample. Moreover, the differentiation of the genera, as well as species or varieties can also used to identify

and establish of the medicinal plants. This method has been served by the means of detecting the adulterants because of containing each structural characteristic for identification [46]. Microscopic analysis carried out using a photomicroscope (detailed below) is possible visual examination usually used for determination. It consists of cross section (transverse section) of midrib, stomatal classification, and leaf measurements that possible to determine and confirm plants species on their specific characters [15]. Moreover, it is not only recommended procedures for preliminary inspection by microscopy because of its simplicity and rapidness, but also the result can be carried out as the average index statistical assessment [47].

Photomicroscope

A modern research about microscopic evaluation use a digital camera attached above the microscope. It is more convenient than *camera lucida* [14]. Anyhow, the specimen has to be very thin and the optical part used should be of very high quality as any defects accentuate in the final print [16]. The photograph is recorded by digital camera attached above the microscope by helping of the scale labeling program as shown in **Figure 1**. The photomicrography is uniquely qualified to be used for routine and advanced microscopic investigation of medicinal plant materials [48].

Figure 1 Microscope (Axioskop, Germany) attached with a digital camera (Cannon Power shot A640) and conjunction using AxioVs40 V.4.6.3.0 program

Clarification reagents of microscopic analysis

The presence of various contents within the cell such as starch grain, plastids, fat and oils etc., may give non-translucent section and obscure certain characteristics. There are some reagents that can dissolve of these contents and have been used to make a penetrating effect. Those sections may be more transparent and reveals details of the structures. Some of the reagents that most frequently used such as Chloral hydrate and Sodium hypochlorite are described below [47].

a) Chloral hydrate solution

Chloral hydrate is colorless hygroscopic crystal with melting point at 55 ºC. It's valuable and widely used as the best for clearing reagent. This solution dissolves starch, proteins, chlorophyll, resins, and volatile oils with the help of gentle warming. It does not dissolve calcium oxalate and causes the shrunken cells to expand without damage of cell wall or other tissue. Chloral hydrate is not only used for cross section, but also for whole leaves, flowers etc. [45].

b) Sodium hypochlorite solution

This solution is useful bleaching agent to remove deeply coloured sections such as many barks as well as for removing chlorophyll from the leaves [14]. The sections are immersed in the solution and leaved for a few minutes or until bleaching. The section should be removed from the solution and then washed with water when bleaching is completed. [47].

Cross section of midrib

Leaf anatomical structure is established by using the microscopic and clearing reagent mentioned above to identify the characteristics of plant species. The transverse section of midribs and main veins demonstrated vascular tissues and particular surface cytomorphological characters such as trichomes, palisade cells, stomata, etc. Each cell type, form, size and its distribution within midrib cross section can provide distinguished identity for plant authentication. Moreover, midrib anatomical character enables to detect the contamination or adulteration in plant materials as well [3, 46].

Stomatal classification

Stomata are frequently present in the lower epidermis of the leaf. A stoma consists of two enclosing guard cells which occurring the stomatal pore or stomatal aperture. There are cells which surround the stomata called subsidiary cells. The structure of guard cells and subsidiary cells are distinguishable into four patterns which used for taxonomic purpose [49]. The structure and shape of the epidermis and stomata are the first investigation in the microscopic analysis of the leaf identification on ventral surface area [14]. Four differentiation types of stoma regarding to guard cells and subsidiary cells of mature leaf are often available as described below and shown in **Figure 2 a)** to **Figure 2 d)** respectively [47].

a) The anomocytic (irregular-celled) types: the stoma is surrounded by varying number of cells, which generally not different from those of the epidermis or may be no subsidiary cells and also called “ranunculaceous” because of frequent occurrence in the Ranunculaceae.

b) The anisocytic or cruciferous (unequal-celled) type: the stoma is usually surrounded by three or four subsidiary cells which one is markedly smaller than the other.

c) The diacytic or caryophyllaceous (cross-celled) type: the stoma is accompanied by two subsidiary cells, the common wall of which is at right angle to the stoma.

d) The paracytic or rubiaceous (parallel-celled) type: the stoma has two subsidiary cells with the parallel to long axis of the stoma.

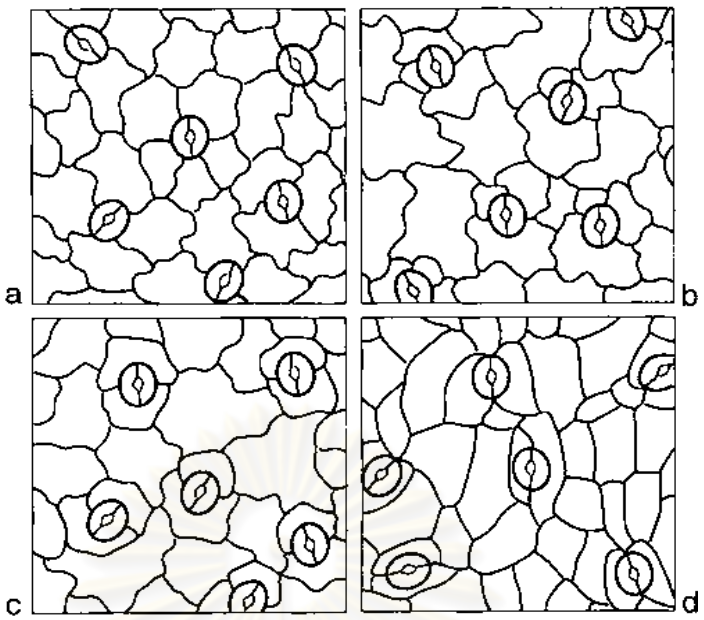

Figure 2 Types of leaf stomata in ventral surface area:
a = Anomocytic type
b = Anisocytic type
c = Diacytic type
d = Paracytic type

Leaf measurement

According to the microscopic determination, leaf measurement is a constant number used to distinguish between some closely related species not easily characterized by general microscope or photomicroscope [48]. Those numerous usefully made such comparison can be applied to the positive identification of the botanical, geographical and other sources of plant. Thereby, it has great value for a quality of the medicinal plants based on their specific characters [16]. Leaf measurement can be studied consisting as follows:

a) Stomatal number and stomatal index

Due to the information of stoma, the two guard cells and the stomatal pores are means as one stoma cell. It is a very specification of criteria for identification and characterization of leafy crude drugs. The average number of stomata cells per a square millimeter (mm^2) calculated from thirty determinations is a parameter as called stomatal number. In addition to stomatal number, the number of epidermal cells per each area is also counted. The stomatal index is a percentage proportion of stomatal

number to epidermal cells plus stomatal number in one mm^2 area [14]. This parameter is more distinguishable characteristic for leafy medicinal plant [16].

b) Palisade ratio

Palisade cell is photosynthetic cell type of the mesophyll characterized by elongated cells with long axes perpendicular to the leaf surface. It contacts with epidermis and position occurs in the adaxial leaf blade part, or presents in both adaxial and abaxial leaf blade parts called “isobilateral mesophyll” [49]. Palisade ratio is the average number of the palisade cells that position at beneath each upper epidermal cell [50]. It is used for identification and evaluation of medicinal plants and also powdered plant. Palisade ratio is a constant number with diagnostic value in differentiating the species. Despite of geographical variation, the palisade ratio is still stable. Hence, it is a very useful diagnostic feature for characterization and identification of different plant species [16].

c) Cicatrix number and cicatrix index

The glossary has been described the word “Cicatrix” as “the scar left by a wound or by the separation of the one plant part from another (as a leaf from a stem) and characterized by substances protecting the exposed surface” [49]. For leaf measurement, cicatrix is a trichome scar which marks on a leaf after trichome detaches on the leaf [14, 51]. The cicatrix number and the cicatrix index are determined using cicatrix instead of stomata and defined in term of cicatrix number and cicatrix index respectively [48]. It has been used as one of the constant numbers for identification of the plant leaves covering trichomes [52].

Therefore, the adulterants or others substituent materials presented in crude drugs can be distinguished by this way with the aid of photomicroscope. Thus, these parameters which when taken together are demonstrated a useful method to support authenticated evidence [16]. Although the microscopic examination is important for identification of medicinal plants, it is not sufficient for a complete identification [47]. In addition, a complementary with other analytical methods such as molecular technology will provide important supporting evidence [11].

Molecular analysis

Regardless of the original species while the demand of market is increasingly grown to treat in their natural activities. Some of plant is not easily characterized by microscopy analysis because the adulterated may lead to confuse or misunderstand by authentication. In addition, DNA-based molecular markers have utility their plants science such as taxonomy, physiology, embryology, and genetics which are possibility as new source of evidences [53]. Moreover, molecular techniques have become widely used not for plant, but also in fungi determination [54]. Analyzing of DNA that is present in all organisms is suitable for identifying plant materials because genetic composition is unique for each individual. DNA extracted from leaves, stems or roots of medicinal plants carried out of the same genetic information without effect by physiological conditions and environmental factors. Until now, various techniques of DNA-based markers are introduced as DNA fingerprinting which categorized into three techniques namely hybridization-based methods, PCR-based methods, and sequencing-based markers [11]. Therefore, these methods are also important for authentication of the medicinal plants [17, 55].

Polymerase chain reaction (PCR)

PCR-based methods are generated by Mullis and coworkers, PCR technique is the amplification of the interested region in the genome which fragments *in vitro* by using thermostable DNA polymerase and either random or specific primers. It is the most interest become of the most popular technique in many researches. The advantage of PCR is requiring tiny amount of DNA samples in experiment effort, for each analysis due to the high sensitivity of PCR and has ability to produce large within short periods. There have been many publications or document books which success of this technique [18]. PCR markers have been introduced with mainly are more sensitivity and reliability techniques such as PCR-based methods include sequence characterized amplified regions (SCAR), amplified fragment length polymorphism (AFLP), or PCR-based methods have include Restriction Fragment Length Polymorphisms (RFLP) or PCR-RFLP is widely used as a molecular marker as described below.

PCR-RFLP

PCR in combination with RFLP uses endonucleases to digest PCR products of regions with sequence polymorphisms. PCR-RFLP has been used for DNA fingerprinting for authentication because its codominance and high reproducibility [18]. A restriction endonuclease recognizes a specific sequence of nucleotide basically used of four or six pairs in length, and then cleaves it. The number or location of recognition sites is varying in each sample. The similarity of the samples is compared by the higher or closer cleavage patterns which possible estimate from proportion between samples [56]. For example, two samples that closely related species when using PCR-RFLP technique for authentication, different banding pattern were obtained. The results from PCR-RFLP can be applied for differentiation between these two samples, as shown in **Figure 3**.

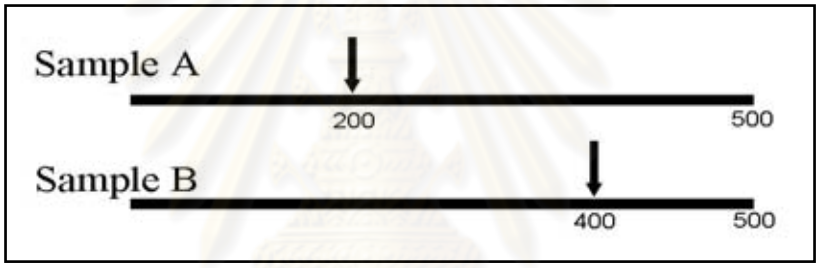

Figure 3 An example of PCE-RFLP of two samples that closely related species sample A and B can differentiate by this technique

Internal Transcribed Spacer (ITS)

Species-specific region, such as internal transcribed spacer (ITS), nuclear ribosomal DNA has been approximate used for species-level identification [57]. The ITS region is commonly found on either side of transcribed region of the 18S, 5.8S and 26S nuclear ribosomal DNA [13] as sown in **Figure 4.** The ITS region is occur highly repeated in the plant nuclear genome which present in up to many thousands of copies long arranged in tandem repeats. Sequence comparison of the ITS region is widely used in molecular phylogeny. Therefore, universal PCR primers designed from highly conserved regions flanking the ITS and its relatively small size (600-700 base pair) enable easy amplification of ITS region and due to a high degree of variation even between closely related species make the ITS region an interest for investigation [58, 59] as **Figure 5**.

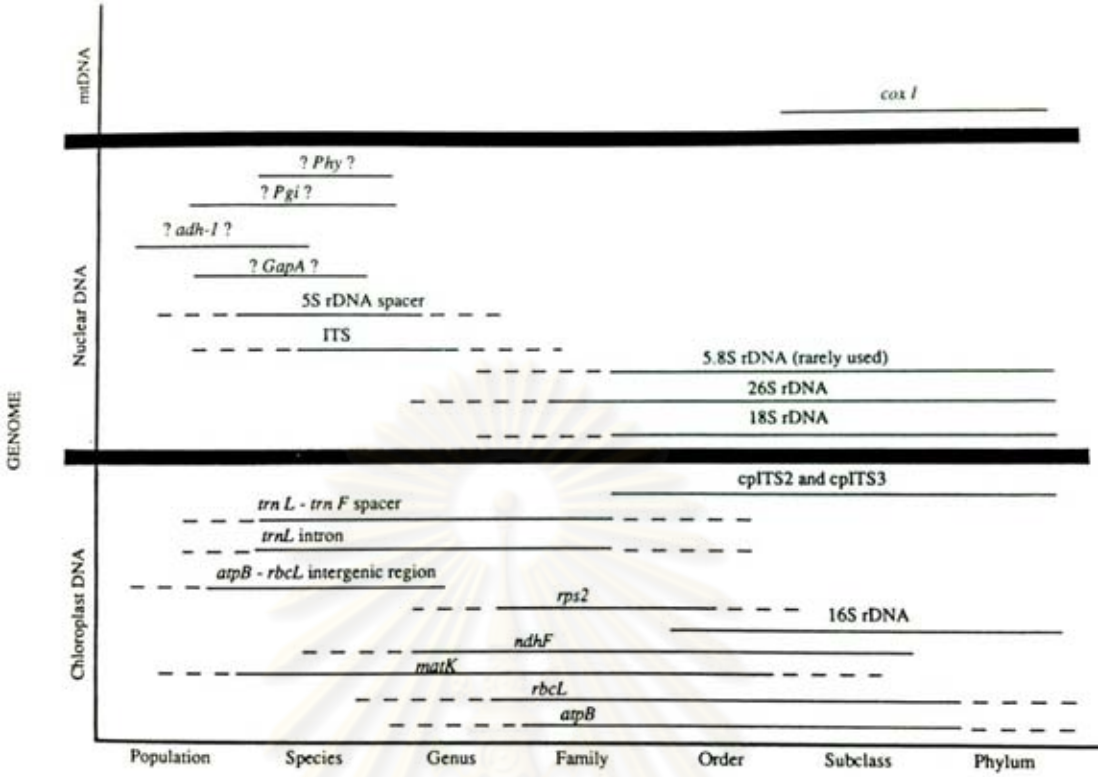

Figure 4 An approximate taxonomic level of utility in various genes and DNA regions used in phylogenetic reconstruction based on angiosperms whereas ITS regions is the best gene when study in species level

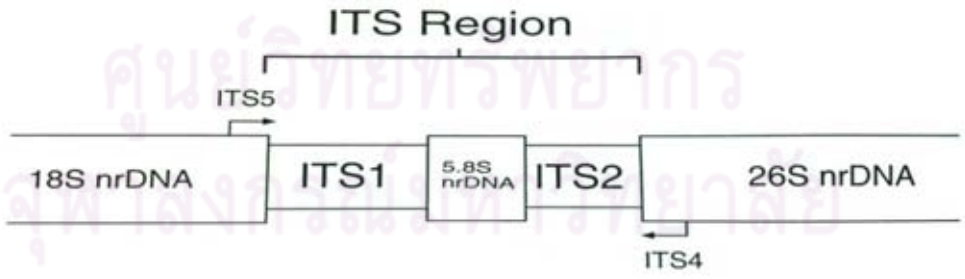

Figure 5 The ITS region of nuclear DNA illustration with flanking subunit and location of primer sites

Previous studied have been recorded of *M. citrifolia* were found incompletely classified the characteristic of each cells type by morphology method, but have a distinct relationship with *M. elliptica* by using AFLP technique [19]. Moreover, *M. citrifolia* in Thailand have been closed relation with the exotic *M. citrifolia* form Hawaiian by using RAPD. Nevertheless, based on using RAPD as a marker to identify the genetic discrimination between different locations of *M. citrifolia*, but also confirmed the distinct relationship with *M. elliptica* [20]. There have been reported based on the similarity on the morphology of head inflorescences and fruits of the tribe Morindeae, and also submitted the DNA sequencing to GenBank database [60].

Using PCR-based method for authentication of the medicinal plants is useful as a powerful new tool which can be use for quality control and quality assessment. Moreover, this efficiently examination is for misidentified raw materials or adulterated medicinal plants [17]. Nevertheless, standardization way of microscopic analysis is also revealed as an individual characteristic which molecular analysis is a new importance with various examinations that both analyses are advantage for plants authenticated methods will continue development [61, 62].

CHAPTER III

MATERIALS AND METHODS

Scope of Investigation

1. Observation of the aerial parts of each investigated species.

2. Study of the microscopic analysis of each investigated species: cross section of midrib, stomatal classification on ventral surface of leaf and leaf measurement including of stomatal number, stomatal index and palisade ratio (cicatrix index and cicatrix ratio for *M. coreia* only).

3. Molecular analysis of each investigated species: DNA extraction and ITS PCR-RFLP.

Samples collection and authentication

Plants authentication

All samples were authenticated by Associate Professor Dr. Nijsiri Ruangrungsi, College of Public Health Sciences and Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, and comparing with the herbarium specimens at Forest Herbarium Thailand (BKF). All samples were deposited at College of Public Health Sciences, Chulalongkorn University.

Collection and observation

Prepared herbarium specimen of the authenticated sample by holding in newspaper to absorb moisture, then rigidly pressed by using corrugated cardboards. The plant specimen press was tightened using straps with 2-side wooden press frames to contain the plant materials. Photographs of the aerial part were taken in various views for the record. Determined the characteristics of each species including leaf, flower and fruit and drew their pictures on the drawing paper in the proportion size related to the original.

Microscopic analysis

Material

Plant materials

The total of twelve authenticated specimens of four investigated *Morinda* species were collected from different locations in Thailand. Their localities and collecting dates were shown in **Table 2**.

Table 2 List of the four investigated *Morinda* samples and their different collecting localities in Thailand

Species	Sample no.	Habitat, (Province)	Collecting date	Voucher ID
Morinda citrifolia	1	Central part, (Bangkok)	July, 2009	CI011
	2	Northern part, (Phayao)	September, 2009	CI031
	3	North-Eastern part, (Yasothon)	July, 2009	CI051
Morinda elliptica	1	Western part, (Kanchanaburi)	November, 2010	EL041
	2	North-Eastern part, (Yasothon)	July, 2009	EL051
	3	Central part, (Nakhon Pathom)	November, 2009	EL031
Morinda umbellata	1	Southern part, (Songkhla)	April, 2010	UM011
	2	Southern part, (Songkhla)	April, 2010	UM012
	3	Southern part, (Songkhla)	September, 2010	UM014
Morinda coreia	1	Central part, (Nakhon Pathom)	November, 2009	CO022
	2	Western part, (Kanchanaburi)	March, 2010	CO011
	3	North-Eastern part, (Kalasin)	September, 2009	CO031
Total number of *Morinda* species, **12 samples**				

Chemicals and Reagents

- Chloral hydrate solution (Ajax Finechem Pty Ltd., Australia)
- Glycerine (Imex glycerine, Honghuat Co., Thailand)

Materials

- Microscope slide (Sail Brand, China)
- Cover slips (Menzel. Glazer)
- Razor blade
- Drawing materials and others

Instrumentations

- Microscope (Carl Zeiss model Axio Lab, Germany) with AxioVision40 V4.6.3.0 software
- Digital camera (Cannon Power shot A640)

Methods for microscopic analysis

All the microscopic specimens were analyzed by a microscope. The images were captured using a digital camera attached to the microscope. AxioVision40 V4.6.3.0 software was used for images alignment and labeling. The individual of each sample was studied under objectives with a 10X, 20X and 40X magnifications.

The fresh mature leaves were cleaned and cut with a razor blade at a right angle to the longitudinal axis of the material into a suitable size. Cross section was prepared by cutting the leaves in parallel including the midrib and lamina into pieces as thin as possible and transferred these tissue sections by a brush moistened with water. Selected satisfactory sections were prepared and mounted onto a slide in glycerin water for microscopic examination under photomicroscope [10]. The characteristic cells and tissues of midrib were photographed using digital camera and scaled for labeling size of each character. This field was specific for each magnification of lens. If the lens were changed, the magnification must be calculated again. Cross sections of midrib were drawn in the proportion size related to the original in drawing paper.

Methods for leaf measurement

a) Preparation of leaves

The fresh mature leaves were cleaned and cut into small pieces (1x1 cm), in the central part of lamina, midway between the midrib and the margin. Small pieces of leaves were cleared by gently warming with chloral hydrate solution (chloral hydrate: distilled water, 9: 1). This solution was rapid removing of chlorophyll from the leaf fragments with frequently stirred. Until the leaf fragments were transparent, and then they were washed with distilled water at least twice times. After washing, leaf fragments were kept in glycerin to maintain the moisture and structure of cells. The leaf fragments were examined under the photomicroscope which each area were taken avoid from the veinlet, margin, or unclear field. Each area was calculated per square millimeter (mm^2) by using scaling program. This field was specific for each

magnification of lens. If the lens were changed, the area and its field must be calculated again. Thirty fields of photographs were determined for each four investigated species with three different locations according to **Table 2**. Stomatal number, stomatal index and palisade ratio (cicatrix number and cicatrix index for only *M. coreia*) were recorded and presented by mean and standard deviation (SD). Then, mean and SD of each sample were averaged as the constant number of each species. The methods were carried out as below.

b) Stomatal number and stomatal index

The photomicroscope and other equipments were already set up for observation. After preparing the leaf, transferred the lower epidermis fragment of the leaf to a slide with a small drop of glycerin to prevent the material from drying. Then, held the fragment by placed a cover slip over it. Stomatal number was calculated by counting the number of ordinary stomata of each field (1 mm^2) and plus with the number of incomplete stomata from two sides of the field border. For stomatal index, the epidermal cells were traced and counted in same field and protocol of stomatal number. Stomatal index was defined as the percentage of stomata from the total number of epidermal cells, which can be explained below:

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

Where S = the total numbers of stomata of leaf

E = the total numbers of epidermal cells in the same area of leaf

C) Palisade ratio

The cleared pieces of leaf fragments on upper epidermis were mounted according to the methodology of stomatal number and stomatal index as leaf preparation. The palisade ratio was examined under the microscope by counting the number of palisade cells which beneath four contiguous upper epidermal cells. For any incomplete palisade cell beneath four contiguous epidermal cells, count as half cell. Combined the number of counted palisade cells, and then divided by 4.

d) Cicatrix number and cicatrix index

Transferred the upper epidermis of cleared leaf on the slide and prepared for observation. According to cicatrix number and cicatrix index, it required the same methods as stomatal number and stomatal index respectively then calculated as follows.

$$\text{Cicatrix index} = \frac{C}{E+C} \times 100$$

Where C = the total numbers of cicatrix of leaf

E = the total numbers of epidermal cells in the same area of leaf

Methods for stomatal classification

The fresh mature leaves were cleaned and cleared with chloral hydrate solution using the same procedure as described in part of leaf measurement. In the other way, ventral surface of leaf which consisted of stomata cells was peeled as a thin membranous layer by pulling. The thin membranous layer which was sufficiently big enough for the field vision were taken on glass slide and mounted in glycerin. A 20X eyepiece was used to determine the character of stomata type with the help of a photomicroscope and drawn in the proportion size related to the original on drawing paper.

Molecular analysis

Material

Plant Materials

Total of 42 *Morinda* samples were collected from different location in Thailand, and a detail of authentication was referred in authentication part. Their collecting locality details were showed in **Table 3**.

Table 3 List of *Morinda* species used for molecular analysis in this study

Species	Sample no.	Locality	Collecting date	Voucher ID
Morinda citrifolia	1	Bangkok 1	November, 2009	CI011
	2	Bangkok 2	November, 2009	CI012
	3	Bangkok 3	November, 2009	CI013
	4	Bangkok 4	November, 2009	CI014
	5	Chachoengsao	November, 2010	CI021
	6	Phayao	September, 2009	CI031
	7	Nakhon Ratchasima 1	November, 2010	CI041
	8	Nakhon Ratchasima 2	November, 2010	CI042
	9	Nakhon Ratchasima 3	November, 2010	CI043
Morinda elliptica	1	Phatthalung 1	October, 2010	EL011
	2	Phatthalung 2	October, 2010	EL012
	3	Phatthalung 3	October, 2010	EL013
	4	Phatthalung 4	October, 2010	EL014
	5	Phatthalung 5	October, 2010	EL015
	6	Songkhla	November, 2009	EL021
	7	Nakhon Pathom	November, 2010	EL031
	8	Kanchanaburi 1	November, 2010	EL041
	9	Kanchanaburi 2	November, 2010	EL042
	10	Kanchanaburi 3	November, 2010	EL043
	11	Kanchanaburi 4	November, 2010	EL044
Morinda umbellata	1	Songkhla 1	August, 2010	UM011
	2	Songkhla 2	August, 2010	UM012
	3	Songkhla 3	September, 2010	UM013
	4	Songkhla 4	September, 2010	UM014
	5	Songkhla 5	September, 2010	UM015
	6	Songkhla 6	September, 2010	UM016
	7	Songkhla 7	September, 2010	UM017
Morinda coreia	1	Kanchanaburi 1	March, 2010	CO011
	2	Kanchanaburi 2	March, 2010	CO012
	3	Kanchanaburi 3	March, 2010	CO013
	4	Kanchanaburi 4	November, 2010	CO014
	5	Kanchanaburi 5	November, 2010	CO015
	6	Kanchanaburi 6	November, 2010	CO016
	7	Kanchanaburi 7	November, 2010	CO017
	8	Nakhon Pathom 1	November, 2009	CO021
	9	Nakhon Pathom 2	November, 2009	CO022
	10	Kalasin 1	September, 2009	CO031
	11	Kalasin 2	September, 2009	CO032
	12	Nong Khai 1	November, 2009	CO041
	13	Nong Khai 2	November, 2009	CO042
	14	Nakhon Ratchasima 1	November, 2010	CO051
	15	Nakhon Ratchasima 2	November, 2010	CO052
Total of 4 investigated *Morinda* species, **42 samples**				

Chemicals and Reagents

- CTAB: Hexadecyltrimethylammonium bromide (Sigma-Aldrich Company Co., St. Louis, MO, U.S.A.)
- Liquid nitrogen
- 2 Mercaptoethanol (Sigma-Aldrich Company Co., St. Louis, MO, U.S.A.)
- Saturated phenol (Amresco, U.S.A.)
- Chloroform (Merck, Darmstadt, Germany)
- Isoamyl alcohol (Sigma-Aldrich Company Co., St. Louis, MO, U.S.A.)
- Absolute ethanol (Merck, Darmstadt, Germany)
- Sodium acetate (BDH Laboratory supplies, Poole, England)
- Tris (hydroxymethly)-aminomethane hydrochloride (Fluka, Biochemika, Germany)
- Orthoboric acid powder (BDH Laboratory supplies, Poole, England)
- EDTA: Ethylene diamene tetraacetic acid (Merck, Darmstadt, Germany)
- NaCl: Sodium hydroxide (BDH Laboratory supplies, Poole, England)
- Agarose (Ultrapure ^{TM}, Life technologies, U.S.A.)
- Ethidium bromine (Sigma-Aldrich Company Co., St. Louis, MO, U.S.A.)
- 1 kb DNA Ladder (Promega. U.S.A.)
- 100 bp DNA Ladder (Promega. U.S.A., Fermentas, U.S.A.)
- Loading Dye (bromophenol blue, Fermentas, U.S.A.)
- *Taq* DNA polymerase (Fermentas, U.S.A.)
- Primer (Operon Biotechnologies, Germany)
- Restriction enzymes; *MseI*, *EcoRI*, and *MspI* (Fermentas, U.S.A., New England BioLabs Inc., U.S.A.)

Materials

- Microcentrifuge tube (Axygen, U.S.A.)
- Pipet tips (Axygen, U.S.A.)
- Micropipette (Eppendorf, Germany)
- Mortar

Instrumentations

- CLC Sequence Viewer 6 version 6.5 software
- UV transilluminator (AutoChem ^{TM} system, U.S.A.)
- LabWorks Analysis software version 4.6.0 gel application
- Thermal cycler (Gene Amp PCR9700, Applied Biosystems)
- Gel electrophoresis apparatus and power supply
- Centrifuge (BiofugePico, Kendro, Germany)
- Vertex mixer, shaking incubator, and etc.

Methods for molecular analysis

Preparation of CTAB buffers

Genomic DNA was individually extracted from the fresh young specimen leaves using a modified CTAB technique [63]. **Table 4** showed the preparation of CTAB buffers (4 µl of 2 mercaptoethanol was immediately added to each 1 ml of CTAB buffers before used).

Table 4 Preparation of CTAB buffers

Stock reagent	Final concentration	Final amount
CTAB	2% (w/v)	2 g
1 M Tris-HCl pH8	100 mM	10 ml
0.5 M EDTA	20 mM	4 ml
5 M NaCl	1.4 M	28 ml
Make up with distilled water to **100 ml**		

Methods for DNA extraction

For DNA extraction procedure, 1.5 ml microcentrifuge tubes, CTAB extraction buffer, and 65 $^{\circ}$C the shaking incubator were prepared and then followed the modified CTAB procedures:

1. Fresh leaves materials were ground with liquid nitrogen to a fine powder in mortar. The ground powders were transferred into 1.5ml microcentrifuge tube with the help of spatula.

2. Then 500 µl of CTAB buffer was added into the tube and vortex. Followed by incubate the CTAB/plant extract mixture at 65 °C for an hour in shaking incubator.

3. After incubation, centrifuge the tube at 13,000 rpm for 10 minutes to spin down cell debris the bottom of tube.

4. Transfer the supernatant (upper phase) to new microcentrifuge tube following with 500 µl of saturated phenol.

5. Vortex the solution through enough emulsify of the phases, then centrifuge at 13,000 rpm for 10 minutes.

6. Transfer the aqueous phase (upper) to a new microcentifuge tube, 500 µl phenol/chloroform/isoamyl alcohol (25:24:1) was added and mixed. After that, the tube was centrifuged at 13,000 rpm for 10 minutes. Depending upon the purification of DNA, the aqueous phase may be re-extracted.

7. Be carefully transferred the final upper aqueous phase to a clean microcentrifuge tube. Then 1:10 volume of 3M sodium acetate pH 5.0 was added following by 2 volume of ice-cold (-20 °C) absolute ethanol, mix by slowly invert the tube.

8. Let the tube stand at -20 °C for an hour to precipitate DNA.

9. After precipitation, centrifuge at 13,000 rpm for 10 min.

10. Remove all of the supernatant. DNA was then washed with 1ml of cold 70% ethanol and gently inverted the tube several times. After the washing, centrifugation at 13,000 rpm for 10 minutes.

11. Discard all the supernatant and allowed the DNA pellet to dry in room temperature, do not leaved the DNA to over dry or it will be hard to re-dissolve.

12. Dissolved DNA in 100 µl TE buffer (10mM Tris pH8, 0.1mM EDTA pH8) and stored at -20 °C further use as templates in PCR amplification. The genomic DNA was then analyzed by 1.5% agarose gel electrophoresis, stained by ethidium bromide and visualized under UV illuminator.

Methods for PCR amplification of ITS region

PCR amplification of ITS region (including ITS1, 5.8S rDNA and ITS2) was amplified by using the extracted DNA as a template. PCR reactions were carried out in 20 µl as the final volume, containing of 1-2 µl of DNA template, 100 mM KCl, 20 mM Tris-HCl (pH 8.0), 0.1 mM dNTPs, 0.1 µM of each primers, 2.5 mM $MgCl_2$, and 0.5 unit of *Taq* DNA polymerase. A pair of universal PCR primers for amplification (ITS4 and ITS5) was designed based on the conserved sequence located on nuclear ribosomal DNA as presented in **Figure5** [64] and nucleotide sequences of these primers were presented in **Table 5**. PCR amplification was performed in PCR Thermal cycler under the following condition: initial denaturation step at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 1 minute, primer annealing at 50 °C for 1 minute, primer extension at 72 °C for 1 minute and following a final extension at 72 °C for 5 minutes, and then held at 4 ºC. 1.5 g of agarose was prepared by adding to 100 ml of 1X TBE buffer and solubilized by heating in microwave. Then, let the gel solution to warm before pouring into a plastic tray and then wait until it was formed. After the gel was solid, removed the comb and put the tray into a gel electrophoresis apparatus fulfilled with 1x TBE buffer in chamber. Five µl of each amplified PCR products were then analyzed in 1.5% agarose gel electrophoresis comparison with 100 bp molecular weight marker. Electrophoresis was performed at constant voltage of 100 volts until the faster migration dye (bromophenol blue) has traveled at a half of gel and then stained with ethidium bromide. The agarose gel was visualized under UV transilluminator and photographed. A negative control which lacking the template DNA was included to each set of PCR reactions for DNA products controlled of possible contamination in the reagents.

Table 5 PCR amplification primers on ITS regions used in this study [64]

Primer name	Sequence (5' - 3')	Length (bp)	Tm (ºC)	Direction
ITS 4	TCC TCC GCT TAT TGA TAT GC	20	55	Reverse
ITS 5	GGA AGT AAA AGT CGT AAC AAG G	22	56	Forward

Methods for Restriction Fragment Length Polymorphism (RFLP)

The restriction enzymes were designed based on the sequence of ITS regions which have been published (NCBI GenBank database) from previous study including the ITS sequences of *M. citrifolia* (accession number FJ907060), *M. elliptica* (accession number FJ907066), *M. umbellata* (accession number FJ907094), and *M. coreia* (accession number FJ907064) details of these sequences were shown in **Appendix B** [60]. ITS sequences of 4 *Morinda* were aligned and predict the restriction map using CLC Sequence Viewer 6 version 6.5 software results shown as **Figure 6** and **Figure 7**.

Figure 6 Predictions of restriction site of *MseI* restriction enzyme on ITS regions of 4 *Morinda* species from GenBank

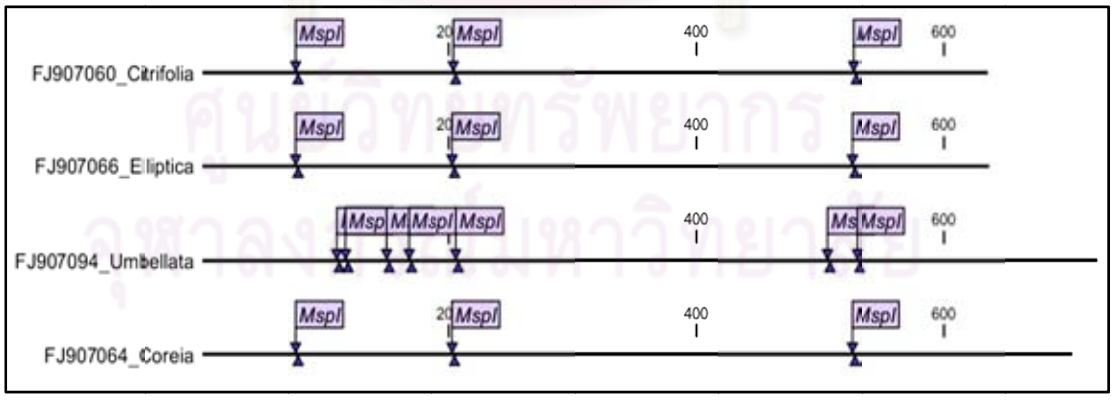

Figure 7 Predictions of restriction site of *MspI* restriction enzyme on ITS regions of 4 *Morinda* species from GenBank

For this study, PCR amplification products were digested using three restriction enzymes, *MseI*, *EcoRI*, and *MspI*, which their recognition site was shown in **Table 6**.

Table 6 restriction endonucleases used for pattern analysis

Restriction enzyme	Genomic source	Sequence	Reaction conditions
MseI	*Micrococcus* spp.	5'…T▼TAA…3' 3'…AAT▲T…5'	37 ºC
EcoRI	*Escherichia coli*	5'…G▼AATTC…3' 3'…CTTAA▲G…5'	37 ºC
MspI	*Moraxella* spp.	5'…C▼CGG…3' 3'…GGC▲C…5'	37 ºC

The digestion of PCR products was performed according to instructions from the manufacturer. The reaction mixture was carried out in 20 µl which consisting of 10 µl of ITS PCR amplification product, 2 µl of restriction buffer, 1 µl restriction enzyme (10 U/µl), and 7 µl of sterilized distilled water. The reaction was incubated to proceed at 37 ºC for at least 5 hours (or overnight) in shaking incubator. Ten µl of the restriction fragment results were then separated through their length by 2.5% agarose gel electrophoresis which comparison with 100 bp DNA ladder. Electrophoresis was performed in 1x TBE buffer at constant voltage of 80 volts until the faster migration dye (bromophenol blue) has traveled to two-third of gel and then stained with ethidium bromide. The *Morinda* ITS region restriction fragment patterns were analyzed under UV transilluminator and photographed.

CHAPTER IV

RESULTS

The results of observation of the aerial parts

Four investigated *Morinda* species were observed and described the aerial parts including leaf, flowers, and fruit which were drawn in **Figure 9, 11, 13, and 15** respectively.

1. ***Morinda citrifolia* L.** (**Figure 8-9**)

Vernacular names: Thailand: Yor, Yor ban (Central), Mata suea (Northern), Yae yai (Karen-Mae Hong Son); **India**: Bartundi, Nagakuda; **English**: Indian Mulberry; **Malay**: Mengkudu; **Philippines**: Tombongaso; **Sanskrit**: Achchhuka, Ashyuka; **Tamil**: Nuna, Manjatbavattai.

Distribution: From tropical Asia, India, Tamil, Thailand, Myanmar, Malay Peninsula, to Philippine and China.

Observation: It is a small glabrous tree with straight trunk and smooth bark, yellowish white and branchlets obtusely four-angled. The leaves are 12.5-20 X 7.5-10 cm with broadly elliptic, acute, acuminate or obtuse which their main nerve have prominently 8-10 pairs. Leaves are glabrous with bright green and shining. The peduncle often suppressed and acute base and petiole is 1.3 cm long. There are short and broad with connate obtusely membranous stipules. The flowers are white and in dense of ovoid head over 2.5 cm long. Peduncles are solitary (rarely 2-3 together) usually 2.5-5 cm leaf-opposed long. Calyx-limb is truncate. There is 1 cm long of infundibuliform corolla tube with the hairy mouth and five lobes, acute lanceolate. Flower with five stamens which are hairy filaments and ½-exserted anthers. Ripe fruits are white, smooth, glossy, and their size as a small egg, there are compressed ovoid pyrenes with concave-convex, and winged in the edge.

Uses: Leaf: infantile diarrhea; decoction with mustard as a favorite domestic remedy, relieve pain; expressed juice of leaves as externally applied to gout, cooling and externally in fever; boiled the leaves can applied in fever and headache. Root: emetic and laxative; decoction of roots.

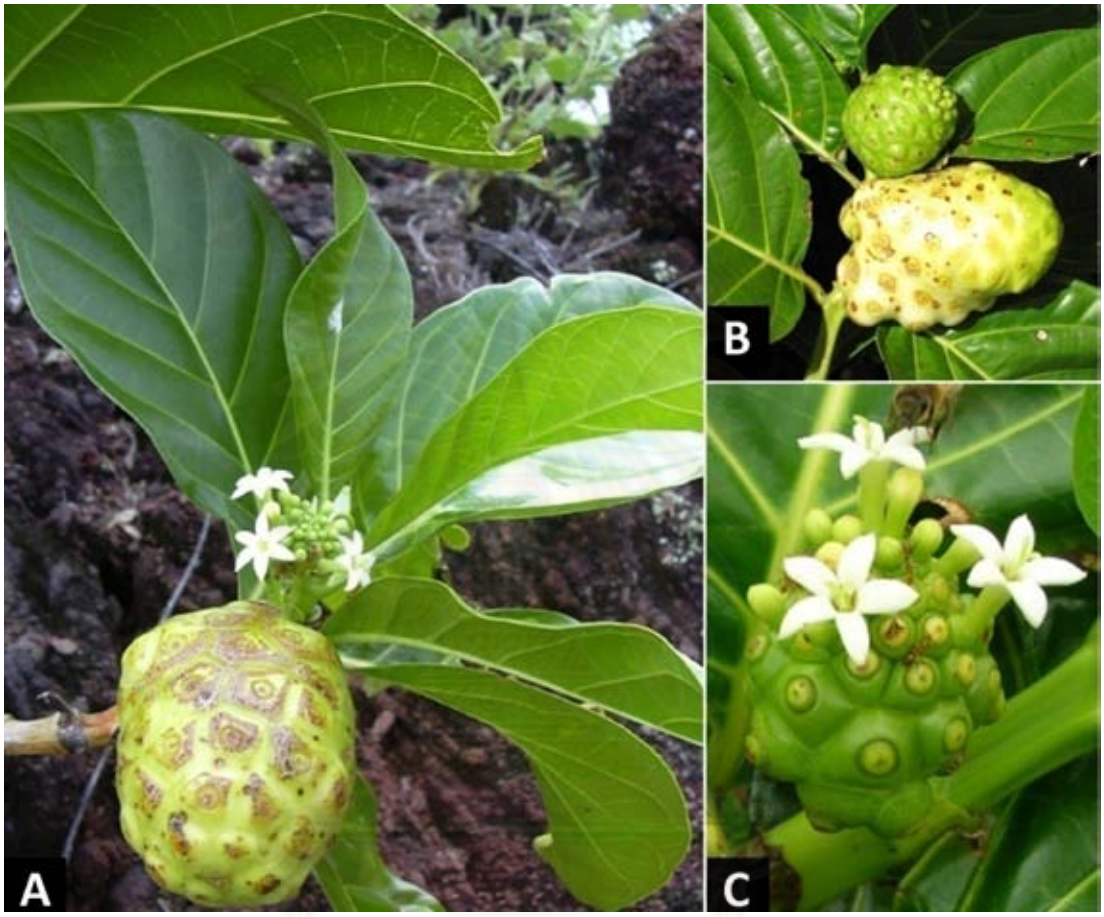

Figure 8 Photography of *Morinda citrifolia* L. [65]
A: Fruiting branch
B: Ripe fruit
C: Multiple fruit and flower

Figure 9 Whole plant of *Morinda citrifolia* L.
A: Fruiting branch
B: Floret with pistil and stamen
C: Multiple fruit

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2. ***Morinda elliptica*** **Ridl.** (**Figure 10-11**)

Vernacular names: Thailand: Yor thuean (Chumphon), Yor pa (Trang, Satun), Ka mu du (Malay-Narathiwat); **Malaysia:** Mengkudu jantan.

Distribution: From Malay Peninsula, Singapore, to Thailand, and Penang.

Observation: It is a small tree usually about 4-5 m. tall with white bark. Leaves are narrow-elliptic or oblanceolate shortly acuminate and long narrowed to base. The petioles are 1 cm long with 0.6 cm slender peduncles. Corolla-tubes are white cylindrical with 1-1.5 cm long of lobes sub-acute. Fruits are oblong head with green and hardly pulpy in 1-1.5 cm long.

Uses: Leaf: eaten to sharpen the appetite, and used in case of fever, headache, cholera, and diarrhea or dysentery. It’s the most medicinally used in Malay Peninsula.

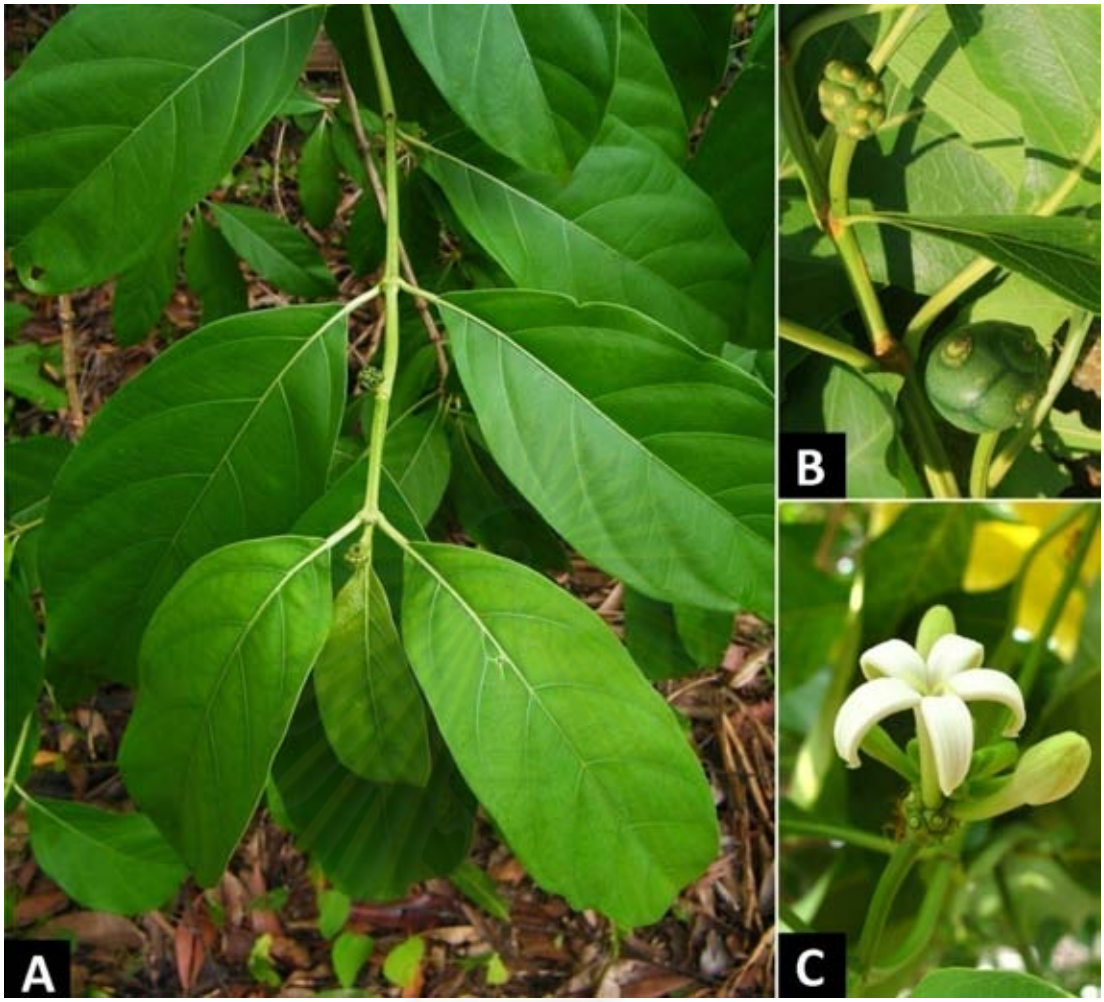

Figure 10 Photography of *Morinda elliptica* Ridl.
A: Fruiting branch
B: Multiple fruit
C: Flower

Figure 11 Whole plant of *Morinda elliptica* Ridl.
A: Fruiting branch
B: Floret with pistil and stamen
C: Multiple fruit
D: Ripe fruit

3. ***Morinda umbellata*** **L.** (**Figure 12-13**)

Synonyms: *Morinda scandens* Roxb. [66]

Vernacular names: Thailand: Yor yan (Peninsular); **Indo China**: Day dat, Doc vo, Khua mac; **Tamil**: Nuna, Surinji, Tanakku; **Sanskrit**: Daruharidra.

Distribution: From Malay Peninsula, Thailand to China and Australia.

Observation: It is a scrambling shrub or climbing by long, slender, sarmentose and divaricate brunches. Bark is gray, striate, long internodes, and young parts are puberulous. The leaves are rather small about 5-10 cm usually oblong lanceolate, but sometimes broadly oval, tapering or acute at base. There are shortly acuminate or acute, glabrous of leaves with dark green color. Leaves venation are finely reticulate and pellucid. The petiole is 6 mm and often twisted. The stipules are connate sheathing, membranous, and subpersistent. The few of flowers are together and their heads are smaller than 1.3 cm with globose stalked. The 4-10 peduncles are together in umbels at the terminal with short of calyx-limb and perfectly truncate. The corolla-tubes are very short, but 4 lobes are oblong-oval which longer than the tube. Head of fruits are small about 8 mm with smooth and scarlet lobulated.

Used: Leaf: used as decoction in cases of diarrhea and dysentery. Root and leaf: used as antidysenteric.

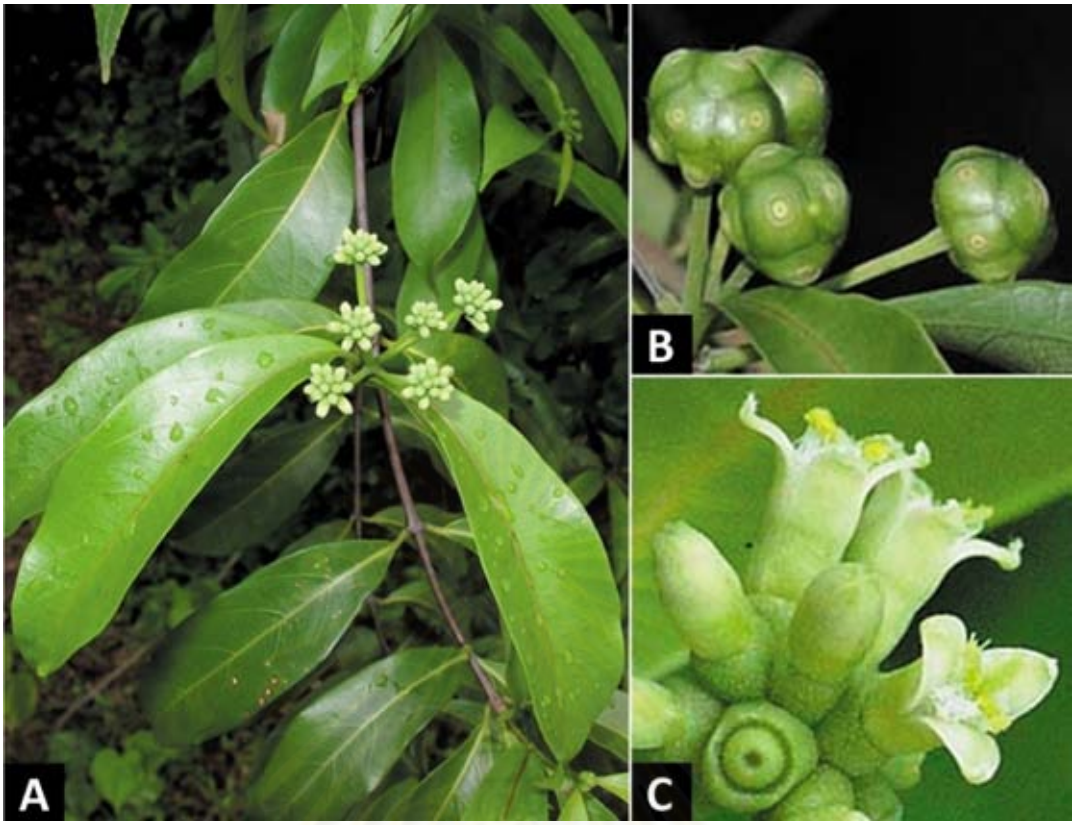

Figure 10 Photography of *Morinda umbellata* L. [67, 68]
A: Fruiting branch
B: Multiple fruit
C: Flower

Figure 13 Whole plant of *Morinda umbellata* L.
A: Fruiting branch
B: Petal with long hair
C: Multiple fruit
D: Ripe fruit

4. ***Morinda coreia*** **Ham.** (**Figure 14-15**)

Synonyms: *Morinda tinctoria* Roxb. [12], *Morinda exserta* Roxb. [69]

Vernacular names: Thailand: Yor pa (General), Salak pa, Salak luang (Northern), Khui (Phitsanulok), Khu (Karen-Kanchanaburi), Kho (Karen); **Myanmar**: Nibase; **Singapore**: Seinglaing; **Tamil**: Nuna, Molugu, Togaru; **Sanskrit**: Achchhuka.

Distribution: From upper and lower Myanmar, Bengal, Indonesia, Thailand to Malay Peninsula and India.

Observation: A middle-sized tree that usually pubescent or tomentose which spongy bark and deeply cracked grayish yellow. Leaves are not shining and shape as elliptic-obovate or lanceolate, blade 4-8 cm with narrowed into petiole 1.3-2.5 cm long. Solitary peduncles, axillary or leaf-opposed, and there are frequently in short trichotomous panicles at the end of branchlets. The flowers scented with 5-merous of corolla which are usually tomentose at the outside. There is 1.3-2 cm long of tubes with exserted or included of anthers. The syncarpium fruits are 2 cm diameter.

Uses: Root or stem: internally astringent and styptic; the infusion of dries boils is tonic as a bath. Root: stop vomiting in cholera; crushed in alcohol to provide a medicine. Leaf and root: treat ague; a decoction is prescribed and their poultice is applied to the spleen. Bark: astringent and febrifuge.

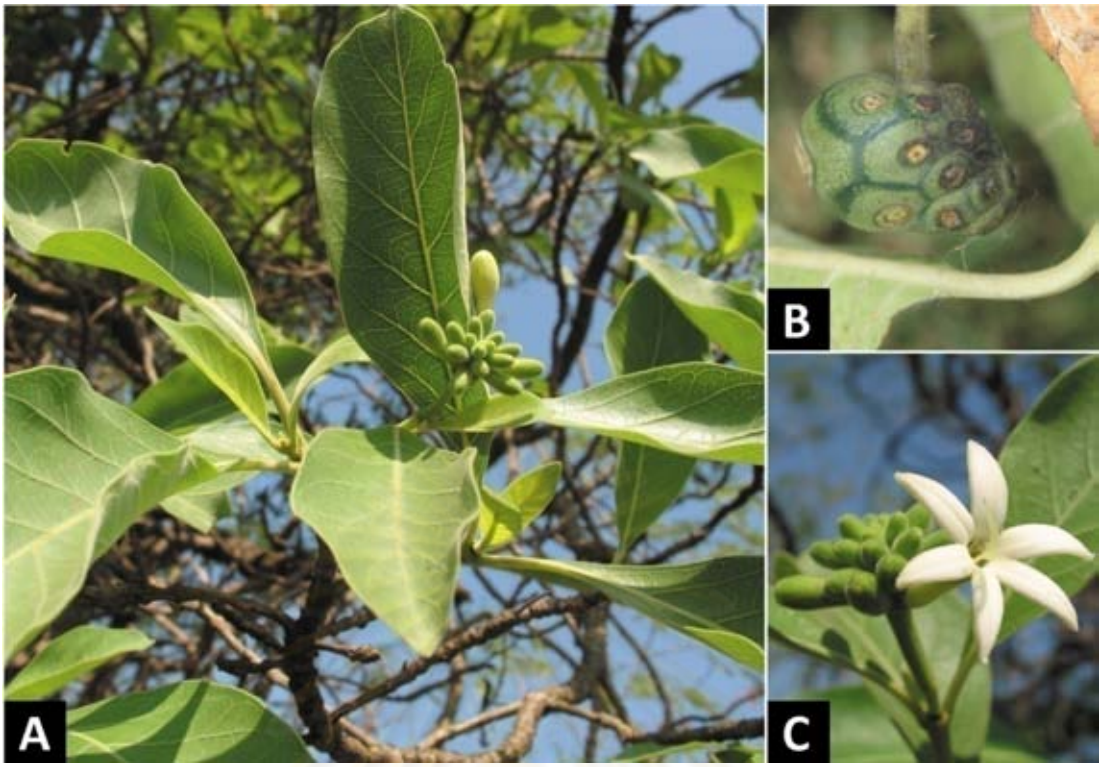

Figure 14 Photography of *Morinda coreia* Ham.
A: Fruiting branch
B: Multiple fruit
C: Flower

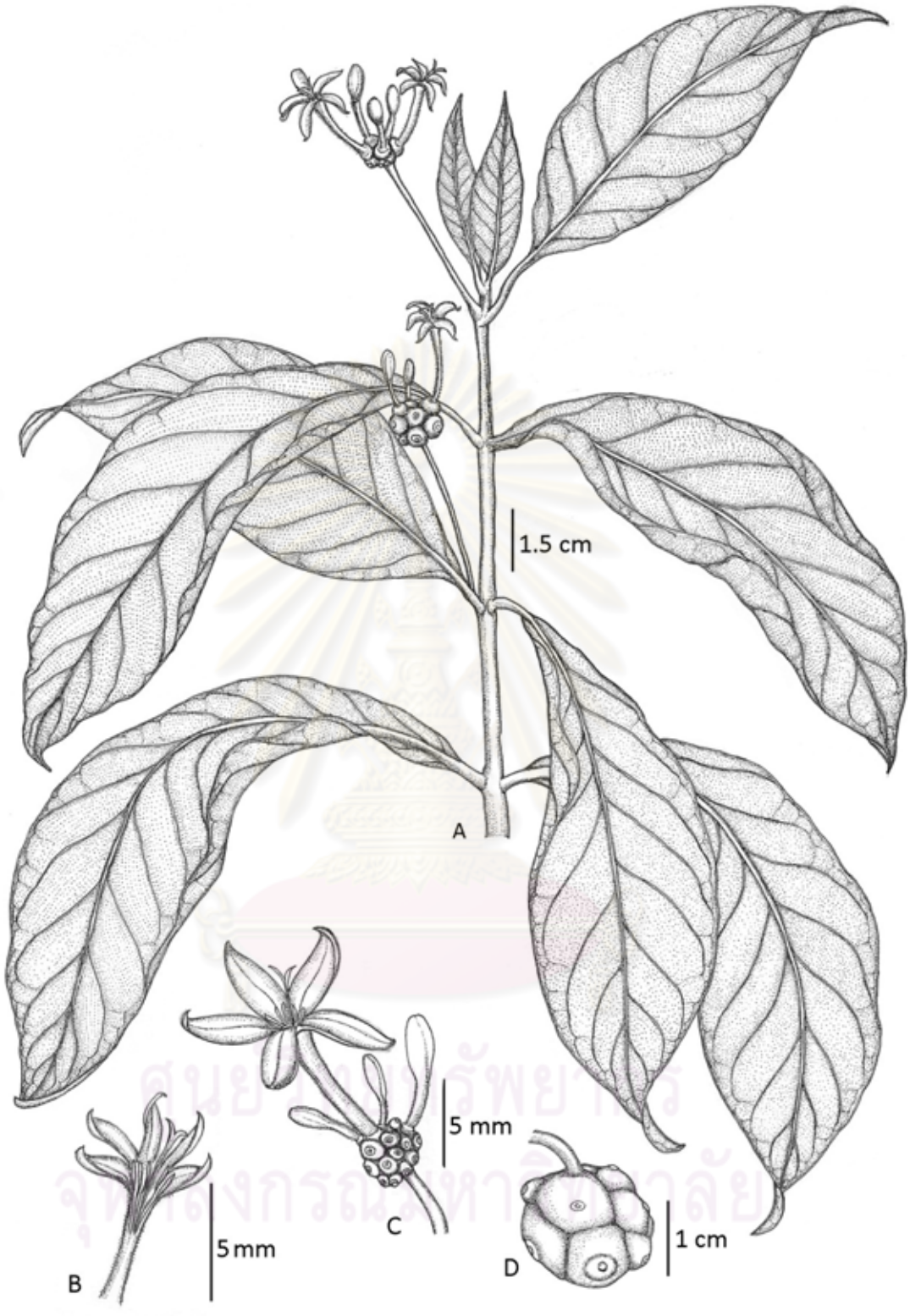

Figure 15 Whole plant of *Morinda coreia* Ham.
A: Fruiting branch
B: Floret with pistil and stamen
C: Multiple fruit
D: Ripe fruit

The results of microscopic analysis

The results of cross section of midrib and stomatal classification

The outline drawings of midrib cross sectional view and stomatal classification on ventral surface area of each species were shown in **Figure 16-19**. The ventral surface of *Morinda* leaves was composed of epidermal cell, stomata cell, and two subsidiary cells showing parallel to the long axis of the guard cells. The stomata structure of four investigated *Morinda* species can be classified as the paracytic or rubiaceous (parallel-celled) type. For midrib cross section of each species, the feature characteristics were found and described as below.

a) *M. citrifolia*: (**Figure 16**) The upper epidermis was absent of stoma cell, but the lower epidermis was composed of numerous paracytic stomatal cells type. The mesophyll was beneath of two palisade layer and consisted of various spongy cells containing chloroplasts. The midrib was composed of three to five collenchyma layer cells underneath the epidermis of both upper and lower epidermal cells. The parenchyma cell comprised raphide calcium oxalate crystals structure. The vascular bundles were arranged showing like a “C” turns up shaped.

b) *M. elliptica*: **Figure 17** showed the slightly wavy upper epidermis which the stoma cell was absent and lower epidermis which the paracytic stomatal cells type was shown. The mesophyll was composed of two palisade layers and various spongy cells containing chloroplasts. The midrib was composed of two to four layers of collenchyma underneath the epidermis of both upper and lower epidermal cells. The parenchyma cell comprised both raphide and rosette aggregate calcium oxalate crystal structures. The vascular bundles were arranged as a turned-up of C shape.

c) *M. umbellata*: **Figure 18** showed the upper epidermis of *M. umbellata* which was rather wavy cutineous than *M. elliptica* and also lacked the stoma cell. The mesophyll was beneath of two palisade layer and consisted of various spongy cells containing chloroplasts. The lower epidermis was composed of numerous stomata cells. The midrib was composed of three to five layers collenchyma underneath the epidermis of both upper and lower epidermal cells. The

parenchyma cell was not presented calcium oxalate crystal structure, but there were the group of fibers surrounding the C shaped of xylem tissue.

d) *M. coreia*: (**Figure 19**) The lower epidermis was also composed of stomatal cells but the upper epidermis was smooth and showed the absence of stoma cell. Moreover, there were covering with trichomes on both surfaces. The presence of double palisade layers and lots of spongy cells were remained in mesophyll. The midrib was composed of two to four layers collenchyma underneath the epidermis of both upper and lower epidermal cells. The parenchyma cells comprised raphide calcium oxalate crystal structures. The vascular bundles were arranged similarly to C shape turned-up.

According to the following characteristic of midrib cross section of each species, dichotomous key could be generated based on the presence/absence of calcium oxalate crystal structures (raphide crystal or rosette crystal), trichome, and fiber layer as **Figure 20**. Calcium oxalate crystal was found in other three species of *Morinda* except *M. umbellata* and the trichome was found only in *M. coreia*. Raphide calcium oxalate crystal was found only in *M. citrifolia* while both raphide and rosette calcium oxalate crystal types were found in *M. elliptica*.

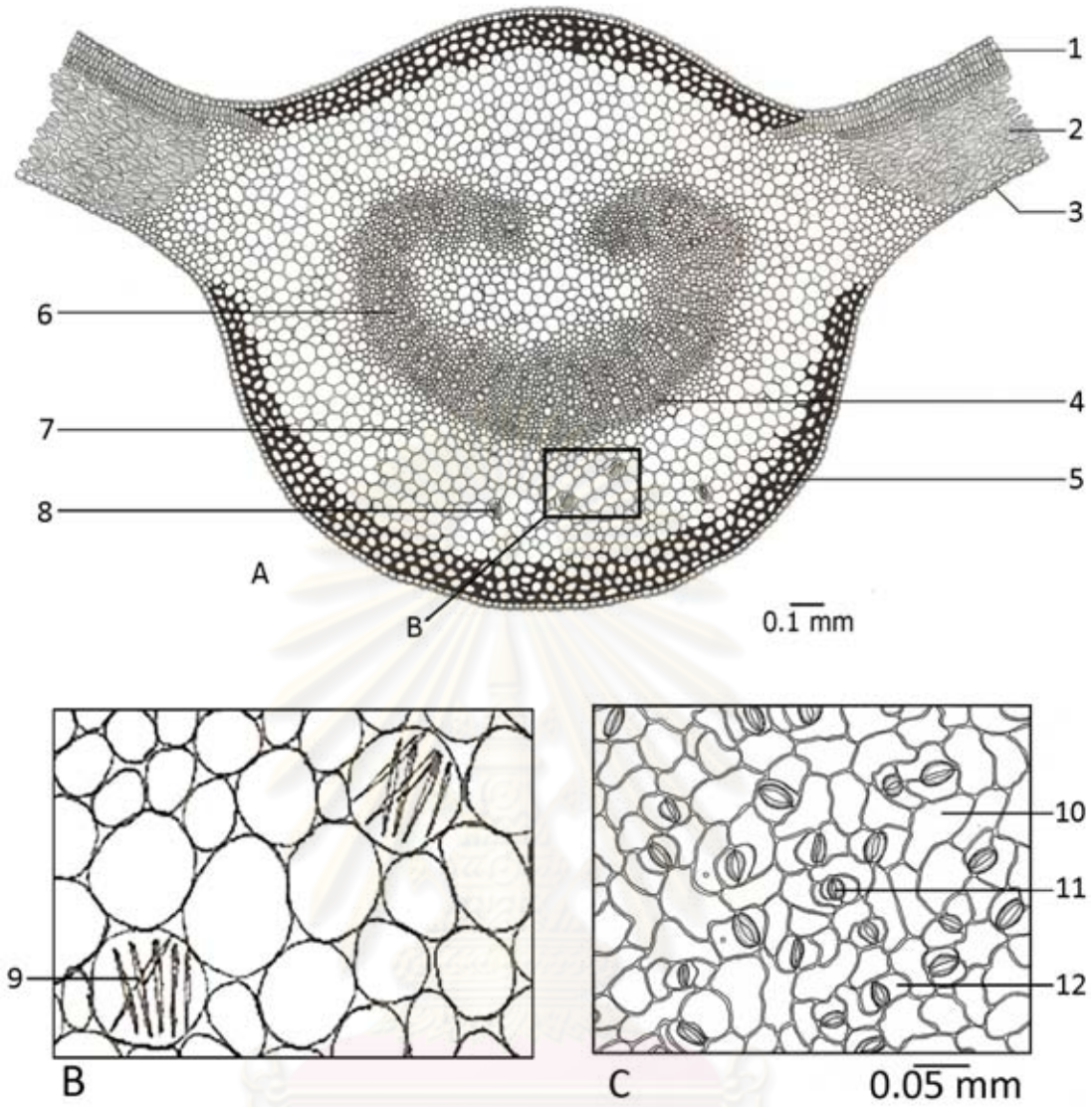

Figure 16 Cross section of midrib and ventral surface of leaf of *M. citrifolia* L.

A = Cross section of midrib: 1. Palisade cell, 2. Spongy cell, 3. Stoma, 4. Phloem tissue, 5. Collenchyma, 6. Xylem tissue, 7. Parenchyma, 8. Raphide crystal

B = Magnify of picture: 9. raphide crystal

C = Ventral surface area of leaf: 10. Epidermal cell, 11. Stoma, and 12. Subsidiary cell

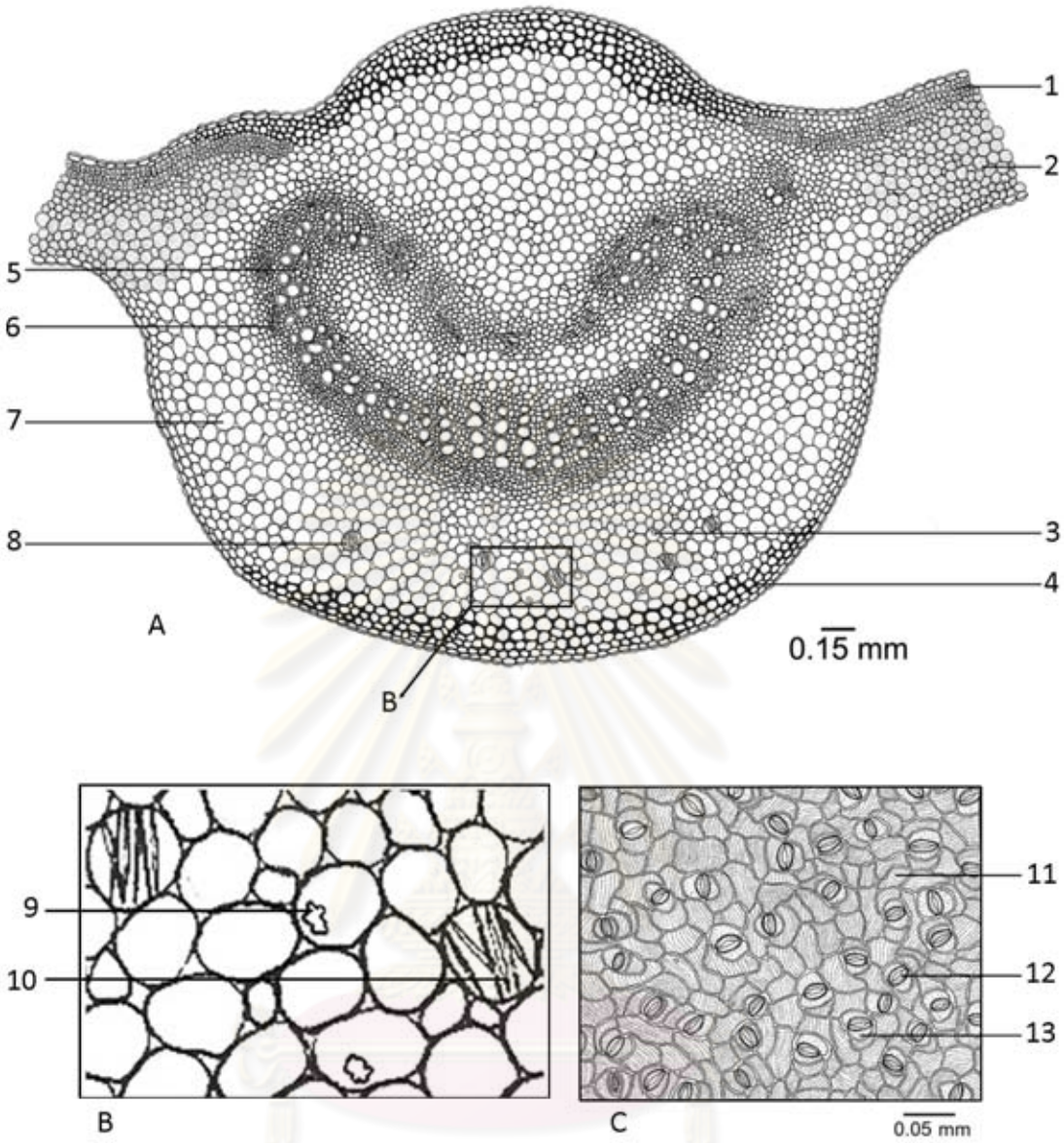

Figure 17 Cross section of midrib and ventral surface of leaf of *M. elliptica* Ridl.

A = Cross section of midrib: 1. Palisade cell, 2. Spongy cell, 3. Rosette aggregate crystal, 4. Collenchyma, 5. Xylem tissue, 6. Phloem tissue, 7. Parenchyma, 8. Raphide crystal

B = Magnify of picture: 9. Rosette aggregate crystal, 10. Raphide crystal

C = Ventral surface area of leaf: 11. Epidermal cell, 12. Stoma, and 13. Subsidiary cell

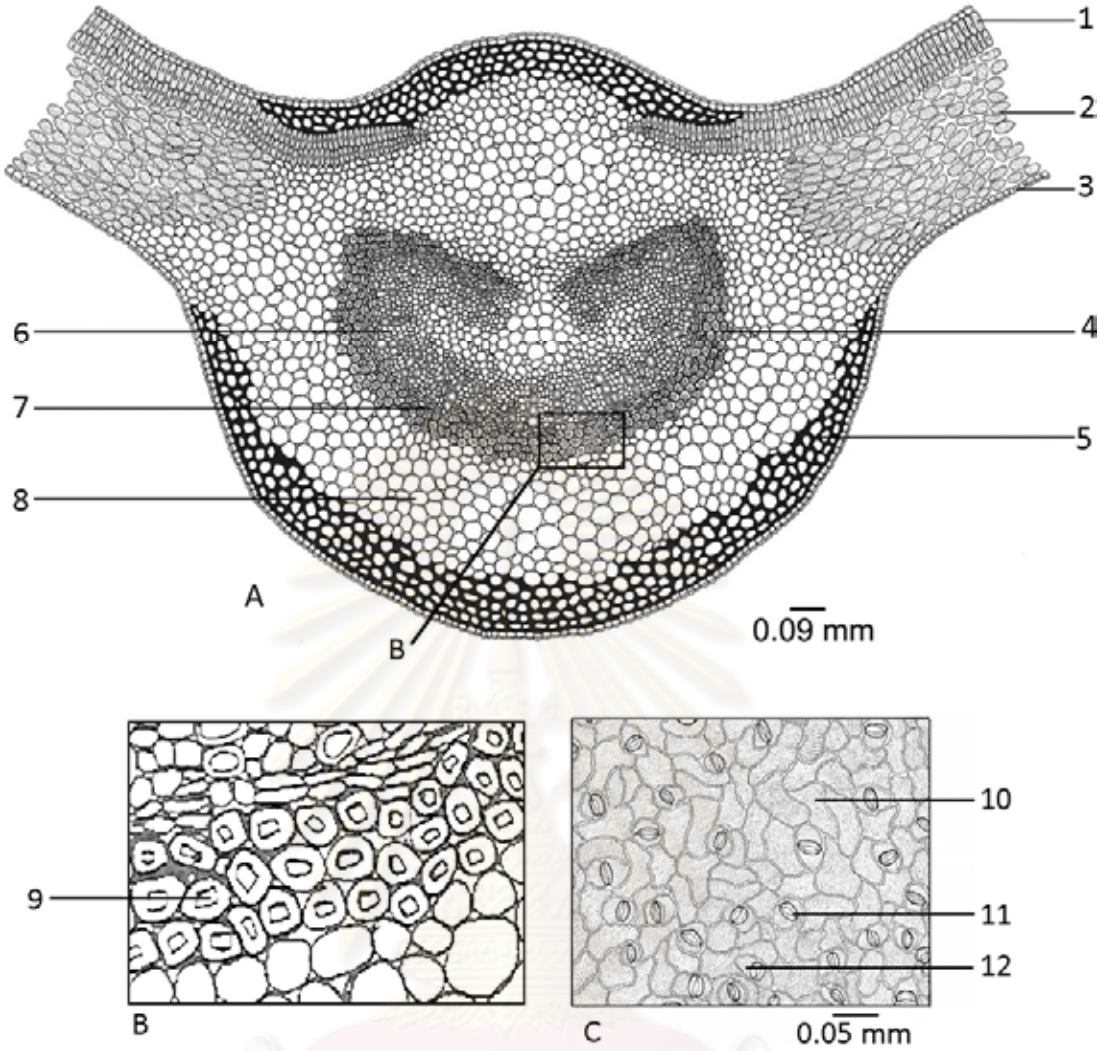

Figure 18 Cross section of midrib and ventral surface of leaf of *M. umbellata* L.

A = Cross section of midrib: 1. Palisade cell, 2. Spongy cell, 3. Stoma, 4. Group of fiber, 5. Collenchyma, 6. Xylem tissue, 7. Phloem tissue, 8. Parenchyma

B = Magnify of picture: 9. Group of fiber

C = Ventral surface area of leaf: 10. Epidermal cell, 11. Stoma, 12. Subsidiary cell

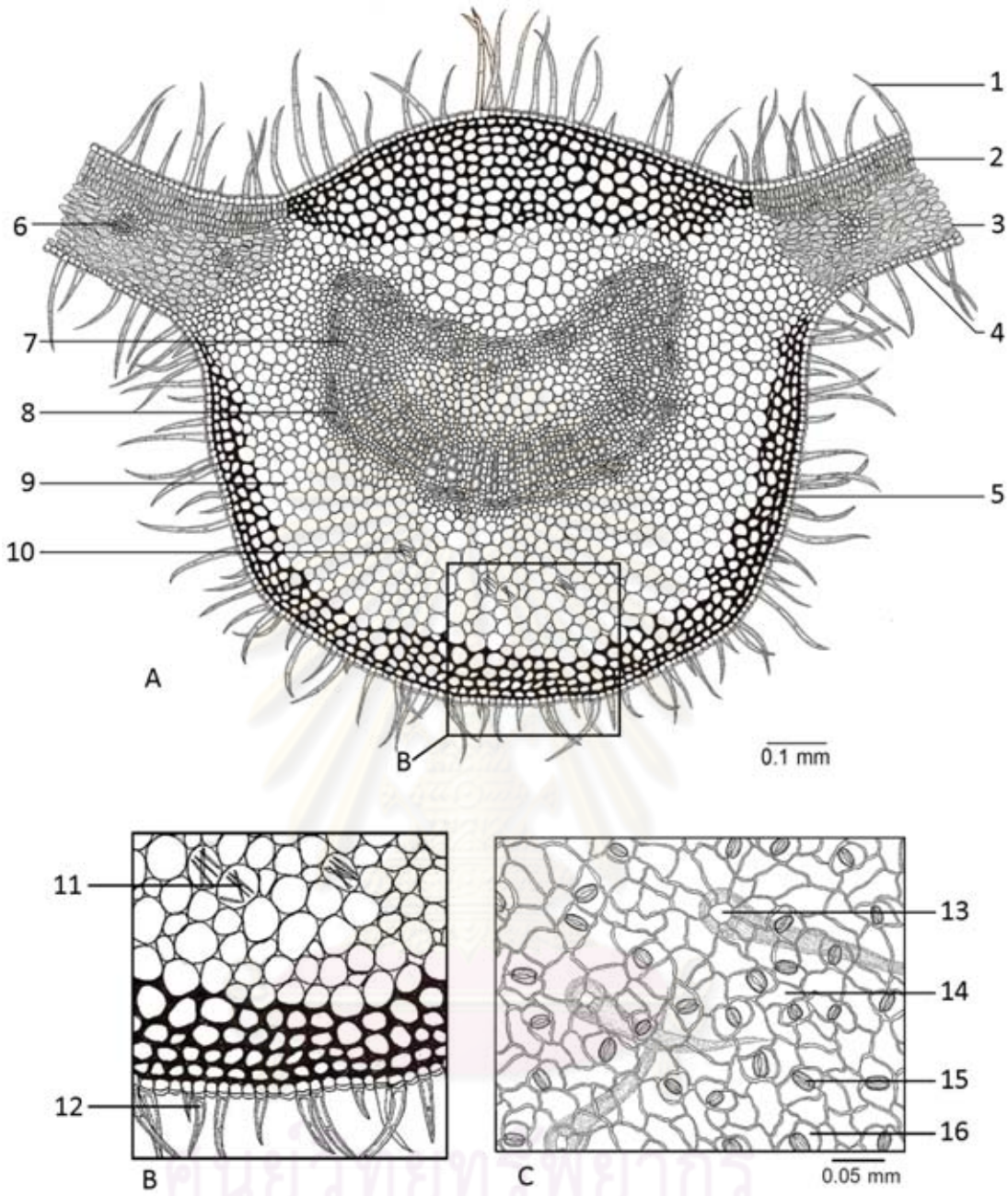

Figure 19 Cross section of midrib and ventral surface of leaf of *M. coreia* Ham.

A = Cross section of midrib: 1. Multicellular trichome, 2. Palisade cell, 3. Spongy cell, 4. Stoma, 5. Collenchyma, 6. Vascular bundle, 7. Xylem tissue, 8. Phloem tissue, 9. Parenchyma, 10. Raphide crystal

B = Magnify of picture: 11. Raphide crystal, 12. Multicellular trichome

C = Ventral surface area of leaf: 13. Cicatrix, 14. Epidermal cell, 15. Stoma, and 16. Subsidiary cell

Dichotomous key of the cross section of midrib in four investigated *Morinda* species.

A. Calcium oxalate crystal present, fiber layer absent
  B. Trichome present..... *M. coreia*
  BB. Trichome absent
    C. Raphide crystal present, rosette absent..... *M. citrifolia*
    CC. Raphide and rosette crystal present..... *M. elliptica*
AA. Calcium oxalate crystal absent, fiber layer present..... *M. umbellata*

Figure 20 Dichotomous key of four investigated *Morinda* species were generated based on the presence of calcium oxalate crystal structure and trichome from the midrib cross section.

The results of Leaf measurement

The constant number of leaf measurements which consisting of stomatal number, stomatal index, palisade ratio, cicatrix number and cicatrix index by microscopic analyses were shown as mean ±SD of each sample in **Table 7** and the raw data in each areas were shown in appendix A. The stomatal number and the palisade ratio of *M. citrifolia* were markedly lower than other species (mean ± SD = 181.33 ±9.78 and 7.17 ±0.53, respectively) whereas the stomatal number and the palisade ratio of *M. elliptica* showed higher value. However, the stomatal index showed no difference among four species. Based on the microscopic investigation, the result indicated that four species of *Morinda* have specific values of leaf measurement which were the important property for identification.

Table 7 Leaf measurement values of *Morinda* species (mean ±SD, n =30)

***Morinda* species**	**Sample no.**	**Stomatal number**	**Stomatal index**	**Palisade ratio**	**Cicatrix number**	**Cicatrix index**
M. citrifolia	1	181.47 ± 9.54	22.01 ± 0.77	7.16 ± 0.51	-	-
	2	183.20 ± 8.94	22.29 ± 0.96	7.20 ± 0.44	-	-
	3	179.33 ± 10.87	21.72 ± 0.88	7.18 ± 0.65	-	-
	avg.	**181.33 ± 9.78**	**22.01 ± 0.87**	**7.17 ± 0.53**	-	-
M. elliptica	1	349.07 ± 16.34	24.47 ± 0.94	17.48 ± 0.45	-	-
	2	348.00 ± 19.14	24.34 ± 1.07	18.28 ± 0.61	-	-
	3	345.73 ± 21.39	24.06 ± 1.01	17.58 ± 0.55	-	-
	avg.	**347.60 ± 18.96**	**24.29 ± 1.01**	**17.78 ± 0.54**	**-**	**-**
M. umbellata	1	265.60 ± 15.17	25.19 ± 1.10	13.37 ± 0.52	-	-
	2	264.27 ± 22.65	25.10 ± 1.33	13.46 ± 0.46	-	-
	3	266.27 ± 15.32	25.40 ± 1.16	13.51 ± 0.49	-	-
	avg.	**265.38 ± 17.71**	**25.23 ± 1.20**	**13.45 ± 0.49**	**-**	**-**
M. coreia	1	348.13 ± 18.72	27.69 ± 1.08	13.42 ± 0.50	29.60 ± 4.28	1.83 ± 0.26
	2	345.73 ± 17.19	27.80 ± 0.99	13.38 ± 0.37	28.40 ± 3.38	1.76 ± 0.20
	3	350.80 ± 13.21	28.16 ± 0.79	13.47 ± 0.45	28.67 ± 4.34	1.78 ± 0.26
	avg.	**348.22 ± 16.37**	**27.88 ± 0.95**	**13.42 ± 0.44**	**28.89 ± 4.00**	**1.79 ± 0.24**

avg = average and - = absent of cicatrix

The results of molecular analysis

The results of DNA extraction

The total genomic DNA was isolated from young leaves of all 42 samples using modified CTAB method as described in chapter 3. The genomic DNA was examined on 1.5% agarose gel electrophoresis after strained with ethidium bromide as shown in **Figure 21** and all illustrations of all specimens were shown in **Appendix C**. The extracted total DNA was then stored at -20 ºC for further use as templates in PCR amplification.

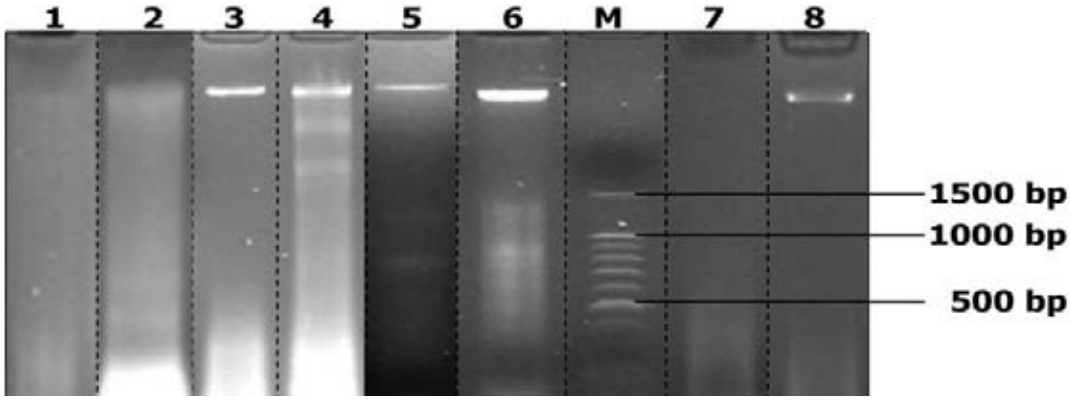

Figure 21 1.5% agarose gel electrophoresis of genomic DNA from some investigated *Morinda* species.
Lane M: 100 bp plus DNA Ladder
Lane 1-2: *M. citrifolia* from Bangkok and Phayao province
Lane 3-4: *M. elliptica* from Phatthalung province
Lane 7-8: *M. coreia* from Nong Khai province and Nakhon Ratchasima province

The results of PCR amplification on ITS region

Forty-two genomic DNA of *Morinda* species were used as PCR template for PCR amplification on the ITS regions by using two universal primers, ITS4 and ITS5. The PCR products were examined on 1.5% agarose gel electrophoresis compared with 100 bp DNA Ladder. An approximately 650 bp in length were obtained from four *Morinda* species as shown in **Figure 22**. For all illustrations of all PCR products are shown in **Appendix C**.

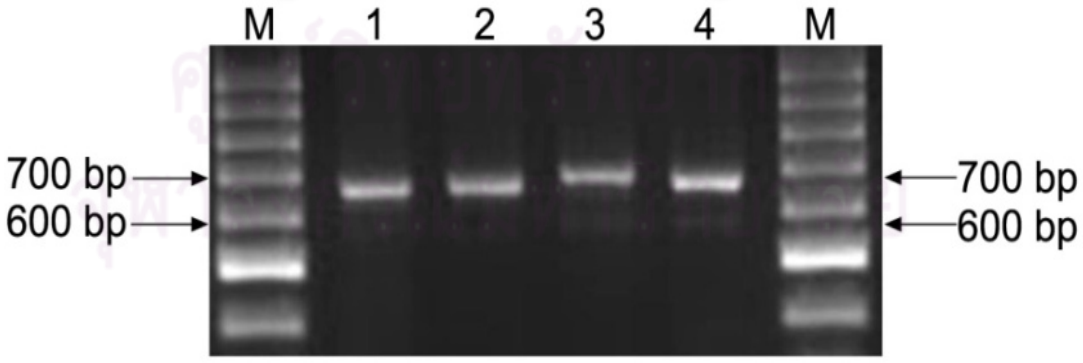

Figure 22 An approximately 650 bp in length of PCR products from the amplification on ITS region of four investigated *Morinda* species.
Lane M: 100 bp DNA Ladder
Lane 1: *M. citrifolia*
Lane 2: *M. elliptica*
Lane 3: *M. umbellata*
Lane 4: *M. coreia*

The results of PCR-Restriction Fragment Length Polymorphism (RFLP)

The PCR products were then digested with three restriction enzymes (*MseI*, *EcoRI* and *MspI*) following the instructions of the manufacturer (Fermentas), fractionated in 2.5% agarose gel electrophoresis, stained with ethidium bromide and then visualized under UV light and photographed. The size of fragments was compaired with 100 bp DNA ladder (Fermentas). The restriction patterns of each enzyme were shown in **Figure 23** and the restriction fragment size after digested with restriction enzymes was shown in **Table 8**.

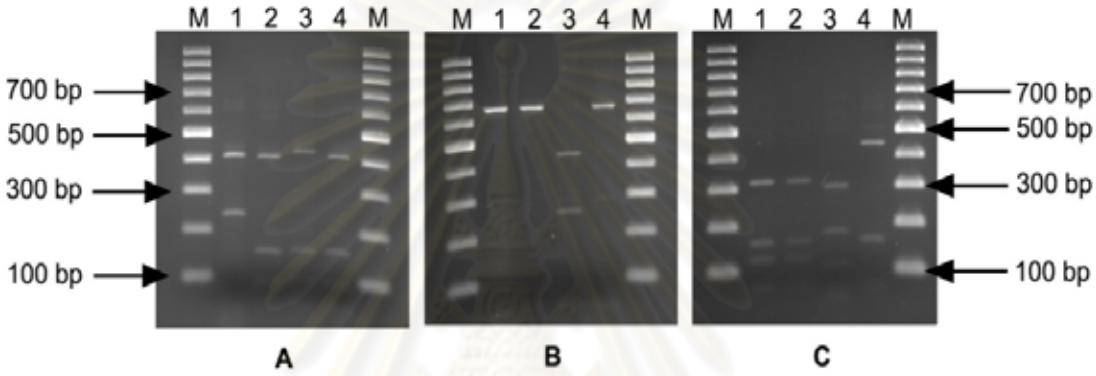

Figure 23 Restriction pattern of four investigated *Morinda* species digested with three restriction enzymes (**A**: *MseI*; **B**: *EcoRI*; and **C**: *MspI*).

Lane M: 100 bp molecular weight marker
Lane 1: *M. citrifolia*
Lane 2: *M. elliptica*
Lane 3: *M. umbellata*
Lane 4: *M. coreia*

Table 8 Restriction fragment size (base pair) of ITS region

***Morinda* species**	**Restriction Enzymes**		
	MseI	***EcoRI***	***MspI***
M. citrifolia	400, 250	650 (undigested)	300, 160, 140, 50
M. elliptica	400, 150, 100	650 (undigested)	300, 160, 140, 50
M. umbellata	400, 150, 100	400, 250	300, 170, 130, 50
M. coreia	400, 150, 100	650 (undigested)	450, 150, 50

The result of PCR product from *M. citrifolia* after digested with *MseI* showed the unique lower band of 250 bp while other three species showed the two lower bands of 150 and 100 bp (**Figure 23A and Table 8**). Only ITS region of *M. umbellata* has an *EcoRI* restriction site, cleavage of PCR product with *EcoRI* obtained an approximately 400 and 250 bp fragments (**Figure 23B and Table 8**) whereas the other three species lacked of *EcoRI* restriction site. Therefore, the banding pattern of the others species showed only one undigested banding pattern of 650 bp as a single band in **Figure 23B**. When digested four species of *Morinda* with *MspI*, *Morinda* species can be divided into three groups. Firstly, the PCR products digestion of *M. citrifolia* and *M. elliptica* had the four similar restriction patterns of approximately 300, 160, 140, and 50 bp (**Figure 23 and Table 8**). Secondly, only *M. coreia* presented the upper banding pattern of an approximately 450 bp while others species showed the same pattern at approximately 300 bp. Lastly, restriction patterns of *M. umbellata* from *MspI* restriction enzyme obtained an approximately 300, 170, 130 and 50 bp as shown in **Figure 23C**.

CHAPTER V

DISCUSSION AND CONCLUSION

The increase in usage of medicinal plant has highlighted the necessity for assurances of safety and administration of plants in the prevention and treatment of human diseases. Thus, identification is also a great importance to ensure highest effectiveness. Based on the morphological characteristics, macroscopic identification is to observe the entire plant or crude drug such as shape, size, colour, texture and odour which require a highly skill or well trained individual whereas microscopic identification is to observe the smaller section of plant such as leaf measurement index which requires a sample preparation and laboratory instrument. Microscopic and molecular analyses can be applied for identification of four investigated *Morinda* species.

The results of stomatal classification of each investigated *Morinda* species showed that they have two subsidiary cells which their common wall parallel to the long axis of the guard cells. The type of stomata of investigated *Morinda* species were classified as paracytic type or rubiaceous type (parallel-celled type), consisting of guard cells and parallel subsidiary cells which the unique characteristic of family Rubiaceae [19]. Based on the cross section of midrib, each investigated *Morinda* species have their prominent characteristics which are distinguishable and can culminate to generate in dichotomous key as shown in **Figure 20**. Based on the microscopic investigation, the results indicated that four species of *Morinda* have specific values which were the important property for identification. However, the stomata index of four species was quite similar. Therefore, the authentic samples of these species from different location would be carried out for its quality controls. The summary of leaf measurements of these *Morinda* species can inform the standardization or quality control of each species as shown in **Table 9.**

Table 9 Summary of leaf measurements of four investigated *Morinda* species (Mean ±SD)

Species	Stomatal Number	Stomatal Index	Palisade Ratio	Cicatrix Number	Cicatrix Index
M. citrifolia	181.33 ± 9.78	22.01 ±0.87	7.17 ±0.53	-	-
M. elliptica	347.60 ±18.96	24.29 ±1.01	17.78 ±0.54	-	-
M. umbellata	265.38 ±17.71	25.23 ±1.20	13.45 ±0.49	-	-
M. coreia	348.22 ±16.37	27.88 ±0.95	13.42 ±0.44	28.89 ±4.00	1.79 ±0.24

- = absent cicatrix

Leaf measurement index was previous used for identification of *Gisekia pharnacioides* from other closely related species [70]. In addition, microscopic identification can use for identification of four species of Folium syringae [71]. Based on the slight difference of morphological characteristics, other traditional methods were mainly used such as high performance liquid chromatography (HPLC) profiling for distinguishing medicinal plant's compounds from adulterants [72, 73]. However, there were some factors that influenced the use of these methods such as the amounts of samples, the stability of chemical constituents and the variable sources of samples. As the result, DNA based techniques have been developed for these limitations. DNA markers are useful for the authentication and standardization of medicinal plant [11]. The ITS region has been widely used in plant molecular phylogeny due to the relatively small size (< 700 bp) and high copy number of ribosomal DNA gene enable easy amplification even from small quantities of DNA or from herbarium materials and due to a high degree of variation even between closely related species made the ITS region was an interesting for phylogenetic investigation [74]. The nuclear DNA ITS region has also been used successfully in various studies as genetic markers for plant identification. ITS region was widely used for identification of Traditional Chinese medicine such as *Angelica sinensis* authentication [75]. PCR-RFLP technique using restriction endonucleases was applied for authentication of *Panax* species which was used as Chinese traditional medicine [76]. Therefore, other genes and non-coding region can also be use as molecular markers for authentication [77]. Compared with the molecular markers in chloroplast, the nuclear DNA based on ITS region is more widely prospect in authentication of medicinal plants. PCR-RFLP is a rapid technique that is based on the detection of restriction pattern which the data was utilized as a very useful marker to identify the Menispermaceae plants that are prescribed in Thai

Folk Medicines [78]. This technique have been wildly applied to DNA marker for discrimination in many medicinal plants [61] and also including other group such as fungi detection in fruits [79] because it is an inexpensive, reliable, repeatable, relative simple, and provides effective and accurate by means of identification [80].

However, the molecular marker of ITS region in *Morinda* species was still limited at the species level. In this present study, on the basis of two universal primers, ITS PCR-RFLP method using restriction enzymes *MseI*, *EcoRI* and *MspI* was established as a convenient and efficient method for distinguish four investigated *Morinda* species: *M. citrifolia, M. umbellata, M. elliptica*, and *M. coreia*. The distance between the restriction sites of restriction enzymes were individuals on the length of the fragmentation of each species and the digested PCR products revealed polymorphisms after separation on agarose gel. In the case of *MseI* restriction enzyme, PCR amplification of ITS gene could discriminate *M. citrifolia* from the others *Morinda* species because of the individual lower banding patterns on agarose gel. In the *EcoRI* digestion, the PCR product of *M. umbellata* showed two fragments, whereas another three *Morinda* species showed undigested fragments as a single band. From the results of digestion with *MspI* restriction enzyme, PCR products fragmentation can be used to distinguish among *M. coreia* from other three *Morinda* species including of *M. elliptica*. By the result, restriction enzyme *MseI*, *EcoRI*, and *MspI* can be used cleavage for discriminate *M. citrifolia, M. umbellata*, and *M. coreia* to *M. elliptica* respectively.

In conclusion, authentication of medicinal plants is important to be developed for more reliability while the biological activities are well known according to chemical constituents and pharmacological studies. The usage of plants without the proper identification or misidentification can cause a risk for practical applications in both agriculture and medicine because of their side-effects and toxicity. Hence, analyses by microscopic and molecular methods are successful as powerful tools for identification of investigated *Morinda* species in Thailand. The characteristics of leaf and of leaf measurement as well as ITS PCR-RFLP markers established in this study will provide an important tool for quality control and standardization for further study and can be effectively used together as an important role in species identifying not only investigated *Morinda* species but also another plant species.

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APPENDICES

APPENDIX A

The raw data of leaf measurement

Table 10 Stomatal number, stomatal index and palisade ratio of *M. citrifolia*; sample collected from College of Public Health Sciences, Chulalongkorn University, Bangkok

Area number	Number of epidermal cells	Stomatal number	Stomatal index	Palisade cell	
				Number beneath four epidermal cells	Palisade ratio
1	668	184	21.60	31	7.75
2	624	172	21.61	33	8.25
3	636	184	22.44	27	6.75
4	672	192	22.22	32	8.00
5	652	188	22.38	29	7.25
6	640	176	21.57	28	7.00
7	656	176	21.15	33	8.25
8	652	192	22.75	26	6.50
9	628	192	23.41	29	7.25
10	620	184	22.89	26	6.50
11	632	188	22.93	30	7.50
12	612	168	21.54	26	6.50
13	624	168	21.21	27	6.75
14	644	196	23.33	29	7.25
15	632	196	23.67	30	7.50
16	676	192	22.12	30	7.50
17	672	192	22.22	27	6.75
18	652	188	22.38	26	6.50
19	660	180	21.43	27	6.75
20	648	180	21.74	27	6.75
21	628	172	21.50	30	7.50
22	644	180	21.84	31	7.75
23	620	168	21.32	29	7.25
24	644	172	21.08	27	6.75
25	636	172	21.29	27	6.75
26	648	168	20.59	30	7.50
27	620	176	22.11	28	7.00
28	632	188	22.93	29	7.25
29	692	192	21.72	27	6.75
30	620	168	21.32	28	7.00
	Mean	**181.47**	**22.01**	**Mean**	**7.16**
	S.D.	**9.54**	**0.77**	**S.D.**	**0.51**

Table 11 Stomatal number, stomatal index and palisade ratio of *M. citrifolia*; sample collected from Chiangkham district, Phayao province

Area number	Number of epidermal cells	Stomatal number	Stomatal index	Palisade cell	
				Number beneath four epidermal cells	Palisade ratio
1	652	188	22.38	29	7.25
2	612	192	23.88	30	7.50
3	632	184	22.55	29	7.25
4	616	180	22.61	31	7.75
5	640	192	23.08	32	8.00
6	664	188	22.07	31	7.75
7	652	184	22.01	28	7.00
8	624	184	22.77	30	7.50
9	616	188	23.38	29	7.25
10	668	172	20.48	30	7.50
11	640	192	23.08	26	6.50
12	644	176	21.46	26	6.50
13	612	180	22.73	32	8.00
14	668	188	21.96	28	7.00
15	616	196	24.14	27	6.75
16	676	180	21.03	30	7.50
17	624	172	21.61	26	6.50
18	632	180	22.17	26	6.50
19	652	200	23.47	29	7.25
20	612	172	21.94	31	7.75
21	668	192	22.33	28	7.00
22	616	180	22.61	27	6.75
23	632	168	21.00	27	6.75
24	628	168	21.11	28	7.00
25	664	176	20.95	31	7.75
26	624	176	22.00	28	7.00
27	644	200	23.70	29	7.25
28	648	176	21.36	29	7.25
29	628	192	23.41	29	7.25
30	656	180	21.53	28	7.00
	Mean	**183.20**	**22.29**	**Mean**	**7.20**
	S.D.	**8.94**	**0.96**	**S.D.**	**0.44**

Table 12 Stomatal number, stomatal index and palisade ratio of *M. citrifolia*; sample collected from Yasothon province

Area number	Number of epidermal cells	Stomatal number	Stomatal index	Palisade cell	
				Number beneath four epidermal cells	Palisade ratio
1	688	184	21.10	28	7.00
2	656	180	21.53	29	7.25
3	672	184	21.50	28	7.00
4	688	208	23.21	27	6.75
5	672	180	21.13	25	6.25
6	652	204	23.83	24	6.00
7	668	180	21.23	29	7.25
8	696	204	22.67	26	6.50
9	636	168	20.90	28	7.00
10	624	168	21.21	29	7.25
11	692	176	20.28	29	7.25
12	640	168	20.79	34	8.50
13	632	188	22.93	28	7.00
14	668	172	20.48	26	6.50
15	652	184	22.01	31	7.75
16	628	168	21.11	28	7.00
17	616	180	22.61	30	7.50
18	640	184	22.33	31	7.75
19	628	172	21.50	32	8.00
20	628	168	21.11	33	8.25
21	620	172	21.72	33	8.25
22	612	168	21.54	24	6.00
23	628	176	21.89	30	7.50
24	644	172	21.08	29	7.25
25	648	176	21.36	33	8.25
26	624	180	22.39	26	6.50
27	620	188	23.27	29	7.25
28	648	172	20.98	28	7.00
29	632	172	21.39	27	6.75
30	636	184	22.44	28	7.00
	Mean	**179.33**	**21.72**	**Mean**	**7.18**
	S.D.	**10.87**	**0.88**	**S.D.**	**0.65**

Table 13 Stomatal number, stomatal index and palisade ratio of *M. elliptica*; sample collected from Bohploy district, Kanchanaburi province

Area number	Number of epidermal cells	Stomatal number	Stomatal index	Palisade cell	
				Number beneath four epidermal cells	Palisade ratio
1	1120	348	23.71	69	17.25
2	1080	352	24.58	68	17.00
3	1056	376	26.26	68	17.00
4	1072	376	25.97	66	16.50
5	1112	368	24.86	71	17.75
6	1140	360	24.00	71	17.75
7	1108	360	24.52	71	17.75
8	1080	364	25.21	69	17.25
9	1060	340	24.29	71	17.75
10	1040	336	24.42	69	17.25
11	1076	348	24.44	74	18.50
12	1136	352	23.66	70	17.50
13	1096	328	23.03	70	17.50
14	1044	340	24.57	71	17.75
15	1068	336	23.93	70	17.50
16	1036	360	25.79	69	17.25
17	1040	320	23.53	69	17.25
18	1032	344	25.00	67	16.75
19	1048	340	24.50	72	18.00
20	1100	336	23.40	69	17.25
21	1116	356	24.18	70	17.50
22	1048	352	25.14	70	17.50
23	1060	320	23.19	70	17.50
24	1044	320	23.46	70	17.50
25	1128	364	24.40	69	17.25
26	1040	364	25.93	69	17.25
27	1080	344	24.16	73	18.25
28	1012	364	26.45	69	17.25
29	1108	332	23.06	74	18.50
30	1148	372	24.47	69	17.25
	Mean	**349.07**	**24.47**	**Mean**	**17.48**
	S.D.	**16.34**	**0.94**	**S.D.**	**0.45**

Table 14 Stomatal number, stomatal index and palisade ratio of *M. elliptica*; sample collected from Yasothon province

Area number	Number of epidermal cells	Stomatal number	Stomatal index	Palisade cell	
				Number beneath four epidermal cells	Palisade ratio
1	1048	364	25.78	74	18.50
2	1092	376	25.61	74	18.50
3	1080	364	25.21	78	19.50
4	1052	336	24.21	71	17.75
5	1052	340	24.43	72	18.00
6	1044	332	24.13	70	17.50
7	1060	348	24.72	70	17.50
8	1096	388	26.15	70	17.50
9	1100	372	25.27	69	17.25
10	1088	364	25.07	75	18.75
11	1080	360	25.00	70	17.50
12	1108	360	24.52	75	18.75
13	1052	352	25.07	72	18.00
14	1048	384	26.82	72	18.00
15	1104	368	25.00	75	18.75
16	1080	348	24.37	71	17.75
17	1088	344	24.02	72	18.00
18	1132	352	23.72	77	19.25
19	1084	320	22.79	76	19.00
20	1064	336	24.00	76	19.00
21	1060	324	23.41	71	17.75
22	1108	348	23.90	71	17.75
23	1048	328	23.84	75	18.75
24	1108	320	22.41	75	18.75
25	1048	328	23.84	75	18.75
26	1132	340	23.10	76	19.00
27	1100	320	22.54	74	18.50
28	1104	332	23.12	71	17.75
29	1112	336	23.20	73	18.25
30	1068	356	25.00	74	18.50
	Mean	**348.00**	**24.34**	**Mean**	**18.28**
	S.D.	**19.14**	**1.07**	**S.D.**	**0.61**

Table 15 Stomatal number, stomatal index and palisade ratio of *M. elliptica*; sample collected from The Sireerukachart Garden, Mahidol University, Salaya Campus, Nakhon Pathom province

Area number	Number of epidermal cells	Stomatal number	Stomatal index	Palisade cell	
				Number beneath four epidermal cells	Palisade ratio
1	1048	336	24.28	69	17.25
2	1128	376	25.00	70	17.50
3	1100	376	25.47	71	17.75
4	1100	332	23.18	74	18.5
5	1136	344	23.24	71	17.75
6	1132	360	24.13	73	18.25
7	1104	324	22.69	73	18.25
8	1072	360	25.14	70	17.50
9	1084	320	22.79	72	18.00
10	1056	376	26.26	70	17.50
11	1064	352	24.86	68	17.00
12	1100	376	25.47	69	17.25
13	1140	388	25.39	69	17.25
14	1132	388	25.53	72	18.00
15	1148	332	22.43	70	17.50
16	1128	356	23.99	75	18.75
17	1096	352	24.31	69	17.25
18	1092	332	23.31	71	17.75
19	1040	328	23.98	67	16.75
20	1108	348	23.90	75	18.75
21	1088	328	23.16	69	17.25
22	1088	320	22.73	71	17.75
23	1056	320	23.26	68	17.00
24	1072	320	22.99	70	17.50
25	1068	336	23.93	68	17.00
26	1064	328	23.56	72	18.00
27	1080	344	24.16	70	17.50
28	1036	324	23.82	68	17.00
29	1068	356	25.00	67	16.75
30	1084	340	23.88	68	17.00
	Mean	**345.73**	**24.06**	**Mean**	**17.58**
	S.D.	**21.39**	**1.01**	**S.D.**	**0.55**

Table 16 Stomatal number, stomatal index and palisade ratio of *M. umbellata*; sample collected from The Prince of Songkla University, Songkhla province

Area number	Number of epidermal cells	Stomatal number	Stomatal index	Palisade cell	
				Number beneath four epidermal cells	Palisade ratio
1	848	256	23.19	53	13.25
2	736	260	26.10	55	13.75
3	820	292	26.26	52	13.00
4	828	292	26.07	55	13.75
5	772	260	25.19	57	14.25
6	736	268	26.69	56	14.00
7	840	296	26.06	56	14.00
8	776	280	26.52	52	13.00
9	748	260	25.79	54	13.50
10	764	240	23.90	51	12.75
11	784	260	24.90	51	12.75
12	808	296	26.81	52	13.00
13	804	256	24.15	57	14.25
14	836	280	25.09	51	12.75
15	756	276	26.74	56	14.00
16	764	268	25.97	54	13.50
17	776	252	24.51	51	12.75
18	780	240	23.53	55	13.75
19	804	272	25.28	51	12.75
20	824	248	23.13	55	13.75
21	836	268	24.28	55	13.75
22	760	264	25.78	53	13.25
23	740	264	26.29	54	13.50
24	776	240	23.62	52	13.00
25	824	268	24.54	50	12.50
26	796	260	24.62	54	13.50
27	788	260	24.81	51	12.75
28	752	264	25.98	52	13.00
29	820	264	24.35	56	14.00
30	772	264	25.48	53	13.25
	Mean	**265.60**	**25.19**	**Mean**	**13.37**
	S.D.	**15.17**	**1.10**	**S.D.**	**0.52**

Table 17 Stomatal number, stomatal index and palisade ratio of *M. umbellata*; sample collected from The Prince of Songkla University, Songkhla province

Area number	Number of epidermal cells	Stomatal number	Stomatal index	Palisade cell	
				Number beneath four epidermal cells	Palisade ratio
1	772	228	22.80	56	14.00
2	736	244	24.90	55	13.75
3	768	232	23.20	52	13.00
4	816	268	24.72	53	13.25
5	784	224	22.22	51	12.75
6	752	248	24.80	52	13.00
7	796	232	22.57	53	13.25
8	804	272	25.28	52	13.00
9	836	292	25.89	54	13.50
10	792	296	27.21	52	13.00
11	832	296	26.24	55	13.75
12	820	272	24.91	51	12.75
13	764	260	25.39	55	13.75
14	764	256	25.10	55	13.75
15	712	236	24.89	51	12.75
16	716	264	26.94	56	14.00
17	772	228	22.80	54	13.50
18	780	248	24.12	52	13.00
19	840	292	25.80	55	13.75
20	804	272	25.28	56	14.00
21	836	276	24.82	56	14.00
22	828	300	26.60	57	14.25
23	772	284	26.89	55	13.75
24	788	280	26.22	56	14.00
25	828	272	24.73	53	13.25
26	824	280	25.36	56	14.00
27	792	288	26.67	55	13.75
28	748	260	25.79	52	13.00
29	764	268	25.97	52	13.00
30	788	260	24.81	53	13.25
	Mean	**264.27**	**25.10**	**Mean**	**13.46**
	S.D.	**22.65**	**1.33**	**S.D.**	**0.46**

Table 18 Stomatal number, stomatal index and palisade ratio of *M. umbellata*; sample collected from The Prince of Songkla University, Songkhla province

Area number	Number of epidermal cells	Stomatal number	Stomatal index	Palisade cell	
				Number beneath four epidermal cells	Palisade ratio
1	792	272	25.56	51	12.75
2	744	248	25.00	55	13.75
3	808	280	25.74	52	13.00
4	756	260	25.59	53	13.25
5	796	276	25.75	51	12.75
6	764	280	26.82	57	14.25
7	744	260	25.90	53	13.25
8	776	284	26.79	56	14.00
9	740	272	26.88	53	13.25
10	768	252	24.71	55	13.75
11	792	264	25.00	56	14.00
12	776	284	26.79	53	13.25
13	812	240	22.81	54	13.50
14	784	264	25.19	57	14.25
15	804	280	25.83	51	12.75
16	772	284	26.89	51	12.75
17	780	252	24.42	54	13.50
18	824	268	24.54	53	13.25
19	824	280	25.36	57	14.25
20	776	236	23.32	53	13.25
21	780	256	24.71	56	14.00
22	764	260	25.39	53	13.25
23	792	248	23.85	53	13.25
24	756	248	24.70	53	13.25
25	788	252	24.23	57	14.25
26	760	280	26.92	56	14.00
27	812	252	23.68	55	13.75
28	796	284	26.30	52	13.00
29	788	288	26.77	56	14.00
30	784	284	26.59	55	13.75
	Mean	**266.27**	**25.40**	**Mean**	**13.51**
	S.D.	**15.32**	**1.16**	**S.D.**	**0.49**

Table 19 Stomatal number, stomatal index, palisade ratio, cicatrix number and cicatrix index of *M. coreia*; sample collected from The Sireerukachart Garden, Mahidol University, Salaya Campus, Nakhon Pathom province

Area number	Stomatal cell			Palisade cell		Cicatrix cell		
	Number of epidermal cells	Stomatal number	Stomatal index	Number beneath 4 epidermal cells	Palisade ratio	Number of epidermal cells	Cicatrix number	Cicatrix index
1	920	336	26.75	53	13.25	1540	28	1.79
2	864	344	28.48	56	14.00	1600	28	1.72
3	972	364	27.25	52	13.00	1604	24	1.47
4	876	332	27.48	53	13.25	1548	24	1.53
5	884	380	30.06	53	13.25	1576	28	1.75
6	904	364	28.71	52	13.00	1564	28	1.76
7	980	360	26.87	56	14.00	1536	28	1.79
8	920	356	27.90	53	13.25	1608	24	1.47
9	920	360	28.13	55	13.75	1556	28	1.77
10	976	368	27.38	51	12.75	1564	24	1.51
11	960	344	26.38	52	13.00	1616	24	1.46
12	964	360	27.19	51	12.75	1576	32	1.99
13	884	328	27.06	55	13.75	1576	28	1.75
14	912	336	26.92	57	14.25	1552	32	2.02
15	868	376	30.23	53	13.25	1540	24	1.53
16	916	340	27.07	51	12.75	1612	28	1.71
17	880	380	30.16	51	12.75	1552	28	1.77
18	904	356	28.25	56	14.00	1552	36	2.27
19	880	332	27.39	57	14.25	1648	24	1.44
20	908	380	29.50	53	13.25	1564	28	1.76
21	884	332	27.30	55	13.75	1676	36	2.10
22	900	336	27.18	56	14.00	1624	36	2.17
23	868	320	26.94	55	13.75	1540	36	2.28
24	896	320	26.32	53	13.25	1564	24	1.51
25	900	340	27.42	53	13.25	1644	36	2.14
26	888	340	27.69	55	13.75	1576	36	2.23
27	900	328	26.71	51	12.75	1560	28	1.76
28	940	364	27.91	53	13.25	1596	24	1.48
29	940	348	27.02	57	14.25	1596	28	1.72
30	864	320	27.03	52	13.00	1624	28	1.69
	Mean	**348.13**	**27.69**	**Mean**	**13.42**	**Mean**	**28.67**	**1.78**
	S.D.	**18.72**	**1.08**	**S.D.**	**0.50**	**S.D.**	**4.34**	**0.26**

Table 20 Stomatal number, stomatal index, palisade ratio, cicatrix number and cicatrix index of *M. coreia*; sample collected from Bohploy district, Kanchanaburi province

Area number	Stomata cell			Palisade cell		Cicatrix cell		
	Number of epidermal cells	Stomatal number	Stomatal index	Number beneath 4 epidermal cells	Palisade ratio	Number of epidermal cells	Cicatrix number	Cicatrix index
1	904	348	27.80	54	13.50	1556	24	1.52
2	900	328	26.71	52	13.00	1560	24	1.52
3	904	328	26.62	55	13.75	1568	24	1.51
4	872	320	26.85	55	13.75	1564	32	2.01
5	912	356	28.08	54	13.50	1568	32	2.00
6	880	352	28.57	51	12.75	1608	24	1.47
7	944	368	28.05	54	13.50	1628	32	1.93
8	920	316	25.57	53	13.25	1608	28	1.71
9	876	344	28.20	53	13.25	1624	32	1.93
10	948	368	27.96	51	12.75	1628	32	1.93
11	872	328	27.33	54	13.50	1592	24	1.49
12	884	320	26.58	53	13.25	1600	28	1.72
13	888	356	28.62	51	12.75	1628	28	1.69
14	892	324	26.64	54	13.50	1624	32	1.93
15	928	340	26.81	53	13.25	1572	24	1.50
16	892	356	28.53	54	13.50	1556	28	1.77
17	908	360	28.39	56	14.00	1588	24	1.49
18	872	340	28.05	52	13.00	1560	32	2.01
19	948	364	27.74	53	13.25	1552	24	1.52
20	876	368	29.58	53	13.25	1560	28	1.76
21	908	336	27.01	54	13.50	1564	32	2.01
22	872	368	29.68	54	13.50	1604	28	1.72
23	896	336	27.27	53	13.25	1540	32	2.04
24	880	364	29.26	53	13.25	1588	32	1.98
25	876	340	27.96	54	13.50	1564	32	2.01
26	936	364	28.00	56	14.00	1564	28	1.76
27	880	352	28.57	53	13.25	1612	24	1.47
28	896	324	26.56	56	14.00	1620	32	1.94
29	872	336	27.81	51	12.75	1604	28	1.72
30	896	368	29.11	56	14.00	1564	28	1.76
	Mean	**345.73**	**27.80**	**Mean**	**13.38**	**Mean**	**28.40**	**1.76**
	S.D.	**17.19**	**0.99**	**S.D.**	**0.37**	**S.D.**	**3.38**	**0.20**

Table 21 Stomatal number, stomatal index, palisade ratio, cicatrix number and cicatrix index of *M. coreia*; sample collected from Kalasin province

Area number	Stomata cell			Palisade cell		Cicatrix cell		
	Number of epidermal cells	Stomatal number	Stomatal index	Number beneath 4 epidermal cells	Palisade ratio	Number of epidermal cells	Cicatrix number	Cicatrix index
1	876	356	28.90	52	13.00	1540	28	1.79
2	884	360	28.94	57	14.25	1600	28	1.72
3	876	352	28.66	54	13.50	1604	24	1.47
4	880	344	28.10	51	12.75	1548	24	1.53
5	904	352	28.03	51	12.75	1576	28	1.75
6	880	360	29.03	55	13.75	1564	28	1.76
7	888	336	27.45	52	13.00	1536	28	1.79
8	892	352	28.30	55	13.75	1608	24	1.47
9	900	356	28.34	54	13.50	1556	28	1.77
10	880	348	28.34	53	13.25	1564	24	1.51
11	876	340	27.96	57	14.25	1616	24	1.46
12	876	336	27.72	54	13.50	1576	32	1.99
13	884	332	27.30	53	13.25	1576	28	1.75
14	916	364	28.44	53	13.25	1552	32	2.02
15	880	368	29.49	54	13.50	1540	24	1.53
16	892	360	28.75	51	12.75	1612	28	1.71
17	880	340	27.87	55	13.75	1552	28	1.77
18	928	368	28.40	54	13.50	1552	36	2.27
19	892	336	27.36	57	14.25	1648	24	1.44
20	920	368	28.57	54	13.50	1564	28	1.76
21	888	340	27.69	56	14.00	1676	36	2.10
22	900	324	26.47	54	13.50	1624	36	2.17
23	892	356	28.53	53	13.25	1540	36	2.28
24	952	352	26.99	55	13.75	1564	24	1.51
25	888	356	28.62	51	12.75	1644	36	2.14
26	876	360	29.13	53	13.25	1576	36	2.23
27	924	368	28.48	55	13.75	1560	28	1.76
28	872	360	29.22	56	14.00	1596	24	1.48
29	900	320	26.23	52	13.00	1596	28	1.72
30	948	360	27.52	55	13.75	1624	28	1.69
	Mean	**350.80**	**28.16**	**Mean**	**13.47**	**Mean**	**28.67**	**1.78**
	S.D.	**13.21**	**0.79**	**S.D.**	**0.45**	**S.D.**	**4.34**	**0.26**

APPENDIX B

Sources of ITS regions sequences which have been published from NCBI GenBank database using in this study

1. *Morinda citrifolia* L.

LOCUS FJ907060 695 bp DNA linear PLN 07-AUG-2009
DEFINITION *Morinda citrifolia* voucher McDowell 5742 (ETSU) 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.
ACCESSION FJ907060
VERSION FJ907060.1 GI:253992851
KEYWORDS .
SOURCE *Morinda citrifolia* (Indian mulberry)
ORGANISM *Morinda citrifolia*
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons; asterids; lamiids; Gentianales; Rubiaceae; Rubioideae; Morindeae; Morinda.
REFERENCE 1 (bases 1 to 695)
AUTHORS Razafimandimbison,S.G., McDowell,T.D., Halford,D.A. and Bremer,B.
TITLE Molecular phylogenetics and generic assessment in the tribe Morindeae (Rubiaceae-Rubioideae): how to circumscribe *Morinda* L. to be monophyletic?
JOURNAL Mol. Phylogenet. Evol. 52 (3), 879-886 (2009)
PUBMED 19394432
REFERENCE 2 (bases 1 to 695)
AUTHORS Razafimandimbison, S. G., McDowell, T. D., Halford, D. A. and Bremer, B.
TITLE Direct Submission
JOURNAL Submitted (08-APR-2009) Department of Botany, Bergius Foundation, Stockholm University, Stockholm 10691, Sweden
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2. *Morinda elliptica* Ridl.

LOCUS FJ907066 696 bp DNA linear PLN 07-AUG-2009
DEFINITION *Morinda elliptica* voucher Larsen et al. 41223 (AAU) 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.
ACCESSION FJ907066
VERSION FJ907066.1 GI:253992857
KEYWORDS .
SOURCE *Morinda elliptica*
ORGANISM *Morinda elliptica*
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons; asterids; lamiids; Gentianales; Rubiaceae; Rubioideae; Morindeae; Morinda.
REFERENCE 1 (bases 1 to 696)
AUTHORS Razafimandimbison, S. G., McDowell, T. D., Halford,D.A. and Bremer, B.
TITLE Molecular phylogenetics and generic assessment in the tribe Morindeae (Rubiaceae-Rubioideae): how to circumscribe *Morinda* L. to be monophyletic?
JOURNAL Mol. Phylogenet. Evol. 52 (3), 879-886 (2009)
PUBMED 19394432
REFERENCE 2 (bases 1 to 696)
AUTHORS Razafimandimbison,S.G., McDowell,T.D., Halford,D.A. and Bremer,B.
TITLE Direct Submission
JOURNAL Submitted (08-APR-2009) Department of Botany, Bergius Foundation, Stockholm University, Stockholm 10691, Sweden
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 421  aacgcaagtt gcgcccgaag ccattaggct gagggcacgt ctgcctgggc gtcacgcatc
 481  gcgtcgccac ccccctcctc gccctgcgcg ggatgaacga gggtgacgga tgttggcctc
 541  ccgtgtccca agcggcgcgg ctggcctaaa tgcgagtcct cggcccggga cgtcacggcg
 601  agtggtggtt gaactcatca actcgagagc cgtcgcgacg acgcccgacg gggaactctc
 661  cgaccctaga gctcttcgcg agcctcgacc cgcacc
```

//

3. *Morinda umbellata* L.

LOCUS FJ907094 783 bp DNA linear PLN 07-AUG-2009

DEFINITION *Morinda umbellata* voucher Takeuchi & Ama 15319 (BR) 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

ACCESSION FJ907094

VERSION FJ907094.1 GI:253992885

KEYWORDS .

SOURCE *Morinda umbellata*

ORGANISM *Morinda umbellata*
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons; asterids; lamiids; Gentianales; Rubiaceae; Rubioideae; Morindeae; Morinda.

REFERENCE 1 (bases 1 to 783)

AUTHORS Razafimandimbison, S. G., McDowell, T. D., Halford, D. A. and Bremer, B.

TITLE Molecular phylogenetics and generic assessment in the tribe Morindeae (Rubiaceae-Rubioideae): how to circumscribe *Morinda* L. to be monophyletic?

JOURNAL Mol. Phylogenet. Evol. 52 (3), 879-886 (2009)

PUBMED 19394432

REFERENCE 2 (bases 1 to 783)

AUTHORS Razafimandimbison,S.G., McDowell,T.D., Halford,D.A. and Bremer,B.

TITLE Direct Submission

JOURNAL Submitted (08-APR-2009) Department of Botany, Bergius Foundation, Stockholm University, Stockholm 10691, Sweden

FEATURES Location/Qualifiers

source 1..783
/organism="Morinda umbellata"
/mol_type="genomic DNA"
/specimen_voucher="Takeuchi & Ama 15319 (BR)"

/db_xref="taxon:268745"
/note="authority: Morinda umbellata L."
misc_RNA <1..>783
/note="contains 18S ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and 28S ribosomal RNA; nrITS"

ORIGIN

```
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   61  atcatttaga ggaaggagaa gtcgtaacaa ggtttccgta ggtgaacctg cggaaggatc
  121  attgtcgaat cctgccccac agacgaccgc gaactcgtta aacaaatgcc ggaggccggc
  181  gggcgcggga aaccctgcct gtcggccccc gggcccaact taactcccgg cgcggaatgc
  241  gccaaggaat actcaaacgg atggccggcc gccctgcgga ttccgcgggg cgagccgagc
  301  gtctgatcgt ttaaccagaa cgactctcgg caacggatat ctaggctctc gcatcgatga
  361  agaacgtagc gaaatgcgat acttggtgtg aattgcagaa tcccgtgaac catcgagttt
  421  ttgaacgcaa gttgcgcccg aagccattag gctgagggca cgtctgcctg ggcgtcacgc
  481  atcgcgtcgc cacccccctcc tcgccttaga aatttggacg gacgggggtg acggatgttg
  541  gcctcccgtg ctctcgcggc gcggccggcc caaatgcgag tcctcggccc gggacgtcac
  601  gacgagtggt ggttgaactc ttcagctcga gtgctgtcgc gacgacgccc gacgtggaac
  661  tctaccgacc ctagggctat cggccccgag aggagccgcg agccttcgac cgcgacccca
  721  ggtcaggcgg gattacccgc tgagtttaag catatcaata agcggaggaa aagaaactaa
  781  cag
//
```

4. *Morinda coreia* Ham.

LOCUS FJ907064 763 bp DNA linear PLN 07-AUG-2009

DEFINITION Morinda coreia voucher Lorence 9460 (PTBG) 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

ACCESSION FJ907064

VERSION FJ907064.1 GI:253992855

KEYWORDS .

SOURCE *Morinda coreia*

ORGANISM *Morinda coreia*

Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons; asterids; lamiids; Gentianales; Rubiaceae; Rubioideae; Morindeae; Morinda.

REFERENCE 1 (bases 1 to 763)

AUTHORS Razafimandimbison,S.G., McDowell,T.D., Halford,D.A. and Bremer,B.

TITLE Molecular phylogenetics and generic assessment in the tribe Morindeae (Rubiaceae-Rubioideae): how to circumscribe *Morinda* L. to be monophyletic?

JOURNAL Mol. Phylogenet. Evol. 52 (3), 879-886 (2009)

PUBMED 19394432

REFERENCE 2 (bases 1 to 763)

AUTHORS Razafimandimbison, S. G., McDowell, T. D., Halford, D. A. and Bremer, B.

TITLE Direct Submission

JOURNAL Submitted (08-APR-2009) Department of Botany, Bergius Foundation, Stockholm University, Stockholm 10691, Sweden

FEATURES Location/Qualifiers

source 1..763

/organism="Morinda coreia"

/mol_type="genomic DNA"

/specimen_voucher="Lorence 9460 (PTBG)"

/db_xref="taxon:659038"
/note="authority: Morinda coreia Buch.-Ham."
misc_RNA <1..>763
/note="contains 18S ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and 28S ribosomal RNA; nrITS"

ORIGIN

```
    1  ggattgcggc gacacgagcg gttcgctgcc cgcgacgtcg caagaagttc gctgaacctt
   61  atcatttaga ggaaggagaa gtcgtaacaa ggtttccgta ggtgaacctg cggaaggatc
  121  attgtcgaat cctgaccggt agaccgcgaa ctcgttaaac aaacttgggg cgctggcgag
  181  cgagggaaac accgcccgtc cgcgcgccaa cccaactaaa ctctcggcgc gggatgcgcc
  241  aaggactact caaacggatt gccggcctcc ccgcggcttc cgcggggcga gctgcgcgtc
  301  tggtcgttta accaaaacga ctctcggcaa cggatatcta ggctctcgca tcgatgaaga
  361  acgtagcgaa atgcgatact tggtgtgaat tgcagaatcc cgtgaaccat cgagtttttg
  421  aacgcaagtt gcgcccgaag ccattaggct gagggcacgt ctgcctgggc gtcacgcatc
  481  gcgtcgccac ccccctcctc gccccgcgcg ggatggtcga gggtgacgga tgttggcctc
  541  ccgtgtccct agcggcgcgg ctggcctaaa tgcgagttct cggcccggga cgtcacggcg
  601  agtggtggtt gaactcatca actcgagagc cgtcgcgacg acgcccgacg gggaactctc
  661  cgaccctaga gctcttcgcg agccttcgac cgcgacccca ggtcaggcgg gattacccgc
  721  tgagtttaag catatcaata agcggaggaa aagaaactaa caa
//
```

APPENDIX C

Figures of agarose gel electrophoresis of *Morinda* species in this study

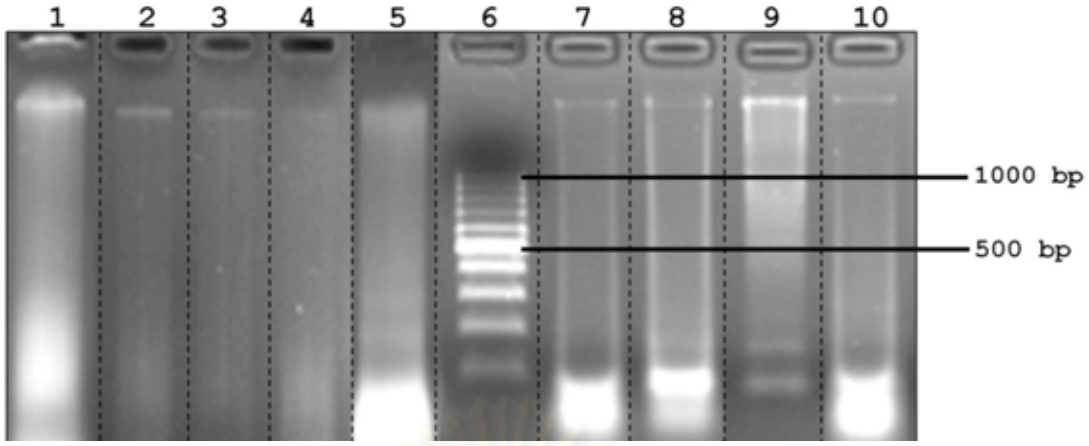

Figure 24 Total genomic DNA isolated from 9 extracted of *M. citrifolia* in different locations

Lane 1-4: Bangkok

Lane 5: Chachoengsao

Lane 6: 100 bp DNA Ladder

Lane 7: Phayao

Lane 8-10: Nakhon Ratchasima

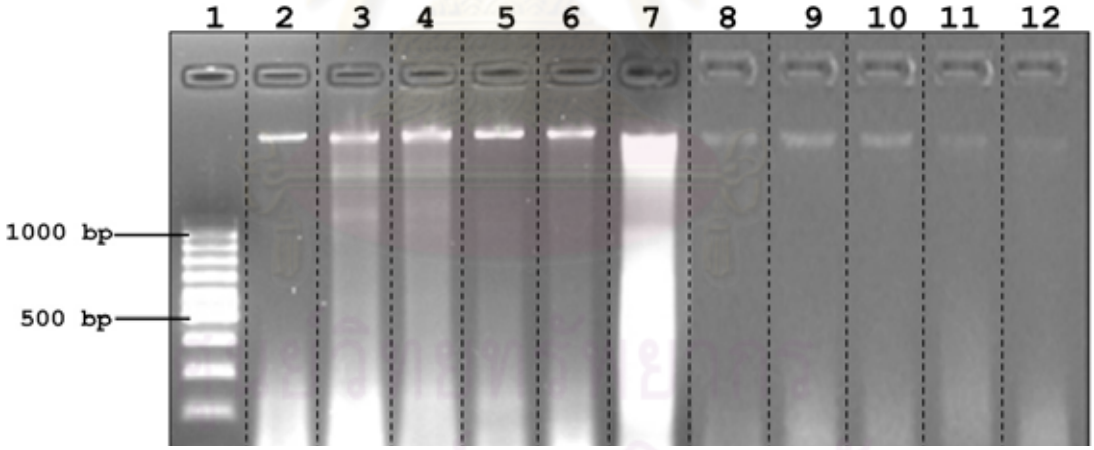

Figure 25 Total genomic DNA isolated from 11 extracted of *M. elliptica* in different locations

Lane 1: 100 bp DNA Ladder

Lane 2-6: Phatthalung

Lane 7: Songkhla

Lane 8: Nakhon Pathom,

Lane 9-12: Kanchanaburi

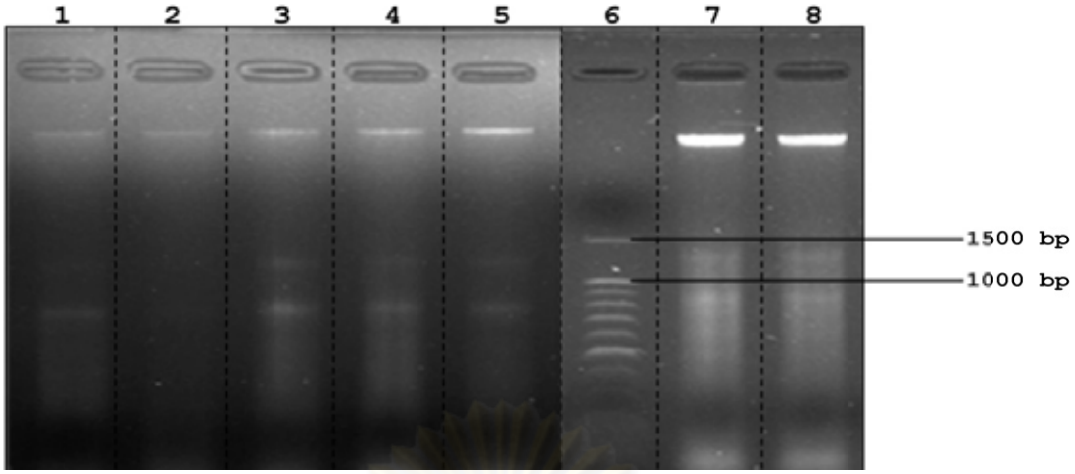

Figure 26 Total genomic DNA isolated from 7 extracted of *M. umbellata* in different locations

Lane 1-5: Songkhla

Lane 6: 100 bp plus DNA Ladder

Lane 7-8: Songkhla

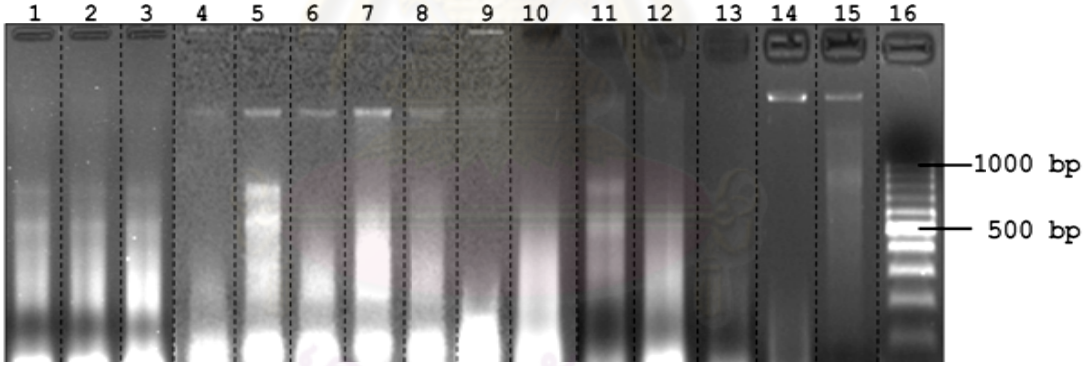

Figure 27 The total genomic DNA isolated from 15 extracted of *M. coreia* in different locations

Lane 1-7: Kanchanaburi

Lane 8-9: Nakhon Pathom

Lane 10-11: Kalasin

Lane 12-13: Nong Khai

Lane 14-15: Nakhon Ratchasima

Lane 16: 100 bp DNA Ladder

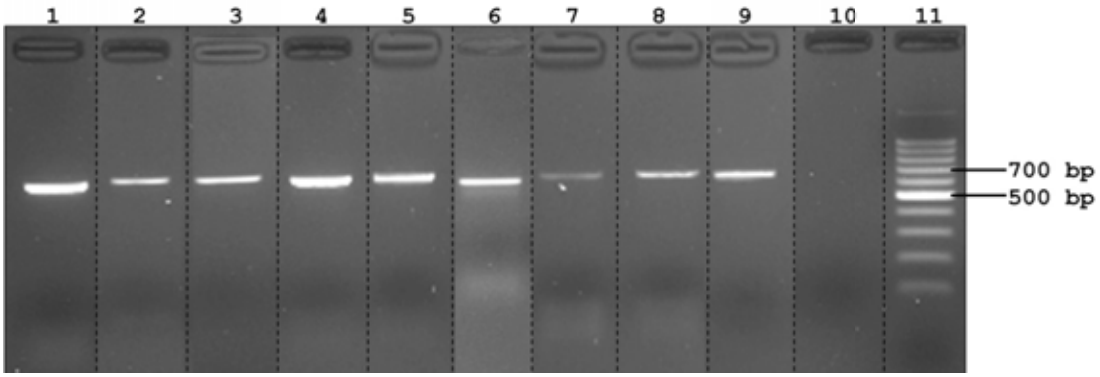

Figure 28 The PCR products of ITS region amplified from each *M. citrifolia* samples

Lane 1-4: Bangkok

Lane 5: Chachoengsao

Lane 6: Phayao

Lane 7-9: Nakhon Ratchasima

Lane 10: Negative control

Lane 11: 100 bp plus DNA Ladder

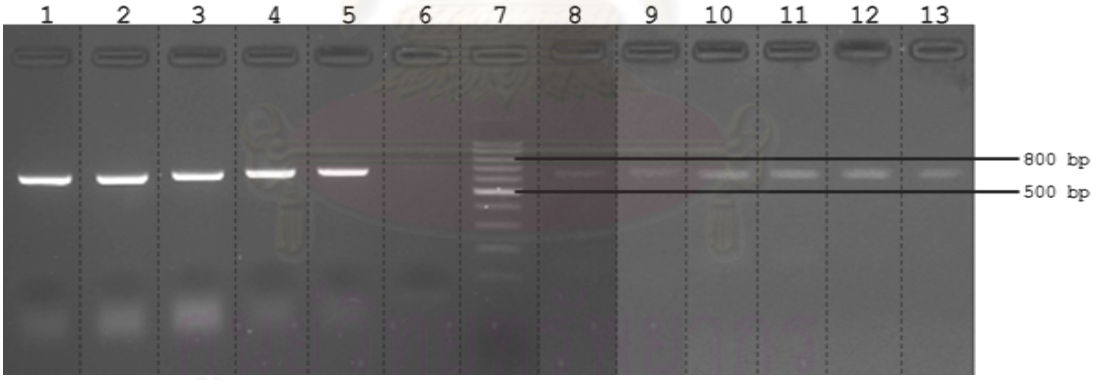

Figure 29 The PCR products of ITS region amplified from each *M. elliptica* samples.

Lane 1-5: Phatthalung

Lane 6: Negative control

Lane 7: 100 bp DNA Ladder

Lane 8: Songkhla

Lane 9: Nakhon Pathom

Lane 10-13: Kanchana Buri

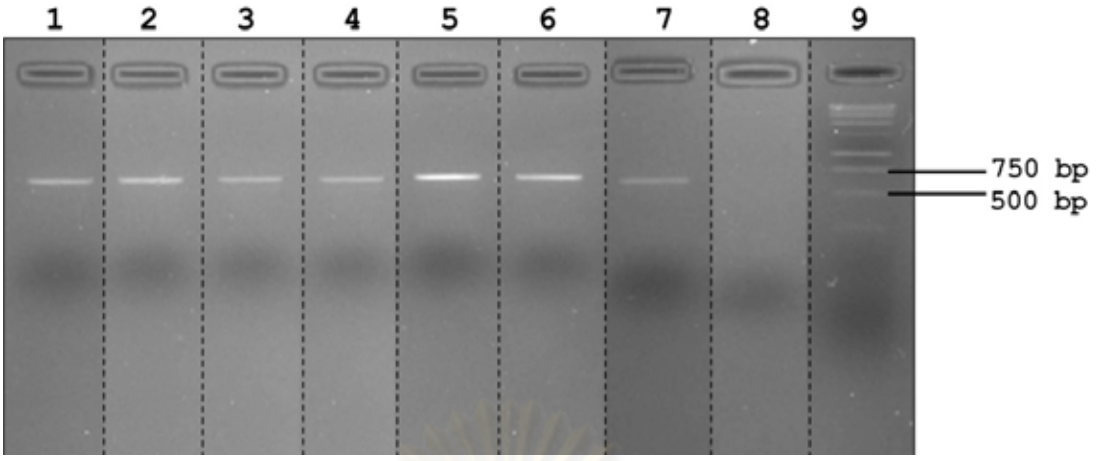

Figure 30 The PCR products of ITS region amplified from each *M. umbellata* samples.

Lane 1-7: Songkhla

Lane 8: Negative control

Lane9: 1 kp DNA Ladder

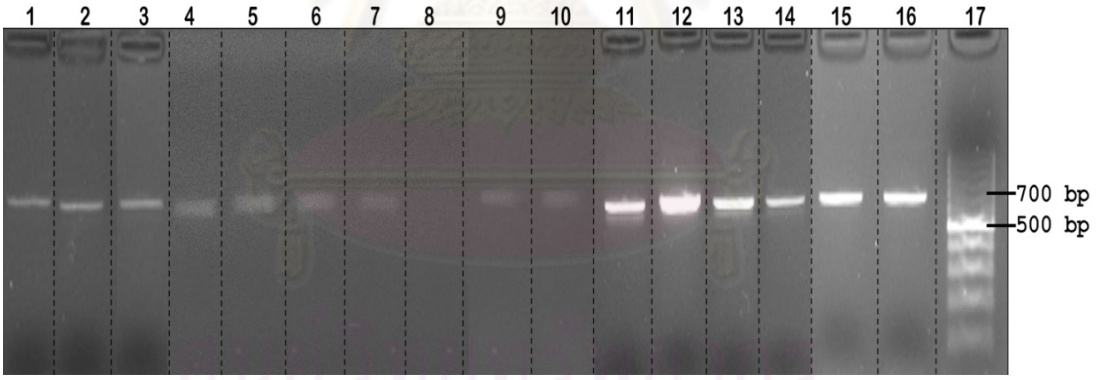

Figure 31 The PCR products of ITS region amplified from each *M. coreia* samples

Lane 1-7: Kanchanaburi

Lane 8: Negative control

Lane 9-10: Nakhon Pathom

Lane 11-12: Kalasin

Lane13-14: Nong Khai

Lane 14-15: Nakhon Ratchasima

Lane 16: 100 bp DNA Ladder

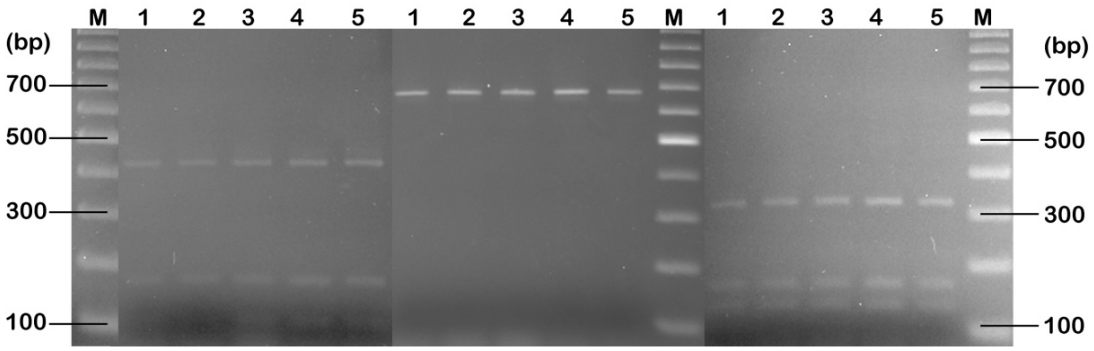

Figure 32 Some of PCR products of *M. elliptica* were digested by restriction enzymes (**A** = *MseI*, **B** = *EcoRI* and **C** = *MspI*)

Lane M: 100 bp DNA Ladder

Lane1-5: *M. elliptica* from Phatthalung province

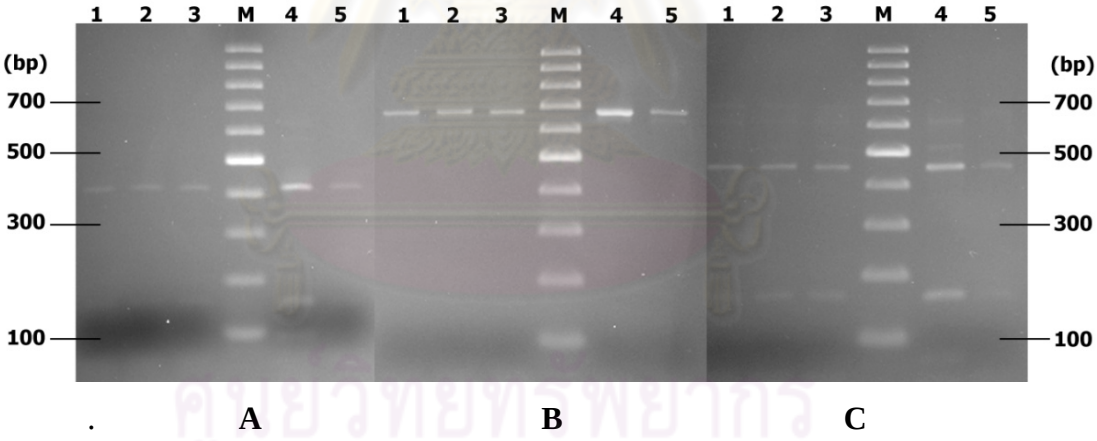

Figure 33 Some PCR products of *M. coreia* were digested by restriction enzymes (**A** = *MseI*, **B** = *EcoRI* and **C** = *MspI*

Lane 1-3: *M. coreia* from Kanchanaburi province

Lane M: 100 bp DNA Ladder

Lane4-5: *M. coreia* from Nakhon Ratchasima province

CIRICULUM VITAE

Miss Woralak Roonyamarai was born on April 8, 1987 in Bangkok, Thailand. She received her Bachelor's degree of Applied Thai Traditional Medicine, Second Class Honors in 2008 from the School of Health Sciences, Mae Fah Luang University, Thailand. She attended to study Master degree of Public Health Sciences in 2009 at College of Public Health Sciences, Chulalongkorn University, Thailand.

Proceedings

Roonyamarai W., Rungsihirunrat K., Vipunngeun N. and Ruangrungsi N. 2010. Microscopic and biomolecular analyses of selected *Morinda* species in Thailand. NRCT-JSPS Core University Program on Natural Medicine in Pharmaceutical Sciences: The 9th NRCT-JSPS Joint Seminar, Natural Medicinal Research for the Next Decade: New Challenges and Future Collaboration. Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand, December 8-9.