ดีเอ็นเอบาร์โค้ดของพืชสกุล *Bacopa* ที่พบในประเทศไทย

นายชยพล ตั้งพัฒน์ทอง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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DNA BARCODES OF *BACOPA* PLANTS FOUND IN THAILAND

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ชยพล ตั้งพัฒน์ทอง : ดีเอ็นเอบาร์โค้ดของพืชสกุล *Bacopa* ที่พบในประเทศไทย (DNA BARCODES OF *BACOPA* PLANTS FOUND IN THAILAND) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ภญ. ร.ต.อ.หญิง ดร.สุชาดา สุขหร่อง, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. วรัญญู พูลเจริญ, 105 หน้า.

พรมมิ (*Bacopa monnieri*) เป็นสมุนไพรที่จัดอยู่ในวงศ์ Plantaginaceae มีสรรพคุณในการฟื้นฟูความจำ และสติปัญญา ปรากฏอยู่ในตำรายาอายุรเวทและตำรายาไทย สารออกฤทธิ์ที่สำคัญคือบาโคไซด์เอ ซึ่งเป็นสารกลุ่มไตรเทอปีนอยด์แซโปนิน ในประเทศไทยพบพืชในสกุล *Bacopa* จำนวนสามชนิด ได้แก่ พรมมิ [*B. monnieri* (L.) Wettst] ลานไพลิน [*B. caroliniana* (Walter) B. L. Rob.] และผักสามหลั่น [*B. floribunda* (R. Br.) Wettst.] เนื่องจากการจำแนกชนิดของพืชในรูปวัตถุดิบแห้งหรือวัตถุดิบผงเป็นไปได้ยาก ดังนั้นการศึกษานี้เป็นการพัฒนาหาวิธีที่มีความแม่นยำสูงในการจำแนกพืชในสกุล *Bacopa* ที่พบในประเทศไทย โดยใช้ดีเอ็นเอบาร์โค้ด ซึ่งบริเวณของดีเอ็นเอใช้ในการศึกษามีทั้งสิ้น 6 บริเวณได้แก่ ITS, *mat*K, *rbc*L, *ycf*1, *psb*A-*trn*H และ *trn*L-F จากการศึกษาพบว่าการใช้ดีเอ็นเอบาร์โค้ดบริเวณ *ycf*1 และ *trn*L-F ร่วมกับเทคนิควิเคราะห์ high resolution melting ประสบความสำเร็จในการแยกชนิดของพืชในสกุล *Bacopa* ได้ โดยอาศัยค่าของอุณหภูมิที่ทำให้ดีเอ็นเอแยกสายได้ครึ่งหนึ่ง (T_m) ที่จำเพาะของพืชแต่ละชนิด ซึ่งเป็นการศึกษาครั้งแรกในการประยุกต์ใช้เทคนิคดีเอ็นเอบาร์โค้ดในการแยกชนิดของพืชในสกุล *Bacopa* และใช้จำแนกสมุนไพรพรมมิออกจากพืชชนิดอื่นที่มีความใกล้เคียงกัน นอกจากนี้ยังสามารถนำไปใช้ในการกำหนดมาตรฐานและการควบคุณคุณภาพสมุนไพรได้อีกด้วย

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CHAYAPOL TUNGPHATTHONG: DNA BARCODES OF *BACOPA* PLANTS FOUND IN THAILAND. ADVISOR: ASSOC. PROF. SUCHADA SUKRONG, Ph.D., CO-ADVISOR: ASST. PROF. WARANYOO PHOOLCHAROEN, Ph.D., 105 pp.

Bacopa monnieri (Family Plantaginaceae) commonly known as 'Brahmi' or 'Prom-mi' is a medicinal plant used for a remedy of cognitive impairment and intelligence in Ayurvedic Materia Medica and Thai traditional medicine. Bacoside A, the major active constituents, is classified as triterpenoid saponin. In Thailand, there are three *Bacopa* species including Prom mi [*B. monnieri* (L.) Wettst.], Lan pailin [*B. caroliniana* (Walter) B. L. Rob.] and Phak sam lan [*B. floribunda* (R. Br.) Wettst.]. Since the dried or powdered raw materials are difficult to identify plant species, this study attempted to develop a method for accurate identification of *Bacopa* spp. found in Thailand by using DNA barcode. Six DNA regions, ITS, *matK*, *rbcL*, *ycf*1, *psb*A-*trn*H and *trn*L-F, were proposed for the study. DNA barcodes of *ycf1* and *trn*L-F coupled with high resolution melting (HRM) analysis were successfully used for species identification. The characteristic of specific melting temperature (T_m) was able to distinguish among the *Bacopa* species. This is the first study using DNA barcode techniques to differentiate *Bacopa* spp. and identify the medicinal plant *B. monnieri* from the related species. Moreover, DNA barcodes are useful asset for standardization and quality control purposes.

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CONTENTS

Page

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

5-HT	=	Serotonin
AFLP	=	Amplified fragment length polymorphism
Akt	=	Protein kinase B
ATP	=	Adenosine triphosphate
bp	=	Base pair
BuOH	=	Butanol
BZD	=	Benzodiazepines
ca	=	Approximately (Circa)
CA	=	Cornu ammonis
CaM	=	Calmodulin
CBOL	=	Consortium for the barcode of Life
CREB	=	cAMP response element-binding protein
°C	=	Degree Celsius
DA	=	Dopamine
DEN	=	Diethylnitrosamine
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxyribonucleotide triphosphate
e.g.	=	For example (Exampli gratia)
ELISA	=	Enzyme-linked immunosorbent assay
ERK	=	Extracellular signal-regulated kinase
ESLD	=	Evaporative light scattering detector
EtOAc	=	Ethyl acetate
FST	=	Forced swim test
GLAST	=	Glutamate aspartate transporter
H_2O	=	Water
H_2O_2	=	Hydrogen peroxide
HPLC	=	High-performance liquid chromatography
HPTLC	=	High-performance thin layer chromatography
H. pyroli	=	*Helicobacter pylori*

HRM	=	High-resolution melting
Hsp70	=	70 kilodalton heat shock proteins
ICV-STZ	=	injections of intracerebroventricular of streptozotocin
IGS	=	Intergenic spacer
iNOS	=	Inducible nitric oxide synthase
IP_3	=	Inositol trisphosphate
IL-6	=	Interleukin 6
ISSR	=	Inter-simple sequence repeats
ITS	=	Internal transcribed spacer
Kb	=	Kilobase
l-NNA	=	Nitro-L-arginine
LPO	=	Lipid peroxidation
LPS	=	Lipopolysaccharide
LTP	=	Long term potentiation
LZP	=	Lorazepam
*mat*K	=	Maturase K
MAP	=	Mitogen-activated protein
mGlu5	=	Metabotropic glutamate receptor 5
min	=	Minute(s)
mm	=	Millimeter
mRNA	=	messenger RNA
NA	=	Noradrenaline
NO	=	Nitric oxide
NMDAR	=	N-methyl-D-aspartate receptor
PBPE	=	Polybrominated diphenyl ether
PCP	=	Phencyclidine
PCR	=	Polymerase chain reaction
pCREB	=	Phosphorylation of cyclic AMP response element binding
PC-SFC-DAD	=	Packed column supercritical-fluid chromatography with photodiode-array detection

PD	=	Parkinson disease
PHT	=	Phenytoin
PGs	=	Pepsinogen
PINK1	=	PTEN-induced putative kinase 1
*psb*A	=	Photosystem II protein D1
RAPD	=	Random amplified polymorphic DNA
RFLP	=	Restriction fragment length polymorphism
ROS	=	Reactive oxygen species
RP	=	Reversed phase
*rbc*L	=	Ribulose bisphosphate carboxylase large chain
rDNA	=	Ribosomal deoxyribonucleic acid
SCAR	=	Sequence characterized amplified region
SDAT	=	Senile dementia-Alzheimer type
SNP	=	Single Nucleotide Polymorphism
SNP	=	Sodium nitroprusside
SOD	=	Superoxide dismutase
spp	=	Species (plural)
TLC	=	Thin layer chromatography
T_m	=	Melting temperature
TST	=	Tail suspension test
*trn*F	=	Transfer RNA of Phenylalanine
*trn*H	=	Transfer RNA of Histidine
*trn*L	=	Transfer RNA of Leucine
TNF	=	Tumor necrosis factor
UV	=	Ultraviolet
ycf1	=	Hypothetical chloroplast open reading frame 1
V	=	Volt
VGLUT	=	vesicular glutamate transporter

CHAPTER I
INTRODUCTION

Medicinal plants have been used in the historical of humanity for a long time. They were used to maintain the health and to treat illness and disease (Ganie *et al.*, 2015). *Bacopa monnieri* (Family Plantaginaceae) commonly known as 'Prom-mi' is a medicinal plant used for a remedy of cognitive impairment and intellect. The aerial part was used in Ayurvedic Materia Medica for three thousand years. Bacoside A and B, the major active constituents, were classified in type of a triterpenoid saponin (Russo and Borrelli, 2005). In Thailand, there are three species of plants in the genus *Bacopa* including *Bacopa monnieri* (L.) Wettst. (Prom mi), *Bacopa caroliniana* (Walter) B. L. Rob. (Lan Pailin) and *Bacopa floribunda* (R. Br.) Wettst. (Phak sam lan) (Department of National Park, 2014).

Nevertheless, the adulteration and substitution of wrong materials have become a crucial concern for customers in sense of safety and efficacy of medicinal plant products (Ganie *et al.*, 2015). Therefore, authentication of medicinal plants is the essential step for medicinal plant manufacturer. In general, the authentication of medicinal plants is divided into four major methods including morphology (eye), cytology (microscope), chemistry and genetic. The morphological and cytological methods were not practically for using in manufacturer because the raw materials were

usually imported as chopped pieces or fined powder. Moreover, an expertise person was needed. The chemical method is the commonly standard technique in the present. Although the chemical analysis technique can be useful for processed materials. The environment was affected to the major chemical constituents and plant in same genus might have similar chemical profile. Therefore, the chemical methods are not always proper for medicinal plant authentication. Due to the plant genome were persisted in plant materials and commercial products, then molecular analysis was a solution for identification of medicinal plants.

DNA barcodes, the short regions from genome used to distinguish organism species, were successful used for identification of plants (Hebert *et al.*, 2003). Candidate barcode resions that have potential for discriminations among plants have been reported in several studies. Six DNA regions, including ITS (nuclear) and *mat*K, *rbc*L, *psb*A-*trn*H, *trn*L-F and *ycf*1 (plastid), were proposed as the candidate barcode for plants identifications (Li *et al.*, 2011; Vijayan and Tsou, 2010). *Ycf*1, the novel candidate DNA barcode was proposed in the recent year, was successfully discriminated the land plants (Dong *et al.*, 2015). Moreover, the DNA barcode can use for phylogeny analysis for genetic relationship of *Bacopa* plant species or apply for the discovery the new sources of bioactive constituents.

High-resolution melting (HRM) analysis is the new technique for analysis of mutations. This technique was applied for screening the samples without probe. This

technique is a method for detection of DNA dissociation and useful method for detection of point mutation and indels. The fluorescence dye was intercalated with DNA duplex for detecting the accumulation of PCR products during the PCR reactions and for monitoring the melting temperature (T_m) of PCR amplicons. HRM analysis is the sequencing-free method that reasonable to identify medicinal plant products (Osathanunkul *et al.*, 2015).

In this study, we aim to establish the DNA barcodes of plants in the genus *Bacopa* found in Thailand and apply the barcoding sequences with HRM analysis for rapid and reliable identification of *Bacopa* plant species and guarantee the medicinal plant *B. monnieri* products for the regulatory authorities in terms of plant identity for quality control and may develop check-points during the long supply chain starting from local collectors to the market shelves.

CHAPTER II

LITERATURE REVIEW

2.1 *Bacopa* plants

The genus *Bacopa,* which classified in family Plantaginaceae, is aquatic or parudal plants consist of approximately one hundred species (Raus, 2003). *Bacopa* plants are distributed in tropical and subtropical regions around the world in wet, damp and marshy regions (Russo and Borrelli, 2005).

In Thailand, there are three species of plant in the genus *Bacopa* including *B. monnieri, B. caroliniana and B. floribunda* (Department of National Park, 2014). (**Table 1**).

Table 1 The list of *Bacopa* species existing in Thailand

No.	Scientific name	General name	Thai name
1	*Bacopa monnieri* (L.) Wettst.	Herpestis, Water hyssop	พรมมิ, ผักมิ
2	*Bacopa caroliniana* (Walter) B. L. Rob.	Giant Red Bacopa, Lemon Bacopa, Water hyssop	ลานไพลิน
3	*Bacopa floribunda* (R. Br.) Wettst.	N/A	ผักสามหลั่น

The characteristics of the genus *Bacopa* have been described in the Flora of Thailand (Yamazaki, 1990) as following ;

"...Annual or perennial herbs; stems prostrate or erect. *Leaves* opposite, sessile, entire or minutely toothed. *Flowers* solitary and axillary. *Bracteoles* 2, persistent. *Calyx* 5-lobes almost to the base; upper lobe largest, ovate or broadly ovate; lateral two lobes smallest, lanceolate or linear. *Corolla* tubular or didynamous; anther-loculi parallel, contiguous. *Ovary* ellipsoidal, glabrous. Capsule ovoid or globose, included within persistent calyx, loculicidally bivalved. *Seeds* numerous, minute, cylindric or oblong, with coarse reticulation and longitudinal ridges..."

The characteristics of *Bacopa monnieri* (**Figure 1A**) and *Bacopa floribunda* (**Figure 1C**) have been described in the Flora of Thailand (Yamazaki, 1990) as following ;

***Bacopa monnieri* (L.) Wettst.**

"...Prostrate herbs; stems 10 – 40 cm, much branched, rooting at the nodes. *Leaves* spathulate or obovate, obtuse at apex, entire, 6 – 20 by 1 – 5 mm. *Pedicels* 6 – 15 mm long, glabrous. *Bracteoles* linear, 2 – 3 mm. *Calyx* 5 – 6 mm long, glabrous; upper one lower two sepal ovate, acute or subacute, 2.5 mm wide; two lateral ones

lanceolate, acuminate, 1.5 mm wide. *Corolla* white or pale violet, 8 – 10 mm long; lobes subequal, oblong-obovate, rounded or subemarginate at apex, ca 4 by 2.5 mm. *Capsule* ovoid, 5 by 3 mm. *Seeds* ca 0.5 – 0.6 by 0.3 mm ..."

***Bacopa floribunda* (R. Br.) Wettst.**

"... Annual herbs; stems erect 20 – 50 cm. *Leaves* linear-lanceolate, acute at apex, narrowly attenuate to base, distally serrulate or entire, 1 – 7 by 0.2 – 0.5 cm; glabrous. *Pedicels* 0.5-2 mm long, scabrid. *Bracteoles* subulate, 1.5 – 2 mm, scabrid. *Calyx* 5 – 6 mm long in flower, 6 – 7 mm long in fruit, sparsely glandularpilose on both surface, scabrid on margins; upper sepal largest, orbicular-ovate, 4 – 5 mm wide; two lower ones ovate, acute, ca 3 mm wide; two lateral ones smallest, linear, acuminate, 0.3-0.5 mm wide. *Corolla* whitish, 4 – 5 mm long, lobes orbicular, ca 0.8 mm long and wide. *Capsule* globose, 4 mm long and wide. *Seeds* ca 0.3 – 0.35 by 0.2 mm ..."

B. caroliniana were not described in Flora of Thailand. This species were not found in Orientals. However, the characteristic of *B. caroliniana* were described in the botanical taxonomic key in western country (North and South America).

The botanical characteristic of *B. caroliniana* (**Figure 1C**) have been described in Southern Wetland Flora (The United States Department of Agriculture, 1999) as following ;

***Bacopa caroliniana* (Walter) B. L. Rob.**

" ... *Stems*: Floating, creeping, or ascending, hairy, the upright part up to 1 foot tall. *Leaves*: Opposite, simple, ovate, rounded at the tip, clasping at the base, dotted, aromatic, up to 1 inch long, up to ½ inch wide, without teeth, sparsely hairy on the veins of the lower surface. *Flowers*: Solitary in the axils of the leaves, the stalks very short at first but lengthening as the fruit develops; each flower with a pair of small bractlets at its base. *Sepals*: 5, green, some of them ovate and up to ¼ inch long, the others linear and shorter. *Petals*: 5, blue, united, up to ½ inch long. *Stamens*: 4, the longer 2 exserted from the corolla. *Pistils*: Ovary superior. *Fruits*: Capsules ovoid, up to ¼ inch long; seeds grayish brown, distinctly veiny ... "

Figure 1 *Bacopa monnieri* (A) *Bacopa caroliniana* (B) *and Bacopa floribunda* (C)

2.2 Chemical constituents of *Bacopa monnieri*

Initially, bacoside A and B, the triterpenoid saponins, have been isolated from in *B. monnieri*. Recently, these two compounds were revealed as a mixture of twenty saponins which classified into two groups based on the aglycone moieties; jujubogenin (**Table 2**) and pseudojujubogenin (**Table 3**)

2.3 Pharmacological activities and clinical study

Pharmacological activities of the three *Bacopa* were investigated by various research laboratories. The summary of biological activities have been investigated in *B. monnieri* and *B. caroliniana*. (**Table 4**)

Standardized extract of *B. monnieri* was studied in clinical trials as herbal supplements for brain booster as summarized. (**Table 5**)

Table 2 Triterpenoid saponins with jujubogenin aglycone units isolated from *B. monnieri*

Name	IUPAC Name	References
Bacoside A_1	jujubogenin 3-O-[α-L-arabinofuranosyl(1⟶3)]-α-L-arabinopyranoside	Jain and Kulshreshtha, 1993
Bacoside A_3	jujubogenin 3-O-α-L-arabinofuranosyl-(1⟶2)-[β-D-glucopyranosyl-(1⟶3)]-β-D-glucopyranoside	Rastogi *et al.*, 1994
Bacopasaponin A	jujubogenin 3,20-di-O-α-L-arabinopyranoside	Garai *et al.*, 1996a
Bacopasaponin E	jujubogenin 3-O-α-L-arabinofuranosyl-(1⟶2)-[β-D-glucopyranosyl-(1⟶3)]-α-L-arabinopyranoside, 20-O-α-L-arabinopyranoside	Mahato *et al.*, 2000
Bacopasaponin F	jujubogenin 3-O-α-L-arabinofuranosyl-(1⟶2)-[β-D-glucopyranosyl-(1⟶3)]-β-D-glucopyranoside,20-O-α-L-arabinopyranoside	
Bacopasaponin G	jujubogenin 3-O-[α-L-arabinofuranosyl-(1⟶2)]-α-L-arabinopyranoside	Hou *et al.*, 2002
Bacopaside III	jujubogenin 3-O-α-L-arabinofuranosyl-(1⟶2)-β-D-glucopyranosyl	Chakravarty *et al.*, 2003
Bacopaside IV	jujubogenin 3-O-β-D-glucopyranosyl-(1⟶3)-α-L-arabinopyranosyl	
Bacopaside IX	jujubogenin 3-O-{β-D-glucopyranosyl(1⟶4)[α-L-arabinofuranosyl -(1⟶2)]-β-D-glucopyranosyl}-20-O-α-L-arabinopyranosyl	Zhou *et al.*, 2009

Table 3 Triterpenoid saponins with pseudojujubogenin aglycone units isolated from *B. monnieri*

Name	IUPAC Name	References
Bacopasaponin B	pseudojujubogenin 3-O-[α-L-arabinofuranosyl-(1⟶2)]-α-L-arabinopyranoside	Garai *et al.*, 1996a,b
Bacopasaponin C	pseudojujubogenin 3-O-α-L-arabinofuranosyl-(1⟶2)-[β-D-glucopyranosyl-(1⟶3)]-α-L-arabinopyranoside	
Bacopasaponin D	pseudojujubogenin 3-O-[α-L-arabinofuranosyl-(1⟶2)]-β-D-glucopyranoside	
Bacoside A_2	pseudojujubogenin 3-O-α-L-arabinopyranosyl-(1⟶5)-[α-L-arabinofuranosyl-(1⟶6)]-α-D-glucofuranoside	Rastogi and Kulshreshtha, 1999
Bacopaside III	pseudojujubogenin 3-O-[6-O-sulfonyl-β-D-glucopyranosyl-(1⟶3)]-α-L-arabinopyranoside	Hou *et al.*, 2002
Bacopaside I	pseudojujubogenin 3-O-α-L-arabinofuranosyl-(1⟶2)-[6-O-sulfonyl-β-D-glucopyranosyl-(1⟶3)]-α-Larabinopyranoside	Chakravarty *et al.*, 2001, 2003
Bacopaside V	pseudojujubogenin 3-O-ß-D-glucopyranosyl-(1⟶3)-α-L-arabinofuranosyl	
Bacopaside II	pseudojujubogenin 3-O-α-L-arabinofuranosyl-(1⟶2)-[β-D-glucopyranosyl-(1⟶3)]-β-D-glucopyranoside	
Bacopasaponin H	pseudojujubogenin 3-O-[α-L-arabinopyranosyl]	Mandal & Mukhopadhyay, 2004
Bacopaside XI	pseudojujubogenin 3-O-[ß-D-arabinofuranosyl (1⟶3)]-6-O-sulfonyl-ß-D-glucopyranosyl	Bhandari *et al.*, 2009
Bacopaside XII	pseudojujubogenin 3-O-{ß-D-glucopyranosyl(1⟶3)[ß-D-arabinofuranosyl(1⟶2)]-ß-D-glucopyranosyl}-20-Oß-D-arabinopyranosyl	

Table 4 Summary of pharmacological activities in *Bacopa* plants

Pharmacological effects	**Plant species**	**Result**	**Reference**
Memory enhancing effect	*B. monnieri*	The antiamnesic effects by gamma-aminobutyric acid–benzodiazepine pathway	Prabhakar *et al.*, 2008
	B. monnieri	*B. monnieri* suppressed the diazepam induced upregulation of MAP kinase, pCREB and iNOS and attenuated the downregulation of nitrite.	Saraf *et al.*, 2008
	B. monnieri	*B. monnieri* significantly attenuated the l-NNA-induced anterograde amnesia, partially reversing l-NNA-induced retrograde amnesia.	Saraf *et al.*, 2009
	B. monnieri	*B. monniera* reversed the scopolamine induced amnesia by significantly improving calmodulin and by partially attenuating protein kinase C and pCREB.	Saraf *et al.*, 2010
	B. monnieri	Antiamnesic effect B. monniera on L-NNA induced amnesia may be mediated by NO pathyway involving CaM, which is required for LTP sustenance	Saraf *et al.*, 2010
	B. monnieri	*B. monnieri* effects on cholinergic system may be helpful for developing alternative therapeutic approaches for the treatment of Alzheimer's disease.	Saraf *et al.*, 2011
	B. monnieri	*B. monnieri* reduces PHT-induced cognitive deficits without affecting its anticonvulsant efficacy	Vohora *et al.*, 2000

Table 4 Summary of pharmacological activities in *Bacopa* plants (continued)

Pharmacological effects	**Plant species**	**Result**	**Reference**
Memory booster	*B. monnieri*	Bacosides facilitate anterograde memory and attenuate anterograde experimental amnesia induced by scopolamine and sodium nitrite possibly by improving acetylcholine level and hypoxic conditions, respectively.	Kishore and Singh, 2005
	B. monnieri	Bacoside A protects the brain from oxidative stress induced by morphine	Sumathi *et al.*, 2011
	B. monnieri	Brahmi before PCP administration can restore the cognitive deficit by decreasing NMDAR1 in brain areas. Brahmi could be a novel neuroprotective agent for the prevention of cognitive deficit in schizophrenia	Piyabhan and Wetchateng, 2014
	B. monnieri	Brahmi could recover the cognitive deficit by increasing VGLUT1 in CA1 and CA2/3 to normal.	Piyabhan and Wetchateng, 2013
	B. monnieri	BM is a powerful antioxidant which prevents cognitive impairment, oxidative damage, and morphological changes in the ICV-STZ-infused rats	Khan *et al.*, 2015
	B. monnieri	BESEB CDRI-08 *(B. monnieri)* possibly acts on serotonergic system, which in turn influences the cholinergic system through 5-HT_3 receptor to improve the hippocampal-dependent task.	Rajan *et al.*, 2011

Table 4 Summary of pharmacological activities in *Bacopa* plants (continued)

Pharmacological effects	**Plant species**	**Result**	**Reference**
Memory booster	*B. monnieri*	CDRI-08 enhances hippocampus-dependent contextual memory by differentially regulating histone acetylation and protein phosphatases in hippocampus.	Preethi *et al.*, 2014
Anti-parkinson	*B. monnieri*	*B. monnieri* significantly improved the climbing ability in a *D. melanogaster* PD model based on loss of function of PINK1.	Jansen *et al.*, 2014
	B. monnieri	*B. monnieri* reduces alpha synuclein aggregation, prevents dopaminergic neurodegeneration and restores the lipid content in nematodes, thereby proving its potential as a possible anti-Parkinsonian agent.	Jadiya *et al.*, 2011
Antistroke	*B. monnieri*	The chlorophyll salt and aqueous extracts of *B. monnieri* prevent ischemia - reperfusion induced cerebral injury with comparable potency.	Rehni *et al.*, 2007
	B. monnieri	*B. monnieri* attenuates the ischemia induced memory and other neurological deficits including infarct size by exerting antioxidant effects	Saraf *et al.*, 2010
	B. monnieri	Chronic oral *B. monnieri* extract increased cerebral blood flow independent of blood pressure, and this effect explains its nootropic and possibly neuroprotective actions.	Kamkaew *et al.*, 2013

Table 4 Summary of pharmacological activities in *Bacopa* plants (continued)

Pharmacological effects	**Plant species**	**Result**	**Reference**
Anticonvulsant	*B. monnieri*	*B. monnieri* extract treatment potentates a therapeutic effect by reversing the alterations in glutamate receptor binding and NMDA R1 gene expression that occur during epilepsy, resulting in reduced glutamate-mediated excitotoxity in the overstimulated hippocampal neurons.	Reas *et al.*, 2008
	B. monnieri	*B. monnieri* treatment to epileptic rats significantly brought the reversal of the down-regulated mgluR8 gene expression toward control level	Paulose *et al.*, 2008
	B. monnieri	The alcoholic extract of *B. monnieri* may function in a similar manner to BZD, given its benzodiazepine-like action, although the specific receptor interactions were not evaluated	Kaushik *et al.*, 2009
	B. monnieri	*B. monnieri* extract treatment potentates a therapeutic effect by reversing the alterations in glutamate receptor binding and NMDA R1 gene expression that occur during epilepsy, resulting in reduced glutamate-mediated excitotoxity in the overstimulated hippocampal neurons	Khan *et al.*, 2008
	B. monnieri	The forced swim test confirmed the depressive behavior pattern during epilepsy that was nearly completely reversed by *B. monnieri* treatment	Krishnakumar *et al.*, 2009a
	B. monnieri	*B. monnieri* extract treatment reverses the 5-HT_{2C} receptor mediated motor dysfunction in epilepsy	Krishnakumar *et al.*, 2009b

Table 4 Summary of pharmacological activities in *Bacopa* plants (continued)

Pharmacological effects	**Plant species**	**Result**	**Reference**
Anticonvulsant	*B. monnieri*	*B. monnieri* have the neuroprotective effect in epilepsy involves the interaction of 5-HT_{2C} and NMDA receptors, with modulation of mGlu5 receptor and GLAST gene expression at the mRNA level and IP_3 activation at the second messenger level.	Krishnakumar *et al.*, 2015
Antidepressant	*B. monnieri*	The methanol extract, EtOAc fraction and n-BuOH fraction of *B. monnieri* produced a significant decrease in immobility times both in FST and TST, without modifying significantly the spontaneous motor activity.	Shen *et al.*, 2009
	B. monnieri	The *B. monnieri* extract was found to have significant antidepressant activity in forced swim and learned helplessness models of depression and was comparable to that of imipramine.	Sairam *et al.*, 2002
	B. monnieri	*B. monnieri* normalizes stress mediated transient deregulation of plasma corticosterone and levels of monoamines like NA, 5-HT and DA in cortex and hippocampus regions of the brain, which are more vulnerable to stressful conditions analogous to the effects of PQ	Sheikh *et al.*, 2007
	B. monnieri	bacopaside I, bacopaside II, and bacopasaponsin C showed antidepressant activity when tested on forced swimming and tail suspension in mice	Zhou *et al.*, 2007
	B. monnieri	80-120 mg/kg doses of *B. monnieri* extract have significantly higher antidepressant-like activity	Banerjee *et al.*, 2014

Table 4 Summary of pharmacological activities in *Bacopa* plants (continued)

Pharmacological effects	**Plant species**	**Result**	**Reference**
Antidepressant	*B. monnieri*	n-butanol extract of *B. monnieri* has an antidopaminergic/serotonergic effect and may have potential beneficial effects in the treatment of morphine dependence	Rauf *et al.*, 2014
Antianxiety	*B. monnieri*	*B. monnieri* has an anxiolytic action qualitatively comparable to that of the BDZ, LZP.	Bhattacharya and Ghosal, 1998
Antioxidant	*B. monnieri*	*B. monnieri* extract supplementation helps to overcome stress by improved ATP production, normalization of brain monoamines levels, enhance antioxidant response and by down-regulating expression of heat shock proteins and iNOS.	Pandareesh and Anand, 2014b
	B. monnieri	*B. monnieri* can mitigate the lead induced-oxidative stress tissue specifically by pharmacologic interventions which encompass both chelation as well as antioxidant functions	Velaga *et al.*, 2014
	B. monnieri	*B. monnieri* extract protects PC12 cells against SNP-induced toxicity via its free radical scavenging and neuroprotective mechanism.	Pandareesh and Anand, 2014a
	B. monnieri	*B. monnieri* plays a neuroprotective role against PBDE-209-induced alterations in oxidative status	Verma *et al.*, 2014
	B. monnieri	*B. monnieri* pretreatment possesses the potential to modulate endogenous levels of oxidative markers and thus preventing the oxidative impairments and neurotoxicity with acute PQ administration	Hosamani *et al.*, 2016

Table 4 Summary of pharmacological activities in *Bacopa* plants (continued)

Pharmacological effects	**Plant species**	**Result**	**Reference**
Antioxidant	*B. monnieri*	*B. monnieri* extract attenuates the oxidative damage induced by H_2O_2 by improving the antioxidant status, mitochondrial membrane integrity and by preventing DNA fragmentation and lipid peroxidation.	Pandareesh *et al.*, 2016
	B. monnieri	*B. monnieri* reduces stress by modulating the expression of Hsp70 and the activity of P450s and SOD, the enzymes known to be involved in the production and scavenging of reactive oxygen species, in different regions of the brain.	Chowdhuri *et al.*, 2002
	B. monnieri	Bacoside A is effective to prevent DEN-induced hepatocellular carcinoma by quenching lipid peroxidation and enhancing antioxidant status through free radical scavenging mechanism and having potential of protecting endogenous enzymatic and non-enzymatic antioxidant activity.	Janani *et al.*, 2010
	B. monnieri	*B. monnieri* extract promotes the anti oxidant status, reduces the rate of lipid peroxidation and the markers of tumor progression in the fibrosarcoma bearing rats	Rohini *et al.*, 2004
	B. monnieri	*B. monnieri* modulates antioxidant activity, and enhances the defense against ROS generated damage in diabetic rats.	Kapoor *et al.*, 2009
	B. monnieri	*B. monnieri* extract might have insulin like activity and the antihyperglycemic effect of the extract might be due to an increase in peripheral glucose consumption as well as protection against oxidative damage in alloxanised diabetes	Ghosh *et al.*, 2010

Table 4 Summary of pharmacological activities in *Bacopa* plants (continued)

Pharmacological effects	**Plant species**	**Result**	**Reference**
Antioxidant	*B. monnieri*	*B. monnieri* treatment potentiates the antioxidant status and suppressed the tissue damage induced by aluminium – intoxication. These findings suggest that *B. monnieri* whole-plant extracts can be considered as a possible remedy to counteract aluminium – associated neurological disorders	Nannepaga *et al.*, 2014
	B. monnieri	*B. monnieri* exerted differential effects on cytokine production and antioxidant enzyme activities of the lymphocytes from the spleens of young, early middle-aged, and old male F344 rats by modulating ERK pathway with specific effects on CREB and Akt	Priyanka *et al.*, 2013
Anti-diarrheal	*B. monnieri*	*B. monnieri* extract showed anti-diarrheal activity on castor oil induced diarrhea in mice by increased mean latent period and decreased the frequency of defecation.	Afjalus *et al.*, 2012
Anti-ulcer	*B. monnieri*	whole plant juice as a potential antiulcer drug, and the protection afforded was mostly due to the augmentation of mucosal defensive factors.	Rao *et al.*, 2000
	B. monnieri	*B. monnieri* extracts the gastric prophylactic and curative due to its activity on defensive mucosal factors with no discernible effect on cell proliferation. The antioxidant activity of *B. monnieri* extracts may be one of the important factors contributing towards its activity	Sairam *et al.*, 2001

Table 4 Summary of pharmacological activities in *Bacopa* plants (continued)

Pharmacological effects	**Plant species**	**Result**	**Reference**
Anti-ulcer	*B. monnieri*	*B. monnieri* extracts has in vitro anti - H. pylori activity and causes an increase in the accumulation of PGs by human mucosal colonic incubates, which could account for its anti-ulcerogenic activity.	Goel *et al.*, 2003
Hepatoprotective	*B. monnieri*	*B. monnieri* extracts exerted a protective effect against morphine-induced liver and kidney toxicity.	Sumathi *et al.*, 2011
Anti-hypothyroid	*B. monnieri*	*B. monnieri* could increase T4 concentration by 41% without enhancing hepatic lipid peroxidation (LPO) suggesting that it can be used as a thyroid-stimulating drug.	Kar *et al.*, 2002
Fertility booster	*B. monnieri*	Brahmi treatment causes reversible suppression of spermatogenesis and fertility, without producing apparent toxic effects.	Singh and Singh, 2009
Antimicrobial	*B. monnieri*	*B. monnieri* ether extract showed antimicrobial activity against four bacteria and one fungus.	Azad *et al.*, 2012
	B. monnieri	Endophyte isolated from *B. monnieri* revealed their potential to yield potent bioactive compounds that can be used in development of drugs against microbial infections and cancer	Katoch *et al.*, 2014
	B. caroliniana	*B. caroliniana* extract has strong antimicrobial effects against the tested microorganisms especially the bacterium *Staphylococcus aureus* and the yeast culture *Candida albicans*.	Dulger and Hacioglu, 2009

Table 4 Summary of pharmacological activities in *Bacopa* plants (continued)

Pharmacological effects	**Plant species**	**Result**	**Reference**
Anti-inflammatory	*B. monnieri*	The ethanol extract of *B. monniera* possesses strong anti-inflammatory activity via prostaglandin inhibition, thus justifying the traditional uses of this plant in the therapy of various inflammatory conditions.	Channa *et al.*, 2006
	B. monnieri	The methanol fraction and aqueous extract of *B. monnieri* acts as potent anti-inflammatory agent in rats in acute inflammation model.	Abhishek *et al.*, 2010
	B. monnieri	The triterpenoid and bacoside-enriched fractions significantly inhibited inflammation through modulation of pro-inflammatory mediator release LPS-activated TNF-α, IL-6 and nitrite production in mononuclear cells.	Viji and Helen, 2011
Spasmolytic effect	*B. monnieri*	*B. monnieri* has spasmolytic activity on intestinal and vascular tissues from rabbit or guinea-pig may occur mainly through interruption of calcium influx via both voltage and receptor operated calcium channels	Dar and Channa, 1997, 1999
	B. monnieri	*B. monnieri* has the relaxation effects induced by possibly involves prostacyclin compounds and beta-adrenoceptors	Dar and Channa, 1999
Bronchodilatory vasodilatory	*B. monnieri*	various fractions derived from *B. monnieri* possess broncho-vasodilatory activity, which is attributed mainly to inhibition of calcium ions.	Channa *et al.*, 2003
Mast cell stabilising	*B. monnieri*	The methanolic extract exhibited a potent mast cell stabilization effect comparable to disodium cromoglycate. *B. monnieri* are useful for leaves the allergic conditions	Samiulla *et al.*, 2001

Table 5 Summary of clinical trial studies of *B. monnieri.*

Subjects	Dosages	Results	References
35 adults	12 gm/day	Reduction anxiety, enhanced memory span and concentration No side effects observed	Singh and Singh, 1980
20 primary school children	350 mg x 3/day	Enhanced memory, learning, perception and reaction times. No side effects observed.	Sharma *et al.*, 1987
40 mentally retarded children with or without epilepsy	CDRI-08 (KeenMind)	Enhanced learning and controlled abnormal behavior	Dave *et al.*, 1993
36 children with attention deficit hyperactivity disorder (ADHD)	50 mg x 2/day	Ameliorated various cognitive assessments. No side effects observed.	Negi *et al.*, 2000
38 healthy subjects	300 mg/day	No improvement in memory performance	Nathan *et al.*, 2001
46 healthy people	300 mg/day	Improved early information processing and verbal learning rate. Consolidated memory and reduced anxiety. Side effects: nausea, dry mouth and fatigue	Stough *et al.*, 2001
76 healthy subjects	300 mg/day	Enhanced retention of new information.	Roodenrys *et al.*, 2002
85 healthy subjects	Combination of standardized CDRI-08 (KeenMind) 300 mg/day and Ginko biloba 120 mg/day	No significant effect on cognition and memory	Nathan *et al.*, 2004

Table 5 Summary of clinical trial studies of *B. monnieri.* (continued)

Subjects	Dosages	Results	References
23 healthy subjects	Bacomind™ capsule 300 mg/day and 450 mg	Improved cognition. Minor gastrointestinal adverse effects.	Pravina *et al.*, 2007
54 healthy subjects	Standardized CDRI-08 (KeenMind) 300 mg/day	Enhanced cognitive performance in the aging	Calabrese *et al.*, 2008
98 healthy subjects	Bacomind™ capsule 300 mg	A significant improvement in memory acquistion and retention was observed. Gastrointestinal side effects reported.	Morgan and Stevens, 2010
465 subjects	300 mg/day	Improvement in memory function	Stough *et al.*, 2012
60 healthy subjects	300 mg/day	Attention, cognitive processing, and working memory improved.	Peth-Nui *et al.*, 2012
109 healthy subjects and 123 SDAT patients	500 mg x 2/day	Improvements in memory performance and reduction in the levels of inflammatory and oxidative stress markers observed in Brahmi treated SDAT patients	Sadhu *et al.*, 2014
104 elderly subjects with mild cognitive impairment	1 Illumina® tablet/day	Cognitive function improved. One non-serious adverse effect reported	Zanotta *et al.*, 2014
17 healthy subjects	320 mg and 640 mg	Brahmi supplementation reduced stress and alleviated mood in these participants.	Benson *et al.*, 2014

2.4 Medicinal plant authentication methods

Authentication methods become a critical process for medicinal plants manufacturer. The medicinal plants materials were substituted or contaminated by the counterfeit materials in in the sense of cost reducing were rapidly occurrence. In order that, the substitution of fake materials may be influence to the therapeutic effects of medicinal plants and have negative influence for consumer wellbeing.

In general, the authentication methods of medicinal plants are including four principle methods; morphological method (plant morphology), cytological method (microscopy), chemical methods (phytochemical analysis) and molecular method (DNA molecular biology) (Zhao *et al.*, 2006).

2.4.1 Morphological method

Morphological method is a simple method to authenticate medicinal plants. This method refers to the examining of morphological characters. Many pharmacopoeias from various countries are list this method for preliminary authentication of medicinal plants such as Japan Pharmacopoeia, Chinese Materia Medica, British Herbal Medicine Pharmacopoeia and American Herbal Pharmacopoeia (Zhao *et al.*, 2006). Although this method is a simple, fast and easy for authentication. However, the required experience of examiner and undistinguished of closely related species are the weak point of this method.

2.4.2 Cytological method

Cytological method refers to analysis of cell structure and internal features using microscope. This method is properly useful for identification of broken medicinal plant materials e.g. dried or powdered materials, and useful for discriminating the similarly morphological features of plant species. This method have been recorded in most pharmacopoeias such as British Herbal Pharmacopoeia, American Herbal Pharmacopoeia, Japanese Pharmacopoeia and Korea Herbal Pharmacopoeia (Zhao *et al.*, 2006). Even though this method can be accurate and effective for quality control. However, the medicinal plant extracts or processed material may not identified using this method.

2.4.3 Chemical analysis method

Chemical method refers to investigate the characteristic of chemical constituents of medicinal plants. For example, the chemical qualitative and quantitative analysis by spectroscopy and chromatography. This method is the commonly standard methods for authentication of herbs. Thin layer chromatography (TLC), the most commonly effective and simply technique for phytochemical analysis which recorded for standard authentication of medicinal plants in many pharmacopoeia (Zhao *et al.*, 2006). However, the chemical constituents could not be used to identify the closely related plants

species because of the similarly chemical constituents. Moreover, the environments conditions may be affected to the phytochemical constituents.

2.4.4 Molecular method

The progression of molecular biology techniques produces an effect to authentication technique for medicinal plants. Based on the identity of DNA in medicinal plants species can be used for medicinal plant identification. This method provides a specific, accurate, stable and convenient for authentication of medicinal plants and useful for the identification of broken, processed and extracted materials. Moreover, the little amount of sample is adequate for DNA analysis and this technique can be identifying the confound usage of closely related medicinal plants.

The DNA-based marker has been developed for a long time to authenticate the medicinal plant. Many techniques was successfully developed for identification of plant base on the differentiation of DNA sequences such as Restriction fragment length polymorphism (RFLP), Random amplified of polymorphic DNA (RAPD), Amplified fragment length polymorphism (AFLP), Simple sequence repeats (SSR), Inter-simple sequnces repeats (ISSR), and Sequences characterization of amplified regions (SCAR) (Hao *et al.*, 2010). However, the nucleotide sequences of powerful discriminative DNA regions was

important for designed the DNA marker for applied to others rapid convenience and reliable methods.

In recently year, DNA barcoding, the sequence-based method has been developed for identification of medicinal plant. Many candidate DNA regions have been proposed for medicinal plant identifications. The Barcode of Life Plant Working Group recommends the genomic regions *rbc*L + *mat*K for barcoding (CBOL Plant Working Group, 2009). Kress et al., 2005 have been propose other nuclear region of the internal transcribed spacer (ITS) and the chloroplast region of the *trn*H–*psb*A intergenic spacer as optional barcodes for identification of plants because of the high sequence variations and interspecific divergence, and are easily amplified across a broad range of its regions (Kress *et al.*, 2005). In recent year, Dong et al. have been propose a new plastid DNA regions, ycf1, as a promising DNA barcode for identifications of land plants. The result show that *ycf1*b slightly better than the combination of *mat*K and *rbc*L and generally can be performed better than any of the *mat*K, *rbc*L and *trn*H-*psb*A (Dong *et al.*, 2015). In this study, we have establish the barcoding of anther DNA regions *trn*L-F. Since the previous study about HRM analysis for identification of medicinal plants. The result show the successful of *trn*L-F DNA regions can applied with HRM analysis for identification of plant species significantly (Buddhachat *et al.*, 2015; Ganopoulos *et al.*, 2012; Madesis *et al.*,

2012). *Trn*L-F have been use for a long time in phylogenetic analysis of plants. It was believed that *trn*L-F is not the most variable DNA regions of plastid. However, the conserved of secondary structure with alternation of variable regions are the unique advantages of this DNA regions. Because of the conserved regions can be facilitated to design the primers to amplify short variable site DNA regions between them (Vijayan and Tsou, 2010).

Moreover, the DNA barcoding can be applied for other rapid, convenient and reasonable cost for identification of medicinal plants species such as melting curve analysis and high-resolution melting analysis.

2.5 Authentication of *Bacopa monnieri*

The morphology of three *B. monnieri* and *B. floribunda* have been described by Yamazaki in the Flora of Thailand. As for *B. caroliniana* has been reported by in South Wetland Flora (The United States Department of Agriculture, 1999). This description of plant morphology in two references are very useful as a taxonomic key for identification of plant in closely related species. However, the plant material usually input to the manufacturer as a reduced form of material which unable to use morphology to identify the plant materials. Therefore, other methods should be developed to solve this problem

the microscopic method for identification of *B. monnieri* and *B. floribunda* have been reported by Gubbannavar et al. for differentiation between *B. monnieri* and *B. floribunda* (Gubbannavar *et al.*, 2013). They found the specific feature characteristic in plant cell of each species. Moreover, the pharmacognostic, physicochemical and phytochemical investigation of *Bacopa monnieri* have been reported by Mishra et al. (Mishra *et al.*, 2015). This method can be advantages for identification of broken or powdered plant material. However, this method is very difficult to identification in the manufacturer because of the raw material often input as processed materials. Therefore, the chemical analysis of plants can be useful for identification of medicinal plant materials and solve the trouble on processed materials.

The chemical analysis has been widely studied in *B. monnieri*. This method is the standard method for identification of medicinal plant is the present. The previous study show the five compounds including Bacoside A_3 (1), Bacopaside II (2), jujubogenin isomer of Bacopasaponin C (3) bacopasaponin C (4) and Bacopaside I (5) are constituted more than 95% of total saponins in *B. monnieri*. Analytical monograph of *B. monnieri* was published in the United State Pharmacopoeia with required the measurement of five saponins. The Indian Pharmacopoeia and Ayuravedic Pharmacopoeia of India have a *B. monnieri* monograph based on the estimation the four saponins of Bacoside A while the British Pharmacopoeia required the estimation of total bacopa saponins content base on the calculated using Bacopaside II as a reference standard (Deepak

and Amit, 2013). Interestingly, the chemical analysis of other species has not been study. Many articles were reported the chemical analysis of *B. monnieri* which can be useful for identification of medicinal plant materials as summary in the **Table 6**. However, the chemical method has a limitation of closely related plant species which may have a same chemical profile. Moreover, the environment might influence to the content of chemical constituents. Therefore, another method such as DNA may be fulfill limitation of this method.

Table 6 Summary of chemical analysis method of *B. monnieri*.

Apparatus	Chemical constituents	Refernces
HPTLC	Bacoside A	Shrikumar *et al.*, 2004
HPLC	Bacoside A_3 (1), Bacopaside II (2), bacopasaponin C isomer (3) and bacopasaponin C (4)	Deepak *et al.*, 2005
RP-HPTLC, PC–SFC–DAD	Bacoside A_3 and Bacopaside II	Agrawal *et al.*, 2006
ELISA	Bacopaside I	Phrompittayarat *et al.*, 2007a
RP-HPLC	Bacoside A3 (1), Bacopaside II (2), Bacopasaponin C isomer (3), Bacopasaponin C (4) and Bacopaside I (5)	Phrompittayarat *et al.*, 2007b
HPLC – ELSD	Bacoside A, Bacopaside I, Bacoside A3, Bacopaside II, Bacopaside X, Bacopasaponin C and Apigenin	Bhandari *et al.*, 2009b
ELISA	Bacoside A_3 Bacopaside X (jujubogenin) and Bacopaside IV (jujubogenin)	Tothiam *et al.*, 2011
HPTLC	Bacoside A	Shinde *et al.*, 2011
HPLC	Bacopaside I and Bacoside A	Srivastava *et al.*, 2012
HPTLC	Bacoside A and Bacopaside I	Christopher *et al.*, 2017

DNA-based method have been developed for identification of *B. monnieri* by various DNA marker. In 2012, Tripathi et al. have reported the molecular analysis for genetic variation of *B. monnieri* in central India using RAPD and ISSR marker (Tripathi *et al.*, 2012). However, this study were use the DNA marker only for determination of genetic relationship among the *B. monnieri* form different accession of collection. Later, the RAPD-based SCAR marker have been developed for the authentication of *B. monnieri* by Yadav et al (Yadav *et al.*, 2012). This method was developed from the RAPD pattern of *B. monnieri* and its adulterants that sold in the name of Brahmi. The specific PCR product from the RAPD marker of *B. monnieri* which absent in other adulterant were cloned and sequenced for analysis of DNA sequences. The specific primer were designed base on the specific DNA sequences for authentication of *B. monnieri*. These methods were developed base on only *B. monnieri* and its adulterants. However, the adulteration of plant materials incline on related species which have a similarly morphology or chemical constituents (Zhang *et al.*, 2015). Therefore, the DNA analysis of closely related plant species should be analyzed.

Recently, DNA barcode, the short length of standard DNA region using for identification of plant species (Hebert *et al.*, 2003). This technique becomes an additional standard for the identification of medicinal plant materials. However, the DNA barcoding of plant in the genus *Bacopa* are not available for identification of *Bacopa* plant species. Moreover, the accurate, rapid, reliable and economical technique should be developed for raw material identification routines in developing

country. Therefore, DNA barcodes of plant in the genus *Bacopa* should be established and the rapid identification technique using DNA marker should be developed.

2.6 Barcoding – high resolution melting (HRM) analysis

Barcoding-HRM is the coupling of DNA Barcoding and High-resolution melting analysis methods. Which the pair of primers us in this method designed based on knowing DNA sequences. HRM is the post-PCR method that was developed for the detection of genetic variation such as mutation and indels in the nucleotide sequences (Simko, 2016). HRM enables rapid, high-throughput identification of variants in the regions of targets DNA without sequencing. HRM is required the DNA intercalation fluorescence dye which intercalated to the double stranded DNA while the PCR still amplifying. (**Figure 2**) This method is detecting the diminishes of fluorescence which the fluorescence dye were release from the dissociation of double stranded DNA when the increment of temperature in the thermal cycler (**Figure 3**). This denaturation of double stranded DNA is based on the binding affinities of each nucleotide pairs which vary due to indels and mutations. These variations inferred to the fluorescence value which collected when the increment of temperature in the thermal cycler which are plotted as the melting curves. The melting curve's shape and peak are the characteristic of each PCR product from each samples (Osathanunkul *et al.*, 2015). The characteristic of melting curve graph can useful for comparison and distinguish the species among samples.

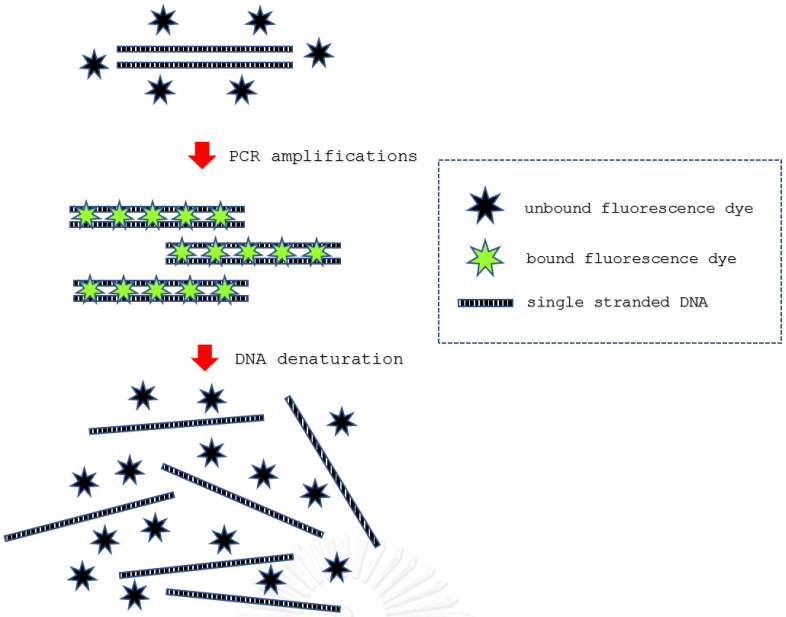


Figure 2 The diagram of fluorescence dye activities while Pre-PCR, PCR (PCR amplification) and post-PCR (DNA denaturation) which inferred to the HRM analysis

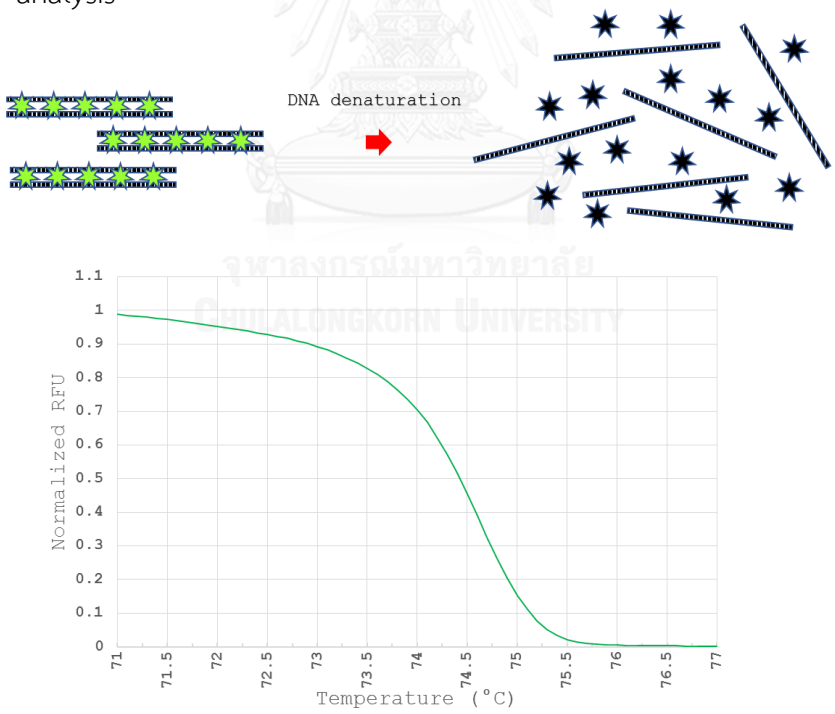


Figure 3 The diminishes of fluorescence intensity during the increment of temperature on DNA denaturation process which inferred the melting curve in HRM analysis

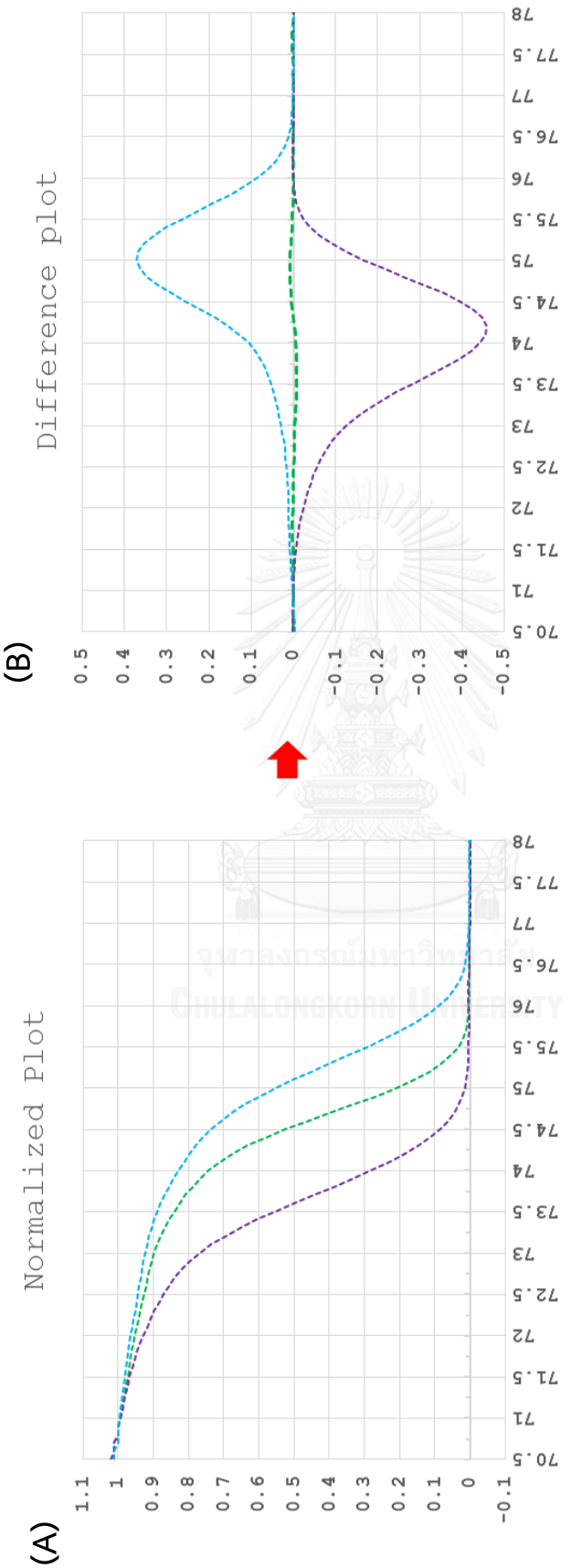


Figure 4 The schematic of melting curve from real-time PCR couple with HRM analysis for plant species identification (A) Normalized Pot (B) Difference Plot

CHAPTER III

MATERIALS AND METHODS

3.1 Plant materials

Total fourteen plant samples from the genus *Bacopa* were collected from the different locations in Thailand including Bangkok, Nakhon Prathom, Phitsanulok, Nakhon Nayok, Chiang Mai and Sakhon Nakhon. (**Table 1**). There were seven sample of *Bacopa monnieri* (Prom mi), five samples of *Bacopa caroliniana* (Lan Pailin) and two samples of *Bacopa floribunda* (Phak Sam Lun). All samples were identified by Associate Professor Thatree Phadungcharoen at the Faculty of Pharmacy, Rangsit University. Herbarium were prepared from each species as voucher specimens and then preserved in the Museum of Natural Medicine at the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

3.2 DNA extraction

Fresh leaf specimens from each species were homogenized into the find powder by mortar and pestle under liquid nitrogen treatment. The powdered samples were transferred to 1.5 ml micro-centrifuge tube and kept in liquid nitrogen prior to DNA extraction. Total genomic DNA were extracted from 100 mg at approximately of each grounded samples by using DNeasy Plant Mini Kit (QIAGEN) following the manufacturer's procedure. In brief, the grounded samples were re-suspended in lysis

Table 7 Plant Material and their accession number for DNA barcodes.

Species	Code	Georgraphic location	Voucher ID	Accession					
				ITS	matK	rbcL	ycf1	psbA-trnH	trnL-trnF
B. monnieri	BM01	Bangkok	CU-MN 20170126	LC214982	LC214984	LC214987	LC214988	LC214981	LC310979
	BM02	Bangkok	CU-MN 20170127	-	-	-	-	-	-
	BM03	Nakhon Pathom	CU-MN 20170128	-	-	-	-	-	-
	BM04	Phitsanulok	CU-MN 20170129	-	-	-	-	-	-
	BM05	Bangkok	CU-MN 20170130	-	-	-	-	-	-
	BM06	Nakhon Nayok	CU-MN 20170131	-	-	-	-	-	-
	BM07	Bangkok	CU-MN 20170132	-	-	-	-	-	-
B. caroliniana	BC01	Bangkok	CU-MN 20170133	LC214983	LC214985	LC214986	LC214989	LC214980	LC310977
	BC02	Phitsanulok	CU-MN 20170134	-	-	-	-	-	-
	BC03	Nakhon Nayok	CU-MN 20170135	-	-	-	-	-	-
	BC04	Chiang Mai	CU-MN 20170136	-	-	-	-	-	-
	BC05	Bangkok	CU-MN 20170137	-	-	-	-	-	-
B. floribunda	BF01	Sakon Nakhon	CU-MN 20170138	LC214990	LC214992	LC214993	LC214994	LC214991	LC310978
	BF02	Phitsanulok	CU-MN 20170139	-	-	-	-	-	-

buffer before incubating the samples at 65 °C for 20 min. The lysates were then transferred to filtering columns and centrifuged at 14000 x g for 1 min to remove cell debris and other precipitates. The flow-through were then transferred to the DNA binding columns and then centrifuged at 14000 x g for 1 min. The flow - through were discarded and the columns were then washed out for the impurities. After drying columns by centrifugation, the genomic DNA were eluted from the columns by elution buffer. The total genomic DNA were determined for the quality by 1% agarose gel electrophoresis, stained with Ultrapower™ Nucleic Acid Staining (E Coli S.R.O.) and visualized under UV light and photographed in GeL-Doc System (Bio-Rad). The purified DNA samples were kept at -20 °C prior to the further analysis.

3.3 PCR amplification, sequencing and data analysis

The universal primer for six loci DNA regions including ITS, *mat*K, *rbc*L, *ycf*1, *psb*A-*trn*H and *trn*L-F were used in PCR reaction (**Table 8**) and the schematic diagram of the amplificated loci and primer pair for PCR and sequencing was shown in **Figure 5** . The PCR reactions were performed in 25 µl amplification volume consisting of 1X PCR buffer with 1 mM $MgCl_2$ (Promega), 0.4 mM dNTP mix (Promega), 1.0 unit of Go*Taq*® DNA Polymerase (Promega) and 20 ng of genomic DNA and carried out in T100™ Thermal Cycler (Bio-Rad) using the cycling condition at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30s, 55 °C for 30s and 72 °C for 1 min for ITS*, ycf*1 *and psb*A-

*trn*H or 72 ℃ for 1 min for *rbc*L and *mat*K, and final extension at 72 ℃ for 10 min. The amplified PCR products were examined by 1% agarose gel electrophoresis in 1% TAE buffer and visualized under UV light by using Ultrapower™ Nucleic Acid Staining in GelDoc™ XR (Bio-Rad) and then photographed.

For DNA sequencing, the amplified products were purified using QIAquick® PCR purification kit (QIAGEN) prior to sequence. According to the manufacture instruction, the amplified DNA were mixed together with binding buffer and transferred to the DNA binding columns. After centrifugation, the flow-through were discarded and the columns were then washed and dried. The PCR product were eluted from the column and prompted to be sequenced. The purified PCR product were bi-directionally sequenced using the pairs of universal primers of each region by ABI 3730XL DNA analyzer at BIONEER Co., ltd. The raw DNA sequences from six regions were aligned, edited, corrected, verified and compiled using MEGA version 7 to confirm the quality of sequences. The consensus sequences of obtained sequences were analyzed by MUSCLE algorithm (Edgar, 2004). All sequences were submitted to GenBank database and their accession number listed in **Table 1**.

Table 8 Primers used for amplification and sequencing

Location	Primer names	Primers sequences (5′⟶3′)	References
ITS	ITS1	TCC GTA GGT GAA CCT GCG G	White et al., 1990
	ITS4	TCC TCC GCT TAT TGA TAT GC	
*mat*K	*trn*K 3914F	TGG GTT GCT AAC TCA ATG G	Johnson et al., 1994
	*trn*K 2R	AAC TAG TCG GAT GGA GTA G	
	*mat*K aF	CTA TAT CCA CTT ATC TTT CAG GAG	Kato et al., 1999
	*mat*K 8R	AAA GTT CTA GCA CAA GAA AGT GCA	
*rbc*L	*rbc*L aF	ATG TCA CCA CAA ACA GAG ACT AAA GC	Levin et al., 2003
	*rbc*L R23	TTT TAG TAA AAG ATT GGG CCG	Ohi-Toma et al., 2006
*psb*A-*trn*H	*psb*A-*trn*HF	GTT ATG CAT GAA CGT AAT GCT C	Sang et al., 1997
	*psb*A-*trn*HR	CGC GCA TGG TGG ATT CAC AAT C	
*ycf*1	*ycf*1bF	TCT CGA CGA AAA TCA GAT TGT TGT GAA T	Dong et al., 2015
	*ycf*1bR	ATA CAT GTC AAA GTG ATG GAA AA	
*trn*L-F	*trn*L c 5 f	CGA AAT CGG TAG ACG CTA CG	Taberlet et al., 1991
	*trn*F f 3 r	ATT TGA ACT GGT GAC ACG AG	

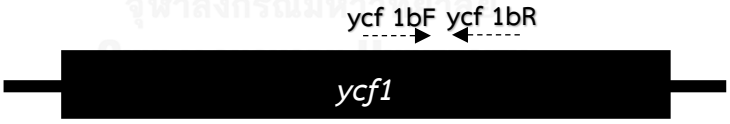
Figure 5 The schematic diagram of the amplificated loci and primer pairs for amplifications and sequencing

(A)

(B)

(C)

(D)



(E)

(F)

3.4 Phylogenetic analysis

For the phylogenetic analysis, the complete sequences of each six loci DNA region, ITS, *mat*K, *rbc*L, *ycf*1, *psb*A-*trn*H and *trn*L-F, were aligned by ClustalW algorithm in MEGA program version 7 (Tamura *et al.*, 2016). All five plastid DNA region sequences, *mat*K, *rbc*L, *ycf*1, *psb*A-*trn*H and *trn*L-*trn*F, were also combined and aligned. Then the aligned data was analyzed for reconstructing phylogenetic trees using MEGA. Some other sequences available in GenBank database were included to this analysis. *Scoparia dulcis* was included as an outgroup and all six loci DNA regions of the plant were sequenced. All phylogenetic trees were inferred using neighbor-joining (NJ) method with maximum composite likelihood model using Kimura-2-parameter nucleotide substitution model with 1,000 replicates of bootstrap supporting analysis. All positions containing gaps and missing data were eliminated by complete deletion.

3.5 Real-time PCR and HRM analysis

The conserved region of *rbc*l, *ycf*1 and *trn*L-F in chloroplast DNA were selected for HRM analysis. The primers sequence for real-time PCR were listed in **Table 9**. The amplification reaction was run for three different regions that indicating as *rbc*L-1P, *ycf*1-1P, *ycf*1-MP and *trn*L-F-MP the product size was 103 bp, 64 bp, 123 bp and 105-126 bp, respectively. The schematic diagram of the amplificated loci and primer pair for real-time PCR was showed in **Figure 6**.

To obtain the DNA melting character of each locus from each species. The real-time PCR and therefore melting step were applied in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) for discriminating species of plants in the genus *Bacopa.* The real-time PCR reaction were carried out in total volume of 20 µl containing of 1X of Ssofast Evagreen Supermix® (Bio-Rad), 0.4 µM of forward primer and reverse primer and 10 ng of plant genomic DNA (Three *Bacopa* species) or the DNA extracted from commercial products. PCR reactions were performed in 96-well plate and the cycling condition consisted of the initial denaturing step at 98 ℃ for 2 min followed by 44 cycles of 98 ℃ for 5 s, 59 ℃ for 30s and 72 ℃ for 20 s. The fluorescent data for PCR amplification were collected during extension step as a green channel. In the final step of amplification, PCR products were denatured at 95 ℃ for 1 min, and then re-annealed at 60 ℃ for 1 min to reform random DNA duplexes. For the melting curve analysis, the PCR product was melted in the ramped steps from 60 ℃ to 90 ℃ in 0.2 ℃ increments and the fluorescence intensity were collected on every increasing point. The melting curves were analyzed using CFX Manager™ Software Upgrade of version 3.1.

Table 9 Designed primer for species identification using real-time PCR coupling with HRM analysis.

Primer set	Primer names	Primers sequences (5′⟶3′)	Amplicon size
*rbc*L-1P	*rbc*L-1PF	ACT TCT TCA CAT TCA CCG TG	103 bp
	*rbc*L-1PR	TCC ACC AGA CAT ACG TAA CG	
*ycf*1-1P	*ycf*1-1PF	TGG CTT TTC TTG AAC GAA TTT GA	64 bp
	*ycf*1-1PR	CAA CAA TCA GGA GAA CGT GAA GA	
*ycf*1-MP	*ycf*1-MPF	CGT TCA AGA AAG GAT CAT ACC C	123 bp
	*ycf*1-MPR	ATC GAA TTG AAG CCC TAG AC	
*trn*L-F-MP	*trn*L-F-MPF	ACA GGA CTT GGA GAA ACC TT	105-126 bp
	*trn*L-F-MPR	CCG ACC ATT ACC AAT GTA AA	

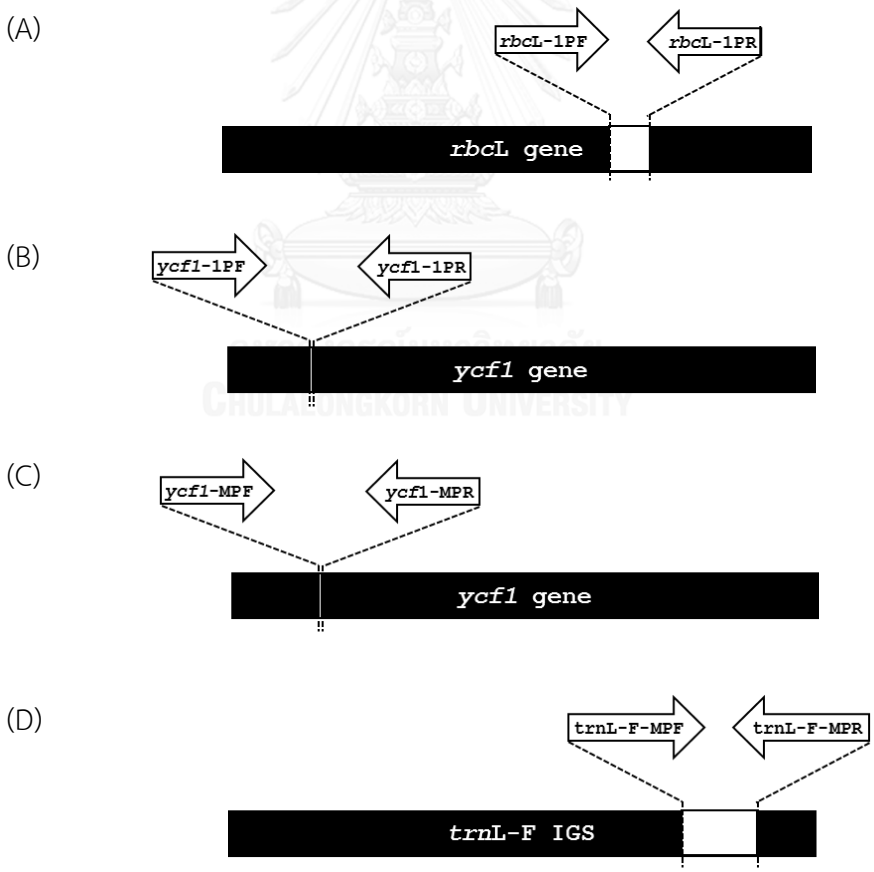


Figure 6 The schematic diagram of the amplificated loci and primer pair for real-time PCR coupling with HRM analysis

CHAPTER IV

RESULTS

4.1 DNA barcoding analysis of *Bacopa* species

The genomic DNA of seven samples of *B. monnieri* (Prom mi), five samples of *B. caroliniana* (Lan Pailin) and two samples of *B. floribunda* (Phak Sam Lun) collected from various locations of Thailand was successfully extracted for DNA barcoding. Six DNA loci were amplified from all genomic DNA samples and subsequently sequenced. The full sequences of five DNA loci from three taxa of *Bacopa* plants including ITS, *mat*K, *rbc*L, *psb*A-*trn*H, *trn*L-F were obtained from the three *Bacopa* plants. The partial sequence of ycf1 region was also retrieved. The lengths of DNA sequences of ITS, *mat*K, *rbc*L, *psbA-trn*H, *trn*L-F were various among the genus *Bacopa*. However, the lengths of *rbc*L gene were similar (**Table 10**). All selected DNA sequences were aligned and subsequently calculated the sequence distances for their relationship (**Table 11**).

Table 10 Sequence analysis of the selected DNA regions of plants in the genus *Bacopa*

Species	DNA region					
	ITS	*mat*K	*rbc*L	*ycf*1	*psb*A-*trn*H	*trn*L-F
Length (bp)	722-731	1521-1536	1434	805-826	405-527	770-955
Variation position (bp)	149	91	32	99	195	228
%Variation	20.5234	5.9438	2.2315	12.1175	42.2078	25.6757
Average %GC content	55.8328	32.7908	43.5379	28.8017	27.3312	35.6383

The complete sequences of ITS region from all *Bacopa* plants were amplified and subsequently bidirectional sequenced. The amplicons were examined by gel electrophoresis and the PCR product sizes were approximately 700 bp. The complete sequences of ITS element from *B. monnieri, B. caroliniana*, and *B. floribunda* were 731, 722, and 725 bp, respectively. The ITS sequences of three *Bacopa* taxa were aligned and the alignment showed the high number variable sites (149 of 731 sites or 20.52%) and high %GC content (55.83%). The ITS sequences of any samples obtained from the same species were identical. A pairwise distance between *B. monnier*i and *B. caroliniana* showed the highest nucleotide sequence divergence (19.6%). A pairwise between *B. caroliniana* and *B. floribunda* representing the lowest sequence divergence (10.2%) (**Table 11A**). The complete ITS sequences of three taxa were submitted to GenBank database and all sequences were shown in **Appendix**.

The complete sequences of maturase K (*mat*K) gene were amplified and sequenced. The PCR products were examined by agarose gel electrophoresis and the PCR product sizes were ranged 1,500 bp. The complete sequences of *mat*K from *B. monnieri, B. caroliniana* and *B. floribunda* were 1536, 1521 and 1536 bp, respectively. To locate the variation sites, the *mat*K sequences of three *Bacopa* taxa were aligned and showed high number of variable sites (91 of 1536 sites or 5.94%). Moreover, %GC content of *mat*K was 32.79% which was lower than that of the ITS aligned. The *mat*K sequences were identical within species. The sequence distances among the three *Bacopa* taxa was analyzed (**Table 11B**). The pairwise distance of *B. monnier*i compared

with *B. caroliniana* revealed the highest value of nucleotide sequence divergence (4.4%) while the sequence divergence between *B. caroliniana* and *B.floribunda* was the lowest (2.7%). All complete *mat*K sequences were submitted to GenBank and the alignment was shown in **Appendix.**

Nevertheless, the complete *mat*K sequences were compiled from the sequencing of two PCR product from four primers including a pair of *trn*K3914F and *mat*K 8R and a pair of *mat*K aF and *trn*K 2R. From the alignment of all *mat*K sequences, two indels were observed in *B. caroliniana* only. There are 6 bp indels from the position 347 to 352 and 9 bp indels from the position 1,525 to 1,533. According to the sequence alignment, the length of complete *mat*K of *B. caroliniana* were shorter (1,521 bp) than others (1,536 bp). The complete sequences of *mat*K gene were successfully amplified and sequenced. The 91 variable sites (5.94%) were observed in the alignment of complete sequences from all species.

The complete sequences of ribulose-1,5-bisphosphate carboxylase/oxygenase large chain gene (*rbc*L) from three *Bacopa* plants were amplified and subsequently bidirectional sequenced. The PCR product were examined by agarose gel electrophoresis and the expected size were approximately 1,500 bp. The complete *rbc*L length of three *Bacopa* taxa were 1,434 bp. All *rbc*L sequences of three *Bacopa* taxa were aligned using MEGA and thee alignment showed 32 positions (2.23%) of variable sites obtained from sequence alignment with 43.54% of GC content. The *rbc*L sequences obtained within same species were identical. The sequences distance

among three *Bacopa* taxa were analyzed (**Table 11C**). Pairwise distances between *B. monnieri* and *B. caroliniana* and between *B. caroliniana* and *B. floribunda* showed the highest value of nucleotide sequence divergences (1.8%). The divergence which pairwise between *B. caroliniana* and *B. floribunda* were the lowest (0.9%). All *rbc*L sequences were submitted to GenBank database. The sequences were shown in **Appendix**.

The partially sequences of hypothetical chloroplast open reading frame 1 gene (*ycf*1) were amplified and subsequently sequenced. The PCR products were examined by agarose gel electrophoresis and the expected sizes were about 800 bp. The length of partial *ycf*1 sequences of *B. monnieri, B. caroliniana* and *B. floribunda* were 805, 820 and 826 bp, respectively. Ninety-nine variable sites (12.12%) with 28.80% of GC content were found in the alignment. All *ycf*1 sequences from the same plant species were identical. The sequences distance among the three *Bacopa* taxa were analyzed (**Table 11D**). Pairwise distance of *B. monnieri* and *B. caroliniana* was 8.1%. The pairwise of *B. caroliniana* and *B. floribunda* were lower at value of 2.2%. All partial *ycf*1 sequences were submitted to GenBank database (**Table 7**). The sequences were shown in **Appendix.**

The complete sequences of photosystem II protein D1- tRNA-Histidine intergenic spacer (*psb*A-*trn*H intergenic spacer) were amplified and sequenced. The PCR products were examined by agarose gel electrophoresis with expected size approximately 500 bp. The length of complete sequences of *psb*A-*trn*H intergenic spacer from *B. monnieri,*

B. caroliniana and *B. floribunda* were 405, 454 and 527 bp, respectively. All *psb*A-*trn*H intergenic spacer sequences were aligned and the alignment showed 195 variation sites (42.21%). with 27.33% of GC content. All *psb*A-*trn*H intergenic spacer sequences within same species were identical. The sequences distance among the genus were analyzed (**Table 11E**). Pairwise distances of *B. monnieri* compare with *B. caroliniana* was 9.3% which greater than the pairwise of *B. caroliniana* and *B. floribunda* (2.5%). All sequences of *psb*A-*trn*H intergenic spacer were submitted to GenBank database (**Table 7**). The sequences were shown in **Appendix.**

The complete sequences of tRNA-Leucine – tRNA-Phenylalanine intergenic spacer (*trn*L-F intergenic spacer) were successfully amplified and sequenced. The amplicons were examined by agarose gel electrophoresis and the expected size were 800 bp. The length of complete sequences of *trn*L-F intergenic spacer from *B. monnieri, B. caroliniana* and *B. floribunda* were 955, 770 and 939 bp, respectively. All *trn*L-F intergenic spacer sequences were aligned and the alignment showed 228 variable sites (25.68%) among the genus with 35.64% of GC content. All samples with the same species revealed the resemblance of *trn*L-F intergenic spacer sequences. The sequences distance among three *Bacopa* taxa was analyzed (**Table 11F**). Pairwise distances value between *B. monnieri* and *B. caroliniana* were 2.4% which greater than the pairwise of *B. caroliniana* with *B. floribunda* 0.8% . All complete *trn*L-F intergenic spacer sequences of three taxa were submitted to GenBank database (**Table 7**)**.** The sequences were shown in **Appendix**.

Table 11 Interspecific distance analysis among three Bacopa plants

(A)

ITS	*B. monnieri*	*B. caroliniana*	*B. floribunda*
B. monnieri			
B. caroliniana	0.196		
B. floribunda	0.102	0.165	

(B)

*mat*K	*B. monnieri*	*B. caroliniana*	*B. floribunda*
B. monnieri			
B. caroliniana	0.044		
B. floribunda	0.027	0.032	

(C)

*rbc*L	*B. monnieri*	*B. caroliniana*	*B. floribunda*
B. monnieri			
B. caroliniana	0.018		
B. floribunda	0.009	0.018	

(D)

*ycf*1	*B. monnieri*	*B. caroliniana*	*B. floribunda*
B. monnieri			
B. caroliniana	0.081		
B. floribunda	0.022	0.080	

(E)

*psb*A-*trn*H	*B. monnieri*	*B. caroliniana*	*B. floribunda*
B. monnieri			
B. caroliniana	0.093		
B. floribunda	0.025	0.084	

(F)

*trn*L-F	*B. monnieri*	*B. caroliniana*	*B. floribunda*
B. monnieri			
B. caroliniana	0.024		
B. floribunda	0.008	0.021	

4.2 Phylogenetic analysis of the three *Bacopa* taxa based on six loci DNA barcodes

Seven NJ trees of three *Bacopa* species collected in Thailand and *Scoparia dulcis* were generated from six DNA loci: ITS, *mat*K, *rbc*L, *ycf*1, *psb*A-*trn*H, and *trn*L-F DNA regions. The tendency of the phylogenetic results was to group *B. monnieri* and *B. floribunda* sisterly with bootstrap supports as 99 %, 84 %, 59 %, 94%, 90%, 96%, and 100% on ITS, *mat*K, *rbc*L *ycf*1, *psb*A-*trn*H, *trn*L-F, and the five-loci combined NJ trees, respectively (**Figure 7, 8, 9, 10, 11, 12, and 13**). *Bacopa monnieri* and *B. floribunda* are the old-world plants distributed in South Asia (india), East Asia (China), South-East Asia (Oriental) and Australia while *B. caroliniana* is the new-world plant which is native to North and South America. Notably, a high length-variation caused by numerous nucleotide insertions and deletions was found in the *psb*A-*trn*H intergenic spacer and would be a caution to use this region for genetic relationship analysis among plants in the genus *Bacopa* without using "complete deletion" option.

The phylogenetic analysis based on ITS region revealed that the ITS sequence of *B. monnieri* available in GenBank (AY492095.1) was classified in the same group as all seven ITS sequences from *B. monnieri* plant materials sampled in this study (with 100% bootstrap). The two samples of *B. floribunda* and five of *B. caroliniana* were put in their own groups with high 99% and 100% bootstrap values, respectively (**Figure 7**).

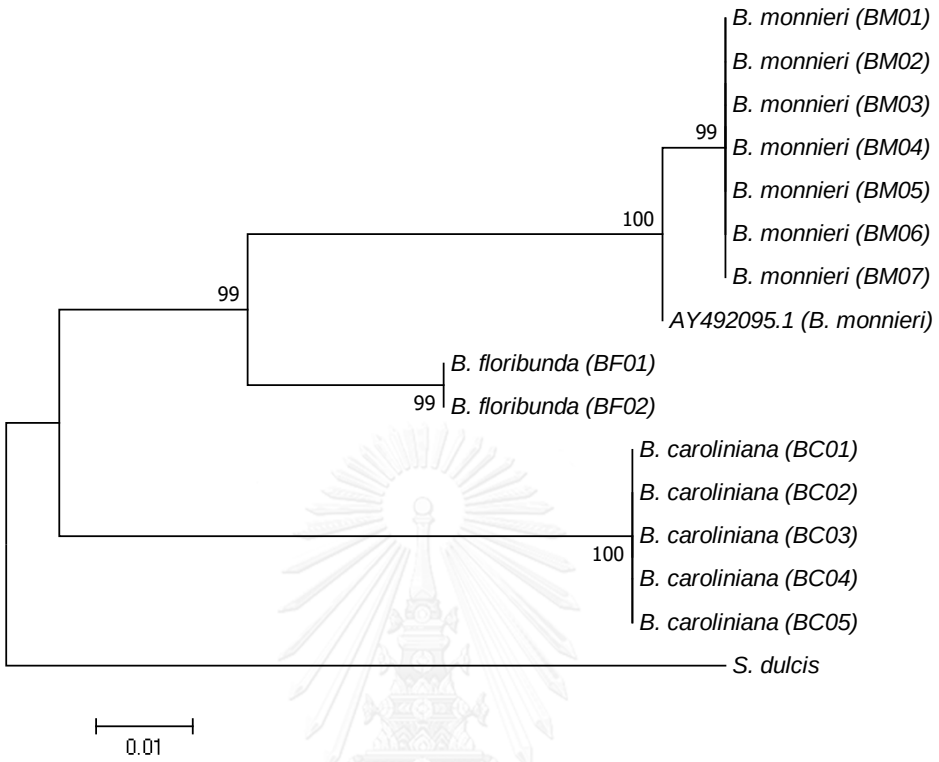


Figure 7 The phylogenetic assessment of plant in the genus *Bacopa* based on ITS region.

The phylogenetic analysis based on *mat*K region showed that the *mat*K sequence of *B. monnieri* from GenBank (AY667458.1) was grouped with all seven *mat*K sequences from plant materials in this study (100% bootstrap). Both samples of *B. floribunda* and five of *B. caroliniana* in this study were classified into their own group with high bootstrap supports of 100% and 100%, respectively (**Figure 8**).

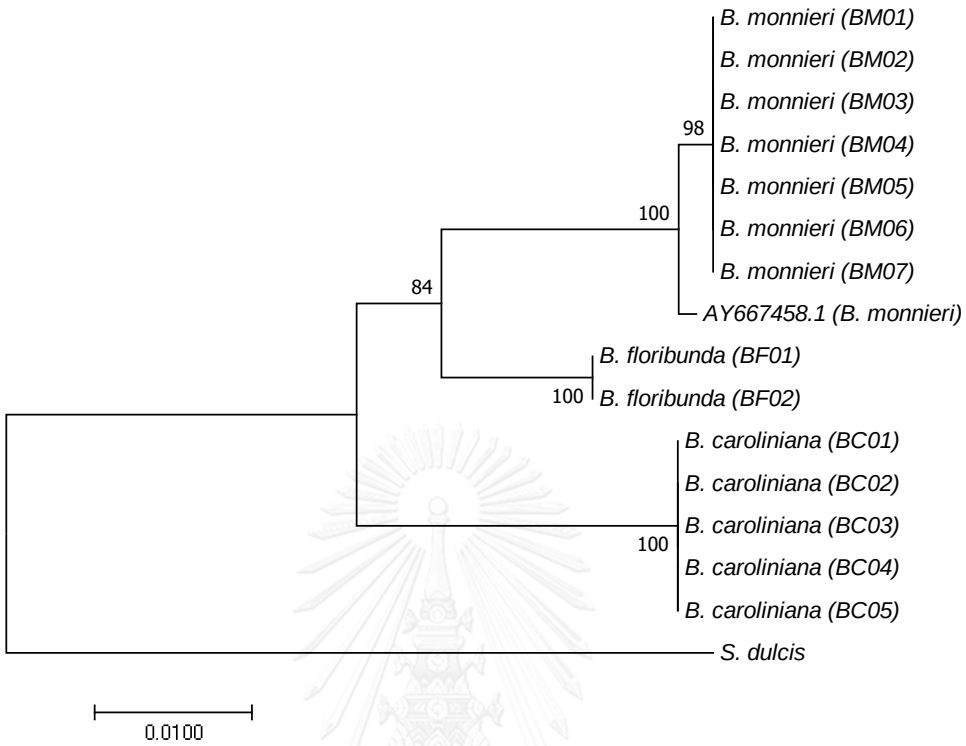


Figure 8 The phylogenetic assessment of plant in the genus *Bacopa* based on *mat*K region.

The phylogenetic assessment of *rbc*L region also put the *rbc*L sequence of *B. monnieri* (KJ773301.1) available in GenBank into the same group of as all seven *B. monnieri mat*K sequences in this study (99 % bootstrap). Likewise, the sequence of *B. caroliniana* accession no. AF123670.1 was grouped with the other five *B. carolinina* with high bootstrap 100%. All seven samples of *B. monnieri,* two of *B. floribunda* and five of *B. caroliniana* were clustered into their own groups with high bootstrap supports of 99%, 99% and 99%, respectively (**Figure 9**).

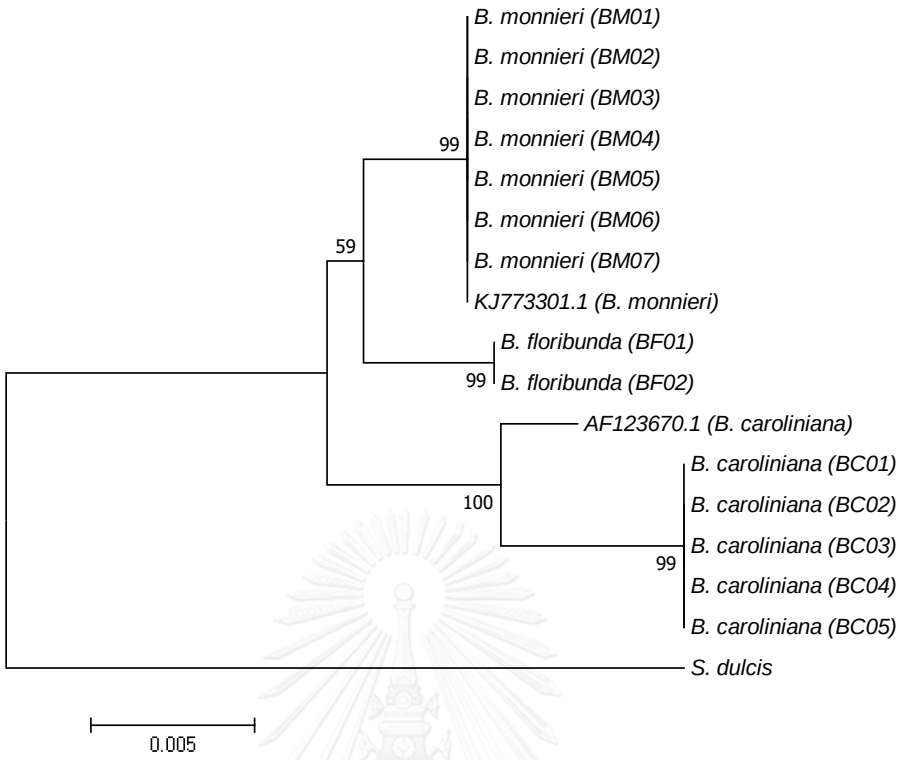


Figure 9 The phylogenetic assessment of plant in the genus *Bacopa* based on *rbc*L region.

Based on the *ycf*1 region, all of the plant samples of *B. monnieri, B. floribunda* and *B. caroliniana* were classified into their own groups with high bootstrap 100% support (**Figure 10**). No sequence of *ycf*1 regions of the genus *Bacopa* had been submitted to GenBank.

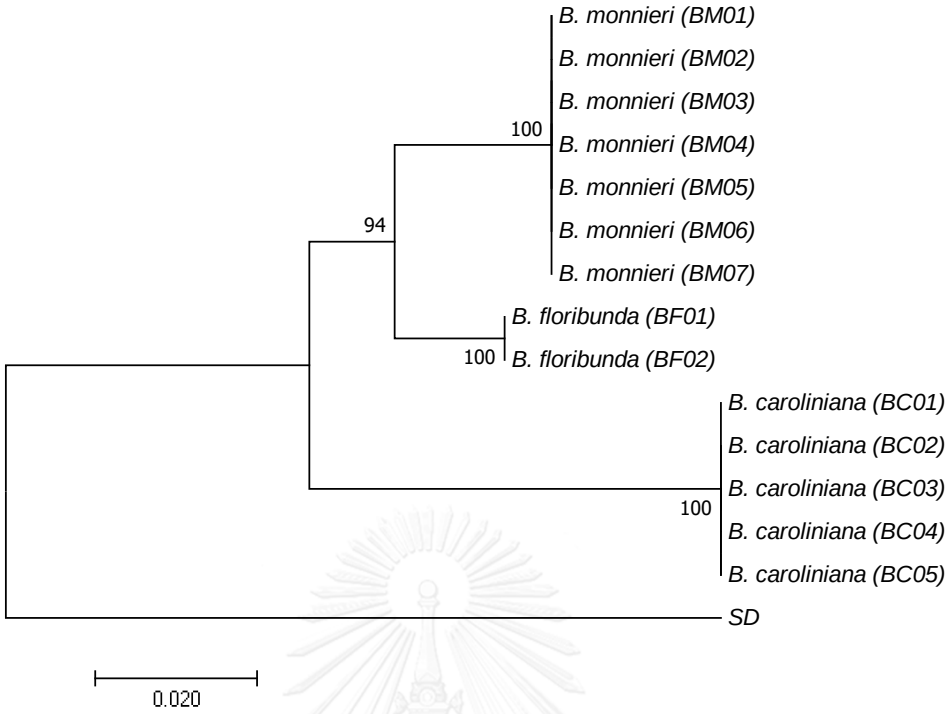


Figure 10 The phylogenetic assessment of plant in the genus *Bacopa* based on *ycf*1 region.

The phylogenetic analysis based on *psb*A-*trn*H region showed that the *psb*A-*trn*H sequence of *B. monnieri* from GenBank (KR108254.1) was grouped with all seven *psb*A-*trn*H sequences from plant materials in this study (99% bootstrap). Both samples of *B. floribunda* and five of *B. caroliniana* in this study were classified into their own group with high bootstrap supports of 73% and 100%, respectively (**Figure 11**).

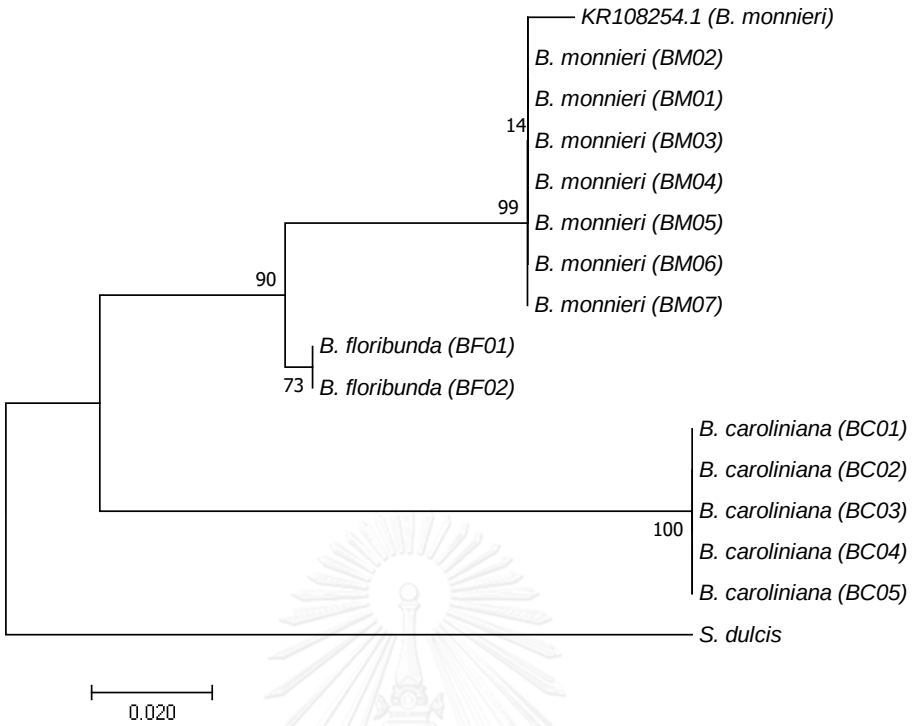


Figure 11 The phylogenetic assessment of plant in the genus *Bacopa* based on *psb*A-*trn*H region.

The phylogenetic relationship of *Bacopa* plant specimens based on *trn*L-F gene revealed that the *trn*L-F sequence of *B. monnieri* from previous study (AY492170.1) was located within the same group as all seven plant materials collected in this study (99% bootstrap). The samples of *B. floribunda* and *B. caroliniana* were classified in their own groups with high bootstrap 88% and 100% supporting values, respectively **(Figure 12).**

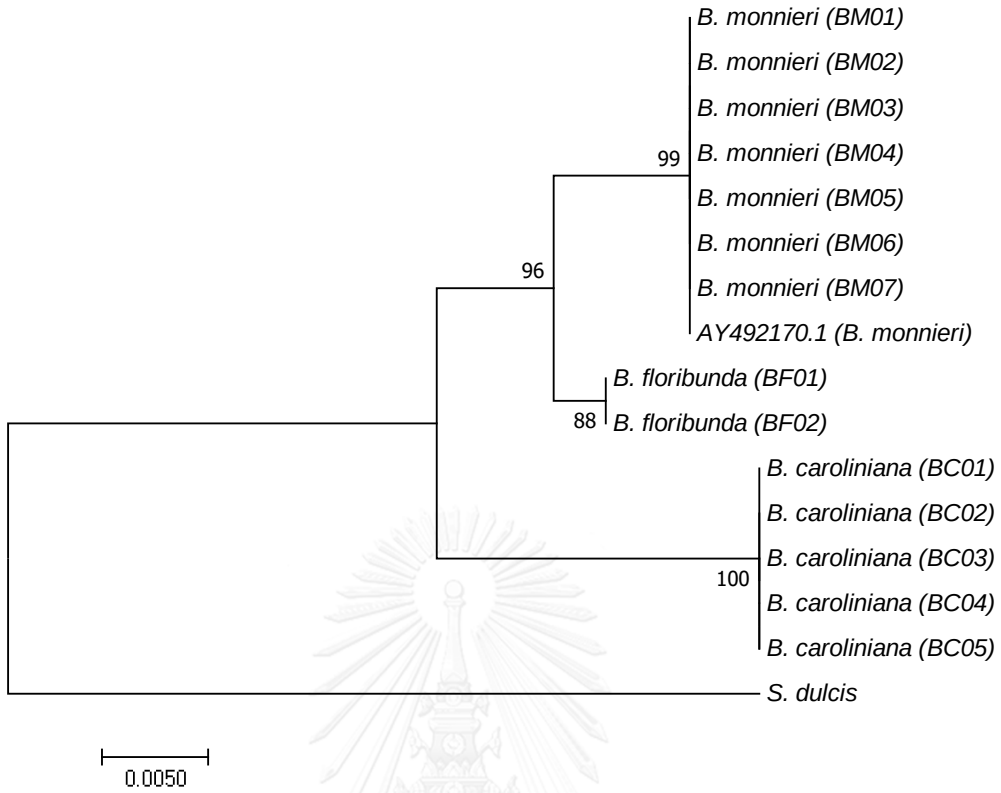


Figure 12 The phylogenetic assessment of plant in the genus *Bacopa* based on *trn*L-F region.

The phylogenetic relationship of *Bacopa* plants based on combined-data of the five chloroplast DNA regions confirmed that all samples of *B. monnieri* was grouped with *B. floribunda* sisterly found the plant materials in same species which used in this study were classified in same group of each species with the high bootstrap 100%. Some morphological characteristics and chemical constituents were then mapped to the tree to suggest the relationship between the three *Bacopa* species. (**Figure 13**).

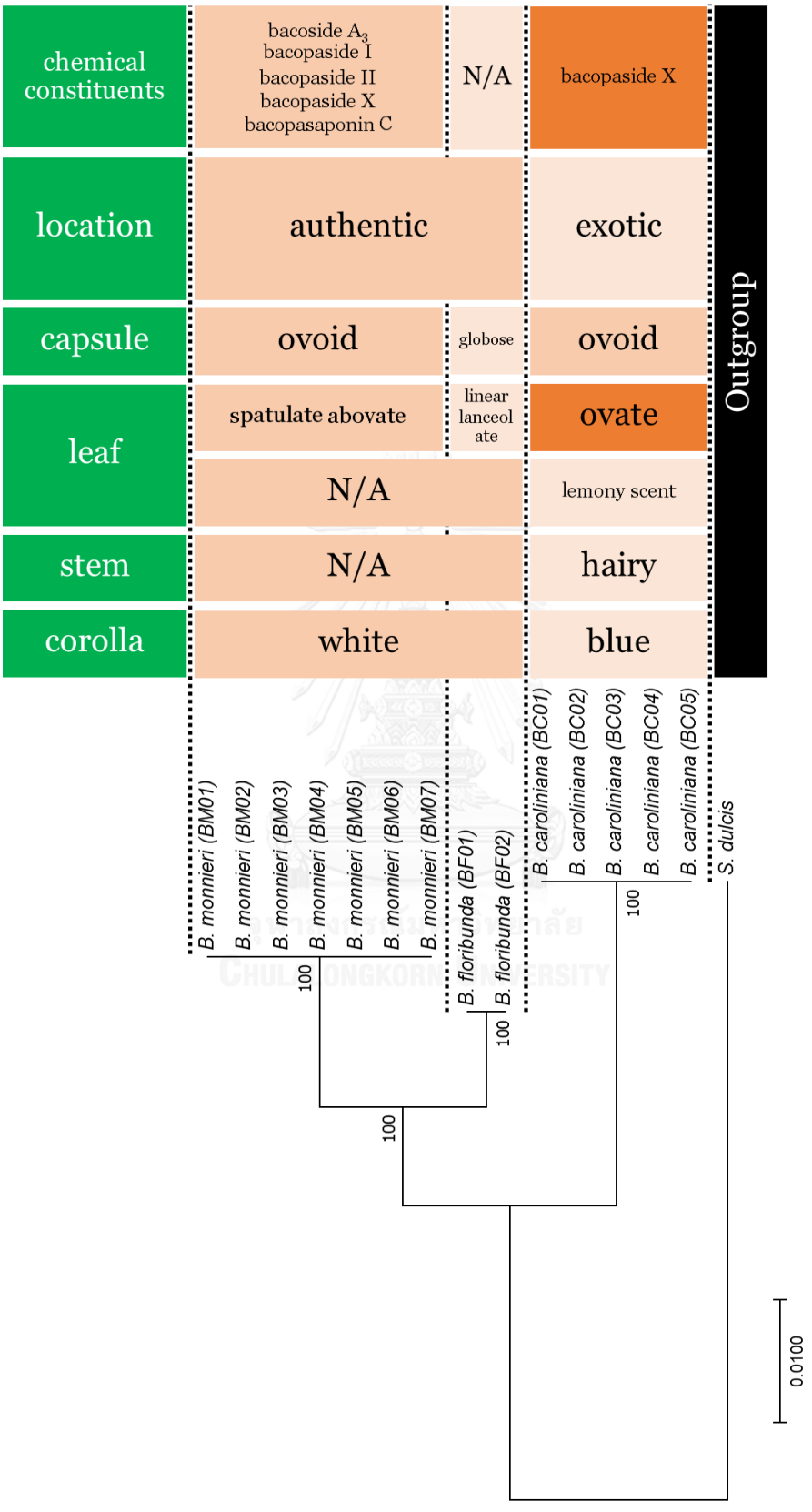


Figure 13 The phylogenetic assessment of plant in the genus *Bacopa* based on five loci plastid genome.

4.3 Barcoding – HRM analysis for identification of the three *Bacopa* plants.

Two primers sets, *rbc*L-1P (**Figure 14**) and *ycf*1-1P (**Figure 15**), were designed to cover the range of single nucleotide polymorphisms (SNPs) of *rbc*L and *ycf*1 of the three *Bacopa* taxa, respectively. The amplicons were produced using real-time PCR couple with HRM analysis. As a result, both of *rbc*L-1P and *ycf*-1P primers can discriminate *B. monnieri* from the others as the melting curve patterns were separated into two groups. The first group is the specific melting curve of *B. monnieri*, and the other group is the similar curve of *B. caroliniana* and *B. floribunda*.

The *rbc*L-1P PCR amplicons in of the three *Bacopa* samples were in the same size as 103 bp. However, one SNP were found at the position 927 of *rbc*L gene aligned in which *B. monnieri* sequence was different from the others. The result showed that the melting curve of *B. monnieri* was isolated from other *Bacopa* species. The melting temperatures (T_m) of *B. monnieri*, *B. caroliniana*, and *B. floribunda* amplicons were 77.8-78.2, 77.4 and 77.4 °C, respectively (**Figure 16**). This method therefore can distinguish *B. monnieri* from related species.

The *ycf*1-1P primer set was designed based on *ycf*1 sequences of three *Bacopa* species and their amplicons were in the same size as 64 bp. But one SNP of *B. monnieri* sequence was found different from the others. These designed primers were investigated on three *Bacopa* species samples to produce melting curves for species discrimination. The result showed that the melting curve of *B. monnieri* was separated

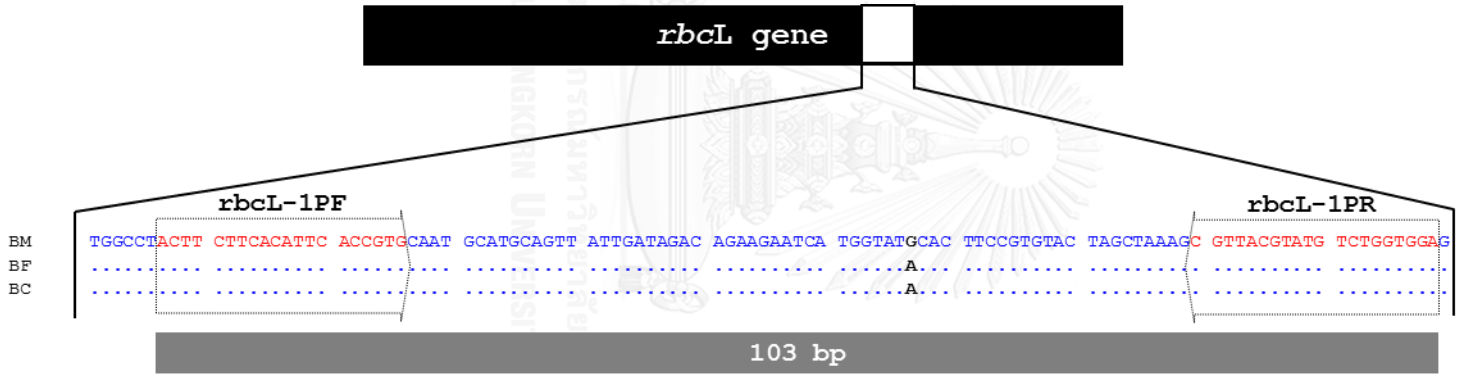


Figure 14 The schematic structure of the amplificated *rbc*L loci and the *rbc*L1-1P primer pairs for barcoding-HRM analysis

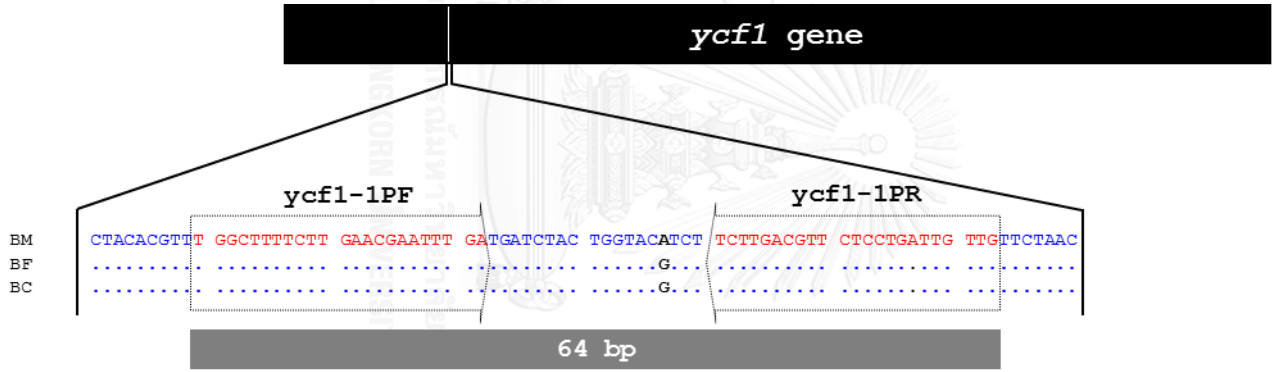


Figure 15 The schematic structure of the amplificated *ycf*1 loci and the *ycf*1-1P primer pairs of for barcoding HRM analysis

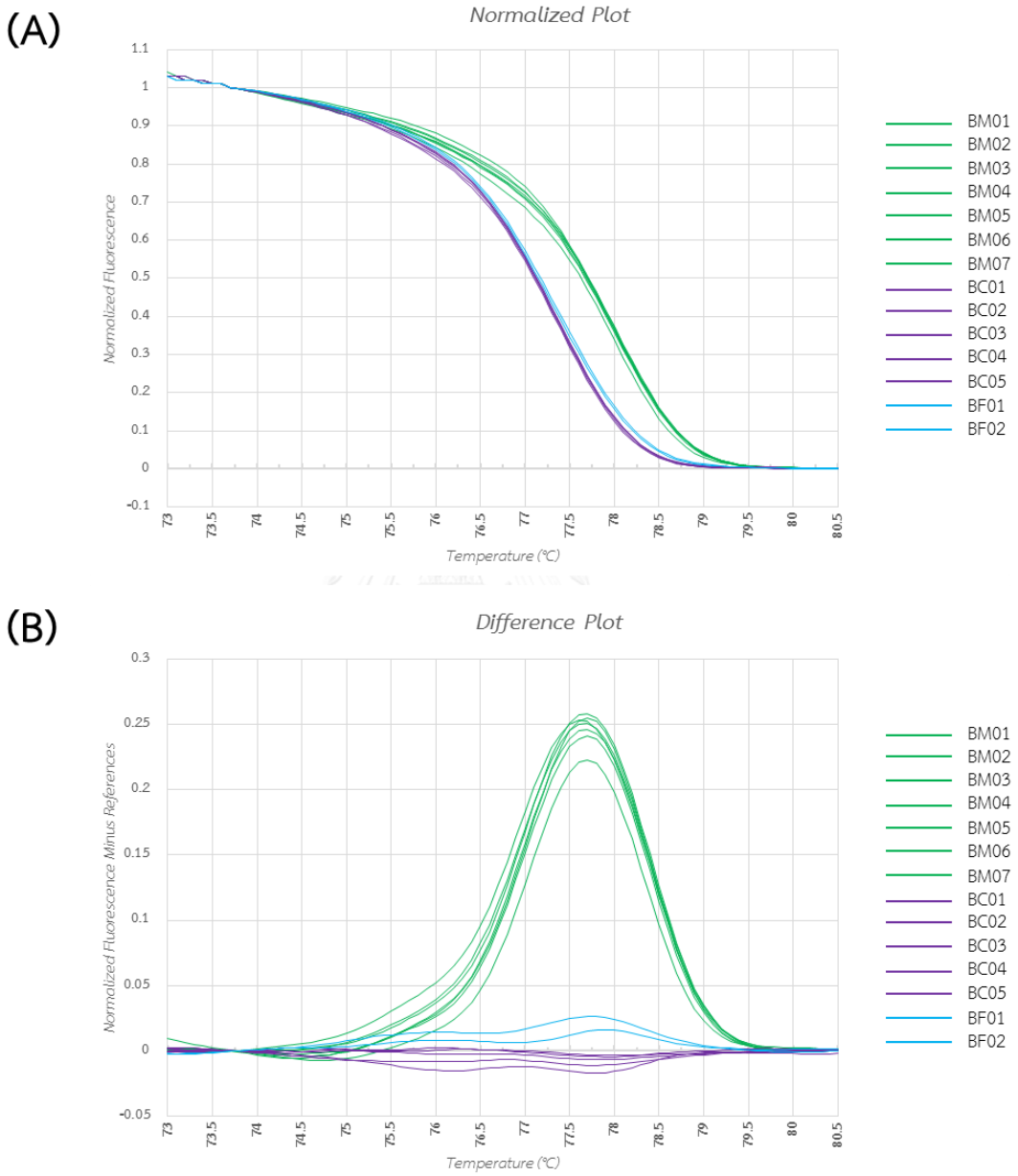


Figure 16 The barcoding-HRM melting curve of PCR amplicons amplified by *rbc*L-1P primer set to identify *Bacopa* plants.

(A) Normalized Plot (B) Difference Plot

from the other two *Bacopa* species. The melting temperature (T_m) of each species were 73.8-74.0, 74.4-74.6 and 74.8 °C, respectively (**Figure 17**). This method can distinguish *B. monnieri* from the related species.

Other two sets of designed primer, *ycf*1-MP (**Figure 18**) and *trn*L-F-MP (**Figure 19**), were designed to cover the range of various SNP of *ycf*1 and *trn*L-F of three *Bacopa* taxa, respectively. The design primer set was evaluated on three *Bacopa* species using real-time PCR couple with HRM analysis. The primer set was evaluated on three *Bacopa* species melting curve pattern. The specific melting curves of each species were separate into three groups which each group is represent there each species. As a result, the *ycf*1-MP can be differentiated each three *Bacopa* species. The melting temperature (T_m) of each species were 74.6, 74.0 and 74.8-75, respectively (**Figure 20**). The *trn*L-F-MP primer set was designed to cover the range of indels and several SNP, the same concept as *ycf*1-MP primer set. These designed primers were investigated on the three *Bacopa* taxa to produce melting curves for species discrimination. The pairs of *trn*L-F-MP primers can be differentiated the species of plant based on the specific melting curve and melting temperature. The melting temperature (T_m) of each species were 74.6-74.8 , 73.8 and 75.4, respectively (**Figure 21**). The specific T_m of each plant obtained from the HRM analysis using *ycf*1-MP and *trn*L-F-MP primer can be used for authentication of *Bacopa* species.

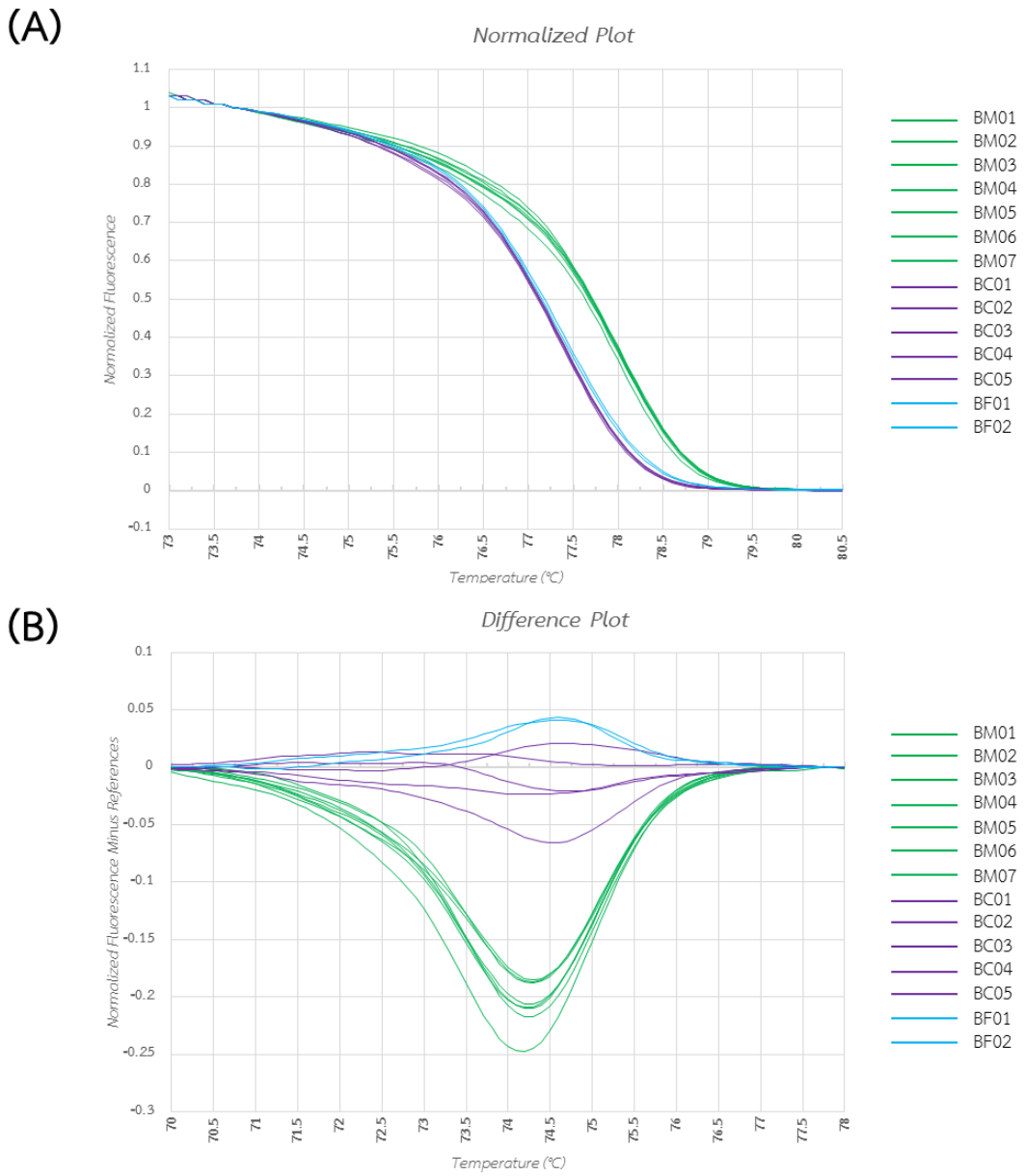


Figure 17 The barcoding-HRM melting curve of PCR amplicons amplified by *ycf*1-1P primer set to identify *Bacopa* plants.
(A) Normalized Plot (B) Difference Plot

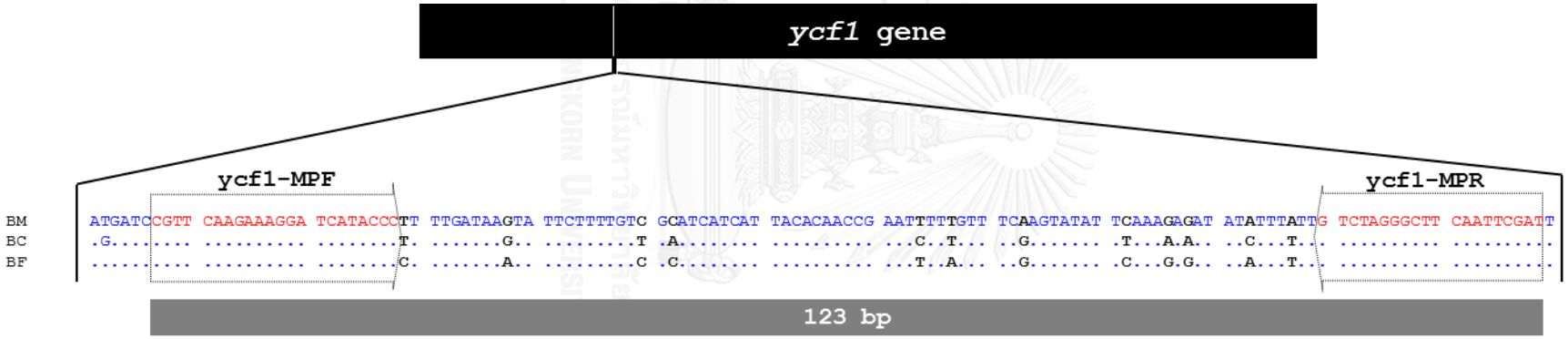


Figure 18 The schematic structure of the amplificated *ycf*1 loci and the *ycf*1-MP primer pair of for barcoding-HRM analysis

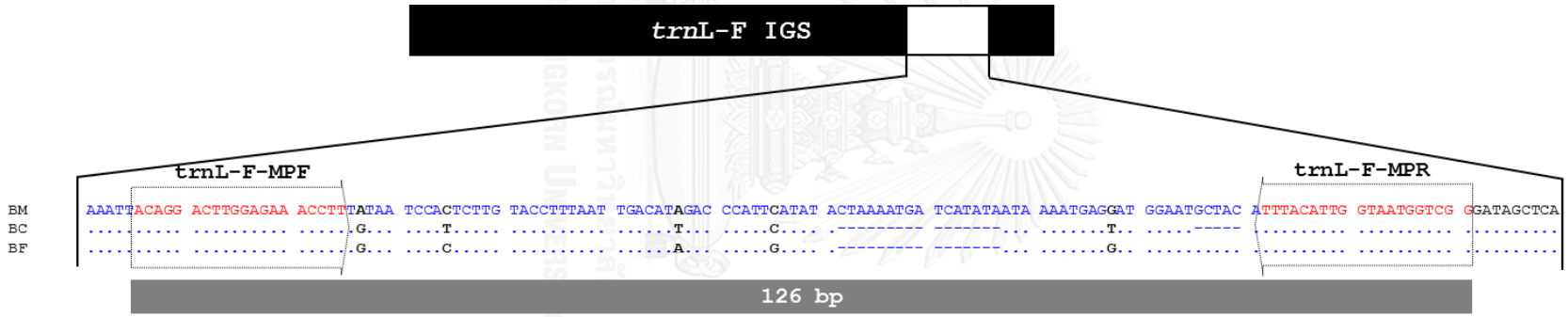


Figure 19 The schematic structure of the amplificated *trn*L-F intergenic spacer loci and the *trn*L-F-MP primer pair of for barcoding HRM analysis

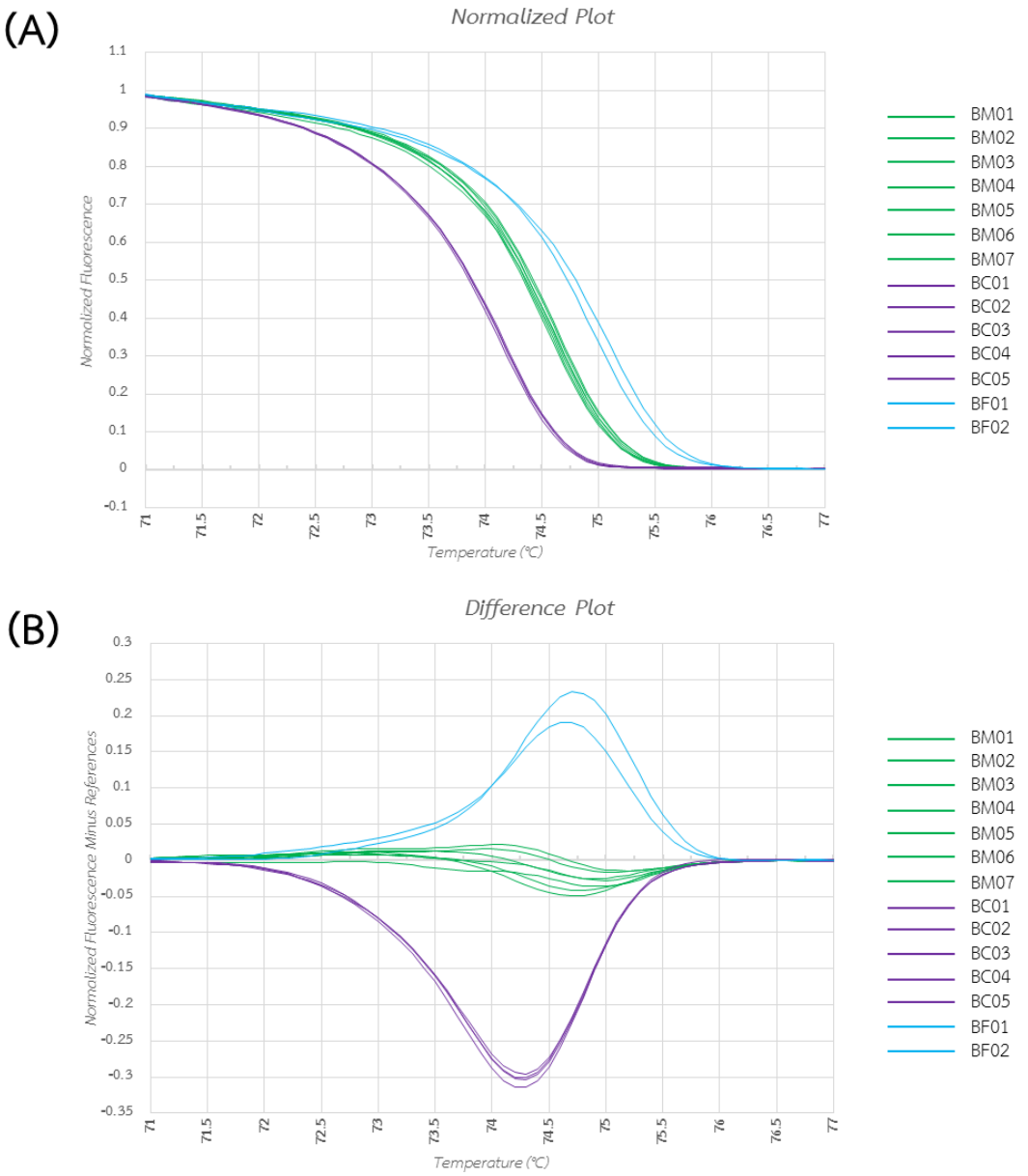


Figure 20 The barcoding-HRM melting curve of PCR amplicons amplified by *ycf*1-MP primer set to identify *Bacopa* plants.

(A) Normalized Plot (B) Difference Plot

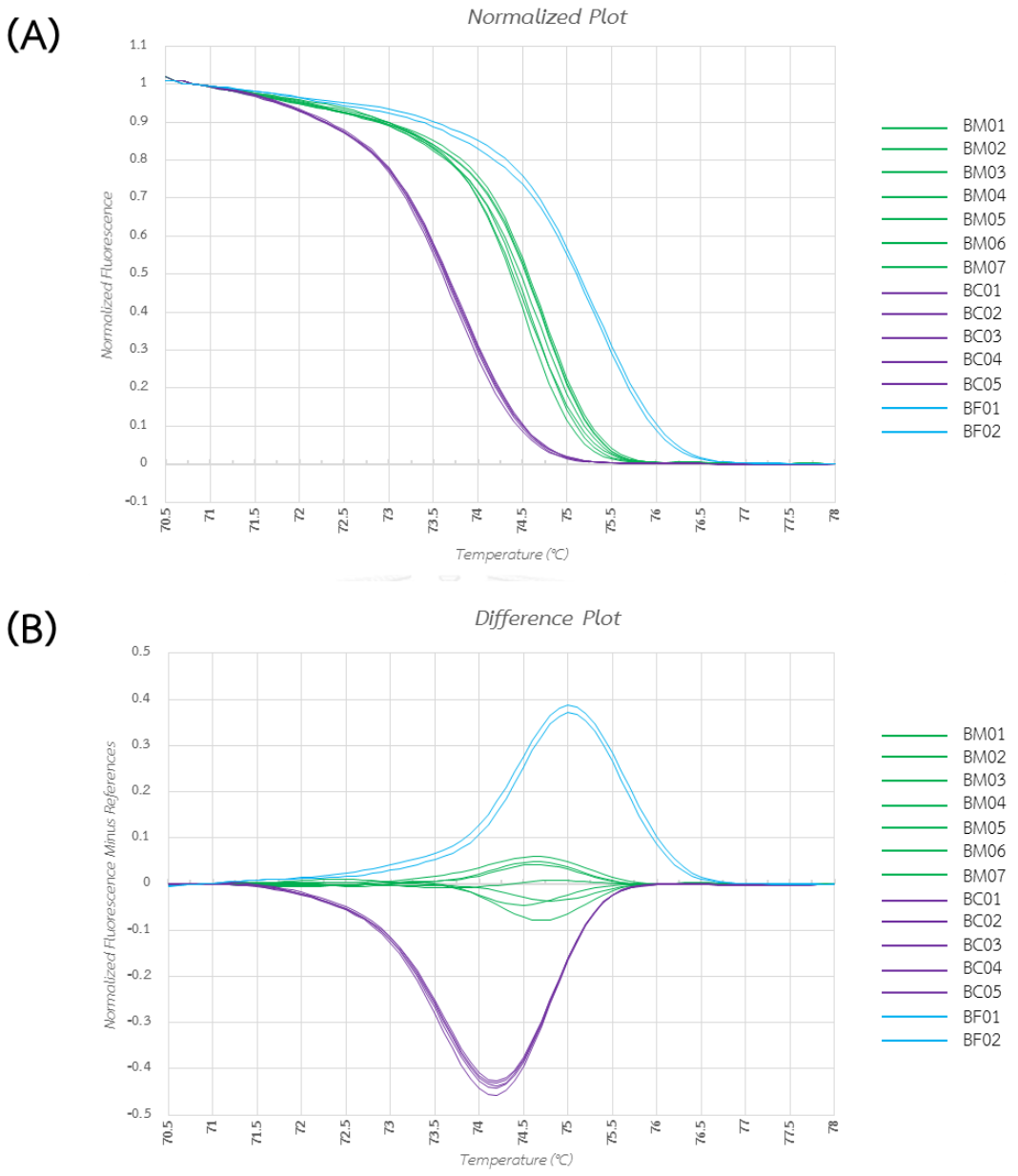


Figure 21 The barcoding-HRM melting curve of PCR amplicons amplified by *trn*L-F-MP primer set to identify *Bacopa* plants.

(A) Normalized Plot (B) Difference Plot

CHAPTER V

DISCUSSIONS

5.1 DNA barcoding analysis for *Bacopa* species

Recently, the herbal authentication is an issue of concern since medicinal plants were often adulterated for the profitable gain with other low-priced plants or unintentional substitution by misidentification of raw materials. The authentication is important for quality control, product efficacy and safety. Various conventional authentication methods were utilized for medicinal plant identifications including, morphological and cytological analysis and chemical approaches (Zhao *et al.*, 2006). However, the discrimination of counterfeit from the genuine plants by the conventional methods is difficult which the requirement of experts and advance equipment. *Bacopa monnieri* or Prommi was manufactured as commercial dietary supplements for cognitive improvement (Russo and Borrelli, 2005). The authentication of this plant by their phenotypic character is difficult since this plant represent a wide variety of structure especially leave size and trait. DNA barcoding is one of DNA-based markers and has application in plant identification. It can be used to identify unknown sample affiliating pre-existing characterization. There are three criteria for selecting the DNA region as an ideal barcode (I) easy to amplify and sequence, (II) the

PCR product not greater than 1 kb in size, (III) representing higher interspecific variation than intraspecific variation.

In this study, ITS, *matK*, *rbcL*, *ycf*1, *psb*A-*trn*H and *trn*L-F, were analyzed from three *Bacopa* existing in Thailand. Selected six loci were successfully performed using the universal primer for each DNA regions. The complete sequences of each DNA region were obtained with high quality of nucleotide chromatogram. After analyzed, the degree of sequence variations of *psb*A-*trn*H, *trn*L-*trn*F, ITS, *ycf*1, *mat*K, *rbc*L among the *Bacopa* species were on the order from 42.21%, 35.64%, 20.52%, 12.12%, 5.94%, 2.23% respectively.

*psb*A-*trn*H is one of most used as DNA barcoding for plants. This study, the length of complete *psb*A-*trn*H sequence from *B. monnieri, B. caroliniana* and *B. floribunda* were 405 bp, 454 bp and 527 bp respectively. The size differences of *psb*A-*trn*H amplicons were easily analyzed by gel electrophoresis. Therefore, the different of *psb*A-*trn*H size were capable to use for the discrimination of *Bacopa* plants. This result was likewise to the study of Vongsak *et al*., they found that the *psb*A-*trn*H size among the plant in genus *Stemona* were different which can analyze by using simple PCR and gel electrophoresis (Vongsak *et al.*, 2008). The *psb*A-*trn*H sequence of *Bacopa monnieri* plant (GenBank No. KR108254.1) was also investigated from the study of the extent of adulteration/substitution for highly traded medicinal plants in crude herbal market using DNA barcodes in South India (unpublished). Moreover, several studies revealed the potential use of *psb*A-*trn*H for the characterization of of Pteridophytes in

Traditional Chinese Medicine (Ma *et al.*, 2010), plant in the family Myristicaceae (Newmaster *et al.*, 2008), Dendrobium species (Yao *et al.*, 2009) and also the identification of the botanical origins of Flos Lonicerae Japonicae and Flos Lonicerae (Sun *et al.*, 2011).

The sequences of *trn*L intron and the intergenic spacer between *trn*L and *trn*F has been consider as the additional barcodes for plant identification, characterization and phylogenetic analysis (Hollingsworth *et al.*, 2011). In this study, the percentage of sequences variations in *trn*L-F region (35.64%) was lesser extent to *psb*A-*trn*H (42.21%). 228 polymorphic sites identified in *trn*L-F reveals to the high sequence variation of this region. The nucleotide polymorphism is beneficial for primer design in real-time PCR and melting curve analysis for plants discrimination (Madesis *et al.*, 2012). As mentioned, the complete sequence of *trn*L-F was available in GenBank only a sequence from *B. monnieri* [AY492170.1]. The sequences of *trn*L-F were used with other regions including ITS, *rps*16 intron and *mat*K-*trn*K intron in the phylogenetic analysis of plant in family Plantaginaceae which the plants from genus Bacopa were included (Albach *et al.*, 2005). Moreover, *trn*L-F and its combinations with ITS were proposed as barcoding system for identification of *Taxus* lineages in Eurasia. This result shows the potential use of *trn*L-F region in plants characterization (Liu *et al.*, 2011).

ITS barcoding is a distinguishable choice for the discrimination of certain organisms. The previous study shown the potential of use of ITS for medicinal plant identification. The ITS2 region was proposed as DNA barcode for the identification of

Gentianopsis paludosa (XUE and LI, 2011), *Boerhavia diffusa* (Selvaraj *et al.*, 2012), *Isatis indigotica* (Chen *et al.*, 2014), rhubarb sources plant (Zhou *et al.*, 2017) and medicinal plant in *Bupleurum* spp. (Chao *et al.*, 2014) and plants from family Euphorbiaceae (Pang *et al.*, 2010). As discussed, ITS sequences were observed only in *B. monnieri* from several studies (GenBank accession No. KP844738.1, KX365333.1, KR215626.1, KM887387.1 and AY492095.1).

Ycf1 is the novel coding gene for using as DNA barcodes for land plants. It has the satisfaction performance for the differentiation of medicinal plants. However, some plants are lacking of ycf1 which is the limitation for using this gene to identify plant (Dong *et al.*, 2015). From DNA sequencing, there were 99 polymorphic sites (12.11%) observed among the three *Bacopa*. Therefore, the sequencing shows the flanking conserved region with were beneficial for downstream applications including HRM analysis.

The sequence analysis of six loci of *Bacopa* plants shows the potential of the individual DNA loci for identification of medicinal plant. However, the DNA marker should be developed based on an advantage of each DNA regions. Therefore, the DNA barcoding should be established in many medicinal plants for the proper DNA barcodes for individual plant genus.

5.2 Phylogenetic analysis of *Bacopa* plants found in Thailand.

All phylogenetic analyses of both nuclear and mitochondrial DNA regions showed similar results that they separated the two old-world *Bacopa* plants (*B. monnieri* and *B. floribunda*) from the new-world plant (*B. caroliniana*). Although *B. monnieri* and *B. floribunda* were different in morphological characteristics, their genetic relationship revealed that they are closely related taxa living in similar oriental areas. On the other way around, *B. caroliniana* which is morphologically resemble to *B. monnieri*, was found to be distantly related to *B. monnieri*. This could be seen as the result of convergent evolution in which both plants have been evolutionarily adapted to similar environmental constraints and therefore have had some similar characteristics of their morphology.

Although most of the DNA sequences used in this study were suitable for phylogenetic analysis of the three *Bacopa* plants, the *psb*A-*trn*H region was found not to be the best one as its nucleotide alignment had too many ambiguous sites and then could give a rather different result from the other regions if not using "complete deletion" option. However, this region had a large 73 – 122 basepair gap difference among the three *Bacopa* species caused by long indels (insertion/deletion) within the DNA sequences and then can be useful for developing a simple fragment-based molecular marker for *Bacopa* plant species identification.

The sisterly phylogenetic grouping of *B. monnieri* and *B. floribunda* was supported by their similar morphology such as leaves, corolla, stem and capsule. The color of corolla in these two species was whitish but that of *B. carolinina* was blue. The stem of *B. caroliniana* was composite with hairy but those of the other two were not. Moreover, the crushed leaf of *B. caroliniana* has a lemon scent but absent in *B. monnieri* and *B. floribunda*. Nevertheless, some morphological characteristics did not show correlation. For example, shapes of the leaves of three taxa were different among species. Moreover, the capsule shapes of *B. monnieri* and *B. caroliniana* were similar but *B. floribunda* capsule had different shape.

The locality distributions of the three *Bacopa* plants suggested the correlation with phylogenetic analysis. *B. monnieri* and *B. floribunda* are the authentic plants distributed in South Asia (India), East Asia (China), and South-East Asia but *B. caroliniana* is distributed in North America. However, the analysis of chemical constituents of three bacopa plants showed uncorrelation of their chemical constituents. *Bacopa monnieri* and *B. caroliniana* consisted of bioactive saponins but *B. floribunda* did not have any saponins.

5.3 HRM analysis for authentication of *Bacopa* plants materials.

Quality and safety are the crucial concerns of medicinal consumable products. The certified product by both assessments can increase products value and the economic gain of manufacturers in trading. The rapid and reliable authentication method has been developed for product accreditation. Barcoding HRM (Bar-HRM) is accurate, reliable and powerful molecular technique that can differentiate the species of related medicinal plants. The Bar-HRM was implemented in rapid analysis of genetic variation (SNP and indel) in commercial dietary and herb products.

HRM analysis was recently applied for the identification plant species in various dietaries products including *Lathyrus clymenum* (Ganopoulos *et al.*, 2012) and *Glycine max* (Madesis *et al.*, 2012), and herbs such as *Phyllanthus* spp. (Buddhachat *et al.*, 2015), *Croton* spp. (Osathanunkul *et al.*, 2015) and almond cultivar (Wu *et al.*, 2008). The specific primers for identification of plant in the genus *Bacopa* were designed based on the DNA sequences of *rbc*L, *ycf1* and *trn*L-F. The three loci were selected for HRM analysis for two reasons. First, the DNA region should have an appropriate polymorphism for the differentiation by T_m. Second, the DNA region should have a conserve sequences within genus for the specific primer design. Four sets of primer were designed for using in HRM analysis to discriminate of *B. monnieri* from others.

PCR primer sets of *rbc*L-1P and *ycf1*-1P were designed cover the single nucleotide polymorphism. The different nucleotide in same position of *B. monnieri ,*

B. caroliniana and *B. floribunda* were G,A,A for *rbc*L and A,G,G for *ycf*1, respectively. A simple nucleotide differences were enough for differentiation by HRM analysis (**Figure 14 and 15**). The *rcb*L gene sequence has the great extent of conserve sequence and the amplification of this DNA region can be easily amplified from most land plants by using universal primers. The sequence conservation can make the falsification when analyzed (Buddhachat *et al.*, 2015). Therefore, *rbc*L region is an improper region for discrimination of plant species using HRM analysis. In this study, the *rbc*L primers for HRM analysis were nonspecifically to Bacopa plants. These primers can also amplify the other *rbc*L sequences which analyzed by Primer BLAST. Interestingly, *ycf*1 region, the novel candidate DNA barcode for land plants with highly DNA polymorphism. The flanking conserved areas which cover variation region were used for the primer design. This primer pairs were successfully used in HRM for the discrimination of *B. monnieri* from the others by the differences of T_m and the melting curve pattern. The T_m of *B. monnieri, B. caroliniana* and *B. floribunda* and BF were 77.8-78.2, 77.4 °C and 74, 74.4.-74.8 °C, respectively. The normalize plot and difference plot were shown in **Figure 16 and 17** The differences of melting plot from others plant were calculated and analyzed relative to the normalization of *B. monnieri*. Although, these two pair of primers can be used to identify *B. monnieri*. However, the specific primers which can separate three species from each other were developed.

To solve this concern, the further designed primers for identification of each three *Bacopa* plants were developed. *Ycf1*-MP and *trn*L-F-MP were designed covered

the multi-variation position. (**Figure 18 and 19**) *Bacopa* plant were successfully discriminated from each other by two sets of primer. The percentage of GC content which calculated from from *B. monnieri, B. caroliniana and B. floribunda* were 31.71, 30.08 and 32.52 respectively. The melting temperature and melting curves show the correlation between percentage of GC content and the specific T_m from each *Bacopa* species. The T_m of amplified PCR product in each *Bacopa* species were on ordered by *B. floribunda* (74.6 ℃) > *B. monnieri* (74.0 ℃) > *B. caroliniana* (74.8-75.0 ℃) (**Figure 20**). The melting curves were obtained from the normalized fluorescence intensity unit and plotted as the regression of fluorescence emission during the denaturation of PCR product when temperature increased. The melting curve was analyzed and differentiated to represent their specific character from each plant by using the data from *B. monnieri* as the baseline. The specific melting curve can be used for the genotyping of plant species for authentication purpose. In addition, the *trn*L-F sequence which was successfully used in HRM analysis for the discrimination of dietary plants in the previous study was used to design the set of specific primer for discrimination of *Bacopa* plants by using HRM analysis. *Trn*L-F was proposed as the suitable DNA region for plants identification (Taberlet *et al.*, 1991). Although the polymorphism of *trn*L-F was not essential for plant identify, this region was extensively used for phylogenetic study of plant and plant identification in closely related species. Moreover, the previous study which using the DNA barcoding couple with the HRM analysis were successfully identify the edible plants (Buddhachat *et al.*, 2015;

Ganopoulos *et al.*, 2012; Madesis *et al.*, 2012). The polymorphism of the selected DNA regions represent the differences in length of amplicon from each plant. From the HRM analysis reveal that, the T_m of PCR product from *B. monnieri, B. caroliniana,* and *B. flotibunda* were 74.6-74.8, 73.8 and 75.4 respectively (**Figure 21**). It was found that the T_m (*B. flotibunda* > *B. monnieri* > *B. caroliniana*) but the length of PCR amplicons (*B. monnieri* > *B. flotibunda* > *B. caroliniana*) were not correlated. From the result, the insertion or deletion of nucleotide sequence may have the inferior affected to the T_m than the proportional of G or C in the gene sequences. Nevertheless, the indels may had an effect to the T_m if the sequence containing the more numbers of G or C in the indel sequences.

From the HRM analysis, there was given the successful discrimination tool for three taxa in the genus *Bacopa* by using two set of designed primer, *ycf1*-MP and *trn*L-F-MP. The result shows the specific melting temperature of PCR amplicons from each plant. The character of melting curves was beneficial for the identification of *Bacopa* plants in final manufacturing products.

CHAPTER VI

CONCLUSIONS

As a human dietary supplement, the quality and safety of medicinal plant products have been focused on contaminants of metals, toxins, especially the non-authentic plant materials which affected to the efficiency of treatment of their consumer wellbeing. Moreover *B. monnieri* has another quality issue as its characteristic phenotype resemble to other *Bacopa* spp., which makes it difficult to differentiate from the others. Therefore, a reliable and rapid method for authentication of *B. monnieri* should be developed to guarantee the quality with accuracy and precision.

In this work, we investigated the DNA sequences of six selected loci DNA barcodes proposed before for identification of plants. The DNA sequences were analyzed and applied with HRM analysis technique to identify *Bacopa* species. *Ycf1* and *trn*L-F turned out to be good DNA regions for HRM analysis because those regions had some inter-species variability from sequencing results. Real-time PCR coupled with HRM analysis of *ycf1*-MP and *trn*L-F-MP were successfully discriminated among *Bacopa* plants. This technique can be applied to identify the raw material in manufacturer and to guarantee the identity of medicinal plants.

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APPENDIX

MULTIPLE SEQUENCE ALIGNMENTS OF THREE *BACOPA* PLANTS

Figure 22 Multiple sequence alignment of ITS of the three *Bacopa* plants

The numbers above the alignment represents the base numbers of sequence alignments. "." Represents nucleotide base that is identical to the nucleotide in the first-row. "-" represents the gap.

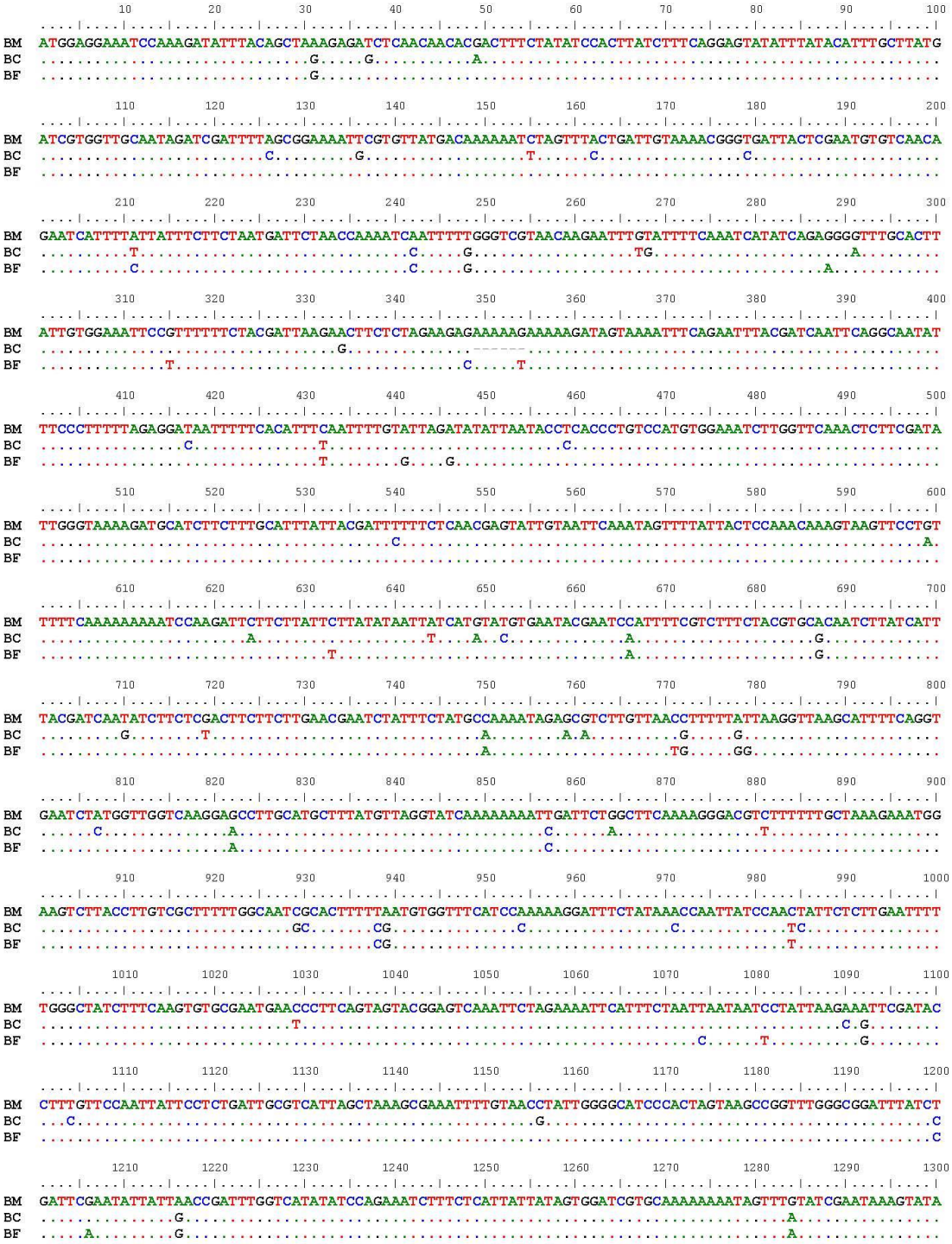

Figure 23 Multiple sequence alignment of *mat*K gene of the three *Bacopa* plants

The numbers above the alignment represents the base numbers of sequence alignments. "." Represents nucleotide base that is identical to the nucleotide in the first-row. "-" represents the gap.

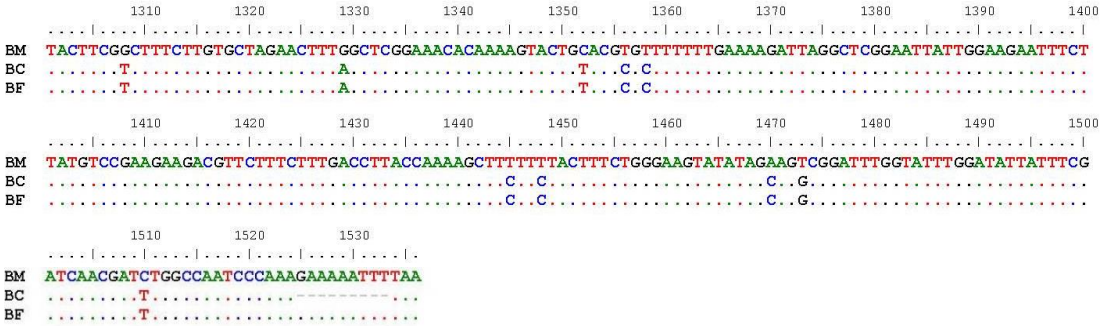

Figure 23 Multiple sequence alignment of *mat*K gene of the three *Bacopa* plants (continued)

The numbers above the alignment represents the base numbers of sequence alignments. "." Represents nucleotide base that is identical to the nucleotide in the first-row. "-" represents the gap.

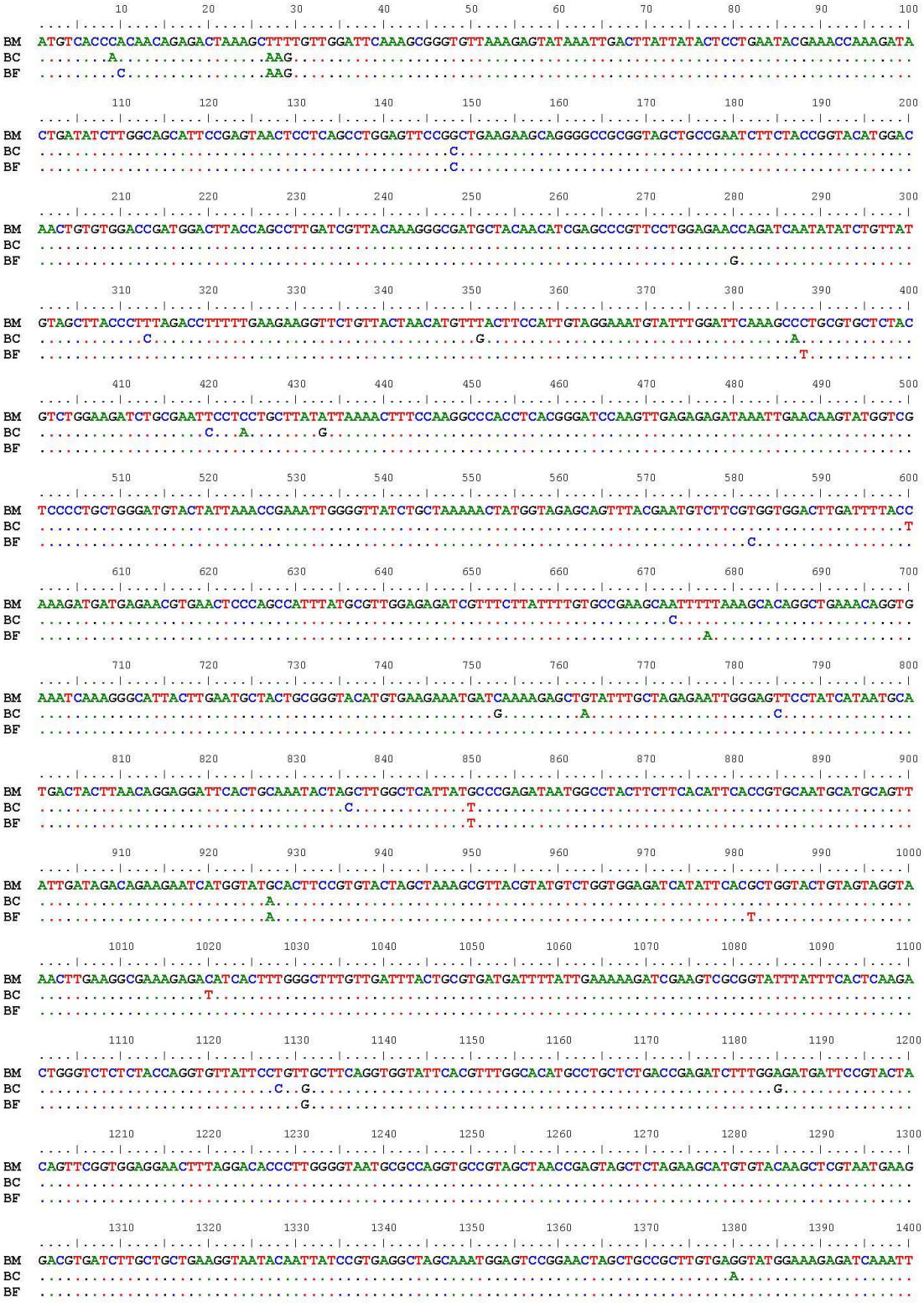

Figure 24 Multiple sequence alignment of *rbc*L gene of the three *Bacopa* plants

The numbers above the alignment represents the base numbers of sequence alignments. "." Represents nucleotide base that is identical to the nucleotide in the first-row. "-" represents the gap.

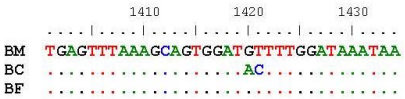

Figure 24 Multiple sequence alignment of rbcL gene of the three *Bacopa* plants (continued)

The numbers above the alignment represents the base numbers of sequence alignments. "." Represents nucleotide base that is identical to the nucleotide in the first-row. "-" represents the gap.

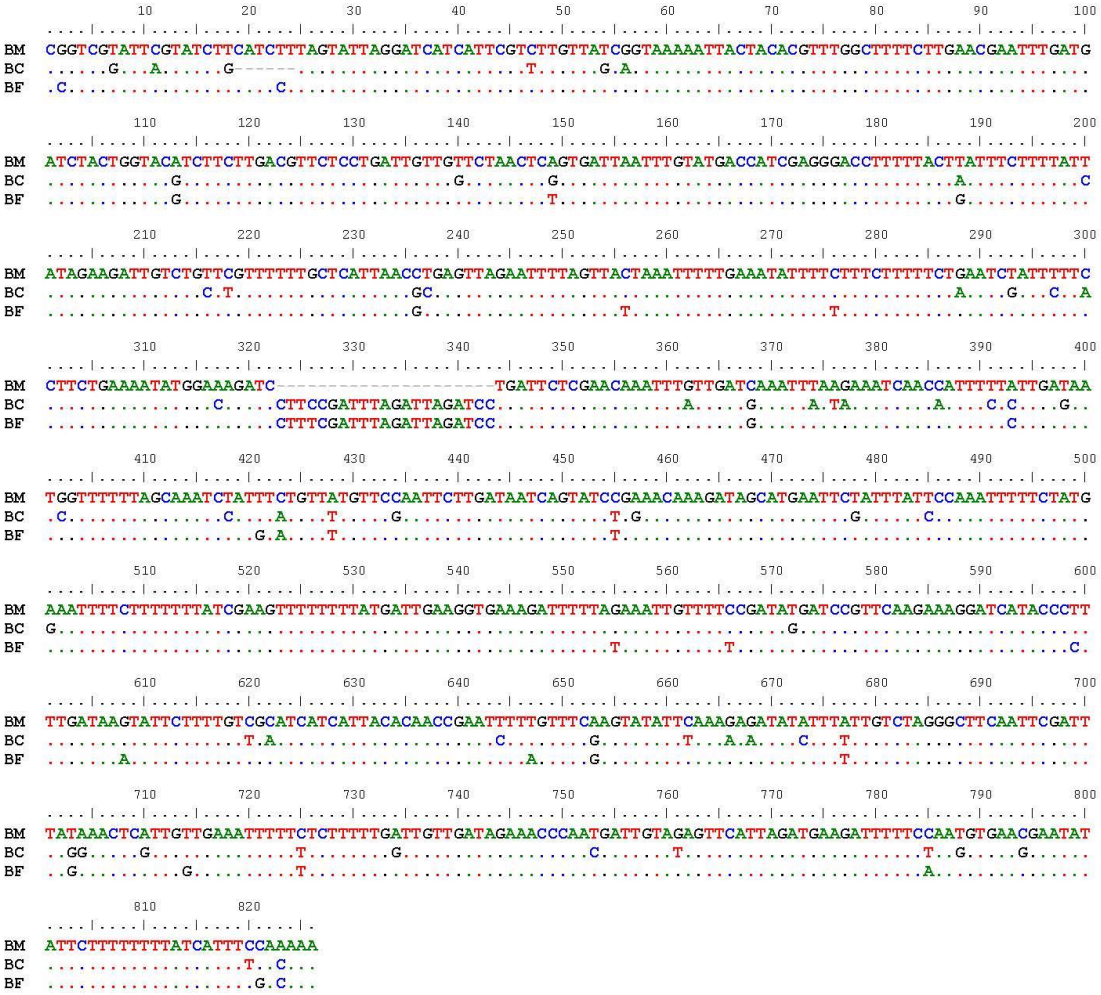

Figure 25 Multiple sequence alignment of *ycf*1 gene of the three *Bacopa* plants

The numbers above the alignment represents the base numbers of sequence alignments. "." Represents nucleotide base that is identical to the nucleotide in the first-row. "-" represents the gap.

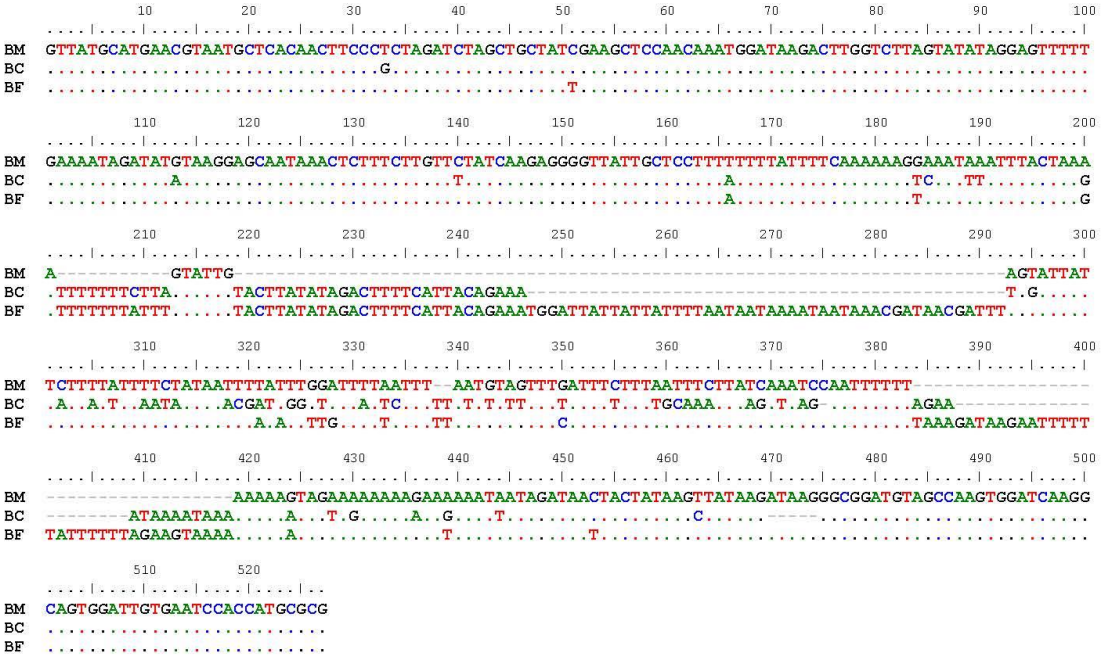

Figure 26 Multiple sequence alignment of *psb*A-*trn*H intergenic spacer of the three *Bacopa* plants

The numbers above the alignment represents the base numbers of sequence alignments. "." Represents nucleotide base that is identical to the nucleotide in the first-row. "-" represents the gap.

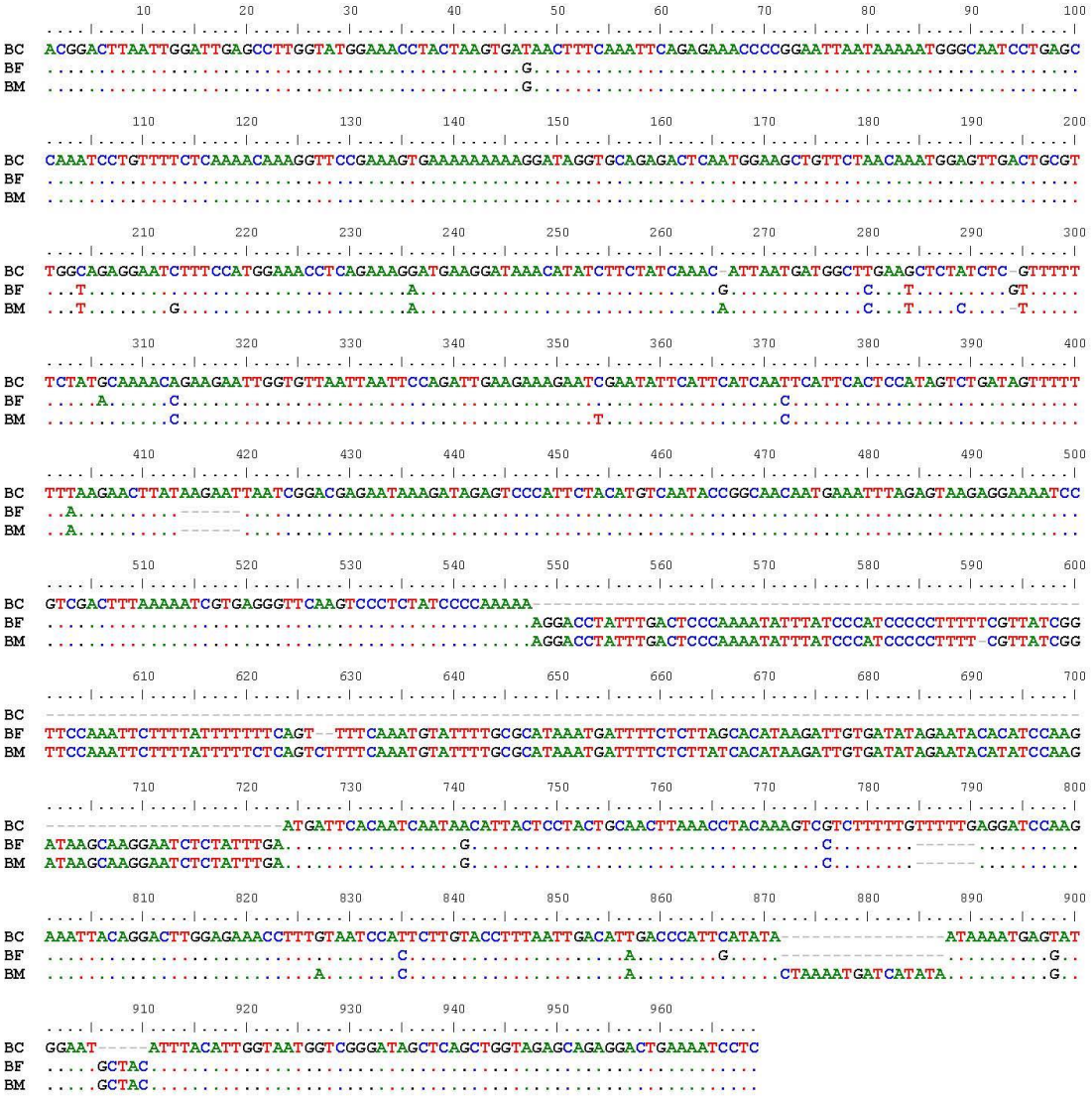

Figure 27 Multiple sequence alignment of *trn*L-F intergenic spacer of the three *Bacopa* plants

The numbers above the alignment represents the base numbers of sequence alignments. "." Represents nucleotide base that is identical to the nucleotide in the first-row. "-" represents the gap.

VITA

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Poster presentation

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