ความหลากหลายภายในสปีชีส์ของมะระขี้นก *Momordica charantia* Linn.
โดยการวิเคราะห์ด้วยอาร์เอพีดี

นาย กิตติพงษ์ พาละออ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีวเคมี ภาควิชาชีวเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2544
ISBN 974-17-0789-4

INTRASPECIFIC VARIATION OF BITTER GOURD *Momordica charantia* Linn. BY RAPD ANALYSIS

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A Thesis Submitted in Partial Fulfillment of the Requirements
for The Degree of Master of Science in Biochemistry
Department of Biochemistry
Faculty of Science
Chulalongkorn University
Academic Year 2001
ISBN 974-17-0789-4

Thesis Title	Intraspecific variation of bitter gourd *Momordica charantia* Linn. by RAPD analysis
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Field of Study	Biochemistry
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กิตติพงษ์ พาละออ : ความหลากหลายภายในสปีชีส์ของมะระขี้นก *Momordica charantia* Linn. โดยการวิเคราะห์ด้วยอาร์เอพีดี (INTRASPECIFIC VARIATION OF BITTER GOURD *Momordica charantia* Linn. BY RAPD ANALYSIS) อาจารย์ที่ปรึกษา: ผศ.ดร. พีรดา มงคลกุล, อาจารย์ปรึกษาร่วม : รศ.ดร. ศิริพร สิทธิประณีต, 94 หน้า, ISBN 974-17-0789-4.

ความหลากหลายทางพันธุกรรมและความสัมพันธ์ภายในสปีชีส์ของมะระขี้นกในประเทศไทยจำนวน 22 สายพันธุ์ซึ่งรวบรวมไว้ที่ศูนย์วิจัยพืชสวนจังหวัดพิจิตร นำมาวิเคราะห์ด้วยเทคนิคอาร์เอพีดีพบว่ามี 6 ไพรเมอร์คือ X-12, X-16, X-18, X-24, X-26 และ X-27 ที่สามารถใช้สร้างเครื่องหมายโมเลกุลแบบอาร์เอพีดีโดยให้รูปแบบคงเดิมเมื่อทำซ้ำ แถบอาร์เอพีดีที่รวบรวมได้มีทั้งสิ้น 109 แถบ ประกอบด้วย แถบดีเอ็นเอที่ไม่มีความหลากหลายจำนวน 54 แถบ(49.5%) และแถบดีเอ็นเอที่มีความหลากหลายจำนวน 55 แถบ(50.5%) เมื่อประมาณค่าความแตกต่างทางพันธุกรรม และสร้างแผนภูมิความสัมพันธ์ทางพันธุกรรมโดยใช้วิธี neighbor-joining พบว่าสายพันธุ์มะระขี้นกประกอบด้วย 2 กลุ่มย่อยซึ่งสอดคล้องกับการจัดกลุ่มโดยใช้ขนาดเส้นผ่านศูนย์กลางของผล อย่างไรก็ตามไม่พบว่าการแบ่งกลุ่มสายพันธุ์มะระขี้นกมีความเกี่ยวข้องหรือสอดคล้องกับการกระจายพันธุ์ทางภูมิศาสตร์ของแต่ละสายพันธุ์แต่อย่างใด

การตรวจหาบริเวณที่มีการแสดงออกของยีน *MAP30* ในบริเวณเนื้อเยื่อปลายยอด ใบ ดอกตูม ดอกเพศผู้ ดอกเพศเมีย เนื้อผลดิบ เมล็ดจากผลดิบ เนื้อผลสุก และเมล็ดจากผลสุก พบการแสดงออกของยีน *MAP30* ในเนื้อเยื่อปลายยอด ใบ และเมล็ดจากผลดิบ และจากการเปรียบเทียบขนาดของยีน *MAP30* ที่เพิ่มปริมาณด้วยวิธีพีซีอาร์โดยใช้จีโนมิกดีเอ็นเอเป็นแม่แบบเทียบกับการเพิ่มปริมาณยีน *MAP30* โดยใช้อาร์เอ็นเอนำรหัสเป็นแม่แบบ พบว่ายีน *MAP30* ที่ได้จากการเพิ่มปริมาณในทั้งสองวิธีมีขนาดเท่ากัน

การโคลนยีน *MAP30* เข้าสู่พลาสมิด pUC18 เพื่อให้สามารถเกิดการแสดงออกได้ใน *Echerichia coli* ไม่พบโคลนใดที่มีชิ้นยีน *MAP30* อยู่เลย ทั้งนี้สาเหตุอาจมาจากไพรเมอร์ที่ใช้สำหรับการเพิ่มปริมาณยีน *MAP30* ด้วยวิธีพีซีอาร์ถูกออกแบบมาผิดพลาดทำให้เอนไซม์ตัดจำเพาะไม่สามารถตัดส่วนปลายของชิ้นยีนได้ ชิ้นยีนดังกล่าวจึงไม่สามารถเชื่อมต่อกับพลาสมิดได้

ภาควิชา.....ชีวเคมี..... ลายมือชื่อนิสิต.....

สาขาวิชา.....ชีวเคมี..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา....2544..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4172222023 : MAJOR BIOCHEMISTER
KEYWORD : BITTER GOURD / *Momordica charantia* Linn. / INTRASPECIFIC VARIATION / RAPD
KITTIPONG PALA-OR : INTRASPECIFIC VARIATION OF BITTER GOURD *Momordica charantia* Linn. BY RAPD ANALYSIS. THESIS ADVISOR : ASSIST.PROF. PEERADA MONGKOLKUL, Ph.D., THESIS CO-ADVISOR : ASSOC.PROF. SIRIPORN SITTIPRANEED, Ph.D., 94 pp. ISBN 974-17-0789-4.

Ramdomly amplified polymorphic DNA (RAPD) analysis was used to determine the levels of genetic diversity and to estimate genetic relationship among 22 accessions of *Momordica charantis Linn*. (Mara-Khee nok) from Pichitr Horticultural Research Center, Thailand. Six of 47 sceened decanucleotide primers (X-12, X-16, X-18, X-24, X-26 and X-27) gave reproducible RAPD patterns. One hundred and nine reproducible RAPD bands were generated across the investigated species of these, 54 bands (49.5%) were monomorphic band and 55 bands (50.5%) were polymorphic band. Genetic variations of 22 accessions of *M. charatia* were analyzed by neighbor-joining program and the dendrogram divided Mara-Khee nok into 2 groups that were correlated diameter size of the fruits. However, the relationship between the division of Mara-Khee nok and their geological distribution was not indicated.

The expression of *MAP30* gene was investigated in apical shoot, leaf, flower bud, male flower, female flower, young fruit tissue, seed of young fruit, ripe fruit tissue and seed of ripe fruit. The *MAP30* gene expression of apical shoot, leaf and seed of young fruit were found. The amplification of *MAP30* gene from genemic DNA template and mRNA template gave PCR product of equal size.

Cloning of *MAP30* gene into plasmid pUC18 for expression in *Escherichia coli* was not successful. The *MAP30* gene fragment was not ligated to plasmid pUC18. It might be caused from wrong designing at the 5'-end restriction site of specific primer for amplification of *MAP30* gene before cloning.

Department...Biochemistry..... Student's signature.....
Field of study...Biochemistry..... Advisor's signature.....…...
Academic year....2001.....…... Co-advisor's signature.....……...

ACKNOWLEDGEMENT

This thesis would not have been completed without a good gesture for many persons. I would like to express my deep gratitude and appreciation to Assistant Professor Dr. Peerada Mongkolkul, my advisor and Associate Professor Siriporn Sittipraneed, my co-advisor for their encouragement, suggestion and discussion through my study.

The special thanks are also extended to Dr. Sirawut Klinbunga and Dr. Tosak Seelanan for this help in data computerized analysis, suggestion, discussion and serving as thesis committee.

My appreciation is also expressed to Associate Professor Dr. Piamsook pongsawasdi for serving as chairman of thesis committee and Mr. Charan Ditchaiwong from Pichit Horticultural Research Center, Thailand for his supporting both specimens and morphological data.

I would like to thanks Chulalongkorn Graduate School for financial supporting on my thesis.

Besides, I wish to extend my gratitude to my mother, my father and my older sister for their love and understanding. And finally, I also appreciate to thanks all teachers and friends in my laboratory room, especially for my lovely younger sisters in room 708; Num, Lee, Cee and Nee, for their help, encouragement and friendship.

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ABBREVIATIONS

A, T, C, G	=	nucleotide containing the base adenine, thymine, cytosine and guanine, respectively
ATP	=	adenosine triphosphate
bp	=	base pair
°C	=	degree celcius
cm	=	centrimetre
CTAB	=	Hexadecyltrimethylammonium bromide
DNA	=	deoxyribonucleic acid
dNTPs	=	deoxyribonucleotide triphosphates (dATP, dTTP, dGTP, dCTP)
EDTA	=	ethylenediamine tetraacetic acid
HCl	=	hydrochloric acid
IPTG	=	isopropyl-thiogalactoside
ITS	=	internal transcribed spacer
kb	=	kilobase
kDa	=	kilo Dalton
KCl	=	potassium chloride
$MgCl_2$	=	magnesium chloride
ml	=	millilitre
mM	=	millimolar
mtDNA	=	mitochondrial DNA
mRNA	=	messenger ribonucleic acid
ng	=	nanogram
OD	=	optical density
PCR	=	polymerase chain reaction

pg	=	picogram
RAPD	=	randomly amplified polymorphic DNA
RFLP	=	restriction fragment length polymorphism
RNA	=	ribonucleic acid
RNaseA	=	ribonuclease A
rpm	=	revolution per minute
Tris	=	tris (hydroxy methyl) aminomethane
U	=	unit
UV	=	ultraviolet
v	=	volume
V	=	volt
W	=	watt
W/ V	=	weight / volume
μg	=	microgram
μl	=	microlitre
μM	=	micromolar
U	=	unit

CHAPTER I

INTRODUCTION

Bitter gourd (*Momordica charantia* Linn) is a member of the Cucurbitaceae (gourd) family, and is a relative of squash, watermelon, muskmelon and cucumber (http://www.island.wsu.edu/crops/bitterme. htm). It is native to Asia with Eastern India and Southern China proposed as the center of domestication (Yang and Walters, 1992). Bitter gourd grows in tropical areas, including parts of the Amazon, East Africa, Asia, the Caribbean, and throughout South America, where it is used as a food as well as a medicine (http://edis.ifas.ufl.edu/scripts/htmlgen.exe?DOCUMENT_FW028). It has other common name, which vary with country such as bitter gourd (U.S.A), balsam pear (U.S.A.), foo gwa (China), kerala (India), nigai uri (Japan), ampalaya (the Philippines) and mara (Thailand). Breeding programs and variety development for bitter gourd have been confined to India and other Asian countries. Many cultivars are available, which vary in fruit size and shape, earliness, yield, fruit quality, and disease resistance (http://www.island.wsu.edu/crops/ bitterme.htm).

Common characters

Momordica is a Latin word that means "to bite" and refers to the look of the uneven skin. The species name *charantia* is unclear as to its meaning but could be Latin and refer to the pointed fruit. Bitter gourd has chromosome number of 2n = 22 (วัชรี, 2541).

For seedling, the stem is ridged and has small hairs. The first leaves are unlobed with broad teeth along the margins and have a heart-shaped base. Mature plant, an annual vine, has a creeping or climbing stem. The alternate leaves have petioles 3-6 cm long, and thin blades. The leaf blades are hairy to smooth, deeply palmately five- to seven-lobed and up to 12 cm wide. The lobes of the blades are rounded to pointed and usually have teeth on the margins. The flowers are usually yellow solitary male and female flowers borne in the leaf axils. The sepals are oval and up to 4.5 mm long. The yellow petals are rounded or indented at the tips and up to 1 cm long. Male and female flowers are borne separately on the same plant (monoecious), and require insects for pollination. The fruits have moderate to very bitter taste, and are characterized by a pebbly surface of smooth warts and smooth lengthwise ridges. They ranges in color from creamy white to dark green, pointed at the blossom end and have white flesh. As the fruit begins to mature, the surface gradually turns yellow or orange. At maturity, it tends to split open, revealing orange flesh and bright red placenta which the seeds are attached. Seeds are tan and oval, with a rough etched surface. There are about 150 to 200 seeds per ounce.The roots are tap root system with ride roots extensive (Figure 1.1).

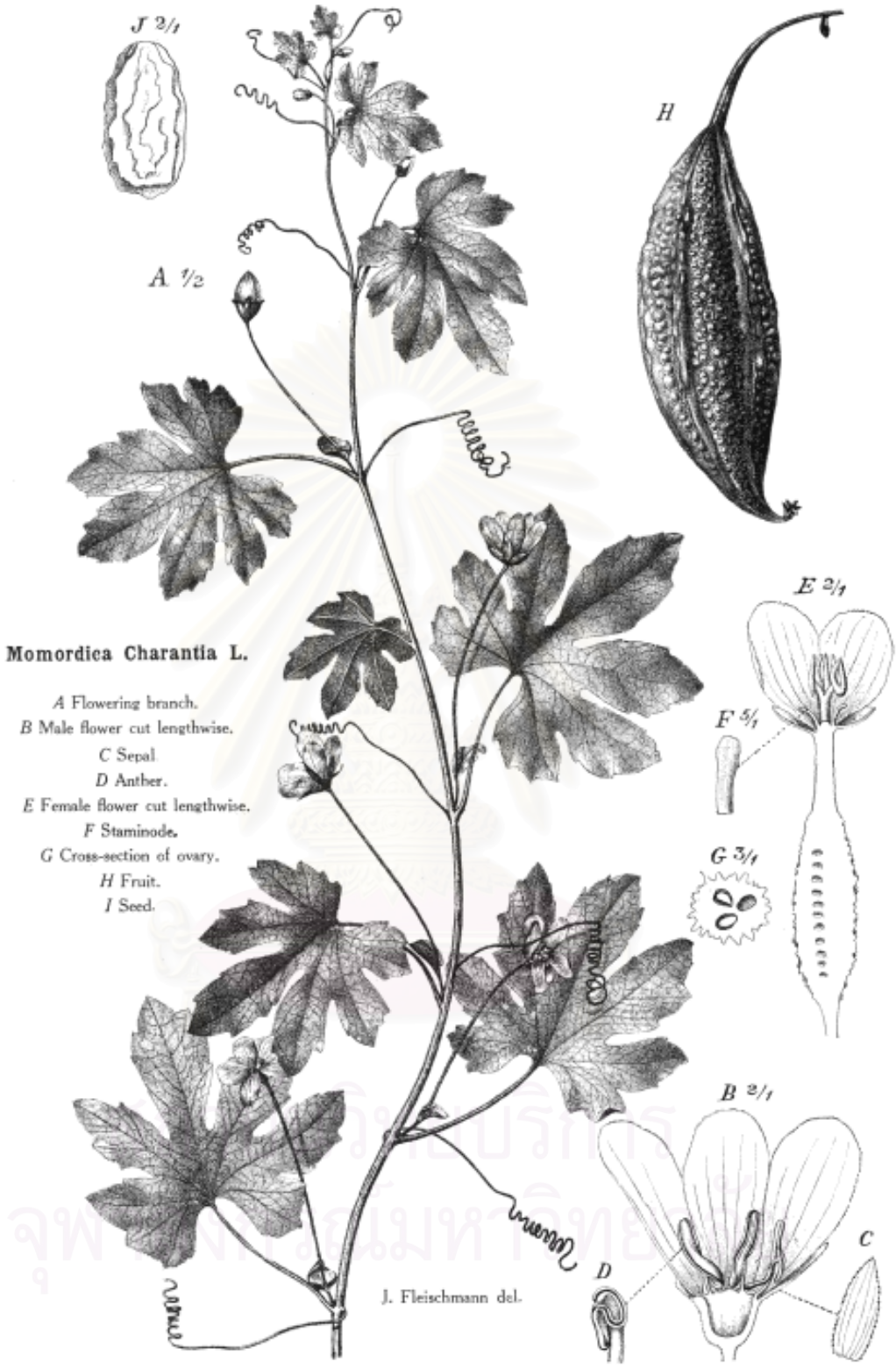


Figure 1.1 General morphology of *Momordica charantia* Linn. (http://www.biodiversity.uno.edu/delta/angio/images/cucur535.gif)

Taxonomy of bitter gourd

The taxonomy of bitter gourd is as follows :

Kingdom Plantae

Division Magnoliophyta

Class Magnoliopsida

Order Cucurbitales

Family Cucurbitaceae

Genus Momordica

Species *Momordica charantia* Linn.

Wide variation of bitter gourd was observed in fruit size (1-9.8 inches), shape (oval, round, oblong, club, etc.) and prominence of tubercles. Variation was also observed in leaf characters such as size, shape and lobing (http://www.Avrdc.Org.tw/pdf/ooprog 08.pdg). Chitenden (1977) divided *Momordica charantia* Linn. into 2 varieties as follows:

1. *M. charantia* Linn. *var. chinensis* : large and long fruit and yellow flower.

2. *M. charantia* Linn. *var. mulicata* : small fruit and pale yellow flower.

Agawal *et al.* (1957) reported that bitter gourd in India has 3 groups; namely,

1. Basakhi: rough fruit surface and oval shape.

2. Asadhi: oval in fruit shape and little rough surface.

3. Wild species: smaller in fruit size than those 2 accessions.

Yang and Walters (1992), however, divided bitter gourd into 3 groups: 1) small fruit type, 10-12 cm long, 0.1-0.3 kg in weight, usually dark green, fruit are very bitter; 2) long fruit type, 30-60 cm long, 0.2-0.6 kg in weight, light green in color with medium size protuberances, and only slightly bitter, most commonly grown in China; and 3) triangular fruit type, cone-shaped, 9-12 cm long, 0.3-0.6 kg in weight, light to dark green with prominent tubercles, moderately to strongly bitter.

In Thailand, bitter gourd is divided into 2 groups by criterion of fruit size. The bigger and longer fruit size is called “Chinese bitter gourd” and the smaller one is called “Mara-Khee-nok”.

Use and nutrition

Bitter gourd is grown entirely for its immature fruits, which are used in oriental cooking. However, in some countries, the young leaves are also harvested and used as potherb (http://www.island.wsu.edu/crops/bitterme.htm). One hundred grams of fresh fruit compose of 83-92 g water, 1.5-2 g protein, 02-1 g lipid, 4-10.5 g carbohydrate, 0.8-1.7 g dietary fiber, 105-250 kJoule energy, 20-30 mg calcium, 1.8-2 mg iron, 38-70 mg phosphorus and 88-96 mg vitamin C. One hundred grams of fresh leaf compose of 82-86 g water, 2.3 g protein, 2.1 g lipid, 17 g carbohydrate, 0.8 g dietary fiber, calcium, iron, phosphorus and vitamin B (วัชรี, 2541). The tender vine tips are an excellent source of vitamin A and a fair source of protein, thiamin, and vitamin C. The bitter flavor in both the fruits and leaves is due to the alkaloid momordicine, which can be reduced somewhat by parboiling or soaking in salt water. Immature fruits are least bitter whereas ripe fruits are extremely bitter and are reported to

be toxic to man and animals (http://www.island.wsu.edu/crops/bitterme.htm).

A review by Suntornsalatul (ปัทมา, 2541) indicated that the chemical component in whole plant of bitter gourd composes of alkaloids, saponin and sterols (charantin). Seed composes of many proteins such as MAP30 (30 kDa), momordin (24 kDa), Momordica charantia agglutinin (32 kDa), Momordica charantia lectin (115 kDa), Momordica charantia inhibitor (23 kDa), Momordica charantia cytostatic factor (40 kDa), α-momorcharin (32 kDa), β-momorcharin (29 kDa), polypeptide-p (p-insulin, 11 kDa), amino acid (aminobutyric acid) and triterpenes (momordicoside A-E and vicine). Fruit composes of alkaloids, saponin, amino acid (citrulline), 5-hydroxytryptamine, sterols (charantin-sitosterol, stigmasterol and acylglucosyl-sterols), triterpenes (cucurbitacin glycosides and momordicoside F1, F2, G, I, K and L) and polypeptide-p (p-insulin). Leaf composes of triterpenes such as momordicine (I, II, III) and cucurbitan triterpenes (III, IV, V). Root composes of alkaloids (momordicine) and saponin.

Medicinal property

The literature on bitter gourd abound with accounts of a wide range of medicinal uses of various plant parts or their extracts. The medicinal properties that are of current interest include hypoglycemic property (anti-diabetic) and activities such as anti-cancer and anti-virus; the latter is primarily concentrated especially for HIV virus. Other properties are summarized in Table 1.1

Bitter gourd has been used as an anti-diabetic home remedy for centuries. Its extracts have been found to reduce sugar level in experimental diabetic animals such as rat. It may block sugar absorption in intestine, or improve the body ability to use sugar. There were also indication that extracts from bitter gourd may enhance the secretion of insulin from pancreas. Recently, a Japanese group of scientist suggested that it also increase the level of HDL-cholesterol while reducing total cholesterol and triglycerides (http://www.sunintegrativehealth.com).

Khanna *et al*. (1981) isolated polypeptide-p protein (p-insulin) from fruit, seed and tissue of bitter gourd. Polypeptide-p has a molecular weight of approximately 11 kDa (166 residues) and it has a very effective hypoglycemic agent when administered subcutaneously to gerbils, langurs and human.

Many proteins from bitter gourd have both anti-cancer and anti-HIV virus properties. One of these is MAP30 (Momordica anti-HIV protein of 30 kDa) which is prominent in ripe seed of bitter gourd. *MAP30* is a member of ribosome inactivating proteins (RIPs, Lee-Huang *et. al.*, 1990) that can inhibit cancer cell proliferation both *in vitro* and *in vivo* (Lee-Huang *et al.*, 2000) and inhibit HIV type 1 infection in T lymphocytes and monocytes. They reported 3 important activities of MAP30 for its anti-viral activities: 1) DNA glycosylase; 2) DNA topoisomerase and 3) RNA N-glycosidase. MAP30, however, is not toxic to uninfected T-cell. It is also shown to inhibit HIV-1 reverse transcriptase (Wang and Ng, 2001). The amino acid sequence of MAP30 is shown in Figure 1.2.

Alpha-momorcharin from seed of bitter gourd has both properties like MAP30 too. Alpha-momorcharin is one of RIPs protein member; it has 263 amino acid residues and molecular mass about 29 kDa

(Ho *et al*., 1991). Alpha-momorcharin can inhibit syncytia formation induced by HIV-1 and reduction of p24 core antigen at the expression level. The core protein is necessary for viral maturation step thereby decreased the number of HIV-infected cells (Zheng *et al*., 1999).

Table 1.1 General medicinal properties and phytochemical of *Momordica charantia* Linn. (http://rain-tree.com/bitmelon.htm).

DESCRIPTION	
Properties or Actions	Antibiotic, Antimutagenic, Antioxidant, Antileukemic, Antiviral, Antidiabetic, Antitumor, Aperitive, Aphrodisiac, Astringent, Carminative, Cytotoxic, Depurative, Hypotensive, Hypoglycemic, Immunomodulatory, Insecticidal, Lactagogue, Laxative, Purgative, Refrigerant, Stomachic, Styptic, Tonic, Vermifuge
Phytochemicals	5-Alpha-Stigmasta-7,25-dien-3-betalOL, 5-hydroxytryptamine, Alkaloids, Alpha-Elaeostearic-acid Ascorbigen, Beta-sitosterol-d-glucoside, Charantin, Citrulline, Cryptoxanthin, Elasterol, Flavochrome, Fluoride, Gaba, Galacturonic-acid, Lanosterol, Lutein, Lycopene, Momordicin, Momordicoside-F-1, Momordicoside-F-2, Momordicoside-G, Momordicoside-I, Mutachrome, Oxalate, Oxalic-acid, Pipecolic-acid, Polypeptide-p, Rubixanthin, Stigmasta-5,25-dien-3-beta-ol, Sugars, Zeaxanthin, Zeinoxanthin
Traditional Remedy	One-half cup leaf decoction 1-2 times daily or 1-3 ml of a 4:1 tincture twice daily. 1 to 2 grams of powdered leaf in tablets or capsules daily can be substituted if desired.

1	MVKCLLLSFL	IIAIFIGVPT	AKGDVNFDLS
31	TATAKTYTKF	IEDFRATLPF	SHKVYDIPLL
61	YSTISDSRRF	ILLNLTSYAY	ETISVAIDVT
91	NVYVVAYRTR	DVSYFFKESP	PEAYNILFKG
121	TRKITLPYTG	NYENLQTAAH	KIRENIDLGL
151	PALSSAITTL	FYYNAQSAPS	ALLVLIQTTA
181	EAARFKYTER	HVAKYVATNF	KPNLAIISLE
211	NQWSALSKQI	FLAQNQGGKF	RNPVDLIKPT
241	GERFQVTNVD	SDVVKGNIKL	LLNSRASTAD
271	ENFITTMTL	LGESVVN	

Figure 1.2 Amino acid sequence of *MAP30* gene described by Lee-Huang *et al.* (1995).

Molecular genetic markers

Molecular markers are useful for various genetic studies. These markers generally include protein and DNA markers. The former refers to markers generally from allozyme and isozyme. The latter is composed of those obtained from several approaches including restriction analysis (restriction fragment length polymorphism, RFLP; DNA fingerprinting and polymorphism of single copy nuclear DNA), PCR-based techniques (e.g. randomly amplified polymorphic DNA, RAPD and microsatellites) and DNA sequencing.

1. Protein markers

Protein polymorphism is regarded as one of the genetic markers detected by electrophoresis of proteins (usually enzyme). Proteins with different net charges migrate at different rate through a gel matrix when exposed to an electric field (Avise, 1994). The protein bands can be visualized by a specific histochemical stain of the particular enzyme.

Analysis of protein polymorphism is a technique of choice to starting with when any molecular data of a species under investigation has not been reported. The majority of protein markers are represented by allozymes, which are different molecular forms of an enzyme coded by different alleles at one gene locus. The allozyme markers are transmitted in a co-dominant manner; therefore, heterozygosity of individuals within populations of species can be determined. The advantages of allozyme approach are its cost-effectiveness, less tedious and time-consuming technique as compared to other molecular genetic techniques. Therefore,

large numbers of samples can be processed within the limiting period of time.

However, allozyme analysis has some limitations. For instance, synonymous mutations can not be detected. Likewise, nucleotide substitution changing one non-polar amino acid to another does not alter the electrophoretic mobility of proteins. Scoring of gels can be complicated and need experienced scientist to interpret the precise results from electrophoresed gels (Kocher and Stephen, 1997).

2. DNA markers

DNA can be compared in several different ways. Genetic variations at the DNA level can be generalized roughly into two categories: base substitutions and insertions/deletions. The polymorphism is usually represented by differences of extrachromosomal DNA and chromosomal DNA

2.1 Extrachromosomal DNA

There are two types of plant extrachromosomal, chloroplast DNA and mitochondrial DNA. In plants, chloroplast DNA (cpDNA) has been the molecular choice for molecular phylogenetic studies (Palmer and Herbon, 1988). Plant cpDNA has the advantage over mtDNA for evolutionary and phylogenetic study because cpDNA is relatively abundant, thus facilitating extraction and analysis. The second advantage is due to extensive background of molecular information of the chloroplast genome. For example, complete DNA sequence of three cpDNA genomes are known (liverwort, *Marchantia polymorpha*; tobacco, *Nicotiana tabacum*; and rice, *Oryza sativa*) (Soltis *et al*., 1992).

Chloroplast DNA of photosynthetic land plants are circular DNA molecules ranging in size from 120 to 217 kb, but plant mtDNAs are abnormally large and variable in size. Plant mtDNAs may have ranging in size from 330 kb (*Citrullus lanatus*) to 2,500 kb (*Cucumis melo*) because it has two regions of homologous repetitive DNA that cause intra- and intermolecular recombination in mtDNA molecule. This phenonemon make mtDNA in plant cell to have more than one size (heterogeneous mtDNA) per individual, this is a major disadvantage for using mtDNA to study plant systematic (Soltis *et al*., 1992).

However, plant mtDNA can be used for study in some case such as cytoplasmic male sterile (CMS). These plants can not produce fertile pollen because of unusual mtDNA. Examples are the study about relationship between normal mtDNA organization and mtDNA organization in cytoplasmic male sterile type T in maize (Faurom *et al*., 1989; Fauron *et al*., 1990) and study of unusual mtDNA organization in cytoplasmic male sterile common bean (Janska and Mackenzie, 1993).

2.2 Chromosomal DNA

Chromosomal DNA contains both unique single copy and repetitive regions. The single copy region generally codes for a particular gene product. The structure of protein coding genes in the nuclear genome consists of coding regions (exons) and non-coding regions (introns or intervening sequences). Typically, exon is highly conserved. In contrast, much higher polymorphism can be observed in introns (Krawczak and Schmidtke, 1994).

Repetitive DNA consists of core sequences that are repeated in varying degrees. They may be made up of coding segments such as the

ribosomal RNA (rRNA) genes, or non-coding tandem repeated units (Parker *et al*., 1998).

Variable number of tandem repeats (VNTRs) are composed of satellite, minisatellite and microsatellite DNA. Large repetitive units of satellite DNA are often associated with heterochromatin located near the centromere of chromosomes. Smaller regions (made up of repeated units < 65 base pairs) are known as minisatellite DNA and can occur throughout the genome (Jeffrey *et al*., 1985) whereas microsatellite DNA has consecutive repeated units of only 1-6 base pairs (Tautz, 1989).

Plant genomes are differed from animal and fungi due to polyploidy which causes it to vary in DNA content. Polyploidy can also occurred during plant development. It has been estimated that 50% of angiosperm were polyploidy resulting from the doubling of chromosomes within a species or the hybridization of two species without chromosome reduction (Foster and Twell, 1996). The second major source of variation in plant chromosomal DNA content comprises a huge number of families of repeated sequences. The proportion of repetitive DNA exceed 50% in genomes larger than 1.5 pg and approach 95% in very large genomes (Flavell, 1980). High proportions of repeats have major implications for exploring genomes via molecular genetics.

DNA techniques commonly used in genetic variation studies

1. Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) analysis is one of the initial techniques widely used to indirectly detect genetic variation at the DNA level. It examines size variation of specific DNA fragment after digestion with restriction enzymes.

Conventional RFLP analysis is carried out by digestion of genomic DNA with a specific endonuclease. Products are then size-fractionated using gel electrophoresis and transferred onto a membrane. The investigated fragment(s) is identified by hybridization with the specific radiolabelled probe. In more comprehensive studies, restriction sites are mapped and the presence or absence of restriction sites, rather than sharing of fragment length are scored (Kocher and Stephen, 1997).

2. PCR-RFLP

The polymerase chain reaction (PCR) is an effective approach for population genetic and systematic studies. The method is based on *in vitro* enzymatic amplification of DNA. Million copies of a particular target DNA fragment are produced from a very low amount of starting template DNA (mostly in the nanogram range). PCR is a simple but powerful technique. The PCR reaction contains the target's double-stranded DNA, two primers that hybridize to flanking sequences on opposing strands of the target sequence, deoxyribonucleotide triphosphates and a *Taq* DNA polymerase. The amplification reaction consists of three steps: denaturation, primer annealing and elongation step. The cycle is repeated for 25-50 times. In each cycle newly synthesized strands act as the templates for subsequent replication resulting in exponential increasing of the specific product within only a few hours. The products obtained are usually electrophoretically analyzed using agarose gels.

The PCR-amplified fragment can be further analyzed for restriction site and/or fragment length polymorphism. After specific DNA is amplified through the PCR, the product is then digested with restriction endonuclease before being electrophoretically analyzed. The most

important advantage of this technique is that hybridization of labeled DNA probes to the target restricted DNA is not necessary. This technique is much simpler than the conventional RFLP approach.

3. DNA sequencing analysis

Polymorphism at the DNA level can be studied by several methods but the most direct strategy is determination of nucleotide sequences of a defined region. The sequences obtained can be aligned by comparing with an orthologous region in the genome of related organisms (or populations).

PCR-amplified fragments can be directly sequenced using the typical chain termination reaction (Sanger *et al*, 1977) or alternatively by cycle-sequencing. Nevertheless, more accurate sequences of DNA fragments are obtained through the cloning approach.

However, DNA sequencing is tedious, time consuming and expensive. The number of base, which can be determined by sequencing usually cover a few hundred bases. Increasing the length of investigated DNA with a large number of samples are prohibited by several factors afore mentioned. With an introduction of automatic DNA sequencers, the experiment can be carried out much faster than that based on manual sequencing thus allowing its wider applications for genetic studies on long DNA sequence, i.e. genome project.

4. Randomly amplified polymorphic DNA (RAPD) analysis

Randomly amplified polymorphic DNA (RAPD) analysis was concurrently developed by Williams *et al*. (1990). Technically, it is a simple method for determination of genetic variations using arbitrarily primer PCR-base technique. The amplification conditions of RAPD differ

from that of the standard PCR in that only single random primer (e.g. 10 mer with GC content usually at least 50 %) is employed. RAPD amplified target DNA on the basis that the nuclear genome contains several priming sites closed to one another that is located in an invert orientation. Accordingly, the primer is utilized to scan genome for the small inverted sequences resulting in amplification of DNA segment of variable length. The amplification products are separated on agarose gels and detected by staining with ethidium bromide.

The advantages of using RAPD markers are as follows: firstly, RAPD analysis is a simple, rapid and inexpensive method for detecting DNA polymorphism. Secondly, RAPD does not require knowledge of the genome under investigation. Thirdly, RAPD is a PCR-based method. It requires small quantity of DNA template per reaction. Fourthly, RAPD does not require the use of radiolabelled probes for hybridization. Finally, unlimited numbers of RAPD primers can be screened for suitable molecular markers of various applications within a short period of time.

There are some disadvantages of the RAPD approach for population genetics, genetic mapping and taxonomic studied too. Fragments (especially those arising from mispairing of a primer with the genomic DNA) may not be reproducible among different laboratories because amplifications are sensitive to slight changes in amplification conditions.

The allele distribution of RAPD amplified fragments is treated in a dominant fashion. Accordingly, the presence of amplified fragment may reflect either a homozygous (AA) or heterozygous (Aa) situation. Only the absence of the fragment reveals the aa genotype. This disadvantage of RAPD results in an inability to estimate heterozygosity

and the actual status of interested alleles because homozygotic can not be dissociated from heterozygotic states.

Genetic studies in bitter gourd

In India, where the plant originated and a lot of researches on its intraspecific variation were done, most of the work were at the morphological and classical genetic levels. Some examples are the relationship between varieties of *M. charantia* and crop yield (Sigh *et al*., 1977) or quantity of protein, carbohydrate and some chemical component in various bitter gourd cultivars (Jaiswal *et al*., 1990). Devedas and Seemanthini (1993) studied the dominant traits of some bitter gourd cultivars and demonstrated that cv. Pusa Bo Mausami had dominant shallow lobed leaves character when crossed with deeply lobed leaves cultivars. Kennedy *et al* (1994) also supported the result using bitter gourd cv. Pusa Visesh. These studies indicated that the shallow lobed leaf trait should be governed by dominant genes. In 1998, Kusamran *et al* showed that fruit extracts from Chinese bitter gourd and Mara-khee-nok had different effects on some hepatic enzymes in rat.

There is still very little molecular genetic information on bitter gourd. Most of the genes studied are of medical importance such as MAP30, α- and β-momorcharin and some protease inhibitors. Lee-Huang *et al*. (1995) had clone, expressed *MAP30* gene and compared the property of recombinant MAP30 and natural MAP30. They found that the complete nucleotide sequence of *MAP30* gene contains a single open reading frame (ORF) and it does not contain any introns. The *MAP30* gene codes for a total of 286 amino acids, including a leader sequence of 23 amino acids, which is not present in the mature protein. The mature

protein consists of 263 amino acids, with a potential N-linked glycosilation site Asn-Leu-Thr at amino acid positions 51-53, and unique Trp^{190} and Met^{254}. MAP30 does not have any Cys residue. Non-glycosylated recombinant MAP30 was as active as the glycosylated natural protein in all bioactivities tested. Similarly, it was also active in topological inactivation of viral DNA, inhibition of HIV-1 integrase and cell-free ribosome inactivation (Lee-Huang *et al.*, 1995).

In addition to MAP30, bitter gourd produces at least eight other related but distinct isoforms (Lee-Huang *et al*, 1995). Two of these have been reported: the α- and β- momorcharins. MAP30 shares about 48% amino acid homology to α-momorcharin. MAP30 and α-momorcharin are distinct molecules, each contains its own unique nucleotide and amino acid sequences.

Ho *et al*. (1991) used a λgt11 cDNA library constructed from fresh seed of the bitter gourd and obtained a full-length cDNA containing the entire sequence of α-momorcharin by immunoscreening. The length of this cDNA is 1044 base pairs long with the nucleotide sequence of the coding region starting from base 322 to 1044. It consists of an open reading frame coding for a polypeptide of 286 amino acids, and 23 amino acids at the N-terminal sequence has high content of hydrophobic amino acids, suggesting that they probably code for a signal peptide which is cleaved off from the mature protein (263 residues, 29 kDa). Amino acid residue 227 is a glycosylation site rendering total molecular mass of 31 kDa. However, glycosylation at this particular site does not appear to be essential for inhibiting protein synthesis and HIV-1 replication.

To date, only one report had used sequence of internal transcribe spacer (ITS1 and ITS2) to study the molecular genetic relationships among some species in family Cucurbitaceae. Jobst *et al*. (1998) had sequenced ITS1 and ITS2 regions of the nuclear ribosomal RNA gene in some different members of Cucurbit family (twenty-six species of different genera including bitter gourd) to compare and estimate phylogenetic relationships among species. ITS1 and ITS2 regions are slightly variable in length, with each length appearing to be genus-specific. Phylogenetic relationships inferred from ITS sequences of some species were in agreement with morphological data. In the genus Cucurbita (cucumber), different types of ITS sequences within one species exist, possibly due to the high frequency of introgression during domestication or due to polyploidization events (Jobst *et al*., 1998). On the contrary, low intraspecific variability was detectable in the genus *Cucumis* (melon), indicating different stages of speciation.

Many studies of genetic variation in family Cucurbitaceae were done by RAPD analysis. Hai Ying *et al* (1998) studied the intraspecific relationship of 34 cucumber (*Cucumis sativar* L.) cultivars by using 20 decanucleotide primers and was able to segregate them into 3 groups. The result was correlated with segregating by other parameters. Horejsi and Staub (1999) studied 118 accessions of cucumber by RAPD technique and the result was similar to that of RFLP. Suk-Yuan *et al* (1998) studied 52 lines of melon (*Cucumis melo* L.) with 12 random primers and divided the melons into 2 large groups, which were also supported by morphometric division. By using RAPD analysis, Stepanley et al (1999) obtains 54 melon accessions from 23 countries, which divided the melon into 2 subspecies.

RAPD is a very useful and widely employed marker for constructing the genetic map (Reiter *et al*., 1992; Park *et al*., 1999), genetic analysis (Rowe *et al*., 1997; Li *et al*., 1998) and molecular taxonomy (Demeke *et al*., 1992; Hadrys *et al*., 1992). In this study, RAPD technique was used for analysis because it did not require any genome data. It was a simple, rapid and inexpensive method for detecting DNA polymorphism. Thus, RAPD was suitable for analyzsis of genetic variation of bitter gourd that had a few genetic information reports.

Objective

The objective of this thesis was to 1) examine the intraspecies diversity and genetic relatedness among the 22 accessions of bitter gourd from Pichitr Horticultural Research Center, Thailand by RAPD analysis, 2) observe the expression of *MAP30* gene from some tissues of bitter gourd and 3) expression of *MAP30* gene in *Escherichia coli* (JM109)

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Instruments

1. Autoclave Model HA-30: Hirayama Manufacturing Co., Japan
2. Automatic micropipette P2, P20, P100 and P1000: Gilson Medical Electronics S.A., France
3. Camera Pentax super A: Asahi Opt. Co. Ltd., Japan
4. Electronic balance Model Alsep EY220A: A&D Co. Ltd., Japan Electrophoresis apparatus: Horizontal gel electrophoresis
   apparatus (9 x 12 cm)
5. -20 °C Freezer: Krungthai Ltd., Thailand
6. -70 °C Biofreezer: REVCO
7. High speed microcentrifuge Model MC-15A: Tomy-Seiko, Japan
8. Centrifuge Model H-103N series: Kokusan, Japan
9. Incubator Model BM-600: Memmert GambH, Germany
10. Microcentrifuge Force 6: Denver Instrument Company, U.S.A.
11. Microwave oven Model TRX1500: Turbora International Co. Ltd., Korea

12. Power supply: BIO-RAD Laboratories, U.S.A.
: Power PAC300
13. Thermal cycler : Gene Amp PCR system Model 2400: Perkin Elmer Cetus, U.S.A.
14. UV transiluminator Model 2011 Macrovue: San Gabriel california, U.S.A.

2.1.2 Inventory supplies

1. Black and white print film TriX-pan 400: Eastman Kodak Company, USA
2. Microcentrifuge tubes, 0.5 and 1.5 ml: Axygen Hayward, USA.
3. Pipette tips, 10, 200 and 1,000 µl: Axygen Hayward, USA.
4. Thin-wall microcentrifuge tubes (domed cap), 0.2 ml: Axygen Hayward, USA.

2.1.3 Chemicals

1. Absolute ethanol: Merck, Germany
2. Agarose: FMC Bioproduct, USA.
3. Ammonium acetate: Univar, Australia
4. 100 base pair DNA ladder: Promega Co., USA.
5. Boric acid: Merck, Germany
6. Bromophenol blue: Merck, Germany
7. Chloroform: Merck, Germany
8. Ethidium bromide: Sigma Chemicai Co., USA.
9. Ethylene diamine tetraacetic acid, disodium salt dihydrate: Fluka, Switzerland
10. 10 mM each dATP, dCTP, dGTP, and dTTP: Perkin Elmer, USA.
11. Ficoll 400: Sigma Chemical Co., USA.

12. GeneAmp PCR core reagents: Perkin Elmer, USA.
    : 10x PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl)
    : 25 mM $MgCl_2$
13. Hexadecyltrimethylammonium bromide: CTAB: Fluka, Switzerland
14. Hydrochloric acid: Merck, Germany
15. Isoamyl alcohol: Merck, Germany
16. 2-Mercapto ethanol: Fluka, Switzerland
17. NucleoSpin Extract 2 in 1 kit: Macherey-Nalgel, Germany
    : NT1 buffer
    : NT2 buffer
    : NT3 buffer
    : NE buffer
    : Nucleospin extract column
    : 2ml collecting tube
18. OneStep RT-PCR Kit: QIAGEN, USA.
    : OneStep RT-PCR enzyme mix (Omniscript Reverse Transcriptase, Sensiscript Reverse Transcriptase, and HotStar*Taq* DNA polymerase)
    : 5x QIAGEN OneStep RT-PCR buffer
    : 10 mM each dNTP mix
    : RNase-free water
19. Phenol crystal: Fluka, Switzerland
20. Polyvinyl-pyrrolidone: PVP-360: Sigma Chemical Co., USA.
21. Sodium chloride: Merck, Germany
22. TRI REAGENT: RNA/DNA/protein isolation reagent kit:

Molecular Research Center Inc., USA.

23. Tris-(hydroxy methyl)-aminomethane: Fluka, Switzerland

2.1.4 Enzymes

1. Ampli *Taq* DNA polymerase: Perkin Elmer, USA.
2. RNase A: Sigma Chemical Co., USA.
3. T_4 DNA ligase (Pharmacia, USA)
4. *Bam*HI (Promega Corporation Medison, Wisconsin)
5. *Eco*RI (New England BioLabs)

2.1.5 Cloning vectors

: pUC18

2.1.6 Bacterial strain

: *Escherichia coli* JM109, genotype: F' [*tra*D36 *pro*AB$^+$ *lac*I^q *lac*ZΔM15] *recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *tri* Δ (*lac-proAB*)

2.2 Sample collections

Leaves from twenty-two accessions of bitter gourd and one accession of *M. cochinchinensis*, which were self-fertilized for 5 generations, were obtained from Pichitr Horticultural Research Center, Thailand with the courtesy of Mr. Charan Ditchaiwong. All samples were stored at -20°C until used for DNA extraction. The details of sample origin were shown in Table 2.1

Table 2.1 Locality of 22 bitter gourds used in this study

Locality	Abbreviation of accession
Rong-Chang district, Muang, Pichitr Province	M001
Samngam district, Samngam, Pichitr Province	M002
Ta-Luang district, Muang, Pichitr Province	M003
RongChang district, Muang, Pichitr Province	M004
Klong-kachain district, Muang, Pichitr Province	M005
Kakang district, Munag, Pichitr Province	M006
RongChang district, Muang, Pichitr Province	M007
Prachinburi Province	M008
RongChang district, Muang, Pichitr Province	M09A
Pichitr Province (separated from M09A by inbreeding)	M09B
Kakang district, Muang, Pichitr Province	M010
Kakang district, Muang, Pichitr Province	M011
Nakhon-Pathom Province	M012
Bang-Bua Tong, Nonthaburi Province	M013
Pichitr Province (separated from M001 by inbreeding)	M014
Pichitr Province (separated from M002 by inbreeding)	M015
Chuar-Yong-Saeng Trade Mark	M016
Nepal (Chinese bitter gourd)	M017
Nakhon-Phanom Province (Nakorn-Viang Jun, Laos)	M019
Haeng-Nguan-Saeng (Dek-Bin) Trade Mark	M020
Nayang distric, Cha-am, Phetchaburi Province	M021
Nonthaburi Province (separated from M013 by inbreeding)	M022
Pichitr Province (*Momordica cochinchinensis* Spreng.)	MF01

Figure 2.1 Locality of samples site

N = Northern

NE = North-Eastern

E = Eastern

SW = South-Western

C = Central

SE = South-Eastern

PEN = Peninsular

Number 14 = Pichitr Province

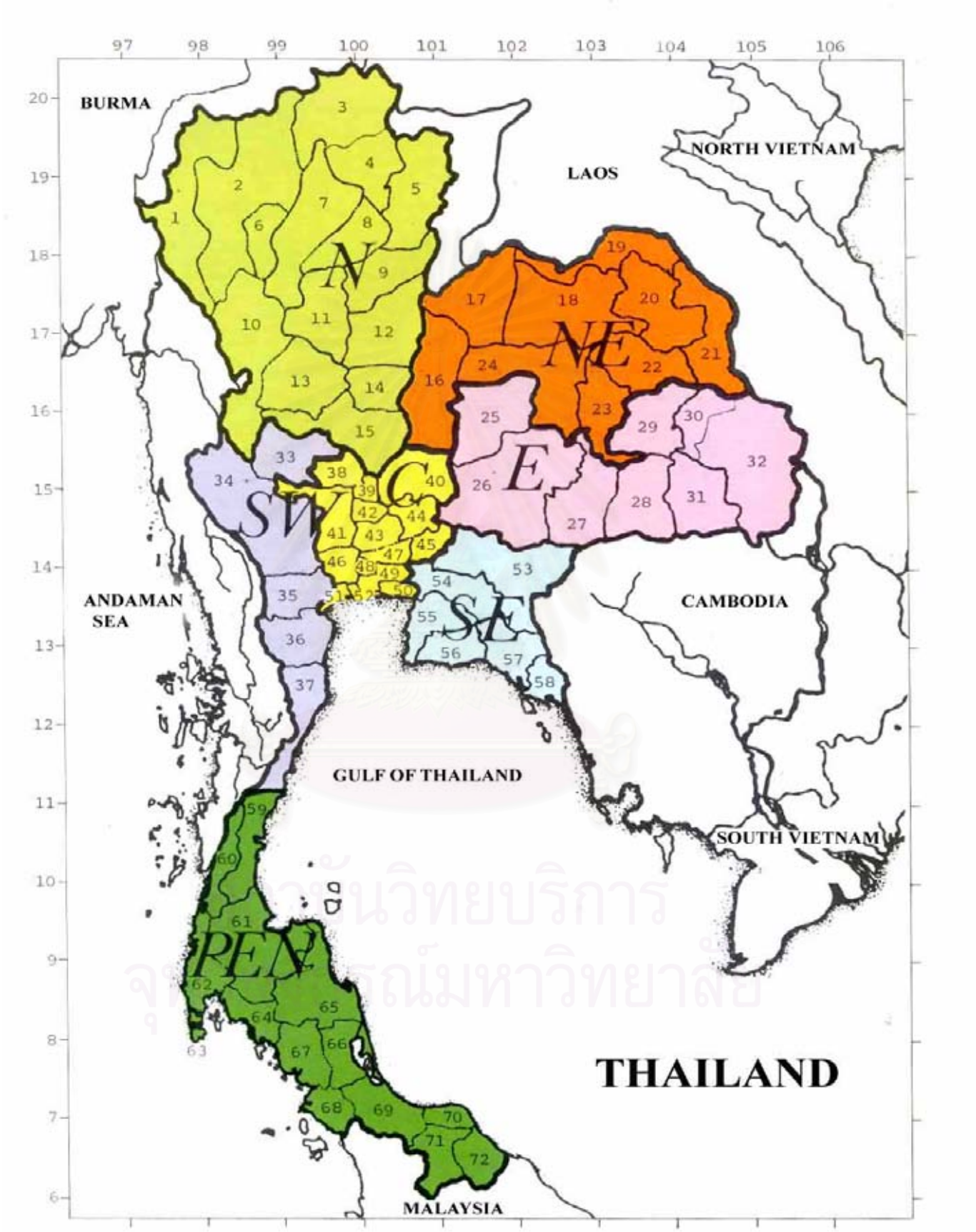
Number 21 = Nakhon-Phanom Province

Number 36 = Petchaburi Province

Number 46 = Nakhon-Pathom Province

Number 48 = Nonthaburi Province

Number 53 = Prachinburi Province


BURMA
LAOS
NORTH VIETNAM
CAMBODIA
SOUTH VIETNAM
ANDAMAN SEA
GULF OF THAILAND
MALAYSIA
THAILAND
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NE
E
SW
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PEN
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2.3 DNA extraction

Genomic DNA was extracted from 1 g of leaf tissue of each bitter gourd accession following the method of National Center for Genetic Engineering and Biotechnology (NCGEB), Thailand (1995).

One gram of leaf tissue was ground to powder in the presence of liquid nitrogen. Immediately after that, the powder was transfered into a 15 ml-tube containing CTAB buffer (2% CTAB, 1.4 M NaCl, 0.2% 2-mercaptoethanol, 0.2 M EDTA, pH 8.0 and 0.1 M Tris-HCl, pH 8.0) and the solution was then incubated at 60°C for 30 minutes. Next, 5 ml of chloroform were added to the solution mixture. The solution mixture was mixed gently and centrifuged at 3,000 rpm for 5 minutes. The upper aqueous phase was transferred to a new 15 ml-tube with a wide-bored pipette. Five milliliters of cold isopropanol were added to the solution, mixed gently and centrifuged at 3,000 rpm for 5 minutes. The aqueous solution was discarded and the pellet was washed with 70% ethanol and dried at room temperature. Then the pellet was dissolved in 0.7 ml of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) and followed by adding 20 μl of 10 mg/ml RNase A. The solution was incubated at 37°C for 30 minutes. After incubation, the solution was transferred to a new 1.5 ml-microcentrifuge tube. Then, phenol/chloroform extraction was performed by adding 250 μl of phenol (saturated with TE buffer) and 250 μl of chloroform to the solution. The solution was mixed gently and centrifuged at 12,000 rpm for 10 minutes. The upper aqueous phase was removed into a new 1.5 ml-microcentrifuge tube and phenol/chloroform extraction was repeated again. Next, a half volume of 7.5 M ammonium acetate solution, pH 7.5, was added and DNA was precipitated by the

addition of two volumes of cold absolute ethanol. The solution was then incubated at -80°C for 20 minutes. The precipitated DNA was recovered by centrifugation at 12,000 rpm for 10 minutes at room temperature. The DNA pellet was washed with 70% ethanol and air-dried. Finally, the pellet was completely redissolved by 300 μl of TE buffer and incubated at 37°C for an hour. The DNA solution was kept at 4°C until further used.

2.4 Measurement of DNA concentrations by agarose gel electrophoresis

Electrophoresis is a standard method used for the estimation of DNA concentration on the basis of its direct relationship between the amount of DNA and the level of fluorescence after ethidium bromide staining. The concentration of DNA was estimated by comparing with the known concentration standard DNA such as λ/*Hin*d III marker. In this study, a 0.7% (w/v) mini-gel was prepared by adding 0.35 g agarose to 50 ml of 1x TBE buffer (85 mM Tris-HCl, 8.91mM boric acid and 2.5 mM EDTA, pH 8.0). The agarose was solubilized by heating in a microwave oven and cooled down to 50-60°C before pouring into a gel mould (9x10 cm) with the presence of well-forming comb. After the gel was set, DNAs premixed with 6x loading dye were loaded into each well and electrophoresis was run at constant voltage (100 volts). Upon completion of electrophoresis, the gel was stained with 2.5 μg/ml ethidium bromide. DNA concentration was estimated from the intensity of the fluorescent band comparing with that of standard λ/*Hin*d III.

2.5 RAPD analysis

2.5.1 Amplification condition

Forty-seven arbitrary primers (Table 2.2) were synthesized by Bioservice Unit (BSU) of the National Center for Genetic Engineering and Biotechnology (NCGEB), National Science and Technology Development Agency, Thailand and were screened by using the modified method of Gomez *et al*., 1996. The amplification reaction was performed in 12.5 μl volume of reaction mixture containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.01% Triton X-100, 2.5 mM $MgCl_2$, 0.2 mM of each dATP, dTTP, dCTP and dGTP, 0.8 μM of a primer, 5-10 mg of genomic DNA and 1 unit of Ampli *Taq* DNA polymerase. The reaction was performed in the DNA Thermal Cycler (Perkin Elmer Model 2400) programmed for 40 cycles consisting of denaturation at 94 °C for 10 seconds, annealing at 36°C for 30 seconds and extension at 72°C for 1 minute. The final extension was carried out at 72°C for 5 minutes.

Table 2.2 Sequence of the 47 RAPD primers

Primer name	Primer Sequence	%GC
RA-01	GTC TGA CGG T	60
RA-02	CAG CTC AAG T	50
RA-03	CGA TCG AGG A	60
RA-04	GCA GAG CAT C	60
RA-05	AAG CAG CAA G	50
RA-06	TCT TCG AGG A	50
RA-07	AGC ACT TCG G	60
RA-08	CAC CGT TCT G	60
RA-09	ACT CCG CAG T	60
RA-10	GTC CTC TGA A	50
X-01	CAG GCC CTT C	70
X-02	CAG CAC CCA C	70
X-03	TTC CCC CGC T	70
X-04	GGA CCC AAC C	70
X-05	ACC CGG TCA C	70
X-06	CGT CTG CCC G	80
X-07	TGA CCC CTC C	70
X-08	GCT CCC CCA C	80
X-09	ACG GCC GAC C	80
X-10	GCC CGA CGC G	90
X-11	GGG CCA CTC A	70
X-12	GGA GAG ACT C	60
X-13	TCC ACT CCT G	60
X-14	CAC AGA GGG A	60
X-15	GGG TTT GGC A	60
X-16	CAA GGG CAG A	60
X-17	GGC AGG CTG T	70
X-18	AAC GGC GAC A	60
X-19	CAC CCC TGA T	60
X-20	CCT TCG GAA G	60
X-21	TTC CCC GCG A	70
X-22	GGG TGT GTA G	60
X-23	AGG ACT GCC A	60
X-24	AAT GCC GCA G	60

Table 2.2 (Continue)

Primer name	Primer Sequence	%GC
X-25	GGA TGC CAC T	60
X-26	GGT GAA CGC T	60
X-27	CCA ACG TCG T	60
X-28	GAT GCC AGA C	60
X-29	GTC CGT ATG G	60
X-30	GAC CAA TGC C	60
X-31	GGT CCC TGA C	70
X-32	GTT TCG CTC C	60
X-33	GTC TTG CGG A	60
X-34	GAA TTT CCC C	50
X-35	GCA CCG AGA G	70
X-36	GTC GAT GTC G	60
X-37	AAG CCT CCC C	70

2.5.2 Agarose gel electrophoresis

RAPD products were analyzed by electrophoresis through 1.5 % agarose gels in 1x TBE buffer. The solution was boiled in a microwave oven to complete solubilization, and cooled down to 60°C before being poured into the chamber set with an inserted comb. When the gel solidified, the comb was carefully removed. The agarose gel was submerged in a chamber containing ample amount of 1x TBE buffer (covering the gel for approximately 0.5 cm).

Twelve and a half microliters of each PCR products were mixed with 1 μl of loading dye (0.25% bromphenol blue and 25% Ficoll in water) and loaded into the well. The λ/*Hind* III and 100 bp DNA ladders were used as standard DNA markers. Electrophoresis was operated at 100 volts until bromphenol blue move to approximately 5 cm from the bottom of the gel. The gel was stained with a solution containing

2.5 μg/ml ethidium bromide for 5 minutes and destained in distilled water for 20 minutes to remove unbound ethidium bromide from agarose gels. The DNA fragments were visualized as fluorescent band under an UV transilluminator and photographed through a red filter using Kodak Tri-X-Pan 400 film. The exposure time was about 10-15 seconds.

2.5.3 Genetic analysis

The RAPD patterns are photographed and evaluated. Each RAPD fragment was treated as an independent character. RAPD bands were scored into a matrix to represent an absence (0) or presence (1) of a particular band. The dominant phenotype produced by a RAPD locus was expressed, on an agarose gel, as the presence of a band and the recessive phenotype was the absence of that band. Thus, pairs of individuals could be compared phenotypically at any locus, based on the shared presence or absence of a band. The shared absence of a band actually provided more information regarding their similarity (both homozygous recessives) than did the shared presence of that band (heterozygous or homozygous dominant). The similarity of pairs of individuals was measured by examining both the shared presence and the shared absence of bands to take advantage of the recessive phenotype. Black (1997) construct the computer program RAPDPLOT version 3.0, for estimating the fraction of matches (M) by using the formula:

$$M = N_{AB} / N_T$$

Where N_{AB} was the total number of matches in individuals A and B (i.e. both bands absent or present) and N_T was the total number of loci scored in the overall study. Unlike the similarity index (S), the denomination for

M was fixed, and the absence was scored because it represented the recessive phenotype at a locus. An M value of 1 indicated that two individuals had identical pattern; a value of 0 indicated that two individuals had completely different patterns.

Finally, M value would be converted to D value (genetic distances between paired samples) by using the equation: D = 1 - M (Black, 1997) for constructing the genetic relationships among bitter gourds studied. The dendrogram was constructed by a neighbor-joining method (Saitou and Nei, 1987) using NEIGHBOR implemented in Phylip version 3.5c (Felsenstein, 1993).

2.6 *MAP30* gene amplification and characterization in bitter gourd

2.6.1 Primer design

Four primers were designed from nucleotide sequences of MAP30 gene reported by Lee-Huang et al. (1995) (Fig 2.1). The primers were synthesized by BSU as mentioned in Section 2.5. The primers composed of two forward primers (S01 and S02) and two reverse primers (AS01 and AS02) and the priming sites were shown in Fig 2.1. Four different products would be obtained by using four sets of primers as shown in Table 2.3.

S01

1 gccgtccgaa **aatggtgaaa tgcttactac tttct**ttttt aattatcgcc atcttcattg

61 gtgttcctac tgccaaaggc gatgttaact tcgatttgtc gactgccact gcaaaaacct

121 acacaaaatt tatcgaagat ttcagggcga ctcttccatt tagccataaa gtgtatgata

181 tacctctgct gtattccact atttccgact ccagacgttt catactcctc aatctcacaa

S02

241 gttatgcata tgaaac**catc tcggtggcca tagatgtgac** gaacgtttat gttgtggcct

301 atcgcacccg cgatgtatcc tacttttta aagaatctcc tcctgaagct tataacatcc

361 tattcaaagg tacgcggaaa attacactgc catataccgg taattatgaa aatctccaaa

421 ctgctgcaca caaaataaga gagaatattg atcttggact ccctgccttg agtagtgcca

481 ttaccacatt gttttattac aatgcccaat ctgctccttc tgcattgctt gtactaatc**c**

AS02

541 **agacgactgc agaagctgca aga**tttaagt atactgagcg acacgttgct aagtatgttg

601 ccactaactt taagccaaat ctagccatca taagcctgga aaatcaatgg tctgctctct

661 ccaaacaaat ctttttggcg cagaatcaag gaggaaaatt tagaaatcct gtcgacctta

721 taaaacctac cggggaacgg tttcaagtaa ccaatgttga ttcagatgtt gtaaaaggta

781 atatcaaact cctgctgaac tccagagcta gcactgctga tgaaaacttt atcacaacca

AS01

841 tga**ctctact tggggaatct gttgtgaatt ga**aagtttaa taatccaccc atatcgaaat

901 aaggcatgtt catgacatg

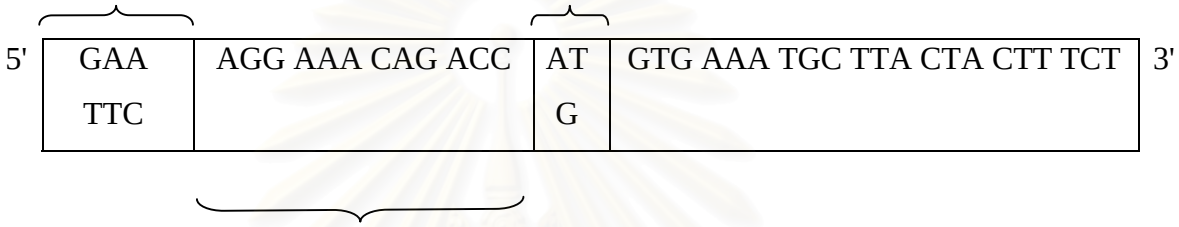
Figure 2.2 Nucleotide sequence of MAP30 gene described by Lee-Huang *et al*, 1995. Boldface and underlined indicate priming sites of primer S01, S02, AS01 and AS02

Table 2.3 Sequences of four sets of oligonucleotide primers designed from the nucleotide sequence of MAP30 gene

No. of Set	Primer Name	Primer Sequence	Primer Size	Product Size
1	S01	5'-GAA TTC AGG AAA CAG ACC ATG GTG AAA TGC TTA CTA CTT TCT-3'	42 base	885 bp
	AS01	5'-GGA TCC TCA ATT CAC AAC AGA TTC CCC AAG TAG AG-3'	35 base	
2	S01	5'-GAA TTC AGG AAA CAG ACC ATG GTG AAA TGC TTA CTA CTT TCT-3'	42 base	570 bp
	AS02	5'-TCT TGC AGC TTC TGC AGT CGT CGT-3'	24 base	
3	S02	5'-CAT CTC GGT GGC CAT AGA TGT GAC-3'	24 base	622 bp
	AS01	5'-GGA TCC TCA ATT CAC AAC AGA TTC CCC AAG TAG AG-3'	35 base	
4	S02	5'-CAT CTC GGT GGC CAT AGA TGT GAC-3'	24 base	307 bp
	AS02	5'-TCT TGC AGC TTC TGC AGT CGT CGT-3'	24 base	

Primer S01 = 42 base

***Eco*RI restriction site** **Start codon of *MAP30* gene**



Primer AS01 = 35 base

***Bam*HI restriction site**

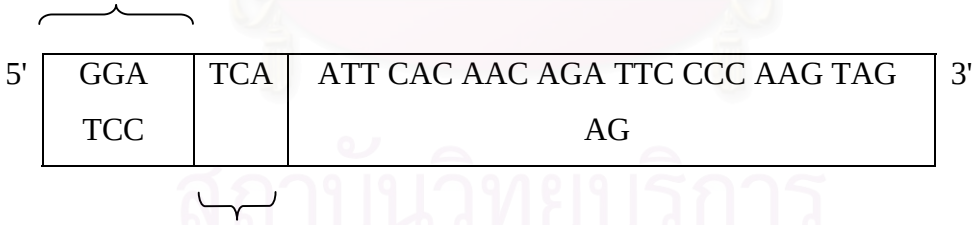


Figure 2.3 Describtion of primer S01 and AS01 nucleotide sequence

2.6.2 Amplification condition

Four sets of primers were used to amplify MAP30 gene according to the method of Lee-Huang *et al*. (1995). The amplification were performed in 20 μl reaction volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 50 pmol of each primer, *Taq* DNA polymerase 0.5 unit and 20-30 ng of bitter gourd genomic DNA. The reaction mixture was performed in a DNA Thermal Cycler (Perkin Elmer model 2400) programmed for 40 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds.

Twenty microlitters of each PCR products were analyzed by electrophoresis through 1.5 % agarose gel by the method as mentioned in Section 2.5.

2.7 Detection of MAP30 mRNA in some organ of bitter gourd

2.7.1 Sample collection

Apical shoot, leaf, flower bud, male flower, female flower, young fruit tissue, seed of young fruit, riped fruit tissue and seed of riped fruit of the unknown bitter gourd were collected from field trial and stored at -70°C until used.

2.7.2 RNA extraction

All instruments that were used to extract RNA were free from RNAse by incubation with diethyl pyrocarbonate (DEPC) overnight and autoclaved twice. TRI reagent kit (RNA/ DNA/ protein isolation reagent)

from Molecular Research Center, Inc. was used for the RNA extraction. The tissue samples (100 mg) was homogenized in the presence of liquid N_2 to powder. The powder was directly scraped into 1 ml of TRI reagent in a 1.5 ml-microcentrifuge tube and the solution mixture was incubated at room temperature for 5 minutes. Two-tenth ml of chloroform solution was added to the mixture, which was shook vigorously for 15 seconds. The solution mixture was stored at room temperature for 10 minutes and then centrifuged at 12,000 rpm for 15 minutes at 4°C. The upper aqueous phase was collected and transferred to a new microcentrifuge tube. Then half millilitre of isopropanol was added to this solution. The solution was stored at room temperature for 10 minutes allowing for the RNA precipitation and was centrifuged at 12,000 rpm for 8 minutes at 4°C. The aqueous phase was discarded and the RNA pellet was washed with 70% ethanol followed by centrifugation at 10,000 rpm for 5 minutes at 4°C. The RNA pellet was dried for 3-5 minutes at 65°C and then dissolved in diethyl pyrocarbonate (DEPC) solution. The concentration of extracted RNA samples were estimated by measuring the absorbance at 260 nm. The purity of RNA samples was determined by calculating a ratio of A260/ A280. The RNA solutions, which were free from DNA and protein contaminations, were indicated by the A260/ A280 ratio between 1.6-1.9.

2.7.3 Reverse transcriptase amplification(RT-PCR)

Onestep RT-PCR Kit (QIAGEN) was used for RT-PCR amplification. Fifty microlitres of RT-PCR reaction mixture contained 1x QIAGEN Onestep RT-PCR buffer containing 12.5 mM $MgCl_2$, 400 μM of each dNTP, 0.6 μM of each primer (primer set1, S01 and AS01), 2.0 μl of QIAGEN Onestep RT-PCR Enzyme mix, 10 Units of RNase inhibitor

and 0.5-0.8 μg of template RNA. Thermal Cycler condition consisted of 50°C for reverse transcription for 30 minutes, 95°C for initial PCR activation for 15 minutes and followed by 40 cycles of 94°C denaturation for 30 seconds, 60°C annealing for 30 seconds and 72°C extension for 30 seconds, Final extension was carried out at 72°C for 10 minutes. The detection of each RT-PCR products were performed by the method as described in Section 2.7.

2.8 Preparation of *MAP30* gene for cloning and expression

2.8.1 Amplification of *MAP30* gene

Amplification of MAP30 gene according to the method of Lee-Huang *et al*. (1995). Primer set 1 (S01 and AS01, Table 2.3) was used for *MAP30* gene amplification. The amplification were performed in 20 μl reaction volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 50 pmol of each primer, *Taq* DNA polymerase 0.5 unit and 20-30 ng of bitter gourd genomic DNA. The reaction mixture was performed in a DNA Thermal Cycler (Perkin Elmer model 2400) programmed for 40 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds.

Primer S01 and AS01 (Table 2.3 and Fig.2.3) were designed as described in Section 2.6.3. Primer S01 was further modified by adding respectively *Eco*RI restriction sequence (GAA TTC) and Shine-Dalgarno sequence of expression vector pTrc99 (5'-AGG AAA CAG ACC-3') at the 5'-P end of the primer before the start codon (ATG) of *MAP30* gene. Primer AS02 was modified by adding *Bam*HI restriction sequence (GGA

TCC) after the stop codon (TGA) of *MAP30* gene. *Eco*RI and *Bam*HI sites were added for cloning the *MAP30* gene into pUC18 vector and Shine-Dalgarno sequence was added for expression of *MAP30* gene in *E. coli* (JM109) host.

2.8.2 The *MAP30* gene fragment preparation

After *MAP30* gene amplification, the 5 reactions of PCR product were pooled (100µl) and completely digested by both *Eco*RI and *Bam*HI. The reaction mixture containing 100µl of gene fragment was composed of 1X reaction buffer for *Eco*RI (50 mM Tris-HCl, pH8.0, 10 mM $MgCl_2$ and 100 mM NaCl), 10 U of *Eco*RI and 10 U of *Bam*HI. The reaction mixture was incubated at 37°C for 18 hours. Complete digested *MAP30* gene was then electrophoresed through 1.5% agarose gel. After electrophoresis, *MAP30* gene fragment was excised from 1.5 % agarose gel using a scalpel and placed in preweighed microcentrifuge tube. DNA fragment was eluted from agarose gel by using Nucleo Spin Extract 2 in 1 kit and Nucleo Spin Extract column (MACHEREY-NAGEL). The method for elution follow the process of this product manual.

Agarose gel containing the fragment of *MAP30* gene was sliced to minimize the gel volume and the weight of gel slice was determined. The gel slice was transferred to clean microcentrifuge tube before adding 300 µl of NT1 buffer for each 100 mg agarose gel. It was then incubated at 50°C for 5-10 min (vortexed the sample every 2-3 min) or until gel slice was completely dissolved. Placed a Nucleo Spin Extract column into a 2 ml collecting tube and the DNA sample was loaded onto the column and centrifuged for 1 minute at 10,000 rpm. The flowthrough was discarded and the column was placed back into the collecting tube,

then added 600 μl NT3 buffer, centrifuged for 1 minute at 12,000 rpm. Discarded flowthrough and placed the column back into the collecting tube followed by adding 200 μl NT3 buffer, centrifuged for 2 minutes at 12,000 rpm. Placed the Nucleo Spin Extract column into a clean 1.5 ml microcentrifuge tube then added 50 μl NE buffer, left at room temperature for 1 minute followed by centrifugation for 1 minute at 12,000 rpm. *MAP30* gene fragment was precipitate by adding 25 μl of 7.5 M ammonium acetate pH 7.5 and followed by adding 150 μl of absolute ethanol into the MAP30 gene fragment solution. The solution was centrifuged at 12,000 rpm for 10 minute and the aqueous phase was discarded. DNA pellet was washed with 70% ethanol, air dried at room temperature, and re-dissolved the DNA pellet with 20 μl of sterile distilled water.

2.8.3 Vector DNA preparation

The pUC18 vector (Appendix B) was completely digested with EcoRI and BamHI. The reaction mixture containing 5 μg pUC18, 1x EcoRI reaction buffer (50mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$ and 100 mM NaCl), 10 U of EcoRI and 10 U of BamHI in total volume of 100 μl was incubated at 37 °C for 18 hours. The linear-form pUC18 was electrophoresed through 0.7% agarose gel and harvested from agarose gel by method as described in Section 2.8.2.

2.8.4 Ligation of MAP30 gene fragment and pUC18 vector

MAP30 gene fragment was ligated to pUC18 vector in a 20 μl reaction volume containing 50 ng of vector, 250 ng of MAP30 gene fragment, 1x ligation buffer (66 mM Tris-HCl, pH 7.6, 6.6 mM $MgCl_2$, 10

mM DTT and 66 μM ATP) and T_4 DNA ligase (Tested User Friendly, USA). Reaction mixture was incubated at 16 °C for 16 hours. The recombinant plasmids were further used for transformation.

2.9 Transformation of ligated products into *E. coli* host cells by electroporation (Dower *et al*., 1988)

2.9.1 Preparation of competent cells

A single colony of *E. coli* JM109 was inoculated in 10 ml of LB-broth (1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl) with vigorous shaking at 37 °C overnight. The starting culture was inoculated into 1 liter of LB-broth and cultured at 37 °C with vigorous shaking until OD_{600} of 0.5 to 0.8. The cells were chilled briefly on ice for 15 to 30 minutes, and harvested by centrifugation in a prechilled rotor at 4000 g for 15 minutes at 4 °C. The pellet were resuspended in 1 liter of cold water and centrifuged as above. After resuspended in 0.5 liter of cold water, the pellet was centrifuged and resuspended in 20 ml of 10% glycerol. The cells were recentrifuged, and finally resuspended in 2 to 3 ml of 10% glycerol. This concentrated cell suspension was divided into 40 μl aliquots. These cells could be used immediately or stored at -70 °C for later used.

2.9.2 Electrotransformation

The recombinant plasmids from Section 2.8.3 were introduced into a competent *rec*A, *end* A *E. coli* strain JM109 by electroporation. One or two microlitres of the ligation mixture from Section 2.8.3 was added to a competent cell solution and left on ice for approximately 2

minutes. The mixture was electroporated in a prechilled 0.2 cm cuvette by using a Gene pulser (Bio-Rad) with the setting parameters of 25 μF, 200 Ω and 2.5 kV. After electroporation, the mixture was immediately removed from the cuvette and added to a new tube containing 1 ml of LB medium (1% tryptone, 0.5% yeast extract and 0.5% NaCl, pH 7.0). The cell suspension was incubated with shaking at 37 °C for 1 to 2 hours. Approximately, 100 μl of this cell suspension were spreaded on a selective LB agar plated containing 50 μg/ml ampicillin, 35 μl (40 mg/ml) of IPTG and 40μl (20 mg/ml) of X-gal and further incubated at 37 °C overnight (Sambrook *et al*., 2001). The recombinant clones containing inserted DNA are white colonies whereas those without inserted DNA are blue colonies.

2.10 Plasmid extraction

A white colony of transformed bacteria was inoculated to 2 ml of LB medium and then cultured overnight at 37 °C with vigorous shaking. Next, the culture was transferred into a microcentrifuge tube and centrifuged at 5,000 rpm for 5 minutes at 4 °C. After that, the supernatant was removed and the cell pellet was resuspended by vortexing in 100 μl of ice-cold Alkaline lysis solution I (50 mM glucose, 10 mM EDTA, pH 8.0 and 25 mM Tris-HCl, pH 8.0). The 200 μl of freshly prepared Alkaline lysis solution II (0.2 N NaOH and 1% SDS) were added and the cell suspension was then mixed by inverting the tube. The viscous bacterial lysated was stored on ice. One hundred and fifty microlitres of ice-cold Alkaline lysis solution III (3 M sodium acetate, pH 4.8) was added and the viscous bacterial lysate was mixed by inverting the tube

several times. The lysate was centrifuged at 4 °C for 5 minutes at 12,000 rpm. The supernatant was transferred to a fresh tube and the phenol/chloroform extraction was performed by adding equal volume of phenol:chloroform (1:1) into the supernatant. The mixture was mixed thoroughly and centrifuged at 10,000 rpm for 5 minutes. The upper phase was transferred into a new tube and equal volume of chloroform was added. Then the mixture was mixed and centrifuged at 10,000 rpm for 5 minutes. The upper phase was transferred into a new tube and the chloroform extraction was performed again. The upper phase of the second time chloroform extraction was transferred into a new tube. The plasmid DNA was precipitated by adding 2 volumes of absolute ethanol at room temperature. The plasmid solution was mixed for 2 minutes and centrifuged at 10,000 rpm for 5 minutes. The pellet was dried and then dissolved in 50 µl of TE buffer containing 20 µg/ml DNase-free RNaseA (pancreatic RNase). The plasmid DNA was stored at –20 °C.

2.11 Detection of recombinant plasmid

For detection of recombinant plasmid, extracted plasmids were cutted by both EcoRI and BamHI restriction enzymes. The cutted products were electrophoresed through 1.5 % agarose gel as described in Section 2.5.2 and estimated for thir sizes by compare with DNA marker (λ/*Hind*III and 100 bp DNA ladder). Plasmid which contain clone of *MAP30* gene should give product of 885 bp in size.

CHAPTER III

RESULTS

3.1 DNA extraction

Total DNA was extracted from leaf tissue of each accession of bitter gourd by CTAB method (Section 2.3). Extracted DNA migrated slower than all fragments of standard λ/*Hin*d III marker indicating that high molecular weight total DNA (> 23.1 kb) was consistently obtained. DNA concentration of 50-100 μg per 1 g of leaf tissue was obtained as estimated by comparing its intensity of EtBr-DNA complex with that of the known amount of λ/*Hin*d III marker in 0.7% agarose gel electrophoresis (Figure 3.1).

3.2 Optimization of RAPD PCR

These experiments were performed to optimize PCR reaction for successful and reproducible amplification of discrete bands from bitter gourd DNA. Major factors that affect more to amplified pattern were concentrations of $MgCl_2$ and template DNA. Various $MgCl_2$ concentrations (1 mM, 1.5 mM, 2.0 mM, and 2.5 mM) and template DNA concentrations (100 ng, 50 ng, 20 ng, and 10 ng) were tested for optimize condition. The result showed that optimal $MgCl_2$ concentration was 2.5 mM and optimal template DNA concentration was 10-20 ng (Fig 3.2).

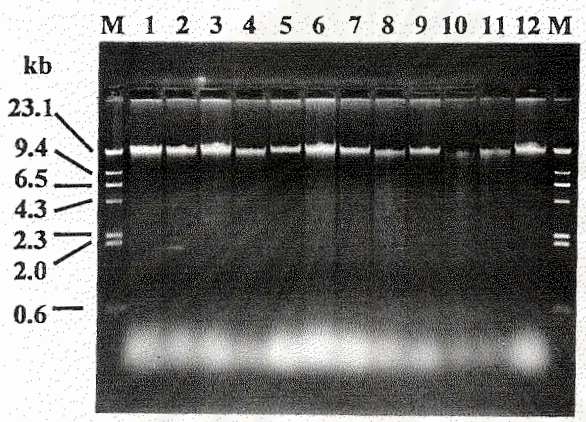


Figure 3.1 A 0.7% ethidium bromide stained-agarose gel showing the quality of high molecular weight DNA extracted from leaf tissue of *Momordica charantia* Linn.

Lane M = λ/*Hin*d III standard DNA marker

Lane 1-12 = total DNA from 12 accessions of *M. charantia*

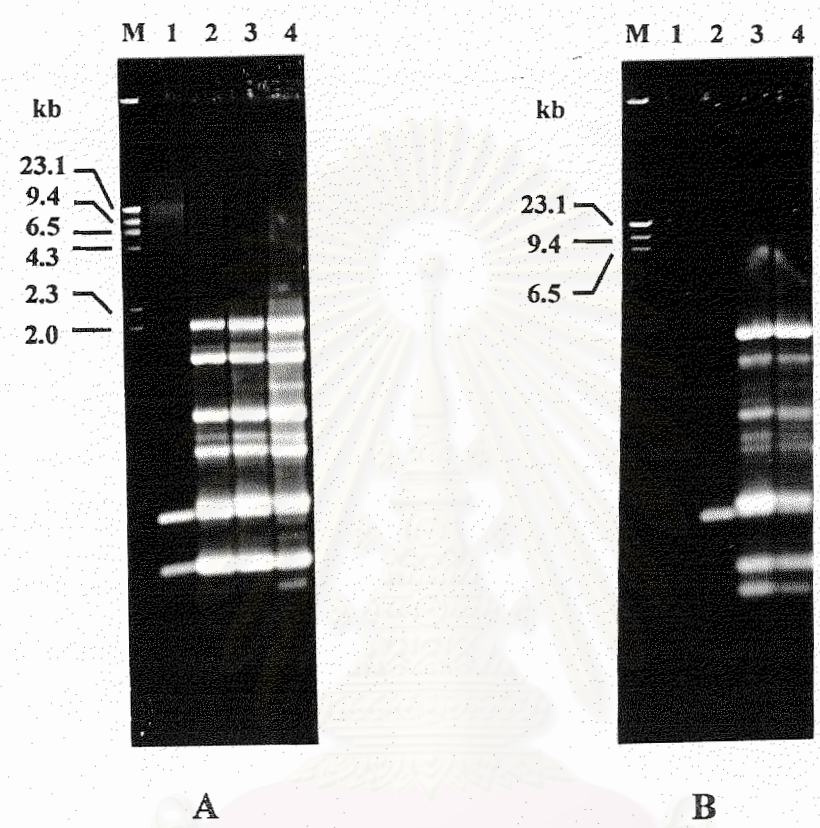


Figure 3.2 Optimization of RAPD-PCR using primer x-16 and bitter gourd DNA code M003

A: variation of $MgCl_2$ concentration. Lane 1-4 are 1.0 mM, 1.5 mM, 2.0 mM, and 2.5 mM $MgCl_2$ concentration, respectively.

B: variation of DNA template concentration. Lane 1-4 are 100 ng, 50 ng, 20 ng, and10 ng DNA template, respectively.

Lane M = λ/*Hin*d III standard DNA marker

3.3 Primer screening

High molecular weight DNA of 22 accessions of *M. charantia* and one of *M. cochinchinensis* (outgroup) were tested for the amplification against 47 decanucleotide primers using RAPD condition from the modified method of Gomez *et al*, 1996. Except primers RA-06 and x-09, all other primers gave successful amplification results. The primers, which gave complicate patterns, low polymorphism or unsuccessful amplification, were excluded. Six primers (X-12, X-16, X-18, X-24, X-26 and X-27; Table 2.2) which gave reproducible RAPD patterns were included for further analysis of genetic diversity of 22 accessions of bitter gourd. Examples of the amplified pattern were shown in Figure 3.3. Figure 3.4 to 3.9 showed the RAPD patterns of those selected primers.

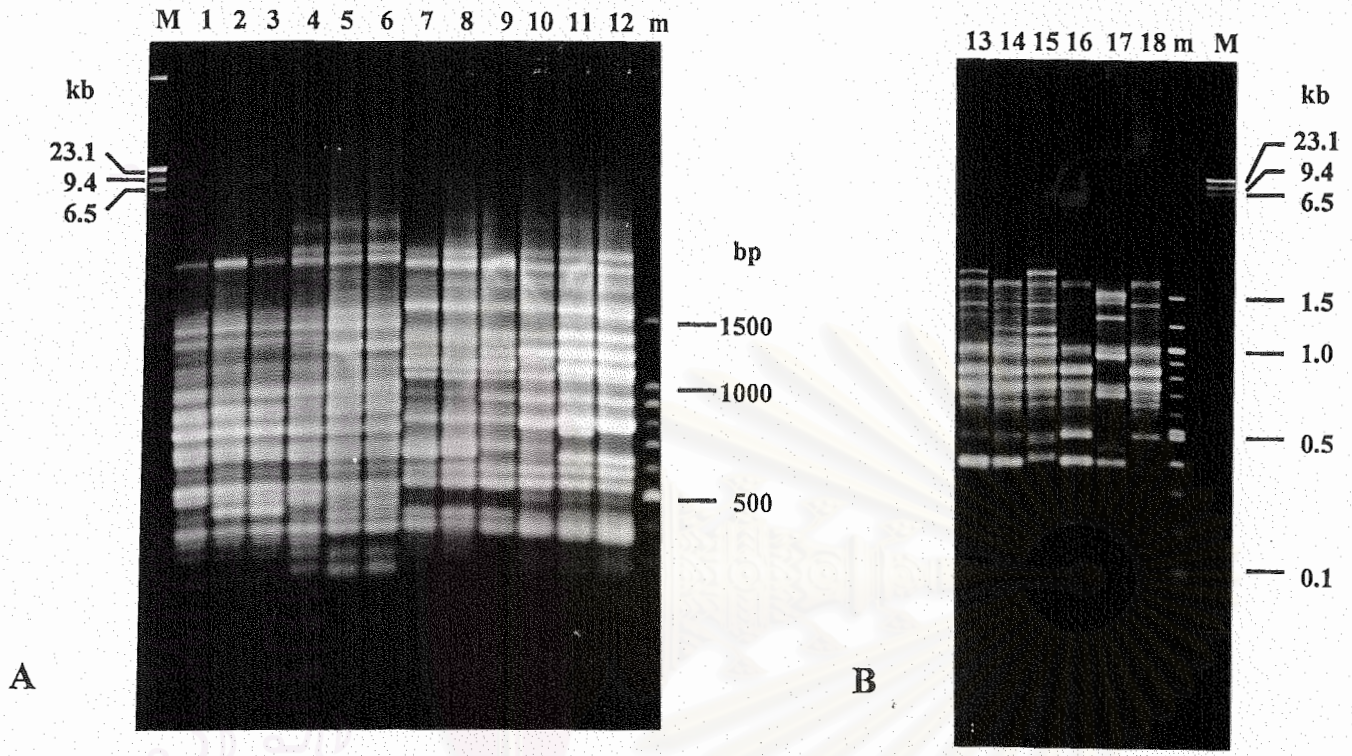


Figure 3.3 RAPD patterns generated from primer excluded (A) and primer included (B) in this study

A = RAPD patterns generated from primer X-02 (lane 1-6) and primer X-05 (lane 7-12)

B = RAPD patterns generated from primer X-26 (lane 13-18)

Lane M= λ/*Hin*d III standard DNA marker

Lane m= 100 bp DNA ladder

Figure 3.4-3.9 RAPD patterns obtained from 22 accession of *M. charantia* and one of *M. cochinchinensis* with primer X-12, X-16, X-18, X-24, X-26 and X-27

Lane M = λ/*Hin*d III standard DNA marker
Lane m = 100 bp DNA ladder
Lane 1-8 = bitter gourd accessions M001-M008, respectively
Lane 9a-9b = bitter gourd accession M09A and M09B, respectively
Lane 10-16 = bitter gourd accessions M010-M016, respectively
Lane 19-22 = bitter gourd accessions M019-M022, respectively
Lane 17 = bitter gourd accessions M017
Lane F1 = *Momordica cochinchinensis* (MF01, outgroup)

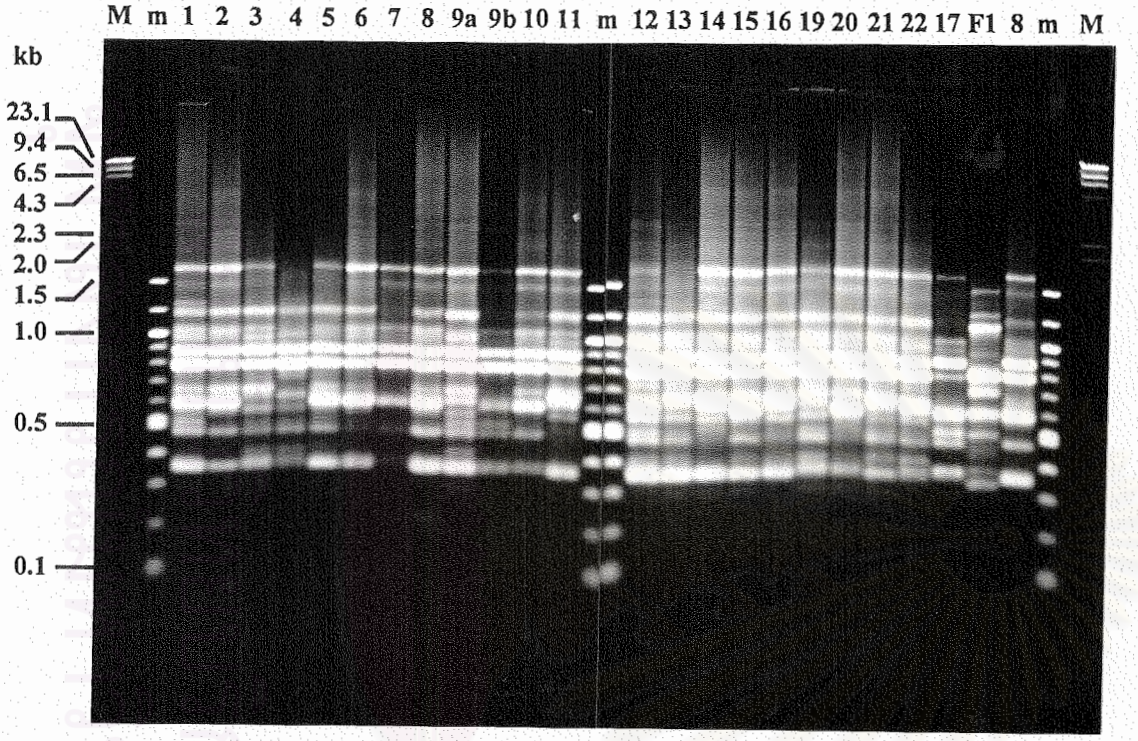


Figure 3.4 RAPD patterns obtained from 22 accession of *M. charantia* and one of *M. cochinchinensis* with the primer X-12

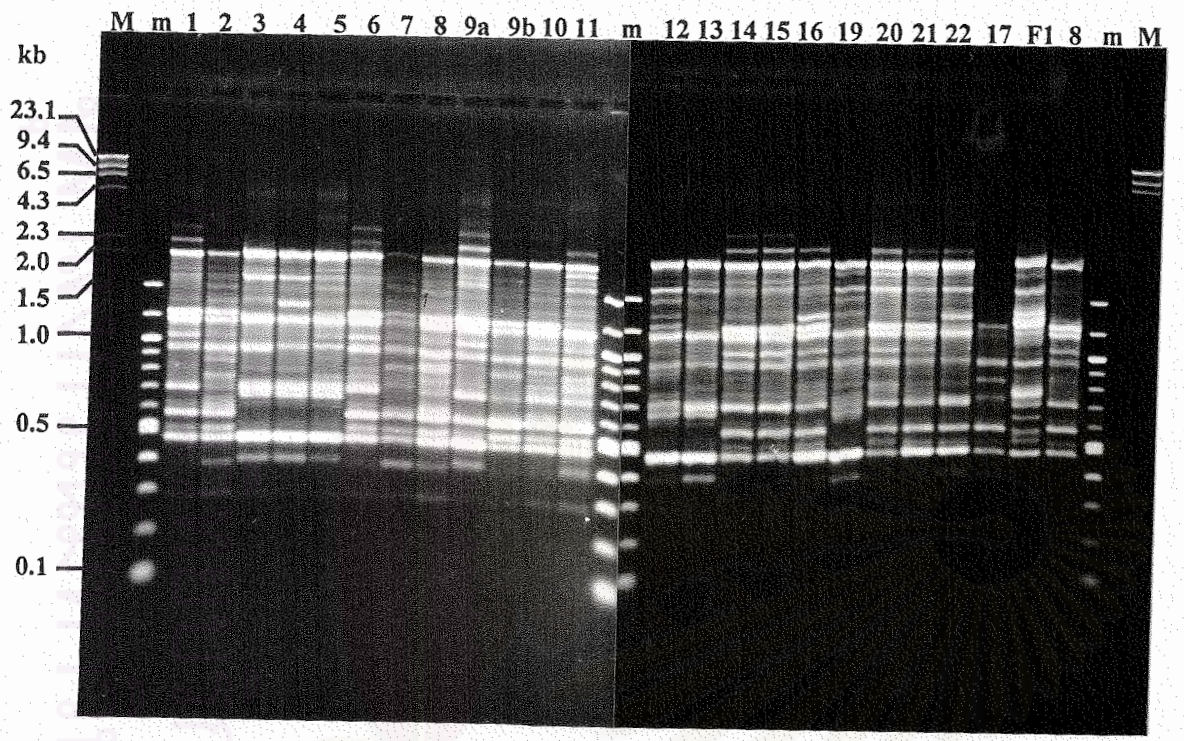


Figure 3.5 RAPD patterns obtained from 22 accession of *M. charantia* and one of *M. cochinchinensis* with the primer X-16

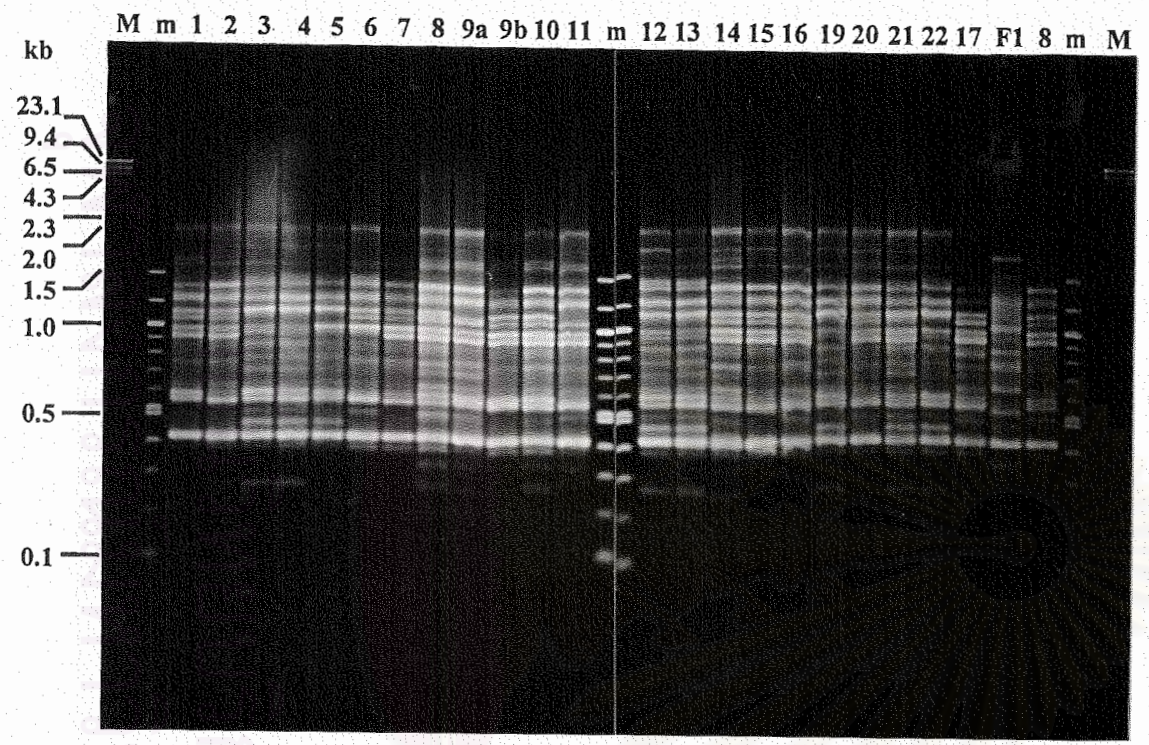


Figure 3.6 RAPD patterns obtained from 22 accession of *M. charantia* and one of *M. cochinchinensis* with the primer X-18

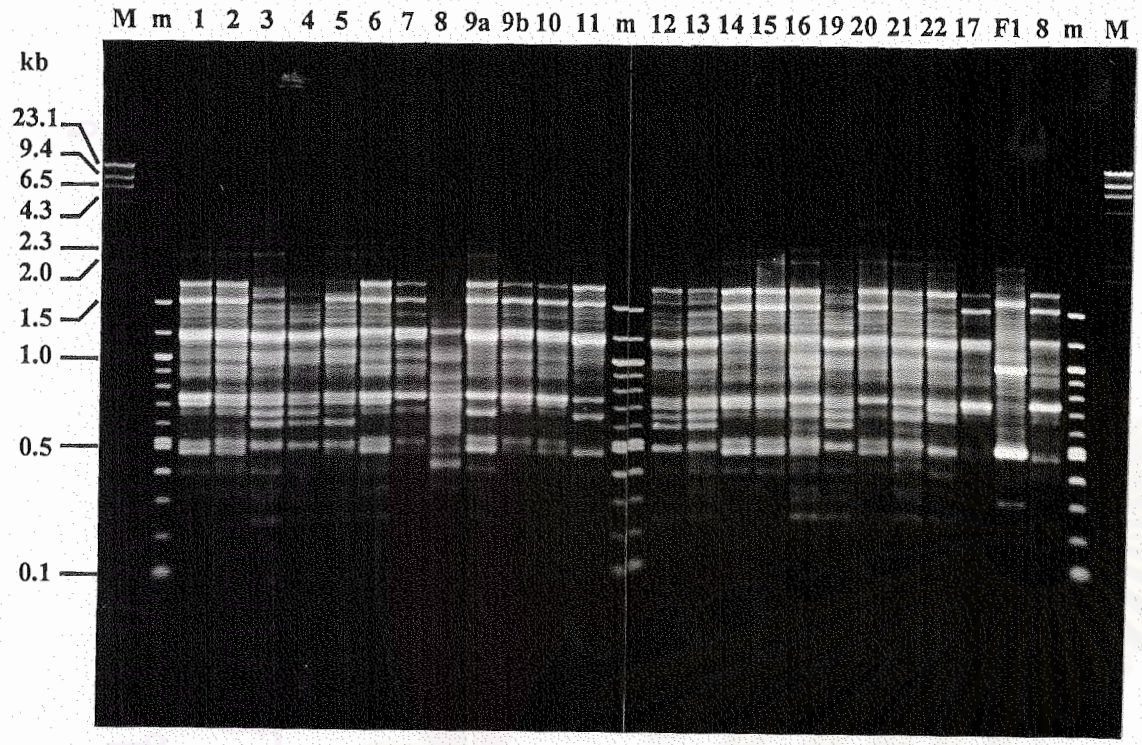


Figure 3.7 RAPD patterns obtained from 22 accession of *M. charantia* and one of *M. cochinchinensis* with the primer X-24

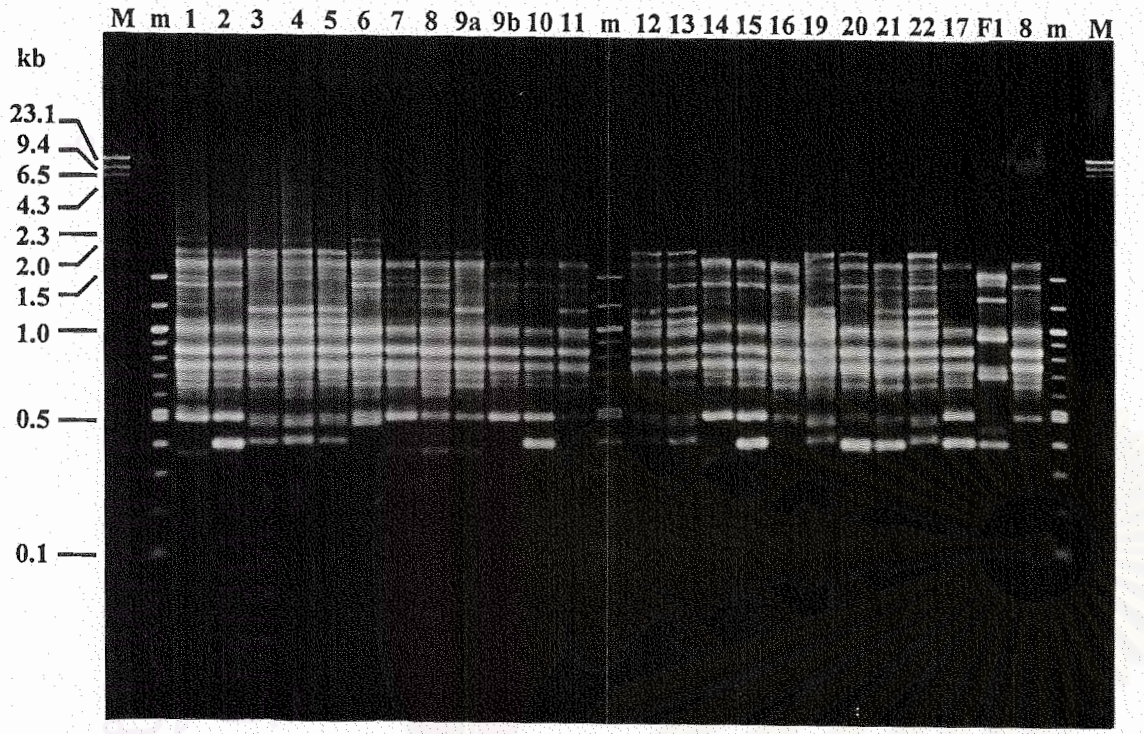


Figure 3.8 RAPD patterns obtained from 22 accession of *M. charantia* and one of *M. cochinchinensis* with the primer X-26

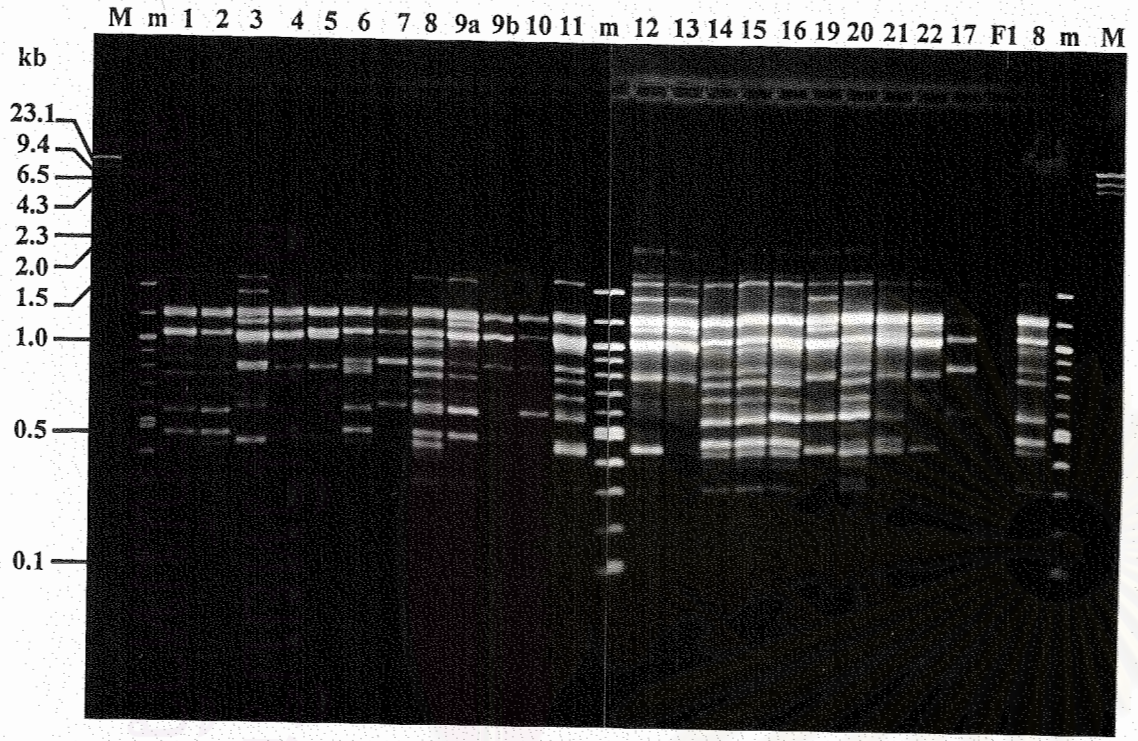


Figure 3.9 RAPD patterns obtained from 22 accession of *M. charantia* and one of *M. cochinchinensis* with the primer X-27

3.4 Determination of genetic diversity of bitter gourd using RAPD analysis

Only reproducible bands were scored for presence (1) or absence (0) in each individual. A total of 109 RAPD fragments from the six primers (X-12, X-16, X-18, X-24, X-26 and X-27) were consistently generated. Primer X-12 gave RAPD bands ranging from 1.7 kb to 350 bp. It gave total 20 consistent and reproducible RAPD bands composed of 9 monomorphic and 11 polymorphic bands. This primer generated a unique RAPD band for bitter gourd that was 0.9 kb in size. Primer X-16 generated RAPD bands ranging from 2.3 kb to 490 bp. It gave total 17 RAPD bands composing of 12 monomorphic and 5 polymorphic bands. Primer X-18 generated RAPD bands ranging from 2.3 kb to 390 bp. It gave total 20 RAPD bands consisting of 13 monomorphic and 7 polymorphic bands. Primer X-24 generated RAPD bands from 1.8 kb to 0.5 kb in range. It gave total 16 RAPD bands composed of 4 monomorphic and 12 polymorphic bands. Primer X-26 generated RAPD bands from 1.8 kb to 370 bp in range. It gave total 22 RAPD bands composed of 13 monomorphic and 9 polymorphic bands. This primer generated 2 unique RAPD bands for bitter gourd that were 750 bp and 850 bp in size. Primer X-27 generated RAPD bands from 1.75 kb to 420 bp in range. It gave total 14 RAPD bands composed of 3 monomorphic and 11 polymorphic bands. This primer generated a unique RAPD band for bitter gourd that was 1.1 kb in size. The numbers of consistent and reproducible bands are summarized in Table 3.1.

Table 3.1 Total number of bands, monomorphic and polymorphic band within 22 accessions of *M. charantia* and the *M. cochichinensis* revealed by RAPD analysis using primers X-12, X-16, X-18, X-24, X-26 and X-27

Primer name	No. of total bands	No. of monomorphic bands	No. of polymorphic bands
X-12	20	9 (45.0 %)	11 (55.0 %)
X-16	17	12 (70.6 %)	5 (29.4 %)
X-18	20	13 (65.0 %)	7 (35.0 %)
X-24	16	4 (25.0 %)	12 (75.0 %)
X-26	22	13 (59.1 %)	9 (40.9 %)
X-27	14	3 (21.4 %)	11 (78.6 %)
Total	109	54 (49.5 %)	55 (50.5%)

3.5 Data analysis on the genetic relationships of similarities and distances in bitter gourd

All investigated taxa were divided into 23 operational taxonomic units (OTUs). The matches between sample (M) of the six primers were calculated as the average of all possible comparisons of individuals between pairs of samples. Genetic distance (D) were converted from the index of similarity between samples (D = 1-M).

A neighbor-joining (NJ) tree, which was constructed from average genetic distance within *M. charantia* species and between *M. charantia* and *M. cochinchinensis* indicated that the separated between the investigated *M. charantia* was clearly separate as shown in Figures 3.10 and 3.11. At the intraspecific level, NJ tree showed that *M. charantia* can be divided into two groups based on RAPD data. Group A (15 accessions) was in the same group as Chinese's bitter gourd from Nepal (M017), while the other 7 accessions were another distinct group, defined as group B. At hhe interspecies level, *M. charantia* were distinctly separated from *M. cochinchinensis*

In addition, a 1,050 bp RAPD band generated from primer X-18 was possibly specific for group A and a 520 bp RAPD band generated from primer X-24 was possibly specific for group B (Figure 3.6 and Figure 3.7, respectively). Both bands may be used as specific markers for differentiating each group of bitter gourd.

Table 3.2 The average genetic distance s within *M.charantia* and between *M. charantia* and *M. cochinchinensis*

	M001	M002	M003	M004	M005	M006	M007	M008	M09A	M09B	M010	M011	M012
M001	0.0000	0.0462	0.3659	0.4167	0.2640	0.0781	0.0635	0.0551	0.1406	0.0394	0.0462	0.1094	0.3607
M002	0.0462	0.0000	0.3659	0.4167	0.3120	0.0781	0.0794	0.0709	0.1875	0.0709	0.0462	0.1406	0.3934
M003	0.3659	0.3659	0.0000	0.0442	0.1017	0.3058	0.3613	0.3667	0.3554	0.3833	0.3659	0.3223	0.0957
M004	0.4167	0.4167	0.0442	0.0000	0.1478	0.3559	0.3966	0.4188	0.3559	0.4359	0.4167	0.3729	0.0893
M005	0.2640	0.3120	0.1017	0.1478	0.0000	0.2520	0.2893	0.2951	0.3008	0.2787	0.2640	0.2520	0.1453
M006	0.0781	0.0781	0.3058	0.3559	0.2520	0.0000	0.1129	0.0880	0.2063	0.1200	0.1094	0.1587	0.3333
M007	0.0635	0.0794	0.3613	0.3966	0.2893	0.1129	0.0000	0.1057	0.1452	0.0732	0.0794	0.1774	0.4068
M008	0.0551	0.0709	0.3667	0.4188	0.2951	0.0880	0.1057	0.0000	0.1520	0.0806	0.0866	0.1200	0.3613
M09A	0.1406	0.1875	0.3554	0.3559	0.3008	0.2063	0.1452	0.1520	0.0000	0.1520	0.1563	0.1429	0.3000
M09B	0.0394	0.0709	0.3833	0.4359	0.2787	0.1200	0.0732	0.0806	0.1520	0.0000	0.0394	0.1520	0.3950
M010	0.0462	0.0462	0.3659	0.4167	0.2640	0.1094	0.0794	0.0866	0.1563	0.0394	0.0000	0.1563	0.3770
M011	0.1094	0.1406	0.3223	0.3729	0.2520	0.1587	0.1774	0.1200	0.1429	0.1520	0.1563	0.0000	0.3167
M012	0.3607	0.3934	0.0957	0.0893	0.1453	0.3333	0.4068	0.3613	0.3000	0.3950	0.3770	0.3167	0.0000
M013	0.3719	0.4050	0.0877	0.0631	0.1379	0.3445	0.4188	0.3729	0.3445	0.4068	0.3884	0.3277	0.0442
M014	0.0543	0.0698	0.3934	0.4454	0.3065	0.1181	0.1200	0.0794	0.1654	0.0635	0.0698	0.1339	0.3884
M015	0.0476	0.0476	0.3950	0.4483	0.3058	0.0806	0.1148	0.0569	0.1935	0.0569	0.0476	0.1452	0.3898
M016	0.0866	0.1181	0.3333	0.3846	0.2623	0.1040	0.1057	0.0806	0.1360	0.0968	0.1024	0.1040	0.3277
M019	0.3934	0.3934	0.0609	0.0357	0.1624	0.3333	0.3729	0.3950	0.3333	0.4118	0.3934	0.3500	0.0877
M020	0.0923	0.0769	0.3659	0.4000	0.2960	0.1250	0.0635	0.1024	0.1719	0.0709	0.0769	0.1875	0.4262
M021	0.1385	0.1538	0.3008	0.3167	0.2480	0.1406	0.1905	0.1496	0.1563	0.1811	0.1538	0.1563	0.2459
M022	0.2846	0.3171	0.1552	0.1504	0.1695	0.2562	0.3277	0.3000	0.2562	0.3167	0.3008	0.2231	0.0957
M017	0.1148	0.0984	0.3739	0.4107	0.3162	0.1333	0.1017	0.1261	0.2333	0.1092	0.1148	0.2000	0.4035
MF01	0.7647	0.7143	0.6964	0.6881	0.7018	0.7436	0.7391	0.7586	0.7778	0.7759	0.7311	0.7436	0.7117

Table 3.2 (continue)

	M013	M014	M015	M016	M019	M020	M021	M022	M017	MF01
M001	0.3719	0.0543	0.0476	0.0866	0.3934	0.0923	0.1385	0.2846	0.1148	0.7647
M002	0.4050	0.0698	0.0476	0.1181	0.3934	0.0769	0.1538	0.3171	0.0984	0.7143
M003	0.0877	0.3934	0.3950	0.3333	0.0609	0.3659	0.3008	0.1552	0.3739	0.6964
M004	0.0631	0.4454	0.4483	0.3846	0.0357	0.4000	0.3167	0.1504	0.4107	0.6881
M005	0.1379	0.3065	0.3058	0.2623	0.1624	0.2960	0.2480	0.1695	0.3162	0.7018
M006	0.3445	0.1181	0.0806	0.1040	0.3333	0.1250	0.1406	0.2562	0.1333	0.7436
M007	0.4188	0.1200	0.1148	0.1057	0.3729	0.0635	0.1905	0.3277	0.1017	0.7391
M008	0.3729	0.0794	0.0569	0.0806	0.3950	0.1024	0.1496	0.3000	0.1261	0.7586
M09A	0.3445	0.1654	0.1935	0.1360	0.3333	0.1719	0.1563	0.2562	0.2333	0.7778
M09B	0.4068	0.0635	0.0569	0.0968	0.4118	0.0709	0.1811	0.3167	0.1092	0.7759
M010	0.3884	0.0698	0.0476	0.1024	0.3934	0.0769	0.1538	0.3008	0.1148	0.7311
M011	0.3277	0.1339	0.1452	0.1040	0.3500	0.1875	0.1563	0.2231	0.2000	0.7436
M012	0.0442	0.3884	0.3898	0.3277	0.0877	0.4262	0.2459	0.0957	0.4035	0.7117
M013	0.0000	0.4000	0.4017	0.3559	0.0973	0.4215	0.2727	0.1053	0.4159	0.7091
M014	0.4000	0.0000	0.0560	0.0952	0.4380	0.1008	0.1628	0.3115	0.1405	0.7627
M015	0.4017	0.0560	0.0000	0.1057	0.4237	0.0952	0.1429	0.3109	0.1017	0.7391
M016	0.3559	0.0952	0.1057	0.0000	0.3613	0.1181	0.1181	0.2500	0.1933	0.7586
M019	0.0973	0.4380	0.4237	0.3613	0.0000	0.3770	0.2951	0.1304	0.3860	0.6937
M020	0.4215	0.1008	0.0952	0.1181	0.3770	0.0000	0.1846	0.3496	0.0984	0.7311
M021	0.2727	0.1628	0.1429	0.1181	0.2951	0.1846	0.0000	0.1707	0.2131	0.7311
M022	0.1053	0.3115	0.3109	0.2500	0.1304	0.3496	0.1707	0.0000	0.3217	0.7143
M017	0.4159	0.1405	0.1017	0.1933	0.3860	0.0984	0.2131	0.3217	0.0000	0.7297
MF01	0.7091	0.7627	0.7391	0.7586	0.6937	0.7311	0.7311	0.7143	0.7297	0.0000

Figure 3.10 A neighbor-joining tree based on genetic distance among 22 accessions of *M. charantia* and *M. cochinchinensis* from Pichitr Horticultural Research Center, Thailand. Genetic distances were estimated from RAPD data using six primers (X-12, X-16, X-18, X-24, X-26 and X-27). *M. cochinchinensis* was used as an outgroup. Detailed information and abbreviations of samples were shown in Table 2.1

Nb = Nontaburi Province

Np = Nakorn-Prathom Province

Pe = Petchaburi Province

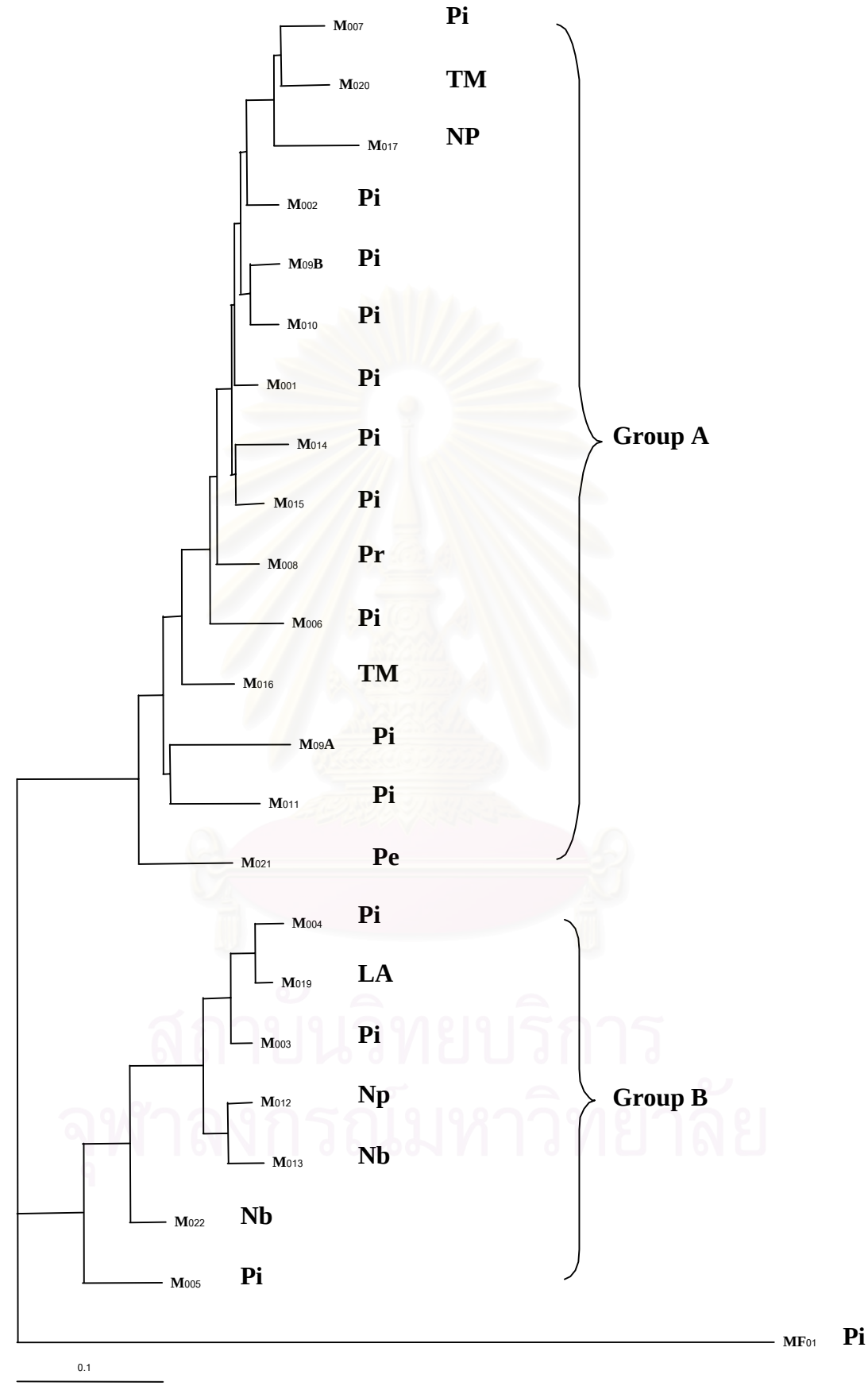
Pi = Pichitr Province

Pr = Prajeenburi Province

LA = Laos

NP = Nepal

TM = Trade Mark



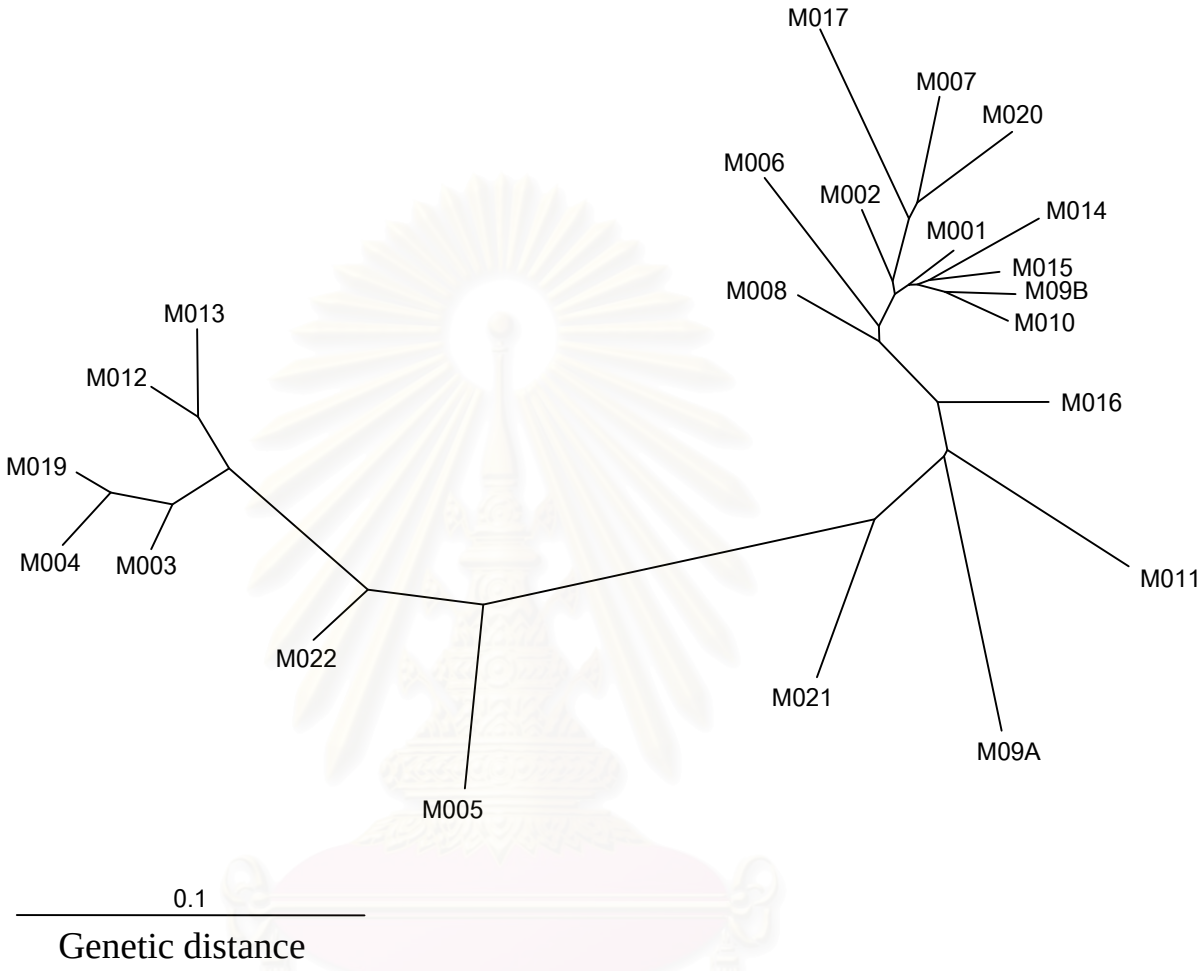


Figure 3.11 An unrooted tree illustrating genetic relationships of 22 accessions of *M. charantia* from Pichitr Horticultural Research Center, Thailand, base on genetic distances estimated from RAPD analysis using six primers (X-12, X-16, X-18, X-24, X-26 and X-27). Detailed information and abbreviations of sample were shown in Table 2.1

3.6 Amplification and characterization of MAP30 gene

Amplification of *MAP30* gene by using primer Set 1 and characterization of MAP30 gene by using 4 sets of primer (Table 2.3) for the amplification of *MAP30* gene from genomic DNA of bitter gourd.

PCR products generated from those sets of primer were shown in Figs 3.12. The results from primer set 1 gave product of 885 bp in size, primer set 2 gave product of 570 bp in size, primer set 3 gave product of 622 bp in size, and primer set 4 gave product of 307 bp in size.

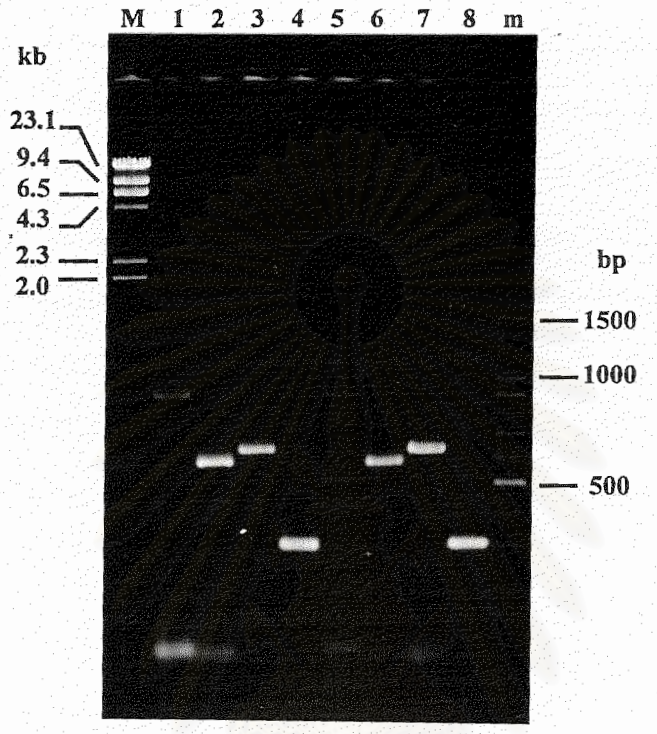


Figure 3.12 *MAP30* gene amplification product using primer Set 1 (885 bp, lane 1 and 5), Set 2 (570 bp, lane 2 and 6), Set 3 (622 bp,lane 3 and 7) and primer Set 4 (307 bp, lane 4 and 8)

Lane M = λ/*Hin*d III standard DNA marker

Lane 1-4 = PCR product from genomic DNA of M002

Lane 5-8 = PCR product from genomic DNA of M003

Lane m = 100 bp DNA ladder

3.7 Reverse transcriptase amplification of MAP30 mRNA from some tissues of bitter gourd

Nine tissue types of one bitter gourd plant (apical shoot, leaf, flower bud, male flower, female flower, young fruit tissue, seed of young fruit, riped fruit tissue and seed of riped fruit) were used for the mRNA extraction. The observed ratio of A_{260}/A_{280} from the mRNA extracts was 2.0-2.3. The 885 bp band was indicated tissue types which had MAP30 mRNA. The result obtained from RT-PCR exhibited successful amplification in apical shoot, leaf and seed of young fruit, albeit very low PCR product was obtained from apical shoot (Fig 3.12). The amplified product of *MAP30* gene from genomic DNA of bitter gourd was used as positive control.

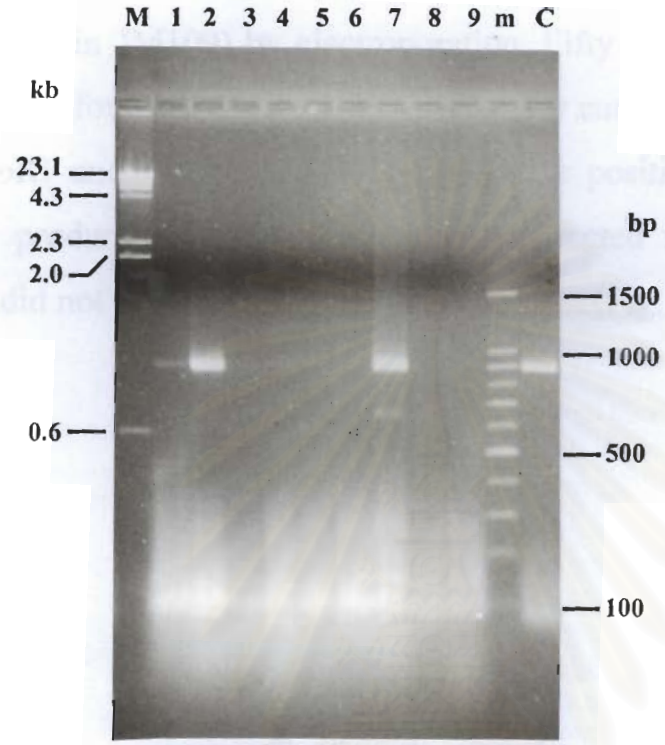


Figure 3.13 RT-PCR amplification product of MAP30 mRNA from apical shoot (1), leaf (2), flower bud (3), male flower (4), female flower (5), young fruit tissue (6), seed of young fruit (7), ripe fruit tissue (8) and seed of ripe fruit (9) from *M. charantia*

Lane M = λ/*Hind* III standard DNA marker
Lane 1-9 = RT-PCR product from each tissue
Lane m = 100 bp DNA ladder
Lane C = positive control of *MAP30* gene (885 bp)

3.8 Cloning of *MAP30* gene and expression

Amplified *MAP30* gene from genomic DNA of bitter gourd from Section 3.6 was cloned into pUC18 vector and introduced into host cell (*E. coli* strain JM109) by electroporation. Fifty white colonies were chosen to verify for successful *MAP30* cloning by cut with both restriction enzyme *Eco*RI and *Bam*HI (Section 2.11). The positive clones should contain cut product of 885 bp in size. All selected 50 clones in this experiment did not show any positive PCR product (Fig. 3.14).

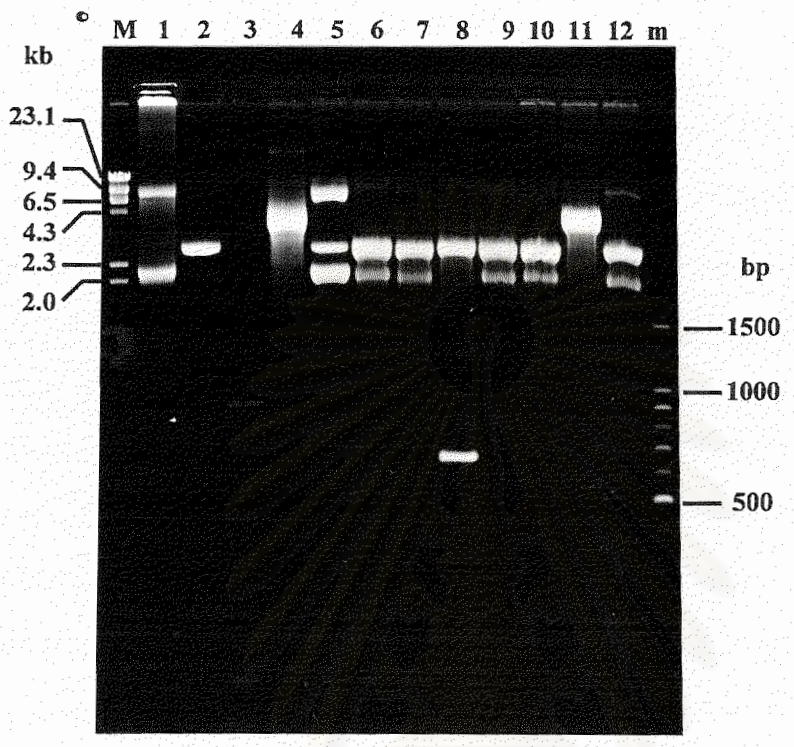


Figure 3.14 PCR amplification products from some white colonies of *E. coli* that contained recombinant pUC18 plasmid.

Lane M = λ/Hind III standard DNA marker

Lane 1 = supercoil form of pUC18

Lane 2 = linear form of pUC18

Lane 3 = fragment of *MAP30* gene (885 bp)

Lane 4-12 = cut product from white colonies of transformed *E. coli*

Lane m = 100 bp DNA ladder

CHAPTER IV

DISCUSSION

Despite the diverse morphology and quality differences in bitter gourd, there is still no information available regards intraspecific variation at the DNA level. The understanding of intraspecific variation at the DNA level of bitter gourd will facilitate the selection and breeding of appropriate germ line or cultivar for further use. It is also a useful information to determine whether the morphology variation is reflected at DNA level. Environmental and genetic factors are important effectors of the morphological variation. Especially for plants, the same species, morphological characters were direct changed by different environment. For examples, *Hieracium umbellatum* occurred at the Swedish West Coast in two different characters. One was a bushy plant with broad leaves and expanded inflorescences growing on rocky cliffs at the sea. The other was a prostrate plant with narrow leaves and small inflorescences that growth on sand dunes (http://www.biologie.uni-hamburg.de/b-online /e37/37b.htm).

The Pichitr Horticultural Research Center, Thailand, has collected and maintained 22 accessions of bitter gourds (Table 2.1). The accessions were mostly derived from Pichitr province, a few from other parts of Thailand, Laos and Nepal. The Pichitr Horticultural Research Center classified the various accessions on morphometric characters. For studies of intraspecific variation in *M. charantia*, the samples from this Pichitr Horticultural Research Center was used because they are pure lines (homozygote) obtained from fifth generation of self-fertilization with

generately stable morphologic characters which is useful for plant breeding.

The RAPD-PCR technique although is a rapid and simple technique, there are many factors that can influence the quality and validity of the data. Some important factors are Mg^{++} concentration; the amount and purity of DNA sample and the quality of the primer used. The reproducibility of RAPD bands was directly affected by the amplification condition. Therefore, the amplified condition was firstly optimized and held constant thoughout the study. The impurity in DNA templates such as polysaccharide and phenolic compound might inhibit PCR amplification. High concentration of Mg^{++} causes nonspecific amplification whereas low concentration of Mg^{++} causes low activity of *Taq* DNA polymerase. The optimum DNA concentration used in this study was 20 ng per reaction. At higher DNA concentration, the bands were absend or present in lower number probably due to the present of remaining impurities. In term of Mg^{++} concentration, 2.5 mM $MgCl_2$ was most suitable.

For better amplification, arbitrary decanucleotide primers with relatively high GC content was chosen. Of the 45 primers tested, 6 primers (X-12, X-16, X-18, X-24, X-26 and X-27; Table 2.2) were chosen for their reproducible RAPD pattern, high polymorphic band and easily to score pattern. A total of 109 reproducible bands were scored. Of these, there are 54 monomorphic bands (49.5%) and 55 polymorphic bands (50.5%) (Table 3.1). A genetic distance matrix was derived from RAPDPLOT v. 3.0 and a neighbor-joining tree was constructed by using PHYLIP v. 3.5c (Figures 3.8 and 3.9). It clearly showed that the 22 accessions of bitter gourds from Pichitr Horticultural Research Center could be divided into 2 subgroups, A (15 accessions) and B

(7 accessions). This grouping is in agreement with fruit size, namely, group A has fruit size diameter > 3 cm whereas group B are < 3 cm (personal communication). The neighbor-joining tree also indicated that bitter gourds has narrow genetic base too. This phenomenon was normally found in cultivated plants that domesticated for long time (Silberstein *et al.*, 1999). However, such grouping was not in any way an indication of accessions difference due to biogeographic distribution from this data, since many varieties from Pichitr province were distributed in both groups A and B. Nevertheless, this results are consistent with that of Wahab and Gopalakrishanan (1993) who studied the genetic divergence in bitter gourd using data from productive and qualitative characters. They concluded that genetic diversity among 50 diverged bitter gourd genotypes were not significantly correlated with geographic diversity. It should be cautioned, though, that the collection at Pichitr Horticultural Research Center contain mostly accessions derived from Pichitr origin. Therefore, it might not actually represent the total genetic resource of bitter gourd in Thailand.

Yang and Walters (1992) proposed that Eastern India and Southern China are native and centers of domestication of bitter gourds. Basing on the origins of sample at Pichitr Horticultural Research Canter, it was noted that the bitter gourds in group A are in the same group with Nepal accession, and those in group B are in the same group with Laos accession. It might be proposed that the group A might originate from India and Nepal whereas group B might originate from southern China through Laos. Because there was only one accession each from Nepal and Laos, the evidence for this proposal was inadequate and need more samples for further verification. The needed samples should be obtained from more area covering all parts of the country and must include samples

from India and China. Many plant samples should be collected at each sampling site to avoid bias of data.

The knowledge obtained from this study can be applied for construction of effective breeding program, selection and management of useful bitter gourd accessions. In addition, with the growing knowledge of its application in the medicinal field, its commercial value, and the importance of gene patenting issue, the collection of Thailand's *M. charantia* genetic information is a necessity.

It is generally known that MAP30 was predominant in seeds of ripe fruit. There are also reports that MAP30 was found in fruit and seed of bitter gourd (Lee-Huang *et al.*, 1990). Thus, the expressions of *MAP30* gene in various tissues of bitter gourd were investigated by RT-PCR technique in this study. The tissues used were apical shoot, leaf, flower bud, male flower, female flower, young fruit tissue, seed of young fruit, ripe fruit tissue and seed of ripe fruit, respectively. The results indicated that *MAP30* gene expressed in apical shoot, leaf and seed of young fruit. However, the result was negative in young fruit tissue, ripe fruit tissue and seed of ripe fruit which were previously to have reported to have the expression of *MAP30* gene (Lee-Huang *et al.*, 1990) since the positive control sample was not performed in the experiment, absence of RT-PCR response does not mean that the tissues did not express *MAP30* gene. There were possibilities that the mRNA of these tissues were degraded during transfered or storage. In the period of sampling and storing, the samples were collected on ice, which temperature was about 0°C. This temperature may not be low enough for preventing mRNA from RNase digest. The samples should be kept in lower temperature such as liquid nitrogen for decreasing the degradation of mRNA. Nevertheless, the result from this study showed that *MAP30* gene could also express in apical

shoot, leaves and seed of young fruit. The result suggested new sources for extraction of MAP30 from tissue of bitter gourd other than ripe seeds.

The *MAP30* gene of some accessions of bitter gourd from Pichitr Horticultural Research Center, Thailand was also characterized by amplification with 4 sets of primer (Table 2.3). Comparing amplification products from primer Set 1 using genomic DNA as DNA template to RT-PCR, both gave the same 885 bp of PCR product sizes. The size corresponded with the report that MAP30 contains 287 amino acids and *MAP30* gene does not have any intron (Lee-Huang *et al*., 1995). Thus, *MAP30* gene was very suitable for gene expression in prokaryotic cell because it could be translated to protein without the need of post-translation modification.

MAP30, a type of ribosome inactivation protein, had the molecular weight 30 kDa. It could inhibit HIV replication and cancer cell division. Although MAP30 was reported to have many kinds of enzyme activities, these activities were also shared by eight other proteins of approximately the same size (Lee-Huang *et al.*, 1995). Thus, MAP30 was difficult to be screened or detected. Since, cloning and expression of *MAP30* gene showed that the anti-viral and anti-tumor properties from recombinant clones were not different from that of wild type (Lee-Huang *et al.*, 1995) and MAP30 from recombinant clones was easier to be extracted and purified, this study also attempted to clone and express the MAP30 in *E. coli*.

In this study, *MAP30* gene was prepared from chromosomal DNA by using PCR technique with specific primers that contained restriction and Shine-Dalgarno sequence as described in Section 2.8.1. The PCR products were cloned into pUC18 plasmid, electroporated into *E. coli* JM109 and screened for the blue/white colonies. Fifty white

colonies were checked for *MAP30* gene insert and none contained the *MAP30* gene recombinant plasmid. The plasmid could not join with inserted DNA (*MAP30* gene) at the *Eco*RI restriction site because the *Eco*RI site at the 5'-end of primer S01 was too short for *Eco*RI binding. As the consequence, the *MAP30* gene fragment was cut by *Bam*HI only. This uncompletely digestion might be corrected by extended the 5'-end of primer S01 at least 3 bases before amplification and cloning to plasmid pUC18. Alternately, *MAP30* gene might be amplified by using primer S01 and was then ligated to plasmid pGEN-T. After that, *MAP30* gene could be double-digested with *Eco*RI and *Bam*HI, ligated to plasmid pUC18 and cloning, respectively.

CHAPTER V

CONCLUSIONS

1. Twenty-two accessions of *Momordica charantia* Linn. from Pichitr Horticultural Research Center, Thailand could be divided into 2 groups (A and B) based on RAPD analysis using primer X-12, X-16, X-18, X-24, X-26 and X-27.
2. Comparing RAPD data with morphological data, all members in group A had more than 3 cm in fruit diameters whereas all members in group B had less than 3 cm in fruit diameter.
3. *MAP30* gene was also expressed in apical shoot, leaves and seed of young fruit.

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APPENDIX A

RAPD genotype generated from six primers in all 22 accessions of *Momordica charantia* Linn. From Pichit Horticultural Research Center, Thailand

A. primer X-12

Size (bp)	01	02	03	04	05	06	07	08	9A	9B	10	11	12	13	14	15	16	19	20	21	22	17	F1
1700	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0
1500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
1250	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
1200	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
1150	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
1010	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0
980	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
920	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
880	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
800	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
720	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
700	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
670	1	1	1	0	1	1	0	1	1	0	1	1	1	1	1	1	1	0	0	1	1	0	0
620	0	0	1	1	0	0	0	0	0	0	0	1	1	1	0	0	0	1	0	1	0	0	0
600	1	1	0	0	1	1	1	1	1	1	1	0	1	0	0	1	0	0	1	0	0	1	1
550	0	0	1	1	0	0	0	0	1	0	0	1	1	1	1	1	1	1	0	0	1	0	0
520	1	0	0	0	1	0	1	0	1	1	0	0	0	0	1	0	0	1	1	0	0	1	1
480	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	0	1	0	1	1	1	0
370	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
350	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0

B. primer X-16

Size (bp)	01	02	03	04	05	06	07	08	9A	9B	10	11	12	13	14	15	16	19	20	21	22	17	F1
2150	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
2100	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
1400	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
1250	1	1	0	0	0	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	0	1	0
1200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
1180	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
1040	1	1	0	0	1	1	1	1	1	1	1	1	0	0	1	1	1	0	1	1	0	0	0
980	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
750	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
700	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
690	0	0	1	1	1	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0
650	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
590	1	1	0	0	0	1	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	1	0
580	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
520	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
515	1	1	0	0	0	1	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	1	0
490	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

C. primer X-18

Size (bp)	01	02	03	04	05	06	07	08	9A	9B	10	11	12	13	14	15	16	19	20	21	22	17	F1
2300	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
1800	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
1700	1	1	0	0	0	0	1	1	1	1	1	1	0	0	1	1	0	0	1	1	0	1	0
1600	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
1450	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0
1400	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1250	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1150	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	0
1050	1	1	0	0	0	1	1	1	1	1	1	1	0	0	1	1	1	0	1	1	0	1	0
1020	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
1000	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
950	0	0	1	1	1	1	0	0	0	0	0	0	1	1	0	0	0	1	0	0	1	0	0
920	1	1	0	0	0	0	1	1	1	1	1	1	0	0	1	1	1	0	1	1	0	1	0
850	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
600	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
560	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
520	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
480	0	0	1	1	1	0	0	0	0	0	0	0	1	1	0	0	0	1	1	1	1	0	1
420	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
390	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0

D. primer X-24

Size (bp)	01	02	03	04	05	06	07	08	9A	9B	10	11	12	13	14	15	16	19	20	21	22	17	F1
1800	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
1780	0	0	1	1	1	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0
1750	1	1	0	0	0	1	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	1	0
1650	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
1500	1	1	0	0	1	1	1	1	1	1	1	1	0	0	1	1	1	0	1	1	0	1	0
1200	1	1	0	0	1	1	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	1	0
1160	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	0	0	1	1	1	1	1	0
1000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
750	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
700	1	1	1	1	1	1	0	1	0	1	1	0	1	1	1	1	0	1	0	0	1	1	0
680	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	0
660	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0
600	0	0	1	1	1	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0
540	1	1	0	0	0	1	1	0	1	1	1	0	0	0	1	1	1	0	1	1	0	0	0
520	0	0	1	1	1	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	1	0	1
500	1	1	0	0	1	1	1	1	1	1	1	1	0	0	1	1	1	0	1	1	0	0	0

E. primer X-26

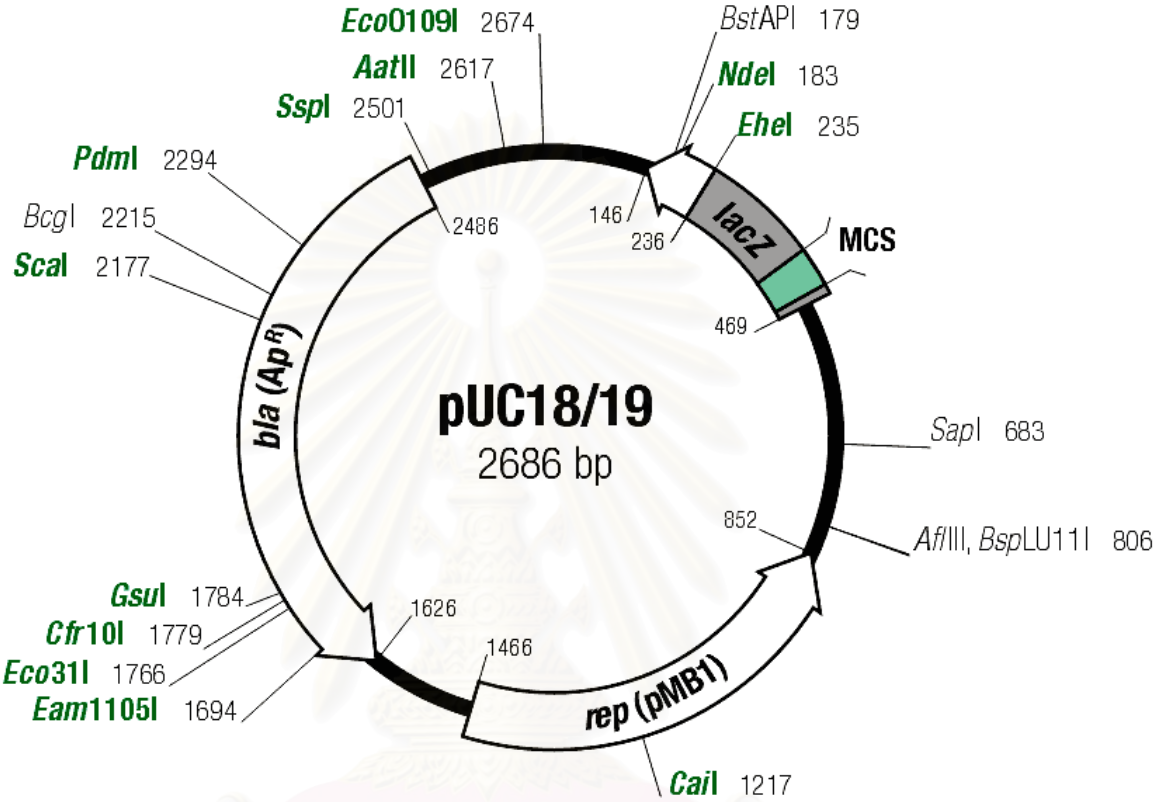
Size (bp)	01	02	03	04	05	06	07	08	9A	9B	10	11	12	13	14	15	16	19	20	21	22	17	F1
1800	1	1	1	1	1	1	0	1	1	0	0	1	1	1	1	1	1	1	1	0	1	0	1
1650	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
1500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
1460	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
1450	1	1	0	0	0	1	1	1	0	1	1	1	0	1	1	1	1	0	1	1	1	1	0
1300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
1150	0	0	1	1	1	0	0	0	1	0	0	1	1	1	0	0	0	1	0	1	1	0	0
1050	1	0	1	1	1	1	1	1	0	0	0	0	1	1	0	0	1	1	0	1	1	1	0
1000	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
950	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
840	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
750	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
740	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
700	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
640	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
500	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
470	0	0	1	1	1	1	0	0	0	0	0	0	1	1	0	0	0	1	0	1	1	0	0
450	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
410	0	0	1	1	1	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	1	0	0
400	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	1	0	1	1
360	1	0	0	0	0	0	1	1	1	1	0	1	0	0	1	0	1	0	0	0	0	0	0
340	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1

F. primer X-27

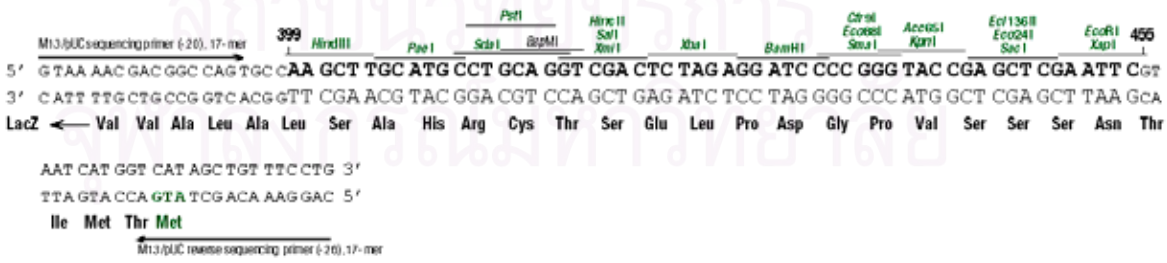
Size (bp)	01	02	03	04	05	06	07	08	9A	9B	10	11	12	13	14	15	16	19	20	21	22	17	F1
1750	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
1650	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
1400	0	0	1	1	0	0	0	1	1	0	0	0	1	1	0	0	0	1	0	0	0	0	0
1200	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
1150	0	0	1	1	0	0	0	0	1	0	0	0	1	1	0	0	0	1	0	1	1	0	0
1050	1	1	1	0	0	1	1	1	1	1	1	1	0	0	1	1	1	0	1	0	0	1	0
980	0	0	1	1	1	1	0	0	0	0	0	1	1	1	0	0	1	1	0	1	1	0	0
820	1	1	1	0	0	1	1	1	1	1	1	1	0	0	1	1	1	0	1	0	0	1	0
800	0	0	1	1	1	0	0	0	0	0	0	0	1	1	0	0	0	1	0	1	1	0	0
760	1	1	0	0	0	1	1	1	1	1	1	1	0	0	1	1	1	0	1	0	0	0	0
560	1	1	0	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	1	1	0	1	0
480	1	1	0	0	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1	1	0	1	0
440	0	0	1	1	1	0	0	0	0	0	0	1	1	1	0	0	0	1	0	0	1	0	1
420	1	1	0	0	0	1	1	1	0	1	1	1	0	0	1	1	1	0	1	1	0	1	0

APPENDIX B

Restriction map of plasmid pUC18



Multiple Cloning Sites



BIOGRAPHY

Mr. Kittipong Pala-or was born on February 11, 1976 in Songkla province. He graduated in field of Botany from Department of Biology, the Faculty of Science, Prince of Songkla University in 1997. In 1998, he had been studying in Master degree of Science at Department of Biochemistry, the Faculty of Science, Chulalongkorn University.