GENETIC DIVERSITY OF THAI BANANA (*Musa*) CULTIVARS USING AFLPs

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ABSTRACT

Bananas (*Musa*) are one of the most important tropical fruit crops in Thailand which believed to be one as the centers of origins. To assess genetic diversity and determine genetic relationships of edible bananas in Thailand, 135 accessions of *Musa* cultivars and species were collected from villages, and natural locations were investigated, with wild *M. itinerans* served as an outgroup taxon. The Amplified Fragment Length Polymorphism (AFLP) analyses of eight primer combinations revealed a total of 730 polymorphic bands and 136 unique marker bands on these *Musa* accessions. UPGMA clustering of numerical data from AFLP patterns showed two large groups that corresponded to genome designations of *M. acuminata* (A) and *M. balbisiana* (B), which were known ancestors of most edible cultivars. The AFLP results can be used to distinguish *M. acuminata* subspecies, and *M. balbisiana*. The AFLP data suggested that Thai bananas, *i.e.* AA, AAA and some AAB cultivars are closely related to *M acuminata* subsp. *malaccensis*. Two forms of this subspecies were found: the typical 'malaccensis' and 'Kra Isthmus' forms. The first was commonly found in southern Thailand and in a new location in the North, while the latter was found only in Ranong and nearby provinces. The ABB, BBA and BBB cultivars were closely related to wild *M. balbisiana* in Thailand and the others have probably been imported from somewhere else such as the Pacific Islands. Unique marker bands showed different characteristics among *M. balbisiana* and related cultivated bananas and the subspecies within *M. acuminata*. The analyses were useful to infer progenitors of these cultivars, pronounce wide genetic diversity of the bananas in Thailand, and encourage for more wild accessions from different geographic origins to be studied.

KEY WORDS: AFLP / GENETIC DIVERSITY / INTERSPECIFIC HYBRIDS / *MUSA* CULTIVARS / DNA FINGERPRINTING

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ความหลากหลายทางพันธุกรรมของพันธุ์กล้วยไทยด้วยเทคนิค AFLPs (GENETIC DIVERSITY OF THAI BANANA (*Musa*) CULTIVARS USING AFLPs)

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บทคัดย่อ

กล้วย (*Musa*) เป็นผลไม้เขตร้อนที่สำคัญชนิดหนึ่งของประเทศไทย เชื่อกันว่าประเทศไทยเป็นหนึ่งในแหล่งกำเนิดของกล้วย เพื่อศึกษาประเมินความหลากหลายและความสัมพันธ์ทางพันธุกรรมของกล้วยกินได้ในประเทศไทย จึงได้มีการเก็บกล้วย 135 ตัวอย่าง จากแหล่งปลูกและป่าธรรมชาติ โดยใช้กล้วยหก *M. itinerans* เป็น outgroup การวิเคราะห์ด้วยเทคนิค Amplified Fragment Length Polymorphism (AFLP) โดยใช้ไพรเมอร์ 8 คู่ ให้แถบดีเอ็นเอแบบ polymorphic 730 แถบและแบบจำเพาะ 136 แถบ การวิเคราะห์ค่าตัวเลขจากผล AFLP ของตัวอย่างด้วยวิธี UPGMA ทำให้จัดกลุ่มตัวอย่างได้ 2 กลุ่มใหญ่ ซึ่งสอดคล้องกับพันธุกรรมกล้วยป่า (*M. acuminata*; A) และกล้วยตานี (*M. balbisiana*; B) และแสดงว่าพันธุ์กล้วยไทยที่มีจีโนมแบบ AA กับ AAA ทั้งหมด, AAB บางส่วน มีความใกล้เคียงทางพันธุกรรมกับกล้วยป่า *M. acuminata* subsp. *malaccensis* ซึ่งชนิดย่อยนี้จำแนกได้ 2 รูปแบบ คือ 1) แบบ ‘malaccensis’ พบทั่วไปทางภาคใต้และพบแหล่งใหม่ในธรรมชาติทางภาคเหนือและ 2) แบบ “คอคอดกระ” ซึ่งพบเพียงที่จังหวัดระนองและจังหวัดใกล้เคียง ส่วนพันธุ์กล้วยไทยจีโนม ABB, BBA และ BBB มีความสัมพันธ์ทางพันธุกรรมใกล้เคียงกับกล้วยตานีที่พบในธรรมชาติของไทย และอีกส่วนหนึ่งเป็นพันธุ์กล้วยที่อาจนำเข้ามาจากที่อื่นเช่นแถบหมู่เกาะแปซิฟิก แถบดีเอ็นเอแบบจำเพาะแสดงให้เห็นถึงความแตกต่างและความสัมพันธ์ทางพันธุกรรมของกล้วยตานี กล้วยปลูก และกล้วยป่าต่างชนิดย่อย การวิเคราะห์นี้มีประโยชน์ในการอนุมานถึงต้นกำเนิดของกล้วยพันธุ์ปลูก แสดงให้เห็นความหลากหลายทางพันธุกรรมกล้วยในประเทศไทย และกระตุ้นให้มีการศึกษากล้วยป่าจากแหล่งกำเนิดอื่นเพิ่มมากขึ้น

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LIST OF ABBREVIATIONS

AFLPs	Amplified Fragment Length Polymorphisms
bp	base pair
CTAB	Cetyl trimethylammonium bromide
DNA	deoxyribonucleic acid
Fig.	Figure
GDE	Genetic diversity estimates
GPS	Global Positioning System
i.e.	Latin 'id est', used to explain exactly what the previous thing that was mentioned means
IRAPs	Inter-Retrotransposon Amplified Polymorphisms
LS	loading solution
mol	mole
nm	nanometer
ng/µl	nanogram per microliter
PCR	polymerase chain reaction
RAPD	Random Amplified Polymorphic DNA
RFLPs	Restriction Fragment Length Polymorphisms
sdH_2O	sterile distilled water
sec	second
sp.	species
SSR	simple sequence repeat
subsp.	subspecies
TBE	Tris-borate or Tris/Borate/EDTA
TE	Tris-EDTA
rpm	round per minute
U	enzyme activity unit

LIST OF ABBREVIATIONS (continued)

UPGMA	Unweighted Pair-Group Method of the Arithmetic average
UV	ultraviolet
var.	variety
VNTR	variable number tandem repeat
w/v	weight by volume
x	a set of chromosome in a haploid genome
µ	micron ($x10^{-6}$)

CHAPTER I
INTRODUCTION

Banana (*Musa*) is one of the most important crops in the world and provides foods for millions of people worldwide, especially in the tropical and subtropical countries. Banana belongs to the family Musaceae, which consists of three genera, *Ensete, Musa* and *Musella*. The *Musa* genus was divided into five sections according to basic chromosome number; *Musa* (x=11), *Rhodochlamys* (x=11), *Callimusa* (x=10), *Australimusa* (x=10) and *Ingentimusa* (x=7) (Simmonds, 1962; Argent, 1976). Most of cultivated bananas are seedless and belongs to the *Musa* section. It is believed that they were derived or originated from parthenocarpy, seed sterility, artificial selection, vegetative propagation, polyploidization, somatic mutation, intra- and interspecific hybridization of two diploid species, *M. acuminata* Colla (AA) and *M. balbisiana* Colla (BB) (Simmonds and Shepherd, 1955; Simmonds, 1962; Valmayor *et al*., 2000). Among wild and cultivated bananas, there are clones and variations. Several attempts have previously been made to classify *Musa* cultivars based on 15 morphological traits and chromosome numbers (Simmonds and Shepherd, 1955; Chomchalow and Silayoi, 1984; Silayoi and Babpraserth, 1983; Valmayor *et al*., 2000). Recently, molecular markers are used not only for classification, but also identification and parental assessment. The markers included isozymes, random amplified polymorphic DNA (RAPD), restriction fragment length polymorphisms (RFLPs), microsatellites, amplified fragment length polymorphism (AFLP) and fluorescence *in situ* hybridization (FISH) probes on chromosome. Among DNA markers, the amplified fragment length polymorphism (AFLP) technique is being widely used for genetic diversity studies because it reveals significant polymorphisms, is a reliable and robust genetic marker assay, and well reproducible (Vos *et al.*, 1995; Mueller and Wolfenbarger, 1999; Crouch *et al*., 1999).

Although there are wide variations of banana cultivars in Thailand, only a few reports on their diversity have been published.

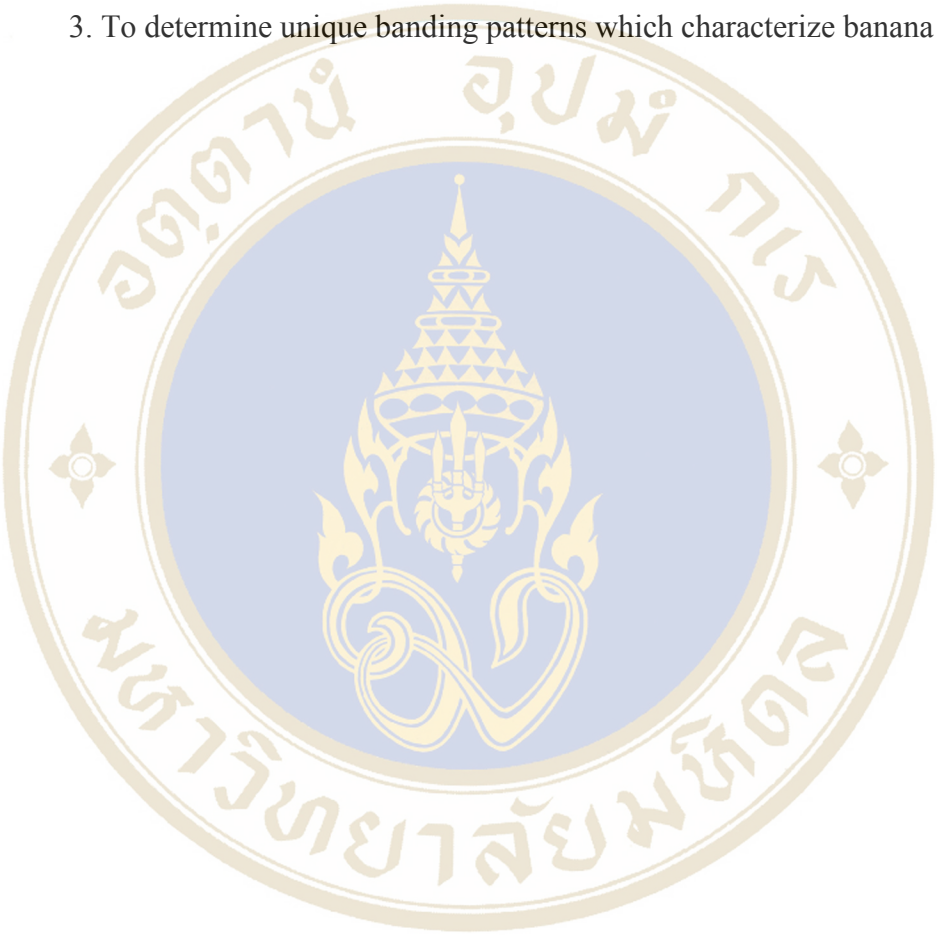
OBJECTIVE

The objectives of this research were divided into three parts as follows:

1. To assess genetic diversity among accessions of the cultivated bananas in Thailand using AFLP markers.

2. To determine genetic relationships among the Thai banana cultivars.

3. To determine unique banding patterns which characterize banana cultivars.



CHAPTER II
LITERATURE REVIEWS

Banana (*Musa*) is one of the most important tropical and subtropical food crops for millions of people worldwide (Loh *et al*., 2000; Noyer *et al*., 2005). World banana production is around 71.2 million tons annually (FAO, 2004), of which that for export trade account for only 15%, the rest was consumed locally. Bananas have been used in everyday life of Thai people *e.g.* foods, ropes, toys, and wrapping materials (Silayoi, 2002). Banana export value ranks fourth among the most important crops in the world after rice, wheat, and maize, while that of Thailand is the third in Southeast Asia. Major export cultivars are *Musa* (AAA) 'Kluai Hom Thong', *Musa* (AA) 'Kluai Khai' and *Musa* (ABB) 'Kluai Namwa' respectively. The major import countries for Thai bananas are Hong Kong, Singapore, Japan and Europe respectively (DOAE, 2003).

Classification and origin of diversity in the genus *Musa*

Banana is a monocotyledonous plant of the family Musaceae, which is composed of three genera *i.e.*, *Ensete, Musa,* and *Musella*. The genus *Musa* is comprised of 30-40 species and is divided into five sections; *Australimusa*, *Callimusa*, *Musa*, *Ingentimusa* and *Rhodochlamys*, on the basis of basic chromosome numbers. The sections *Australimusa* and *Callimusa*, comprised of about six species each, possess a basic chromosome number of $x = 10$, while *Musa* and *Rhodochlamys*, contained about 15 and six species, respectively, possess that of $x = 11$ (Simmonds, 1962, Argent, 1976). *Musa ingens* is the only member in the section *Ingentimusa* with $x = 7$ (Argent, 1976).

The *Musa* section is the largest, most diversified in the genus. It is the most geographically widespread, found throughout South and Southeast Asia, from India to the Pacific Islands (Simmonds, 1962). The majority of cultivated bananas arose from two members of this section *i.e. M. acuminata* (AA) Colla and *M. balbisiana* (BB)

Colla (Simmonds and Shepherd, 1955; Ude *et al*., 2002b, 2003). *M. acuminata* originated in the Southeast Asian region and evolved into a complex of diploid (AA) subspecies (Simmonds, 1962). *M. acuminata* is classified into eight subspecies: subsp. *banksii, burmanica, burmannicoides, malaccensis, microcarpa, siamea, truncata, errans* and *zebrina* (Häkkinen and De Langhe, 2001; Daniells *et al*., 2001). Four subspecies were found in Thailand, *i.e.*, subsp. *siamea* found in the Northern, Northeastern, and central Thailand, subsp. *burmanica* is found in the western part close to Myanmar border, subsp. *malaccensis* found in the South, and subsp. *microcarpa* found in western and southern part of the country (Simmonds, 1962; Chomchalow and Silayoi, 1984). *M. balbisiana* is native to Southeast Asia and the western Pacific (Simmonds, 1962; Wang *et al.*, 2007) and, in the contrary to general opinion, recent reports on diversity within the species have indicated a wide morphological variation (Sotto and Rabara, 2000). However, *M. balbisiana* has no record of subspecies classification (Shepherd, 1990; Ude *et al*., 2002b; Wang *et al*., 2007).

The Evolution of Edibility

Seven processes may have involved in evolution of all edible bananas: parthenocarpy, seed sterility, artificial selection, vegetative propagation, polyploidization, somatic mutation, and intra- and interspecific hybridization between *M. acuminata* and *M. balbisiana* (Simmonds, 1962). It is widely accepted that edible bananas originated mainly from intra- and interspecific hybridizations between two wild diploid species, *M. acuminata* (‘A’ genome) and *M. balbisiana* (‘B’ genome) (Simmonds and Shepherd, 1955). Both species occur endemically in Southeast Asia and Western Pacific (Simmonds, 1962; Valmayor *et al*., 2000; Wang *et al*., 2007). Crossing among species and subspecies has resulted in the appearance of sterility, a trait that was selected during domestication, together with parthenocarpy and vegetative propagation (Simmonds, 1962). The hybrids that evolved from the two natural species include diploids, triploids and a few tetraploids in various genome combinations (Simmonds, 1962; Valmayor *et al*., 2000). Most of banana crops in local and international markets are triploid, and sometimes diploid or tetraploid

(Creste *et al*., 2004). Fig. 1 shows various pathways leading to the development of edible bananas proposed by Simmonds and Shepherd (1955).

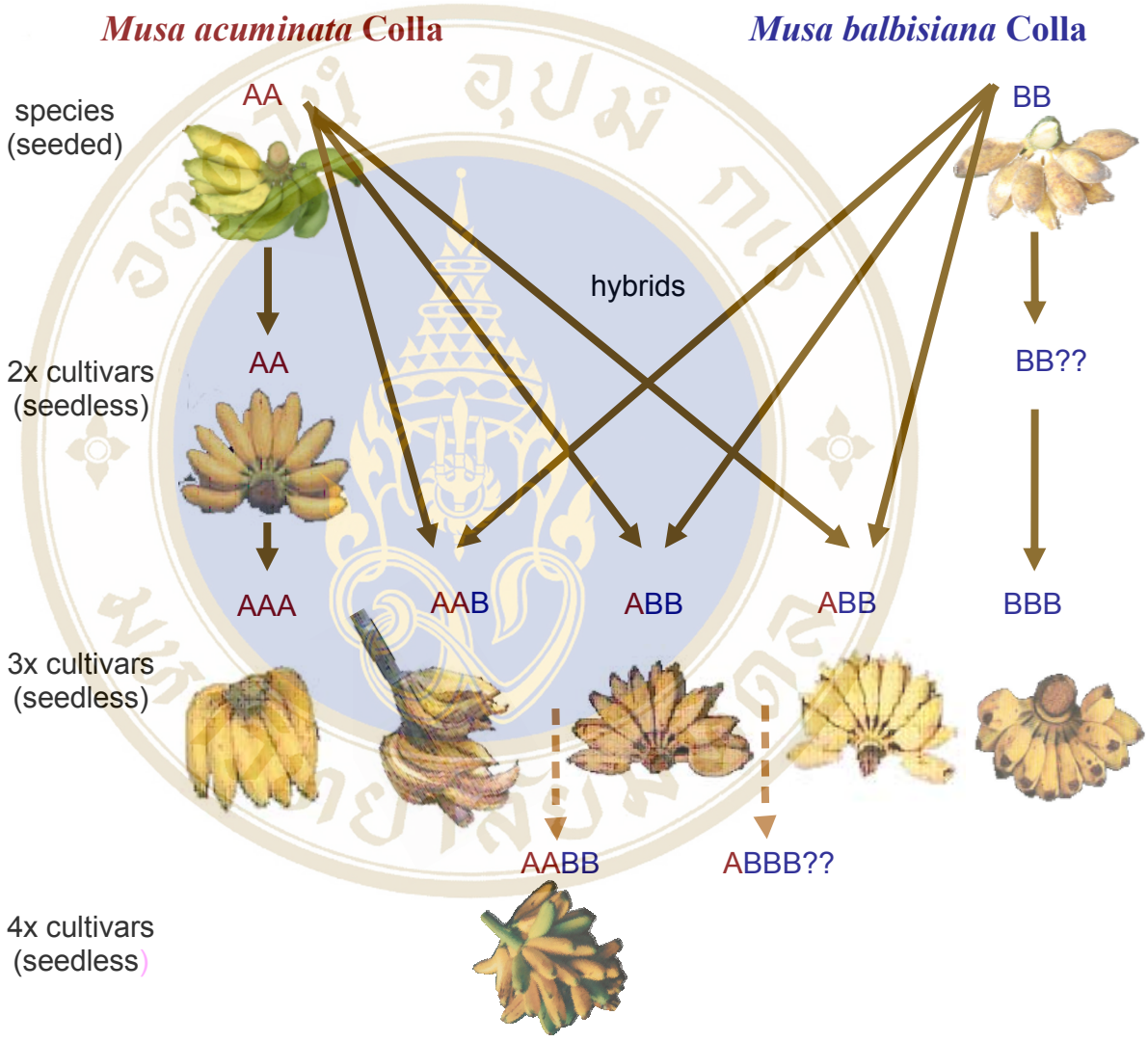


Figure 1 Diagram showing various pathways leading to the development of edible bananas. (Modified from Simmonds and Shepherd, 1955, by S. Swangpol)

Simmonds (1962) summarized the known facts about the geographical distribution of the major groups of edible bananas (Fig. 2). He believed that the *acuminata* cultivars (AA and AAA) are primarily Malayan and the hybrid groups are peripheral, being diversified in India and, to a less extent, in Indochina and eastern Malayan areas, from which wild *M. acuminata* is locally absent, but in which *M. balbisiana* is native (Southeast Asia and the western Pacific) (Wang *et al*., 2007). The dispersal of edible bananas outside Asia was accomplished solely by transport of vegetative planting materials by human agencies. The secondary diversification, however, was caused by somatic mutation (Simmonds, 1962). Mutations affecting traits of economic or horticultural interest have been selected by farmers over the years and multiplied by vegetative propagation to produce morphotypes (Daniells *et al*., 2001).

Figure 2 Geographical distribution of the edible bananas.
(Source: Simmonds, 1962; Marin, 1998)

Classification of banana cultivars

The conventional classification of *Musa* cultivars is based on 15 morphological characters and chromosome counting (Simmonds and Shepherd, 1955). The cultivars were assigned a score of 1 for each character that adheres closely to wild *M. acuminata* and 5 for characters with extreme *M. balbisiana* expression. Intermediate expressions of the characters were assigned scores ranging from 2, 3, or 4 depending on intensity (Simmonds and Shepherd, 1955; Chomchalow and Silayoi, 1984; Silayoi and Chomchalow, 1987). Six genome groups assigned for Thai banana cultivars *i.e.* AA/AAA, BB/BBB, AB/AABB, AAB, ABB, and ABBB (Silayoi and Babpraserth, 1983).

***Musa* classification and identification using molecular techniques**

Genetic diversity and phylogenetic relationship of bananas has been assessed using different molecular techniques, such as Restriction Fragment Length Polymorphisms (RFLPs) (Gawel *et al*., 1991 and 1992; Jarret *et al*., 1992; Fauré *et al*., 1993; Bhat *et al*., 1994; Baurens *et al*., 1997; Carreel *et al*., 2002; Nwakanma *et al*., 2003a, b; Ge *et al*., 2005), Random Amplified Polymorphic DNA (RAPD) (Howell *et al*., 1994; Crouch *et al*., 2000; Pillay *et al*., 2001; Birmeta *et al*., 2004; Onguso *et al*., 2004; Uma *et al*., 2004), simple sequence repeat (SSR) (Bhat *et al*., 1995), Genomic *In Situ* Hybridization (GISH) (Hont *et al*., 2000), microsatellite markers (Creste *et al*., 2003 and 2004), Inter-Retrotransposon Amplified Polymorphisms (IRAPs) (Nair *et al*., 2005). Although these have provided a general understanding of *Musa* classification, the question of the validity of the *Musa* classification system is still unresolved.

The Amplified Fragment Length Polymorphism (AFLP) technique

AFLP (The Amplified Fragment Length Polymorphism) technique, a PCR-based molecular marker, was first developed by Zabeau and Vos, researchers of Keygene N.V. company in Netherlands, and registered a patent in 1993 (Vos *et al*., 1995). AFLP analysis was clearly a powerful technique in terms of its ability to identify a large number of polymorphic bands without any prior knowledge of the

organisms. The ability of this technique to generate many markers with minimum primer testing and the system's high resolution (*i.e.*, band clarity and relative low lane background) are features that make AFLP attractive as genetic markers (Crouch *et al*., 1999). Moreover, AFLP analysis is used in variety of fields, including plants, animal breeding, medical diagnostics, forensic analysis, microbial typing, *etc*.

AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA (Vos *et al*., 1995). The technique combined the reliability of the Restriction Fragment Length Polymorphism (RFLP) analysis with the power of the PCR technique. The technique involves three steps (Fig. 3). The first step is restriction endonuclease digestion of genomic DNA with a restriction enzyme that cuts frequently (*Mse*I, 4 bp recognition sequence) and the other that cuts less frequently (*Eco*RI, 6 bp recognition sequence). The following step is ligation of double stranded adapters to ends of the restriction fragments. The frequent cutter is used to generate small fragments which able to be amplified in the optimal size range for separation on a polyacrylamide gel. The rare cutter is used to limit the number of fragments to be amplified and the ligation of adapters is used to position the attachment of two primers in the next PCR amplification. Selective amplification of sets of restriction fragments using selective AFLP primers is performed. This step increases the number of fragments by selective primers which are the same base sequence with adapters. The PCR amplification uses primer to add nucleotides at 3' ends, which adds two more nucleotides with two steps selective amplification. The first step is preselective amplification using one nucleotides to further additional primer bases and complementary to each of the two adaptor sequences. The second step is selective amplification using three nucleotides containing further additional primer bases. This step uses selective amplification of sets of restriction fragments and reduces the number of fragments because a single 3'-extension on both primers reduce the number of fragments amplified by a factor of 16, a two-base extension reduce the number by 256, and a three-base extension reduces it by 4,096. The last step, analysis of the amplified fragments on polyacrylamide gels and DNA band visualization is generated.

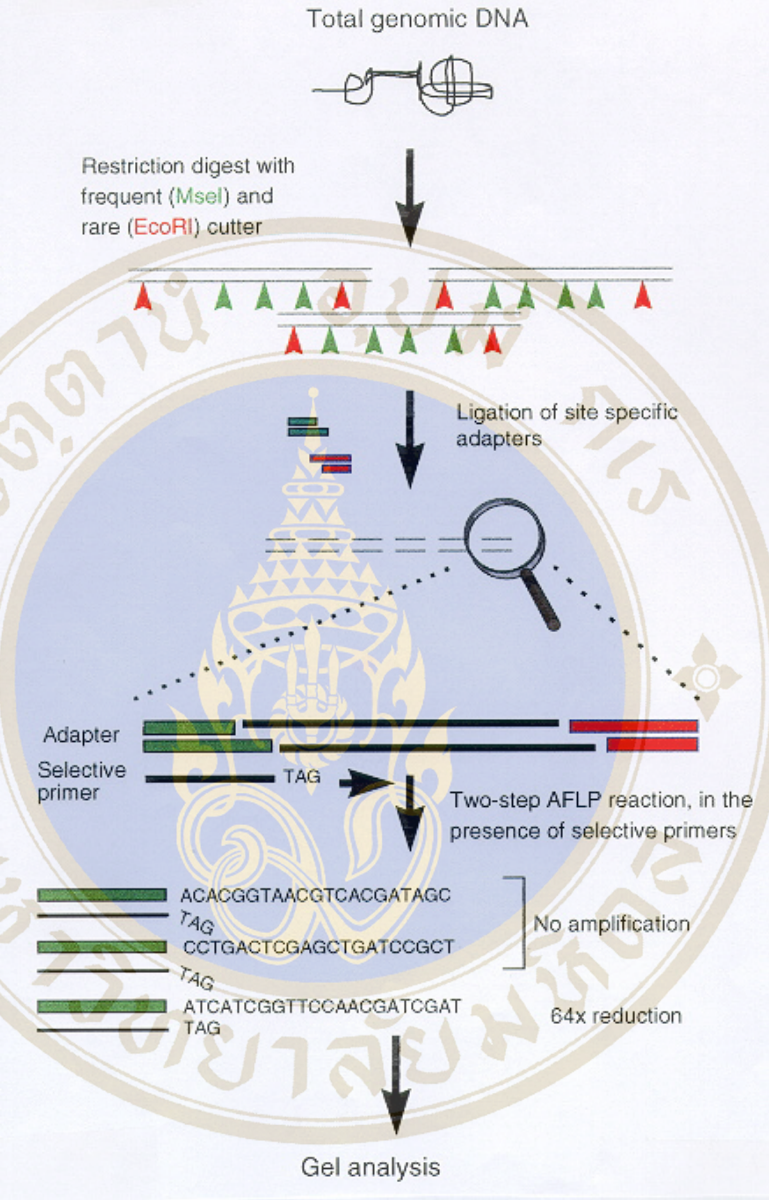


Figure 3 The process of Amplified Fragment Length Polymorphism (AFLP) technique. (Source: Breyne *et al*., 1997)

AFLP markers are extensively used for studying genetic diversity in different plant species (Vos *et al*., 1995; Steiger *et al*., 2002). Comparative studies using restriction RFLPs, RAPD, AFLP and microsatellite techniques have shown that AFLP method is the most efficient method to estimate genetic diversity because of its high reproducibility, high quantity of information throughout multiple loci on the genome, high resolution enough to determine some small genetic differences and generate

multiplex ratio of data for numerical analysis. RAPD technique has the high variable of replicability, but easy to use. RFLP technique provides low quantity of information, but has higher replicability and resolution of genetic differences when compare to RAPD method, but lower than AFLP and microsatellite techniques. Microsatellite method has good qualification as well as AFLP, but needs some knowledge about genetic information which takes development time, difficult to use and develop the process. However, AFLP and microsatellite markers, coupled with sequencing information for systematic analyses, could be synergized as main tools for the analysis of genetic variation (Mueller and Wolfenbarger, 1999).

AFLP markers are extensively used for studying genetic diversity in different plant species (Vos *et al*., 1995; Steiger *et al*., 2002). Comparative studies using restriction RFLPs, RAPD, AFLP and microsatellite techniques have shown that AFLP method is the most efficient method to estimate genetic diversity because of its high reproducibility, quantity of information, resolution of genetic differences and multiplex ratio. RAPD technique has the high variable of replicability, but easy to use. RFLP technique provides low quantity of information, but has higher replicability and resolution of genetic differences when compare to RAPD method, but lower than AFLP and microsatellite techniques. Microsatellite method has good qualification as well as AFLP, but use a long development time, difficult to use and develop the process. However, AFLP and microsatellite markers, coupled with sequencing information for systematic analyses, could surface as main tools for the analysis of genetic variation (Mueller and Wolfenbarger, 1999).

AFLP technique is being widely used for genetic diversity studies because it reveals significant polymorphisms and is a reliable and robust for molecular genetic marker assay. AFLP analysis has been used to discriminate between accessions of a number of plants at species level *i.e.* red alga (Donaldson *et al*., 1998); variety level including barley (Schut *et al*., 1997), rice (Singh *et al*., 1999), olive (Angiolillo *et al*., 1999), globe artichoke (Lanteri *et al*., 2004), and cultivar level including soybean (Lin *et al*., 1996, Ude *et al*., 2003a), sunflower (Hongtrakul *et al*., 1997), potato (Milbourne *et al*., 1997), coffee (Steiger *et al*., 2002), ginger (Wahyumi *et al*., 2003), peanut (Chen *et al*., 2003), white yam (Mignouna *et al*., 2003), sugarcane (Aitken *et al*., 2005; Reffay *et al*., 2005) and cotton (Zhang *et al*., 2005).

The AFLP data clearly distinguished different sections of the genus *Musa* (*Musa, Australimusa, Rhodochlamys* and *Callimusa*) as well as genomic groups within the section *Musa* (Wong *et al*., 2001a and 2002; Ude *et al*., 2002a). AFLP could be used to identify 16 banana cultivars by their unique banding patterns and develop specific probes for identification purposes (Loh *et al*., 2000). Wong *et al*. (2001b) showed AFLP results that subsp. *truncata* was less related to subsp. *malaccensis* and *microcarpa*, both of which clustered together. An AFLP study of 28 *Musa* accessions (Ude *et al*., 2002b) produced four major clusters that closely corresponded to the genome composition of the accessions (AA, BB, AAB and ABB) and the data suggested that three subspecies within the *M. acuminata* complex (subsp. *burmannica*, *malaccensis*, and *microcarpa*), 'Tjau Lagada' (subsp. *microcarpa*), 'Truncata' (subsp. *truncata*) and 'SF247' (subsp. *banksii*) clustered very closely with 'Gros Michel' (AAA) and 'Km 5' (AAA). The fact indicated that more than one *M. acuminata* subspecies may be involved in the origin of triploid AAA bananas (Ude *et al*., 2002b). *M. acuminata* 'Calcutta 4' (subsp. *burmanicoides*) and 'Long Tavoy' (subsp. *burmanica*) were closely related and could have belonged to the same subspecies (Ude *et al*., 2002b). The study of Ude *et al*. (2002b) also showed that there is wide diversity within *M. balbisiana*, as confirmed by Wang *et al*. (2007). A comprehensive study involving both the RAPD and AFLP techniques on 25 plantain cultivars, believed to represent the total diversity of the African plantains, was carried out by Ude *et al*. (2003b). An AFLP study of 28 *Musa* accessions (Ude *et al*., 2002b) and 25 African plantains (De Langhe *et al*., 2005) could separate four large clusters of plantain (AAB) cultivars, i.e. Asamiensa ('Horn'), Agbagba ('False Horn'), Obino l'Ewai ('French') and Batard ('French-Horn'), which correlated morphological character of bunch type. Rubaihayo *et al*. (2002) studied the genetic diversity of 115 East African Highland bananas which separated four major clusters of the AAA accessions (*Musakala*, *Nfuuka*, *Nakitembe,* and *Nakabululu*). AFLP analysis of a full-sib tetraploid hybrid population confirmed previous reports based on Variable Number of Tandem Repeat (VNTR) analysis, of a high frequency of recombination during 2*n* (3*x*) gamete formation by a triploid plantain landrace (Crouch *et al*., 1999). All AFLP analyses indicated that plantain landraces genetic diversity is very narrow (Rubaihayo *et al*., 2002; Ude *et al*., 2003b; De Langhe *et al*., 2005; Noyer *et al*., 2005).

CHAPTER III
MATERIALS AND METHODS

1. Plant materials

A total of 135 *Musa* species and cultivars (Appendix A) from wild habitat and villages in Thailand were collected. The *Musa* species, identified based on Simmonds (1959, 1962), Argent (1976), De Langhe *et al.* (2002) as of Athawongsa (2008), included 17 accessions of *M. acuminata* subsp. *siamea*, 21 accessions of *M. acuminata* subsp. *malaccensis,* two accessions of *M. acuminata* subsp. *microcarpa*, and one accession of *M. acuminata* subsp. *truncata*, three accessions of *Musa* sp., and 18 accessions of *M. balbisiana*. Sixty-nine accessions of the banana cultivars were from six genome designations, *i.e.* AA, AB, AAA, AAB, ABB/BBA, and BBB. *M. itinerans* Cheesman was used as outgroup. Each accession was located using a Global Positioning System (GPS) device. Morphological characterization was done at site. Dry and in-spirit specimens were collected and deposited at Suan Luang Rama IX Herbarium. Living specimens were planted at a private garden. Cigar (youngest unfurled) leaf was collected for DNA extraction. Digital photos and line drawings were kept along with specimens.

2. Genomic DNA extraction

Approximately 5 g of cigar leaf was ground in liquid nitrogen with mortar and pestle. Isolation of total DNA followed the protocol described by Doyle and Doyle (1990), which is a CTAB-based extraction. DNA concentration was estimated using spectrophotometric measurement of UV absorbance at 260 nm (Hoefer DQ 200 fluorometer) (Appendix C).

3. AFLP procedure

The AFLP procedure was carried out as reported by Vos *et al*. (1995) with few modifications (Appendix C). Approximately 100 ng/µl of DNA was digested by two restriction enzymes, *i.e. Eco*RI and *Mse*I or *Tru*9I in 10x buffer A (Promega) and incubated for 2 hr at 37°C or 1 hr at 37°C and 1 hr at 65°C if digest with *Tru*9I respectively. Finally, 10 µl of the restriction reaction was evaluated in 1% agarose gel in 0.5x TBE for completion of digestion. The restricted DNA fragment was ligated to *Eco*RI-adapter and *Mse*I-adapter (Table 1) by adding of 10 µl of ligation master mix and incubated at least 3 hr at 37°C. Each ligation reaction was diluted at the ratio 1:10 with sdH_2O and stored at -20°C. PCR was performed in two consecutive reactions. Five microliters of the diluted DNA template generated was first pre-amplified using *Eco*RI + A and *Mse*I + C primers (Table 1). Each reaction was composed of 50 µl pre-amplification primer mix. PCR was done in a Px2 Thermal Cycler (Thermo Electron Corporation, USA). The pre-amplified DNA was diluted to 1:9 with sdH_2O, vortexed and placed in -20°C or proceeds to next step. Three microliters of the diluted pre-amplified PCR product were used for selective amplification in a reaction tube containing 20 µl of selective amplification mixtures. Eight primer pairs (Table 1) were used for the selective amplification. The PCR product was added with 10 µl of sequencing dye and investigated the selective amplified PCR product using 1% agarose gel electrophoresis in 0.5x TBE. The final PCR products were run on a 4.5% denaturing polyacrylamide gel electrophoresis.

4. Polyacrylamide gel electrophoresis

AFLP product was separated by 4.5% denaturing polyacrylamide gel electrophoresis in 1x TBE buffer in a Sequi-Gen GT Sequencing Cell (Bio-Rad, USA) (Appendix C).

5. Silver nitrate staining

After the electrophoresis, DNA fragments were visualized using silver nitrate staining protocol (Bassam *et al.*, 1991). The gel was rinsed with distilled water and air-dried (Appendix C).

6. Data analysis

For genetic diversity analysis, a band was considered polymorphic if it presented in at least one genotype and absent in others. Each band was scored as a "1" if present and as "0" if absent. A genetic similarity matrix was computed according to Nei and Li's similarity index (Nei and Li, 1979). Genetic diversity estimates (GDEs) were calculated as 1 minus Nei and Li Similarity Coefficient and multiplying the result by 100 [$(1-S_{ij}) \times 100$]. A dendrogram was constructed from the matrix of similarity coefficients using the Unweighted Pair-Group Method of the Arithmetic average (UPGMA) technique (Sneath and Sokal, 1973) of the NTSYS-pc software package version 2.11T (Rohlf, 2000). A particular DNA band size was calculated by interpolation with standard curve from standard DNA ladder (Promega, USA). Curve equation was calculated by Sigma Plot-pc software package version 10.0 (Systat Software, USA). DNA size estimation was done by comparing with 726 bp ladder (Promega, USA).

Table 1 Sequences of the primers and adapters used for AFLP analyses (Vos *et al*., 1995; Loh *et al*., 2000; Wong *et al*., 2001a, b and 2002; Ude *et al*., 2002a, b and 2003).

Primer pairs	Name/Abbreviation[1]	Type	Sequence (5'-3')
	*Eco*RI-adapter	Adapter +	CTCGTAGACTGCGTACC
		Adapter -	AATTGGTACGCAGTCTAC
	*Mse*I-adapter	Adapter +	GACGATGAGTCCTGAG
		Adapter -	TACTCAGGACTCAT
	E+A	Primer +1	GACTGCGTACCAATTCA
1	E+AAC	Primer +3	GACTGCGTACCAATTCAAC
2	E+AAG	Primer +3	GACTGCGTACCAATTCAAG
3	E+ACA	Primer +3	GACTGCGTACCAATTCACA
4	E+ACT	Primer +3	GACTGCGTACCAATTCACT
5	E+ACC	Primer +3	GACTGCGTACCAATTCACC
6	E+ACG	Primer +3	GACTGCGTACCAATTCACG
7	E+AGC	Primer +3	GACTGCGTACCAATTCAGC
8	E+AGG	Primer +3	GACTGCGTACCAATTCAGG
	M+C	Primer +1	GATGAGTCCTGAGTAAC
1	M+CAA	Primer +3	GATGAGTCCTGAGTAACAA
2	M+CAC	Primer +3	GATGAGTCCTGAGTAACAC
3	M+CAG	Primer +3	GATGAGTCCTGAGTAACAG
4	M+CAT	Primer +3	GATGAGTCCTGAGTAACAT
5	M+CTA	Primer +3	GATGAGTCCTGAGTAACTA
6	M+CTC	Primer +3	GATGAGTCCTGAGTAACTC
7	M+CTG	Primer +3	GATGAGTCCTGAGTAACTG
8	M+CTT	Primer +3	GATGAGTCCTGAGTAACTT

[1]E or *Eco*RI and M or *Mse*I are bases of the selective nucleotide (s) added at 3' end.

CHAPTER IV
RESULTS

AFLP profiles of *Musa* accessions

The AFLP analyses was performed on 135 *Musa* accessions using eight primer combinations (Table 2) yielded a total of 730 (83%) polymorphic bands and 136 (17%) monomorphic bands. All bands, ranging from 39 to 730 bp, were scored. The average number of polymorphic bands per primer was 89.25, while the amount for the eight primers ranged from 68 to 111 bands. The AFLP profiles can be used to distinguish between the *Musa* accessions by their unique banding patterns (Fig. 4-7).

A total of 136 unique genetic markers were observed for the *Musa* accessions which clustered into groups corresponding to the genome designation of *Musa* (wild AA, wild BB, AA, AAA, AB?, AAB, ABB, and BBB). Unique makers and their molecular sizes for *M. acuminata*, *M. balbisiana*, and *Musa* sp. were shown in Table 2 and Fig. 4-7. Higher number of unique bands was found in *M. acuminata* than in *M. balbisiana* and *Musa* sp., respectively. Among the *M. acuminata* accessions, unique bands were presented in each subspecies. Combination primer of E-AGC and M-CTG revealed the highest number of unique bands while E-ACT and M-CTC revealed the lowest. Unique maker bands, ranging from 39 to 650 bp were scored. The outgroup species, *M. itinerans*, clearly formed distinct cluster and separated from *M. acuminata*, *M. balbisiana*, and other cultivated bananas.

Genetic diversity between *Musa* accessions

The genetic diversity estimates (GDEs) were used for UPGMA cluster analysis which generated a dendrogram (Fig. 8). The GDE between the 135 accessions was 48.2%, that between *M. acuminata* accessions was 43.6% and between *M. balbisiana* accessions was 25.7%.

The means of the AFLP-based pairwise GDE among *Musa* accessions in this study are presented in Table 3. The pairwise GDE values between AI (*M. acuminata* subsp. *siamea*) and AII (*M. acuminata* subsp. *microcarpa*), AIII (*M. acuminata* subsp. *malaccensis* and cultivated bananas), and AVI (*M. acuminata* subsp. *truncata*) were 54.7, 59.7, and 61.4%, respectively. The value within AI was 46.4% while that among *M. acuminata* subsp. *malaccensis* and cultivated bananas within AIII was 56.5%. The GDE within the AII and within the AIV cannot be determined because they were the only accession of each group. The GDEs within the last group (AV) and between it and the rest of the *M. acuminata* were 17.5% and 66.4%, respectively. Meanwhile, the GDEs within the BI, the BII, and between them were 47.8%, 35.4%, and 53.1%, respectively.

Genetic relationship between *Musa* accessions

UPGMA method assigned the *Musa* accessions into two major clusters (Fig. 8) based on polymorphic bands (Fig. 4-7), *i.e.* cluster A (*M. acuminata*-containing accessions) and cluster B (*M. balbisiana*-containing accessions).

Cluster A

Cluster A can be separated into five groups (AI, AII, AIII, AIV and AV) (Fig. 8) and identified based on Atawongsa (2008). All, but one accession in the AI group belonged to *M. acuminata* subsp. *siamea*. The only one accession, SS & JS 136, was from Prachuap Khiri Khan and identified as *M. acuminata* subsp. *microcarpa* (Athawongsa, 2008).

Within the AI group, two clusters could be recognized, namely AI-1 and AI-2. Another accession of *M. acuminata* subsp. *microcarpa*, SS & JS 131, collected also from Prachuap Khiri Khan, was the only member in the AII group.

The third group (AIII) was composed of *M. acuminata* subsp. *malaccensis* and cultivated bananas of wide variation. The AA and AAA cultivars dominated this AIII group with only one AB, one ABB and three AAB cultivars. This AIII group was composed of two subgroups, the AIII-1 and AIII-2. While AIII-1 group were mainly cultivated ones, the AIII-2 were dominated by *M. acuminata* subsp. *malaccensis*, except one AA cultivated banana ‘Nio Nang Ram’.

M. acuminata subsp. *truncata* was the only member in the fourth group (AIV), while the last group (AV), *Musa* sp., was surprisingly showed unique AFLP bands well-distinguished from other *Musa* species.

Cluster B

The cluster B was separated into two groups (Fig. 8). The first group (BI) comprised of wild *M. balbisiana* and cultivated bananas (ABB/BBA and BBB). One (BBB) banana cultivar, 'Tani Kio Chan' collected from Thailand-Laos border in Nan, was nested with wild and cultivated *M. balbisiana* (BB) within group BI-1. Meanwhile the ABB/BBA and BBB cultivars clustered together in BI-2 group without any related BB species.

. The BII group included AAB- and ABB-designated cultivars, was composed of AAB and ABB cultivars, BBA 'Namwa' accessions, and closely related group of AAB cultivars, 'Klai' and 'Nga Chang'. Eleven clones of 'Namwa' (BBA) were separated into the BI-2 and BII groups. While 'Namwa Sai Dang', 'Namwa Khieo', and 'Namwa Nuan Chan' were among mostly AAB group of the BII, the rest of 'Namwa' were in the BI-2.

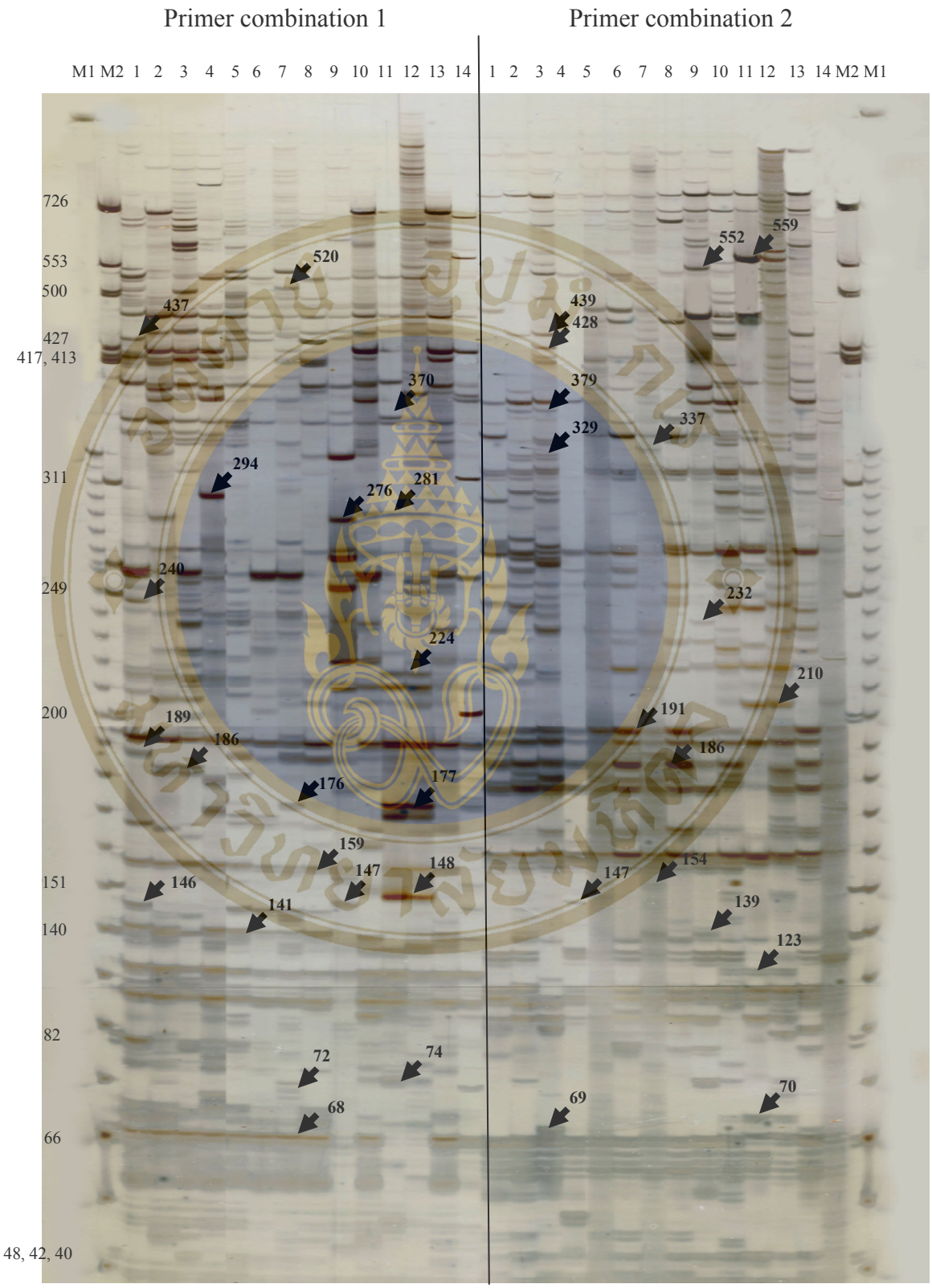


Figure 4

(continue in p. 20)

Figure 4 AFLP profile generated by primer combination 1 (E-AAC, M-CAA) (left), and primer combination 2 (E-AAG, M-CAC) (right). Arrow indicates unique bands as in Table 3. Lane designations with accession number in parentheses are as following.

Lane M1: Marker 10 bp.

Lane M2: Marker 24-726 bp.

Lane 1: *M. acuminata* subsp. *siamea* 'Khae' (SS & JS 001)

Lane 2: *Musa* (AA) 'Nio Nang Ram' (SS & JS 156)

Lane 3: *M. acuminata* subsp. *malaccensis* 'Pa Tha-le Ban' (SS & JS 210)

Lane 4: *M. acuminata* subsp. *truncata* 'Pa U-mong (Tunnel) Piyamit' (SS & JS 206)

Lane 5: *M. acuminata* subsp. *siamea* 'Pa Phanoen Thung 1' (SS & JS 325)

Lane 6: *M. acuminata* subsp. *siamea* 'Pa Phanoen Thung 2' (SS & JS 326)

Lane 7: *M.acuminata* subsp. *microcarpa* 'Pa La-u' (SS & JS 131)

Lane 8: *M. acuminata* subsp. *microcarpa* 'Pa Namtok Kha-On' (SS & JS 136)

Lane 9: *Musa* sp.'Pa Muang Phaem 2' (SS & JS 127)

Lane 10: *Musa* (AAB) 'Nga Chang' (SS & JS 186)

Lane 11: *M. balbisiana* 'Tani Sawankhalok' (SS & JS 003)

Lane 12: *M. balbisiana* 'Tani Pa Tat Luang' (SS & JS 012)

Lane 13: *Musa* (BBA) 'Namwa Nuan' (SS & JS 064)

Lane 14: *M. itinerans* 'Hok Khao Kho' (SS & JS 100)

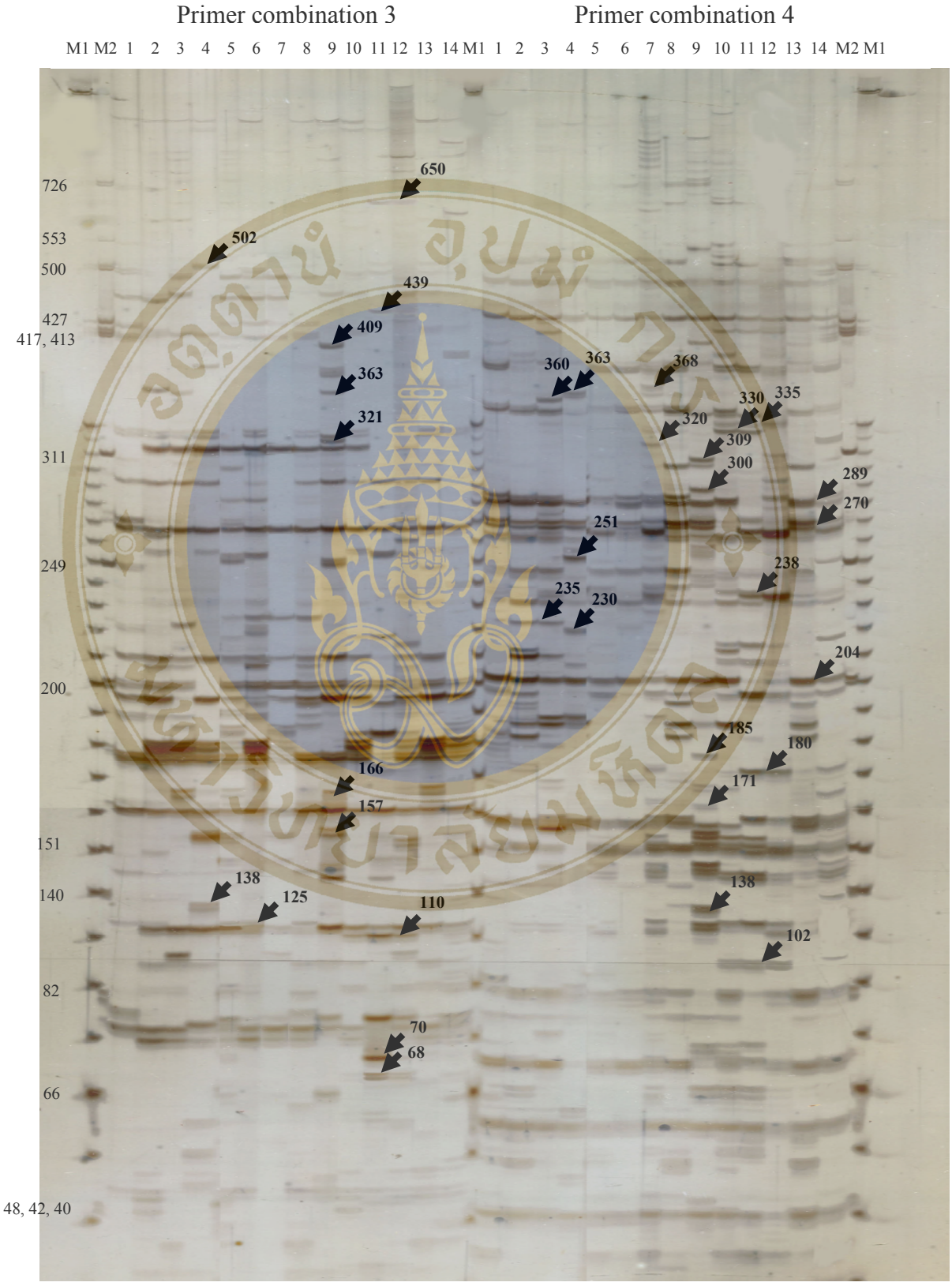


Figure 5

(continue in p. 22)

Figure 5 AFLP profile generated by primer combination 3 (E-ACA, M-CAG) (left), and primer combination 4 (E-ACT, M-CAT) (right). Arrow indicates unique bands as in Table 3. Lane designations with accession number in parentheses are as following.

Lane M1: Marker 10 bp.

Lane M2: Marker 24-726 bp.

Lane 1: *M. acuminata* subsp. *siamea* 'Khae' (SS & JS 001)

Lane 2: *Musa* (AA) 'Nio Nang Ram' (SS & JS 156)

Lane 3: *M. acuminata* subsp. *malaccensis* 'Pa Tha-le Ban' (SS & JS 210)

Lane 4: *M. acuminata* subsp. *truncata* 'Pa U-mong (Tunnel) Piyamit' (SS & JS 206)

Lane 5: *M. acuminata* subsp. *siamea* 'Pa Phanoen Thung 1' (SS & JS 325)

Lane 6: *M. acuminata* subsp. *siamea* 'Pa Phanoen Thung 2' (SS & JS 326)

Lane 7: *M.acuminata* subsp. *microcarpa* 'Pa La-u' (SS & JS 131)

Lane 8: *M. acuminata* subsp. *microcarpa* 'Pa Namtok Kha-On' (SS & JS 136)

Lane 9: *Musa* sp.'Pa Muang Phaem 2' (SS & JS 127)

Lane 10: *Musa* (AAB) 'Nga Chang' (SS & JS 186)

Lane 11: *M. balbisiana* 'Tani Sawankhalok' (SS & JS 003)

Lane 12: *M. balbisiana* 'Tani Pa Tat Luang' (SS & JS 012)

Lane 13: *Musa* (BBA) 'Namwa Nuan' (SS & JS 064)

Lane 14: *M. itinerans* 'Hok Khao Kho' (SS & JS 100)

Figure 6

(continue in p. 24)

Figure 6 AFLP profile generated by primer combination 5 (E-ACC, M-CTA) (left), and primer combination 6 (E-ACG, M-CTC) (right). Arrow indicates unique bands as in Table 3. Lane designations with accession number in parentheses are as following.

Lane M1: Marker 10 bp.

Lane M2: Marker 24-726 bp.

Lane 1: *M. acuminata* subsp. *siamea* 'Khae' (SS & JS 001)

Lane 2: *Musa* (AA) 'Nio Nang Ram' (SS & JS 156)

Lane 3: *M. acuminata* subsp. *malaccensis* 'Pa Tha-le Ban' (SS & JS 210)

Lane 4: *M. acuminata* subsp. *truncata* 'Pa U-mong (Tunnel) Piyamit' (SS & JS 206)

Lane 5: *M. acuminata* subsp. *siamea* 'Pa Phanoen Thung 1' (SS & JS 325)

Lane 6: *M. acuminata* subsp. *siamea* 'Pa Phanoen Thung 2' (SS & JS 326)

Lane 7: *M.acuminata* subsp. *microcarpa* 'Pa La-u' (SS & JS 131)

Lane 8: *M. acuminata* subsp. *microcarpa* 'Pa Namtok Kha-On' (SS & JS 136)

Lane 9: *Musa* sp.'Pa Muang Phaem 2' (SS & JS 127)

Lane 10: *Musa* (AAB) 'Nga Chang' (SS & JS 186)

Lane 11: *M. balbisiana* 'Tani Sawankhalok' (SS & JS 003)

Lane 12: *M. balbisiana* 'Tani Pa Tat Luang' (SS & JS 012)

Lane 13: *Musa* (BBA) 'Namwa Nuan' (SS & JS 064)

Lane 14: *M. itinerans* 'Hok Khao Kho' (SS & JS 100)

Figure 7

(continue in p. 26)

Figure 7 AFLP profile generated by primer combination 7 (E-AGC, M-CTG) (left), and primer combination 8 (E-AGG, M-CTT) (right). Arrow indicates unique bands as in Table 3. Lane designations with accession number in parentheses are as following.

Lane M1: Marker 10 bp.

Lane M2: Marker 24-726 bp.

Lane 1: *M. acuminata* subsp. *siamea* 'Khae' (SS & JS 001)

Lane 2: *Musa* (AA) 'Nio Nang Ram' (SS & JS 156)

Lane 3: *M. acuminata* subsp. *malaccensis* 'Pa Tha-le Ban' (SS & JS 210)

Lane 4: *M. acuminata* subsp. *truncata* 'Pa U-mong (Tunnel) Piyamit' (SS & JS 206)

Lane 5: *M. acuminata* subsp. *siamea* 'Pa Phanoen Thung 1' (SS & JS 325)

Lane 6: *M. acuminata* subsp. *siamea* 'Pa Phanoen Thung 2' (SS & JS 326)

Lane 7: *M.acuminata* subsp. *microcarpa* 'Pa La-u' (SS & JS 131)

Lane 8: *M. acuminata* subsp. *microcarpa* 'Pa Namtok Kha-On' (SS & JS 136)

Lane 9: *Musa* sp.'Pa Muang Phaem 2' (SS & JS 127)

Lane 10: *Musa* (AAB) 'Nga Chang' (SS & JS 186)

Lane 11: *M. balbisiana* 'Tani Sawankhalok' (SS & JS 003)

Lane 12: *M. balbisiana* 'Tani Pa Tat Luang' (SS & JS 012)

Lane 13: *Musa* (BBA) 'Namwa Nuan' (SS & JS 064)

Lane 14: *M. itinerans* 'Hok Khao Kho' (SS & JS 100)

Table 2 Size of unique molecular marker bands specific to each *Musa* taxa detected upon AFLP analyses using eight primer combinations.

	Size of unique molecular marker bands (bp)								Total
Primer Combinations* / *Musa* taxa	1 E+AAC/ M+CAA	2 E+AAG/ M+CAC	3 E+ACA/ M+CAG	4 E+ACC/ M+CAT	5 E+ACG/ M+CTA	6 E+ACT/ M+CTC	7 E+AGC/ M+CTG	8 E+AGG/ M+CTT	
M. acuminata subsp. *siamea*	437, 240, 189, 146	-	125	-	99	405, 70	309, 249	413, 208	12
M. acuminata subsp. *microcarpa*	520, 176, 72	337, 154	-	368, 320	-	140	528, 352	429	11
M. acuminata subsp. *malaccensis*	186	439, 428, 379, 329, 69	-	360, 235	465, 321, 248, 159	334, 160	429, 343	427, 255	18
M. acuminata subsp. *truncata*	294	147	502, 139	363, 251, 230	550, 244, 156	-	372, 306, 276, 177, 158	226	16
M. acuminata	159, 141, 68	191, 186	-	289, 270, 204	310, 209	-	109, 65	-	12
Musa sp.	276, 147	552, 232, 139	409, 363, 321, 166, 157	309, 300, 185, 171, 138	478, 225, 195, 78	355, 317	527, 268, 225, 152	396, 183, 170, 154, 75	30
M. balbisiana	370, 281, 224, 177, 148, 74	559, 210, 123, 70	650, 439, 110, 70, 68	335, 330, 238, 180, 102	402, 250, 170, 155	426	555, 264, 233, 188, 182, 156	554, 361, 111, 96, 52, 39	37
Total	20	17	13	20	18	8	23	17	136

*Primer combination abbreviation is indicated as of Table 1.

Table 3 Means of the AFLP-Based Pairwise Genetic Diversity Estimates (GDEs) among *Musa* accessions

	AI	AI-1	AI-2	AII	AIII	AIII-1	AIII-2	AIV	AV	BI	BI-1	BI-2	BII	*M. itinerans*
AI	-													
AI-1	0.269	-												
AI-2	0.365	0.464	-											
AII	0.547	0.490	0.576	-										
AIII	0.597	0.588	0.601	0.625	-									
AIII-1	0.600	0.591	0.604	0.630	0.411	-								
AIII-2	0.589	0.579	0.593	0.610	0.516	0.565	-							
AIV	0.614	0.604	0.620	0.678	0.641	0.647	0.623	-						
AV	0.664	0.649	0.671	0.709	0.651	0.649	0.655	0.706	-					
BI	0.743	0.729	0.750	0.711	0.710	0.705	0.723	0.755	0.722	-				
BI-1	0.786	0.765	0.796	0.734	0.752	0.745	0.770	0.793	0.762	0.261	-			
BI-2	0.696	0.690	0.699	0.685	0.662	0.659	0.671	0.713	0.678	0.393	0.478	-		
BII	0.652	0.643	0.657	0.661	0.577	0.559	0.624	0.679	0.678	0.531	0.535	0.527	-	
M. itinerans	0.775	0.777	0.774	0.766	0.774	0.775	0.770	0.808	0.789	0.816	0.830	0.799	0.764	-

GDEs represent 1- Nei and Li similarity coefficience

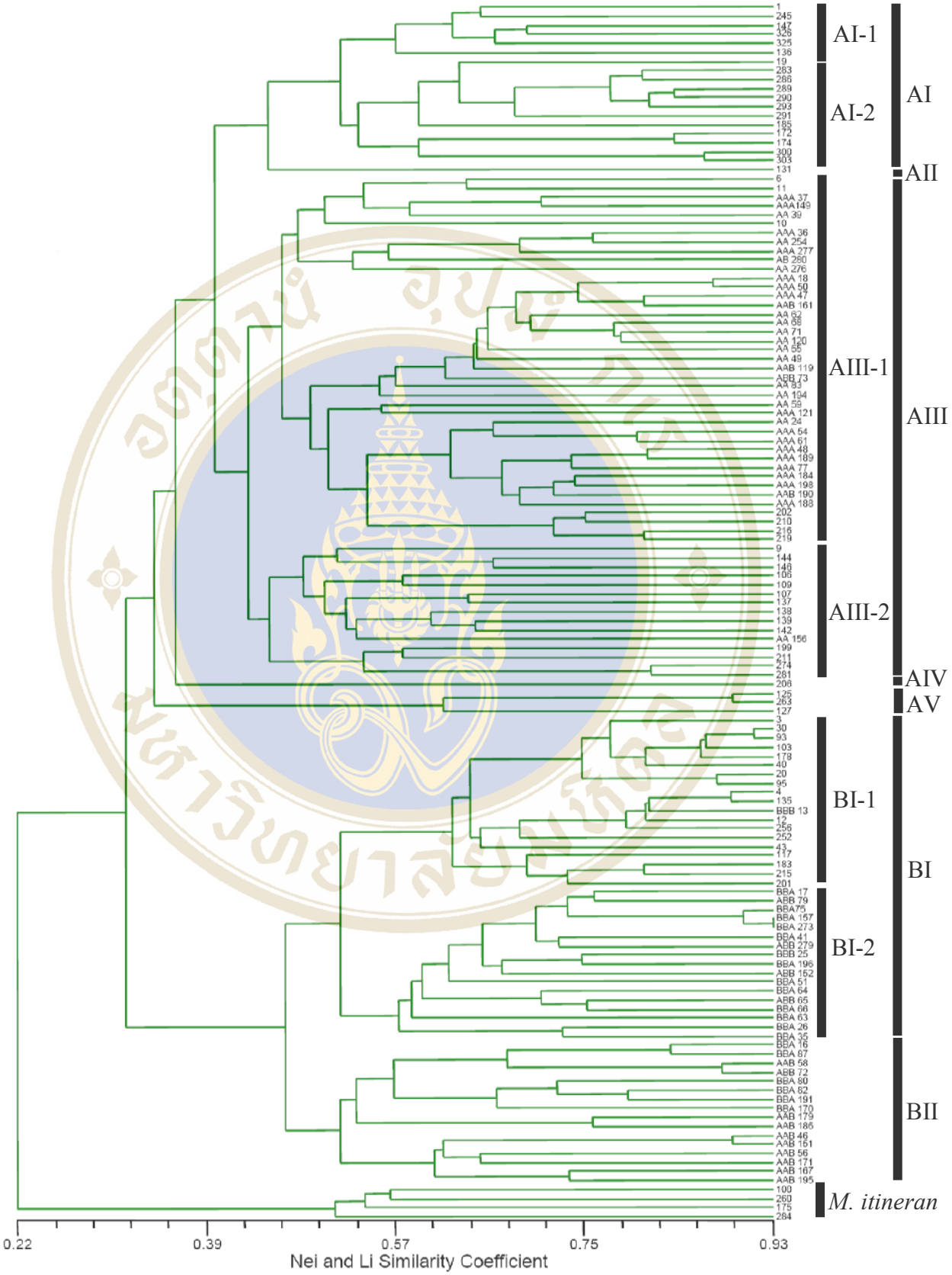


Figure 8 UPGMA cluster analysis of AFLP data generated eight primer combinations for 135 *Musa* accessions depicting patterns of genetic diversity.

CHAPTER V
DISCUSSIONS

Genetic diversity and relationship within wild *M. acuminata* accessions

AFLP analyses well separated *Musa* accessions into their specific status. While *M. itinerans* was distinguished as outgroup, *M. acuminata* and *M. balbisiana* were dissimilar and well clustered in their own group.

M. acuminata accessions contained A genome (A group) which showed higher genetic diversity estimates (GDEs) than *M. balbisiana*, *i.e.*, 43.6% and 25.7% within the *M. acuminata* and the *M. balbisiana* accessions, respectively. The GDE among the *M. acuminata* subsp. *siamea* was 34.5%, while higher GDE, 40.1%, was found within *M. acuminata* subsp. *malaccensis* (data not shown). AFLP analyses correlated with the subspecific status within *M. acuminata* as previously identified using morphological characters (Athawongsa, 2008 based on Simmonds, 1959, 1962; Argent, 1976; De Langhe *et al*., 2002).

Accessions in AI groups distributed in upper part of the country, from Chiang Rai to Prachuap Khiri Khan and from Kanchanaburi to Loei and Sakaeo, AII members of this group was identified as *M. acuminata* subsp. *siamea* except one accession, SS & JS 136. The latter accession was found to be genetically closely related to *M. acuminata* subsp. *siamea* than to *M. acuminata* subsp. *microcarpa* with a GDE of 34.9% and 51.3%, respectively, although it was collected from Prachuap Khiri Khan and identified as *M. acuminata* subsp. *microcarpa*, along with SS & JS 131 in the AII (Athawongsa, 2008).

M. acuminata subsp. *malaccensis* (AIII group) is commonly found in the southern part of Thailand. The AFLP analyses separated the group into two subgroups, AIII-1 and AIII-2 (Fig. 8), with mean of GDE values within group of 36.6% and 34.8%, respectively. This AFLP result agreed with morphometric analysis, which showed two forms of the subspecies *i.e.* typical 'malaccensis' form found commonly in the region and 'Kra Isthmus' form found only in Ranong and

neighboring provinces (Athawongsa, 2008). Surprisingly, the AFLP analyses confirmed the first records of *M. acuminata* subsp. *malaccensis* of typical form in northern Thailand (SS & JS 274 and 281 in AIII-2). The GDE also indicated that these accessions were less similar to other subspecies than to *M. acuminata* subsp. *malaccensis* (data not shown).

New record of *M. acuminata* subsp. *truncata* collected from mountainous area of the Yala province at 670 m above sea level was noted by AFLP analysis (this study and Athawongsa, 2008). Population of the subspecies grew along side to the *M. acuminata* subsp. *malaccensis* (Athawongsa, 2008). Our study, based on GDE, confirmed Wong *et al*. (2001), that the three subspecies of *M. acuminata* found in Thai Peninsula, *i.e.*, *malaccensis*, *microcarpa* and *truncata*, were distinctive.

Interestingly, a group of an unknown *Musa* species was found morphologically and genetically different from *M. acuminata*. GDE values (Table 3), showed that it was least related to other *M. acuminata* accessions. This *Musa* sp. distributes in northwestern provinces of Thailand. The AFLP data indicated clearly its distinctive status of a separated species.

Genetic diversity and relationship within wild *M. balbisiana* accessions

The AFLP data showed low level of genetic variability within *M. balbisiana* accessions (GDE = 25.7%; range = 17.7 - 42.8%) found in Thailand which was similar to the report of Swangpol *et al*. (2007) on DNA sequences. The results disagreed with previous works in other countries (Philippines, Sotto and Rabara, 2000; Nigeria, Ude *et al*., 2002b; China, Wang *et al*., 2007). The analyses clustered 18 *M. balbisiana* accessions into two groups. The first group of BI distributed in the central and northern Thailand, while the second group of BII distributed in the southern part of the country. The low GDE value among the two *M. balbisiana* groups (53.1%) did not support the separation of this species into lower taxonomic rank, though the intraspecific variation has been mentioned previously in Thailand (B. Silayoi, pers. comm. and Swangpol, 2004), and other countries (Ude *et al*., 2002b; Wang *et al*., 2007).

Genetic diversity and relationship among *Musa* accessions (*M. acuminata*, *M. balbisiana*, and the cultivated banana accessions)

AFLP analyses divided cultivated banana accessions into two major clusters; those contained *M. acuminata* (A) genome and the one with *M. balbisiana* (B) genome. The GDEs clearly showed that the banana cultivars in A group were genetically most closely related to *M. acuminata* subsp. *malaccensis* (Table 3). The first group of A-genome containing cultivars comprised of diploid (AA), and triploid (AAA, AAB and some ABB) accessions. This type was also more closely related to *M. acuminata* subsp. *malaccensis* than the other subspecies (Table 3). Surprisingly, subsp. *siamea*, *microcarpa*, and *truncata* were not related to any banana cultivars under this study. Diversity of cultivated bananas related to the *M. acuminata* subsp. *malaccensis* group was observed. Low GDEs between *M. acuminata*-genome containing cultivars and 'Nio Phaya Salawin' (AB), 'Khieo' (AAB), 'Nio Chorakhe Ampawa' (AAB), and 'Nam Lawo' (AAB) of 41.1, 19.0, 36.0, and 26.9% (data not shown), respectively, could indicate their close relationships. The analysis, therefore, suggested that these cultivars may not have been interspecific hybrids, and should be re-investigated for genomic status as of AA and AAA, respectively. Meanwhile, the Nam Chiang Rai (ABB) cultivar with 44.1% GDEs to other A cultivars (data not shown) may have become AAA or AAB. On the other hand, the GDE (34.9% to AIII-2; data not shown) showed that *Musa* 'Nio Nang Ram' in AIII-2 group was closely related to typical 'malaccensis' found commonly in southern Thailand as shown in Fig. 8. Meanwhile, the cultivated bananas as in BI-2 and BII, which clustered in their own clusters without any wild accessions may be imported from the Pacific as proposed by Swangpol *et al*, (2007).

On the other hand, edible bananas which mainly contained B genome, *i.e.* triploid BBA/ABB, AAB and BBB, were closely related to *M. balbisiana* from central, northern, and southern part of the country. The AFLP data showed that cultivated *Musa* (BBB) 'Tani Kio Chan' was closely related to wild *M. balbisiana*, 'Tani Pa Tat Luang', with a GDE of 19.2%, both of which were collected in the Nan Province. The result was in agreement to that of Swangpol *et al*. (2007), who found the relationship of this wild *M. balbisiana* accession to the other triploid BBB in the area. The AFLP technique distinguished *Musa* 'Klai' and 'Nga Chang' which, though

closely related with a GDE value of 23.8%, showed uniquely different AFLP profiles (data not shown).

In the B clusters, the AFLP results grouped the AAB and ABB/BBA cultivars into two clusters. Cultivated bananas in the BI-2 group clustered closely with cultivated and wild *M. balbisiana* in Thailand in the BI-1 group with GDE = 39.3% than those in the BII group, GDE = 53.1%, which proposedly introduced from the Pacific Islands, along with imported *Musa* (ABB) 'Saba'and 'Thep Phanom' cultivars (data not shown).

AFLP profiles of *Musa* accessions in this study were comparable to those in Ude *et al*. (2002a, b) that diploid AA, triploid AAA and AAB cultivars were closer related to *M. acuminata,* and triploid BBA/ABB, ABB and BBB cultivars were closer related to *M. balbisiana*. Swangpol *et al* (2007), in contrast to this experiment, showed that the AAB and ABB cultivars were among the A genome-containing accessions and BBA were among the B genome containing accessions. The study which based on chloroplast DNA sequences may be the results of one-sided maternal inheritance and did not represent the whole genome.

Musa accessions identification using AFLP marker

The AFLP analyses of eight primer combinations on 135 *Musa* accessions revealed 136 unique molecular marker bands which separated clearly on four subspecies of *M. acuminata* (subsp. *malaccensis*, *microcarpa*, *truncata* and *siamea*), *Musa* sp., and *M. balbisiana*. The molecular markers distinguished each *Musa* accessions by size of unique bands (Table 2). These markers, in some extent, related banana cultivars to their wild relatives. This research showed that *M. acuminata* subsp. *malaccensis* have contributed their genomes to the cultivars in AIII group, *i.e*., *Musa* (AA) 'Nio Nang Ram', (AAA) 'Nak', (AAB) 'Nam Lawo', (ABB) 'Nam Chiang Rai', and others. Previous reports offered the unique molecular markers to identify three taxa of *M. acuminata*, *i.e.*, subsp. *malaccensis*, *microcarpa,* and *truncata* (Wong *et al*., 2001). Meanwhile, not only agree, but this study of *Musa* diversity in Thailand based on AFLP analysis also add on more markers to identify more taxa in Thailand, *i.e.*, *M. acuminata* subsp. *siamea*, *Musa* sp., and *M. itinerans*. It will definitely be useful to develop specific probes for *Musa* identification as of Loh *et al*. (2000) and others.

CHAPTER VI
CONCLUSIONS

The analyses of 135 *Musa* accessions using AFLP markers showed the relationship between *M. acuminata*, *M. balbisiana*, and Thai cultivated bananas. The Thai banana cultivars, AA, AAA and some AAB genomes related closely to *M acuminata* subsp. *malaccensis*, while ABB, BBA and BBB cultivars were closely related to wild *M. balbisiana* in Thailand and the others may have been imported from the Pacific Islands.

The *M. acuminata* subsp. *malaccensis* showed two forms; 1) typical 'malaccensis', distribute commonly in the southern and in new locations in northern part of the country, and 2) 'Kra Isthmus' found only in Ranong and nearby provinces. The AFLP results could be used to clearly identify subspecies of *M acuminata* and also separated *Musa* sp. and *M. balbisiana*.

Eight combination primers of AFLP analyses revealed 136 unique molecular marker bands on this *Musa* accessions. These bands showed the different characteristics of the subspecies within *M. acuminata*, *Musa* sp. and *M. balbisiana* and related cultivated bananas. They have high potential of developing as taxa-specific markers for the identification of the bananas in Thailand.

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APPENDIX

APPENDIX A

Table 4 Plant materials of the *Musa* cv. accessions used in this study

No.	Acsn no.[1] SS & JS	Genome dsgntn[2]	Scientific name[3]	Thai name[4]		Source Location[5]
1	001	AA	*M. acuminata* subsp. *siamea*	Khae	แข้	Khao Kho, Phetchabun
2	003	BB	*M. balbisiana*	Tani Sawankhalok	ตานีสวรรคโลก	Sawankhalok, Sukhothai
3	004	BB	*M. balbisiana*	Tani Nu	ตานีหนู	Bang Bua Thong, Nonthaburi
4	006	AA	*M. acuminata* subsp. *malaccensis*	Pa Khao Lak	ป่าเขาหลัก	Takua Pa, Phangnga
5	009	AA	*M. acuminata* subsp. *malaccensis*	Pa Ranong 1	ป่าระนอง 1	Kra Buri, Ranong
6	010	AA	*M. acuminata* subsp. *malaccensis*	Pa Phangnga 1	ป่าพังงา 1	Khuraburi, Phangnga
7	011	AA	*M. acuminata* subsp. *malaccensis*	Pa Bang Hin Pli Tang	ป่าบางหินปลีตั้ง	Kapoe, Ranong
8	012	BB	*M. balbisiana*	Tani Tat Luang	ตานีตาดหลวง	Pua, Nan
9	013	BBB	*Musa* cv.	Tani Kiu Chan	ตานีกิ่วจันทร์	Chaloem Prakiat, Nan
10	016	ABB	*Musa* cv.	Tip Mukdahan	ตีบมุกดาหาร	KTM Collection
11	017	ABB	*Musa* cv.	Theppharot	เทพรส	KTM Collection
12	018	AAA	*Musa* cv.	Hom Khieo Khom	หอมเขียวค่อม	Lak Si, Bangkok
13	019	AA	*M. acuminata* subsp. *siamea*	Pa Huai Rai	ป่าห้วยไร่	Den Chai, Phrae
14	020	BB	*M. balbisiana*	Tani Phu Phiang	ตานีภูเพียง	Phu Phiang, Nan
15	024	AA	*Musa* cv.	Thong Khi Maeo	ทองขี้แมว	QSG Collection
16	025	BBB	*Musa* cv.	Lep Chang Ku	เล็บช้างกุด	KTM Collection
17	026	ABB	*Musa* cv.	Hin	หิน	KTM Collection

Table 4 Plant materials of the *Musa* cv. accessions used in this study (continue).

No.	Acsn no.[1] SS & JS	Genome dsgntn[2]	Scientific name[3]	Thai name[4]		Source Location[5]
18	030	BB	*M. balbisiana*	Tani Lang Suan	ตานีหลังสวน	Lang Suan, Chumphon
19	035	ABB	*Musa* cv.	Hak Muk Nuan	หักมุกนวล	KTM Collection
20	036	AAA	*Musa* cv.	Nak	นาก	KTM Collection
21	037	AA	*Musa* cv.	Khai Kamphaeng Phet	ไข่กำแพงเพชร	KTM Collection
22	039	AA	*Musa* cv.	Pa Rayong	ป่าระยอง	KTM Collection
23	040	BB	*M. balbisiana*	Tani Dam	ตานีดำ	KTM Collection
24	041	ABB	*Musa cv.*	Namwa Ngoen	น้ำว้าเงิน	KTM Collection
25	043	BB	*M. balbisiana*	Tani Kip Ma	ตานีกีบม้า	KTM Collection
26	046	ABB	*Musa* cv.	Nang Klai Surin	นางกลายสุรินทร์	KTM Collection
27	047	AAA	*Musa* cv.	Man	มัน	KTM Collection
28	048	AAA	*Musa* cv.	Kung Khieo	กุ้งเขียว	KTM Collection
29	049	AA	*Musa* cv.	Sa	สา	KTM Collection
30	050	AAA	*Musa* cv.	Hom Khieo Ton Sung	หอมเขียวต้นสูง	KTM Collection
31	051	ABB	*Musa* cv.	Namwa Mali-Ong	น้ำว้ามะลิอ่อง	KTM Collection
32	054	AA	*Musa* cv.	Thong Khak	ทองแขก	KTM Collection
33	055	AA	*Musa* cv.	Nam Nom	น้ำนม	KTM Collection
34	056	AAB	*Musa* cv.	Thong Som	ทองส้ม	KTM Collection
35	058	AA	*Musa* cv.	Khai Boran	ไข่โบราณ	KTM Collection

Table 4 Plant materials of the *Musa* cv. accessions used in this study (continue).

No.	Acsn no.[1] SS & JS	Genome dsgntn[2]	Scientific name[3]	Thai name[4]		Source Location[5]
36	059	AA	*Musa* cv.	Hom Chan	หอมจันทน์	KTM Collection
37	061	AA	*Musa* cv.	Hom Thong Pa	หอมทองป่า	KTM Collection
38	062	AA	*Musa* cv.	Lep Mue Nang	เล็บมือนาง	KTM Collection
39	063	ABB	*Musa* cv.	Namwa Maharat	น้ำว้ามหาราช	KTM Collection
40	064	ABB	*Musa* cv.	Namwa Nuan	น้ำว้านวล	KTM Collection
41	065	ABB	*Musa* cv.	Hak Muk Thong	หักมุกทอง	KTM Collection
42	066	ABB	*Musa* cv.	Namwa Luk Sai Dam	น้ำว้าลูกไส้ดำ	KTM Collection
43	068	AAA	*Musa* cv.	Nua Thong	เนื้อทอง	KTM Collection
44	071	AA	*Musa* cv.	Thong Dok Mak	ทองดอกหมาก	KTM Collection
45	072	ABB	*Musa* cv.	Khai Si Sa Ket	ไข่ศรีสะเกษ	KTM Collection
46	073	ABB	*Musa* cv.	Nam Chiang Rai	น้ำเชียงราย	KTM Collection
47	075	ABB	*Musa* cv.	Namwa Khom	น้ำว้าค่อม	KTM Collection
48	077	AAA	*Musa* cv.	Hom Thong Phon San	หอมทองผลสั้น	KTM Collection
49	079	ABB	*Musa* cv.	Hak Muk Khieo	หักมุกเขียว	KTM Collection
50	080	ABB	*Musa* cv.	E-Ngao Ubon	อีง้าวอุบล	KTM Collection
51	082	ABB	*Musa* cv.	Namwa Sai Dang Nakhon Phanom	น้ำว้าไส้แดงนครพนม	KTM Collection
52	083	AA	*Musa* cv.	Sae Ma	แส้ม้า	KTM Collection
53	087	ABB	*Musa* cv.	Tip Yai Chanthaburi	ตีบใหญ่จันทบุรี	KTM Collection

Table 4 Plant materials of the *Musa* cv. accessions used in this study (continue).

No.	Acsn no.[1] SS & JS	Genome dsgntn[2]	Scientific name[3]	Thai name[4]		Source Location[5]
54	093	BB	*M. balbisiana*	Tani Chiang Klom	ตานีเชียงกลม	Pak Chom, Loei
55	095	BB	*M. balbisiana*	Tani Pak Chom	ตานีปากชม	Pak Chom, Loei
56	100	II	*M. itinerans*	Hok Khao Kho	หกเขาค้อ	Khao Kho, Phetchabun
57	103	BB	*M. balbisiana*	Tani Phra Phuttha Chai	ตานีพระพุทธฉาย	Phuttha Chai, Saraburi
58	106	AA	*M. acuminata* subsp. *malaccensis*	Pa Pang Wan	ป่าปังหวาน	Pato, Chumphon
59	107	AA	*M. acuminata* subsp. *malaccensis*	Pa Pato	ป่าพะโต๊ะ	Pato, Chumphon
60	109	AA	*M. acuminata* subsp. *malaccensis*	Pa Wat Tham Sua	ป่าวัดถ้ำเสือ	Muang, Krabi
61	117	BB	*M. balbisiana*	Tani Bang Marot	ตานีบางมะรอด	Muang, Surat Thani
62	119	AAB	*Musa* cv.	Niu Chorake Amphawa	นิ้วจระเข้อัมพวา	STP Collection
63	120	AA	*Musa* cv.	Sae Lo	แซลอ	STP Collection
64	121	AAA	*Musa* cv.	Ta Kui Noe Mu	ตะกุ่ยเหน่อมู่	STP Collection
65	125	SS	*M. acuminata* subsp. *siamea*	Pa Huai Nam Dang	ป่าห้วยน้ำดัง	Mae Taeng, Chiang Mai
66	127	SS	*Musa* sp.	Pa Muang Pam 2	ป่าเมืองแพม 2	Pang Ma Pha, Mae Hong Son
67	131	AA	*M. acuminata* subsp. *microcarpa*	Pa La-u	ป่าละอู	Hua Hin, Prachuap Khiri Khan
68	135	BB	*M. balbisiana*	Tani Huai Mae Phriang	ตานีห้วยแม่เพรียง	Kang Krachan, Phetchaburi
69	136	AA	*M. acuminata* subsp. *microcarpa*	Pa Namtok Kha-On	ป่าน้ำตกขาอ่อน	Bang Saphan, Prachuap Khiri Khan
70	137	AA	*M. acuminata* subsp. *malaccensis*	Pa Ranong 2	ป่าระนอง 2	Kra Buri, Ranong
71	138	AA	*M. acuminata* subsp. *malaccensis*	Pa Khao Lak 2	ป่าเขาหลัก 2	Takua Pa, Phangnga

Table 4 Plant materials of the *Musa* cv. accessions used in this study (continue).

No.	Acsn no.[1] SS & JS	Genome dsgntn[2]	Scientific name[3]	Thai name[4]		Source Location[5]
72	139	AA	*M. acuminata* subsp. *malaccensis*	Pa Tap Kut	ป่าทับกุด	Muang, Phangnga
73	142	AA	*M. acuminata* subsp. *malaccensis*	Pa Khlong Nakha	ป่าคลองนาคา	Suk Samran, Ranong
74	144	AA	*M. acuminata* subsp. *malaccensis*	Pa Sai Daeng	ป่าทรายแดง	Muang, Ranong
75	146	AA	*M. acuminata* subsp. *malaccensis*	Pa Hong Charoen	ป่าหงษ์เจริญ	Tha Sae, Chumphon
76	147	AA	*M. acuminata* subsp. *malaccensis*	Pa Dan Singkhon	ป่าด่านสิงขร	Muang, Prachuap Khiri Khan
77	149	AAA	*Musa* cv.	Hom Thong Phetchaburi	หอมทองเพชรบุรี	KU Collection
78	151	ABB	*Musa* cv.	Khai Maha Sarakham	ไข่มหาสารคาม	KU Collection
79	152	ABB	*Musa* cv.	Niu Mue Nang	นิ้วมือนาง	KU Collection
80	156	AA	*Musa* cv.	Niu Nang Ram	นิ้วนางรำ	KU Collection
81	157	ABB	*Musa* cv.	Namwa Sai Luang (Ubon)	น้ำว้าไส้เหลือง (อุบล)	KU Collection
82	161	AAA	*Musa* cv.	Khieo	เขียว	KU Collection
83	167	AAB	*Musa* cv.	Nam A-chan Chalongchai	น้ำ อ.ฉลองชัย	KU Collection
84	170	ABB	*Musa* cv.	Namwa Khieo	น้ำว้าเขียว	KU Collection
85	171	AAB	*Musa* cv.	Wan	หวาน	KU Collection
86	172	AA	*M. acuminata* subsp. *siamea*	Pa Ban Pa Kluai	ป่าบ้านป่ากล้วย	Pak Chong, Nakhon Ratchasima
87	174	AA	*M. acuminata* subsp. *siamea*	Pa Pang Sida	ป่าปางสีดา	Muang, Sa Kaeo
88	175	II	*M. itinerans*	Hok Khao Soi Dao	หกเขาสอยดาว	Soi Dao, Chanthaburi
89	178	BB	*M. balbisiana*	Tani Khlong Tani	ตานีคลองตานี	Pong Nam Ron, Chanthaburi

Table 4 Plant materials of the *Musa* cv. accessions used in this study (continue).

No.	Acsn no.[1] SS & JS	Genome dsgntn[2]	Scientific name[3]	Thai name[4]		Source Location[5]
90	179	AAB	*Musa* cv.	Klai	กล้าย	Muang, Chanthaburi
91	183	BB	*M. balbisiana*	Tani Thong Suk College	ตานีวิทยาลัยทองสุข	Thawi Watthana, Bangkok
92	184	AAA	*Musa* cv.	Nam Amphawa	น้ำอัมพวา	Lak Si, Bangkok
93	185	AA	*M. acuminata* subsp. *siamea*	Pa Lap Lae	ป่าลับแล	Lap Lae, Sukhothai
94	186	AAB	*Musa* cv.	Nga Chang	งาช้าง	KTM Collection
95	188	AAB	*Musa* cv.	Chi	จี	KTM Collection
96	189	AAA	*Musa* cv.	Nak Yak Thong Pha Phum	นากยักษ์ทองผาภูมิ	KTM Collection
97	190	ABB	*Musa* cv.	Nam Lawo	น้ำละโว้	KTM Collection
98	191	ABB	*Musa* cv.	Namwa Nuan Chan	น้ำว้านวลจันทร์	KTM Collection
99	194	AA	*Musa* cv.	Khai Thong Ruang	ไข่ทองร่วง	KTM Collection
100	195	AAB	*Musa* cv.	Nam Fat	น้ำฝาด	KTM Collection
101	196	ABB	*Musa* cv.	Chang	ช้าง	KTM Collection
102	198	AAA	*Musa* cv.	Chai	ไช้	STP Collection
103	199	AA	*M. acuminata* subsp. *malaccensis*	Pa Khlong Sai	ป่าคลองทราย	Na Thawi, Songkhla
104	201	BB	*M. balbisiana*	Tani Chanae	ตานีจะแหน	Saba Yoi, Songkhla
105	202	AA	*M. acuminata* subsp. *malaccensis*	Pa Tanopute	ป่าตะเนาะปูเต๊ะ	Ban Nang Sata, Yala
106	206	AA	*M. acuminata* subsp. *truncata*	Pa Umong (Tunnel) Piyamit	ป่าอุโมงค์ปิยะมิตร	Be-tong, Yala
107	210	AA	*M. acuminata* subsp. *malaccensis*	Pa Tha-le Ban	ป่าทะเลบัน	Khuan Don, Songkhla

Table 4 Plant materials of the *Musa* cv. accessions used in this study (continue).

No.	Acsn no.[1] SS & JS	Genome dsgntn[2]	Scientific name[3]	Thai name[4]		Source Location[5]
108	211	AA	*M. acuminata* subsp. *malaccensis*	Pa Na Thon	ป่านาทอน	Thung Wa, Satun
109	215	BB	*M. balbisiana*	Tani Khuan Khanun	ตานีควนขนุน	Khuan Khanun, Phatthalung
110	216	AA	*M. acuminata* subsp. *malaccensis*	Pa Khao Pu Khao Ya 1	ป่าเขาปู่เขาย่า 1	Si Banphot, Phatthalung
111	219	AA	*M. acuminata* subsp. *malaccensis*	Pa Thung Pho	ป่าทุ่งโพธิ์	Chulabhorn, Nakhon Si Thammarat
112	245	AA	*M. acuminata* subsp. *siamea*	Pa Tha Khanun	ป่าท่าขนุน	Thong Pha Phum, Kanchanaburi
113	252	BB	*M. balbisiana*	Tani Hat Wichittra	ตานีหาดวิจิตรา	Khong Chiam, Ubon Ratchathani
114	254	AAB?	*Musa* cv.	Sai Namphueng	สายน้ำผึ้ง	Suan Phueng, Ratchaburi
115	256	BB	*M. balbisiana*	Tani Phu Phing	ตานีภูพิงค์	Muang, Chiang Mai
116	260	II	*M. itinerans*	Hok Khun Chae	หกขุนแจ	Wiang Pa Pao, Chiang Mai
117	263	SS	*Musa* sp.	Pa Phrao	ป่าพร้าว	Phrao, Chiang Mai
118	273	ABB	*Musa* cv.	Namwa Dam	น้ำว้าดำ	KTM Collection
119	274	AA	*M. acuminata* subsp. *malaccensis*	Pa Doi Musoe 2	ป่าดอยมูเซอ 2	Mae Sot, Tak
120	276	AA	*Musa* cv.	Kai Dam	ไก่ดำ	Tha Song Yang, Tak
121	277	AAA	*Musa* cv.	Hom Proi	หอมโปรย	Tha Song Yang, Tak
122	279	ABB	*Musa* cv.	Hak Muk Som	หักมุกส้ม	Sop Moei, Mae Hong Son
123	280	AB	*Musa* cv.	Nio Phaya Salawin	นิ้วพญาสาละวิน	Sop Moei, Mae Hong Son
124	281	AA	*M. acuminata* subsp. *malaccensis*	Pa Salawin	ป่าสาละวิน	Sop Moei, Mae Hong Son
125	283	AA	*M. acuminata* subsp. *siamea*	Pa Doi Pha Hom Pok	ป่าดอยผ้าห่มปก	Fang, Chiang Mai

Table 4 Plant materials of the *Musa* cv. accessions used in this study (continue).

No.	Acsn no.[1] SS & JS	Genome dsgntn[2]	Scientific name[3]	Thai name[4]		Source Location[5]
126	284	II	*M. itinerans*	Hok Mae Chan	หกแม่จัน	Mae Chan, Chiang Rai
127	286	AA	*M. acuminata* subsp. *siamea*	Pa Doi Tung	ป่าดอยตุง	Mae Fa Luang, Chiang Rai
128	289	AA	*M. acuminata* subsp. *siamea*	Pa Doi Phu Nang	ป่าดอยภูนาง	Chiang Muan, Phayao
129	290	AA	*M. acuminata* subsp. *siamea*	Pa Nanthaburi	ป่านันทบุรี	Ban Luang, Nan
130	291	AA	*M. acuminata* subsp. *siamea*	Pa Du Phong	ป่าดู่พงษ์	Santisuk, Nan
131	293	AA	*M. acuminata* subsp. *siamea*	Pa Thung Lang	ป่าทุ่งแล้ง	Long, Phrae
132	300	AA	*M. acuminata* subsp. *siamea*	Pa Dong La-khon	ป่าดงละคร	Muang, Nakhon Nayok
133	303	AA	*M. acuminata* subsp. *siamea*	Pa San Chao Pho Kun Dan	ป่าศาลเจ้าพ่อขุนด่าน	Muang, Nakhon Nayok
134	325	AA	*M. acuminata* subsp. *siamea*	Pa Phanoen Thung 1	ป่าพะเนินทุ่ง 1	Kaeng Krachan, Phetchaburi
135	326	AA	*M. acuminata* subsp. *siamea*	Pa Phanoen Thung 2	ป่าพะเนินทุ่ง 2	Kaeng Krachan, Phetchaburi

[1] Accession numbers were based on S. Swangpol and J. Somana (2008, unpublish data).

[2] Genome designations were as of "Genome size of Thai Bananas (MUSACEAE) Based on Flow Cytometry" by Pornpravee Choksuwanlert and Yadnapha Choeito (2008, unpublished data).

[3] Identification to species and subspecies was based on Athawongsa (2008).

[4] Thai names of banana cultivars are based on S. Swangpol and J. Somana (2008, unpublish data).

[5] Source locations were collection sites (S. Swangpol and J. Somana, 2008, unpublish data), except for those of accessions which were obtained from organization or private collections are indicated as following, KTM Collection = Kamphaeng Phet Thai House Museum, Muang, Kamphaeng Phet, QSG Collection = Queen Sirikit Garden, Chatuchak, Bangkok, STP Collection = Suan Ta Phuk Rang Nai Roi (Orchard), Damnoen Saduak, Ratchaburi, and KU Collection = Pakchong Research Station, Kasetsart University, Pak Chong, Nakhon Ratchasima.

APPENDIX B

Chemicals

40% (w/v) Acrylamide

Acrylamide	190	g
bisacrylamide	10	g
dH_2O	500	ml

This stock solution should be protected from light.

0.8% (w/v) Agarose gel

Agarose	0.8	g
0.5X TBE	100	ml

0.5 M EDTA, pH 8.0

EDTA (ethylenediamine tetraacetic acid) 186.1 g in 800 ml H_2O

Adjust pH to 8.0 with 10 M NaOH

Add H_2O to 1 l

Chloroform : isoamyl (24:1 v/v)

Chloroform	960	ml
Isoamyl alcohol	40	ml

3 M Sodium acetate, pH 5.2

Sodium acetate	24.61	g

Adjust pH to 5.2 with 10% acetic acid

Add dH_2O to 100 ml

10X Loading dye

Bromphenol blue	0.6	g
Glycerol	50	ml
dH_2O	50	ml

Boil 10 min and store at 4°C

Sequencing dye

Formamide	10	ml
Bromphenol blue	2.5	mg
Xylene Cyanol	2.5	mg
0.5 M EDTA pH 8.0	200	µl

10X TBE (Tris-borate)

Tris base	54	g
Boric acid	27.5	g
0.5 M EDTA pH 8.0	40	ml

Add H_2O to 1 l

5X TBE (Tris-borate)

Tris base	27	g
Boric acid	13.75	g
0.5 M EDTA pH 8.0	20	ml

Add H_2O to 1 l

TE Buffer, pH 8.0

1 M Tris-HCl (pH 8.0)	1	ml
0.5 M EDTA (pH 8.0)	0.2	ml
dH_2O	98.8	ml

1 M Tris-HCl, pH 8.0

Tris base	121.1	g in 800 ml H_2O
Adjust pH to 8.0 with 10 M HCl		
Add H_2O to 1 l		

Assay Solution for Determination DNA Quality

Assay solution for determinate concentration of DNA (10-500 ng/µl)

H 33258 stock solution	10	µl
10x TNE	10	ml
Distilled filtered water	90	ml

Silver staining

10% Acetic acid (1 l.)

dH_2O	900	ml
Acetic acid	100	ml

Silver stain (1 l.)

dH_2O	1000	ml
Silver Nitrate	1	g
Formaldehyde 37%	1.5	ml

Deverloper (1 l.)

dH_2O	1000	ml
Na_2CO_3	30	g
Formaldehyde 37%	1.5	ml
Sodiumthiosulfate (10 mg/ml)	200	µl

Acrylamide gel

4.5% Acrylamide gel	50	ml
10% Ammonium persulfate	300	µl
TEMED (N,N,N',N'-Tetramethylethylenediamine)	100	µl

APPENDIX C
Materials and Methods

Quality Determination of DNA

This technique measures the total amount of nucleic acid in sample including DNA, RNA, oligo-, and mononucleotide. Therefore, it is useful for pure DNA preparation of reasonable concentration.

1. Set "zero" or "0" with assay solution and press cal.
2. Set standard 100 ng/µl of DNA and press cal.
3. Wash with distilled filtered water.
4. Add 2 ml of assay solution and set "0".
5. Add 2 µl of DNA and mix by gentle inversion.
6. Put a cuvette on spectrophotometer.
7. Read concentration of DNA on digital screen.

Absorbance reading at 260nm allows the calculation of concentration of nucleic acid: An OD of 1 corresponds to approximately:

* 50 ug/ml of DNA for double-stranded DNA
* 40 ug/ml for single-stranded DNA and RNA
* 20 ug/ml for oligonucleotides.

Ratio of readings at 260nm and 280nm [A 260/ A 280] provides an estimate of Purity of the nucleic acid. Pure preparations of DNA and RNA have ratios of 1.8 – 2.0. If there is significant contamination with protein or phenol, the ratio will be lower and accurate quantitation is not possible. ds DNA OD=1 => 50 ug/ml extinction coefficient = 1/ 50 = 0.02

Digestion of Genomic DNA

1. Approximately 100 ng/µl of DNA was digested.

2. Digest genomic DNA with two restriction enzymes such as *Eco*RI and *Mse*I or *Tru*9I in 10x buffer A.

3. Incubate at least 2 hr at 37°C for *EcoR*I and *Mse*I or 1 hr at 37°C and 1 hr at 65°C for *EcoR*I and *Tru*9I.

4. Investigate digestion completeness by gel analysis (1% agarose gel) in 0.5x TBE using 10 µl of the restriction reaction.

Table 5 Reaction mixture for digesting genomic DNA with restriction enzymes

Solution	Volume (µl)
100 ng/µl DNA	5.00
10 unit/µl *Eco*RI (Promega)	0.50
10 unit/µl *Mse*I (BioLabs) or *Tru*9I (Promega)	0.50
10x Buffer A (Promega)	4.00
sdH_2O	30.00
Total	40.00

Ligation

1. The restricted DNA fragment was ligated to *Eco*RI-adapter and *Mse*I-adapter by adding 10 µl of ligation master mix.

2. Incubate at least 3 hr (preferably overnight) at 37°C.

3. Investigate completeness of ligation by gel analysis (1% agarose gel) in 0.5x TBE using 9 µl of ligation reaction and 1 µl of 6x loading dye.

4. Dilute each ligation reaction at the ratio 1:10 with sdH_2O.

5. The aliquots were stored at -20°C.

Table 6 Reaction mixture for nucleotide adapter ligation

Solution	Volume (µl)
5 pmol/µl *Eco*RI-adapter (Bioservice unit)	1.00
50 pmol/µl *Mse*I-adapter (Bioservice unit)	1.00
T4 DNA ligation (Promega)	0.40
10x ligation buffer (Promega)	1.00
sdH_2O	6.60
Total	10.00

Pre-selective amplification

1. Five microliters of diluted DNA solution were pre-amplified using *Eco*RI+A and *Mse*I+C primers.

2. Each reaction was composed of 50 µl pre-amplification primer mixture (Table 7).

3. PCR was done in a Px2 Thermal Cycler (Thermo Electron Corporation, USA) and performed the following pre-selective PCR program.

4. The pre-amplified PCR product was diluted to 1:9 with sdH_2O, vortex and place in -20°C or proceeds to next step.

Table 7 Reaction mixture for pre-amplification reaction

Solution	Volume (µl)
DNA	5.00
70 ng/µl *Eco*RI+A* (Bioservice unit)	1.00
70 ng/µl *Mse*I+C* (Bioservice unit)	1.00
10x PCR buffer	5.00
1 mM dNTP (Promega)	10.00
50 mM $MgCl_2$ (Promega)	1.50
5 U/µl Taq polymerase (Invitrogen)	0.20
sdH_2O	26.30
Total	50.00

Pre-selective PCR Program (Thermo Electron Corporation, USA**)**

Step 1:	2 min	94 °C	(extended initial denaturation step)
2:	30 sec	94 °C	(denaturation step)
3:	1 min	56 °C	(primer annealing step)
4:	1 min	72 °C	(extension step)
5:	20 times to 2		(step 2 to 4, 20X)
6:	20 min	60 °C	(final extension step)
7:	∞	4 °C	
8:	End		

Selective amplification

1. Three microliter of the diluted pre-amplified PCR product were used for selective amplification in a reaction tube containing 20 µl selective amplification mixtures (Table 8).

2. Eight primer pairs were used for the selective amplification (Table 1).

3. Selective amplification mixtures were performed the following selective PCR program.

4. The PCR product was added with 10 µl of sequencing dye.

5. Investigate the selective amplified PCR product using 1% agarose gel electrophoresis in 0.5x TBE.

6. The final PCR products were run on a 4.5% denaturing polyacrylamide gel electrophoresis.

Table 8 Reaction mixture for selective amplification reaction

Solution	Volume (µl)
Diluted DNA (from 4.4)	3.00
30 ng/µl *Eco*RI+AXX* (Bioservice unit)	1.00
30 ng/µl *Mse*I+CXX* (Bioservice unit)	1.00
10x PCR buffer	2.00
1 mM dNTP (Promega)	4.00
50 mM $MgCl_2$ (Promega)	0.60
5 U/µl Taq polymerase (Invitrogen)	0.10
sdH_2O	8.30
Total	20.00

Selective PCR Program (Thermo Electron Corporation, USA**)**

Step 1:	30 sec	94 °C	(denaturation step)
2:	30 sec	65 °C -1°C/cyc	(primer annealing step, starting at 65°C and reducing each step 1°C till 56°C)
3:	1 min	72 °C	(extension step)
4:	12 times to 1		(step 1 to 3, 12X)
5:	30 sec	94 °C	(denaturation step)
6:	30 sec	56 °C	(primer annealing step)
7:	1 min	72 °C	(extension step)
8:	23 times to 1		(step 5 to 7, 23X)
9:	30 min	60 °C	(final extension step)
10:	∞	4 °C	
11:	End		

4.5% Denatruring Polyacrylamide Gel Electrophoresis

AFLP product was separated by denaturing polyacrylamide gel electrophoresis which was done in a Sequi-Gen GT Sequencing Cell (Bio-Rad, USA).

1. Preparation of 4.5% denatured polyacrylamide gel electrophoresis

1.1 Thoroughly wipe the Sequi-Gen GT glass plates (IPC and outer plates) with 95% alcohol, 3 times.

1.2 After, thoroughly wipe IPG plate with 2 ml of repel-silane and outer glass plate with 1 ml of bind silane (1.5 µl of bind silane and 1 ml of 0.5 % acetic acid) each 1 time.

1.3 After, thoroughly wipe outer glass plate with 95% alcohol, 3 times.

1.4 Assemble gel sandwich with IPC, outer glass plate, and spacers in between the glass plates.

1.5 Slide and secure full-length lever clamps over gel sandwich.

1.6 Insert gel sandwich assembly into precision caster base.

1.7 Prepare mix the gel solution (300 µl of 10% ammonium persulfate solution (APS), 100 µl of TEMED and 50 ml of 4.5% polyacrylamide gel) in syringe which the gel solution is degassing.

1.8 Lay assembly flat on lab bench and attach syringe tubing and slowly inject gel solution between glass plates until the gel solution emerges a few centimeters from the top of the notched (shorter) glass plate (across the entire width of the gel).

1.9 Insert the comb(s) between the plates to the desired depth. After, inject gel solution until end of glass plate.

2.0 Let the gel polymerize for 1-2 hour or overnight.

2. Analysis of DNA fragment using 4.5% denaturing polyacrylamide gel electrophoresis

2.1 Remove the precision caster base from the IPC assembly.

2.2 Pre-run the polyacrylamide gel with 1 l of 1x TBE at 50 watt, 50°C, 45-55 min, and clean the well using Pasteur pipette of syringe to remove any unpolymerized acrylamide gel.

2.3 PCR product was added with 10 µl of sequencing dye and was denatured at 94°C for 3 min and place them on ice.

2.4 Turn off the power supply, and remove the top safety cover.

2.4 Load PCR product 5.0 µl into each well.

2.5 Put the cover on the apparatus and connects it to a high voltage power supply around 50 watt, 50°C.

2.6 Stop running the polyacrylamide gel electrophoresis when a xylene cyanol band has migrated 2/3 of the gel or bromophenol blue band has migrated off the bottom of the gel.

2.7 Remove the connecting equipment of electrophoresis, and remove top safety cover and lever clamps from the IPC assembly. After, take an outer glass plate which the polymorphic bands can be appeared using silver nitrate staining method.

Silver nitrate staining

After electrophoresis DNA fragment can be detected by silver nitrate staining. The silver nitrate staining was followed the protocol described by Bassam *et al.* (1991).

1. Fixation of polyacrylamide gel on outer glass plate with 10% of acetic acid (1 l), 20 min. (keep 10% of acetic acid)
2. Wash a polyacrylamide gel with distilled filtered water 3 times/ 2 l, 2 min.
3. Shake with silver nitrate staining (1 l) slowly, 30 min.
4. Rinse a polyacrylamide gel with distilled filtered water 1 sec.
5. Shake with cool developer solution slowly.
6. Stop reaction when gel has clear dark polymorphic bands with 10% acetic acid.
7. Wash a polyacrylamide gel with distilled filtered water 1 time/2 l, 2 min
8. Dry a polyacrylamide gel in the air.
9. The a polyacrylamide gel can scan, make a copy and analyze AFLP results.

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