การตรวจหาเครื่องหมายดีเอ็นเอจำเพาะและปริมาณกลูโคแมนแนนของ
บุก *Amorphophallus* spp. ในประเทศไทย

นางสาวอรชร เมฆเกิดชู

จุฬาลงกรณ์มหาวิทยาลัย
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DETERMINATION OF SPECIFIC DNA MARKERS AND GLUCOMANNAN CONTENT OF *Amorphophallus* spp. IN THAILAND

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จุฬาลงกรณ์มหาวิทยาลัย
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อรชร เมฆเกิดชู : การตรวจหาเครื่องหมายดีเอ็นเอจำเพาะและปริมาณกลูโคแมนแนนของบุก *Amorphophallus* spp. ในประเทศไทย (DETERMINATION OF SPECIFIC DNA MARKERS AND GLUCOMANNAN CONTENT OF *Amorphophallus* spp. IN THAILAND) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. ชาลีดา บรมพิชัยชาติกุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. ชื่นจิต ประกิตชัยวัฒนา, ดร. จอร์จ เชริดนิกสกี้, 229 หน้า.

พืชสกุลบุกในประเทศไทยถูกนำมาศึกษาหาความสัมพันธ์ระหว่างปริมาณสารกลูโคแมนแนนและลักษณะทางพันธุกรรมของบุก รวมถึงการตรวจหาเครื่องหมายดีเอ็นเอจำเพาะต่อบุกพันธุ์ที่มีกลูโคแมนแนนสูง โดยอาศัยการศึกษาลำดับนิวคลีโอไทด์และลายพิมพ์ดีเอ็นเอชนิดอาร์เอพีดี จากการวิเคราะห์ปริมาณกลูโคแมนแนนในหัวบุกพบว่าหัวบุกพันธุ์เนื้อทราย *(A. muelleri)* มีปริมาณกลูโคแมนแนนสูงสุดที่ร้อยละ 68.93 ของน้ำหนักแห้ง และแสดงความแปรผันของปริมาณกลูโคแมนแนนในระดับต่ำในแต่ละตัวอย่างของสายพันธุ์เดียวกัน โดยสามารถจำแนกกลุ่มของสายพันธุ์ตามปริมาณกลูโคแมนแนนในแต่ละตัวอย่างได้เป็นสามกลุ่ม คือ กลุ่มบุกพันธุ์ที่มีปริมาณกลูโคแมนแนนสูง (ร้อยละ 40-70), บุกพันธุ์ที่มีปริมาณกลูโคแมนแนนปานกลาง (ร้อยละ 20-39) และบุกพันธุ์ที่มีปริมาณกลูโคแมนแนนต่ำ (ต่ำกว่าร้อยละ 20) จากนั้นศึกษาความสัมพันธ์ทางวงศ์วานวิวัฒนาการของบุกโดยใช้ข้อมูลจากลำดับนิวคลีโอไทด์ในตำแหน่ง *trnL-trnF* ของคลอโรพลาสต์ดีเอ็นเอ ribosomal internal transcribed spacer (ITS) และ second intron of *LEAFY* (*FLint2*) ของนิวเคลียสดีเอ็นเอนอกจากนั้นชุดข้อมูลถูกนำมาวิเคราะห์ด้วยวิธีการเปรียบเทียบลำดับนิวคลีโอไทด์ (alignment) ที่แตกต่างกันคือ BMGE Alignment (Block Mapping and Gathering with Entropy) และโปรแกรม PRANK multiple aware-alignment ซึ่งผลการทดลอง ชี้ให้เห็นถึงความคงตัวของแต่ละกิ่งวิวัฒนาการของบุก โดยลำดับนิวคลีโอไทด์ในตำแหน่ง ITS แสดงความสัมพันธ์เชิงวิวัฒนาการของแต่ละตัวอย่างของบุกได้อย่างชัดเจนที่สุด นอกจากนั้นยังได้วิเคราะห์ความแปรผันทางพันธุกรรมด้วยเทคนิคอาร์เอพีดี โดยใช้ไพร์เมอร์ทั้งหมดสิบสามคู่ไพร์เมอร์ พบแถบดีเอ็นเอที่มีความหลากหลายจำนวน 269 แถบและค่าเฉลี่ยของระยะห่างทางพันธุกรรมอยู่ในช่วง 0.075 ถึง 0.0949 โดยผลความสัมพันธ์เชิงวิวัฒนาการของทั้งสองวิธีมีความสอดคล้องกันและยังมีความสอดคล้องกับลักษณะทางกายภาพของบุกและพบความสัมพันธ์เชิงวิวัฒนาการร่วมกันกับปริมาณกลูโคแมนแนนในหัวบุก โดยจากการศึกษาลำดับนิวคลีโอไทด์พบกิ่งวิวัฒนาการร่วมกันของบุกพันธุ์ที่มีกลูโคแมนแนนสูงแบ่งเป็นสองกลุ่ม และสามารถพัฒนาเป็นเครื่องหมายทางพันธุกรรมที่จำเพาะกับกลุ่มบุกพันธุ์ที่มีกลูโคแมนแนนสูง คือ *A. muelleri*, *A. bulbifer* และ *A. xiei* ในขณะที่การวิเคราะห์ด้วยเทคนิคอาร์เอพีดี พบแถบดีเอ็นเอที่มีความจำเพาะต่อบุกพันธุ์ที่มีกลูโคแมนแนนสูงและปานกลางที่ 600 คู่เบส ซึ่งแถบดีเอ็นเอจำเพาะนี้จะถูกพัฒนาเป็นเครื่องหมายพันธุกรรมชนิด SCAR จากการพัฒนาเครื่องหมายทางพันธุกรรมที่จำเพาะกับบุกพันธุ์ที่มีกลูโคแมนแนนสูงสามารถออกแบบไพรเมอร์จำนวน 7 คู่ ได้แก่ HKGM-4F/ HKGM-595R และ MUE-129F/MUE-490R จากเครื่องหมายพันธุกรรมชนิด SCAR; *FLint2 F1/ MUBX236_ Flint2, FLint2 F1/ MUBX253_Flint2*, MUBX520_ITS/26S-82R, MUBX551_ITS/26S-82R และ P17/MUBX994_ ITS จากเครื่องหมายพันธุกรรมในลำดับนิวคลีโอไทด์ โดยได้แถบดีเอ็นเอจำเพาะที่ขนาด 600, 350, 200, 200, 600, 600 และ 900 คู่เบสตามลำดับ จากนั้นทำการตรวจสอบความจำเพาะเบื้องต้นของไพรเมอร์ โดยทดสอบความจำเพาะของ ไพรเมอร์กับตัวอย่างบุกจำนวน 84 ตัวอย่าง พบว่าไพรเมอร์ที่ออกแบบมีความจำเพาะต่อบุกพันธุ์ที่มีกลูโคแมนแนนสูง และจากการตรวจสอบความว่องไวของไพรเมอร์กับดีเอ็นเอต้นแบบพบว่า ไพรเมอร์สามารถให้ผลบวกเมื่อใช้ดีเอ็นเอต้นแบบต่ำสุดประมาณ 0.3 ng/ml นอกจากนี้พบว่าไพรเมอร์เหล่านี้สามารถตรวจสอบชิ้นส่วนที่แตกต่างกันของบุก และมีความสามารถในการทำซ้ำได้ ดังนั้นเครื่องหมายดีเอ็นเอจำเพาะที่พัฒนานี้สามารถนำมาใช้เป็นเครื่องมือในการคัดกรองเบื้องต้นสำหรับบุกพันธุ์ที่มีกลูโคแมนแนนสูง เพื่อนำไปใช้ในการปรับปรุงอุตสาหกรรมการผลิตแป้งบุกในประเทศไทย นอกจากนี้ผลการวิจัยมีศักยภาพที่จะนำมาใช้ในการพัฒนาทางการเกษตรและโปรแกรมการปรับปรุงพันธุ์เพื่อเพิ่มการผลิตกลูโคแมนแนนต่อไป

สาขาวิชา เทคโนโลยีชีวภาพ
ปีการศึกษา 2558

ลายมือชื่อนิสิต .....
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ORACHORN MEKKERDCHOO: DETERMINATION OF SPECIFIC DNA MARKERS AND GLUCOMANNAN CONTENT OF *Amorphophallus* spp. IN THAILAND. ADVISOR: ASST. PROF. CHALEEDA BOROMPICHAICHARTKUL, Ph.D., CO-ADVISOR: ASSOC. PROF. CHEUNJIT PRAKITCHAIWATTANA, Ph.D., GEORGE SRZEDNICKI, Ph.D., 229 pp.

The relationship between konjac glucomannan (KGM) content and genetic characters of *Amorphophallus* species found in Thailand was investigated Specific DNA markers for characterization of high and low KGM content species were developed by using nucleotide region sequencing and Randomly Amplified Polymorphic DNA (RAPD) analysis. Determination of KGM content indicated that the KGM content in Thai species such as *A. muelleri* is as high as 68.93% (dry weight) with small variation among this group. KGM contents found in Thai *Amosphophallus* samples can be divided into high (40-70%), medium (20-39%) and low (<20%), based on the dry weight. Phylogeny reconstructions were studied using the chloroplast *trnL-trnF* spacer, nuclear ribosomal internal transcribed spacer (ITS) sequences and the second intron of *LEAFY* (*FLint2*). Moreover, same data sets were analyzed with BMGE Alignment (Block Mapping and Gathering with Entropy) and PRANK multiple aware-alignment program that indicated a strong performance of phylogenetic clade. Among three nucleotide regions, ITS showed the highest resolution of in-group relationship within this species. Furthermore, RAPD technique together with thirteen primers was used to investigate the genetic variation. From RAPD result, a total of 269 polymorphic bands were generated. The average of genetic distance was varied from 0.075 to 0.949. Genetic relationship trees based on these two techniques were in agreement with each other and related to morphological characters of *Amorphophallus* plants. Moreover, phylogeny result showed evolutionary relationship with KGM content. This study found two monophyletic clades of high KGM content species while in RAPD analysis primer AC-10 could generate specific band at 600 bp which is specific only to high and medium KGM content. Specific band from RAPD analysis was converted to Sequence Characterized Amplified Region (SCAR) markers. In addition, nucleotide sequencing data of each region was used to generate specific marker for high KGM content group of *A. muelleri, A. bulbifer* and *A. xiei*. Seven pairs of specific primers were designed for marker development including HKGM-4F/ HKGM-595R and MUE-129F/MUE-490R from SCAR marker and *FLint2 F1/ MUBX236_Flint2, FLint2 F1/ MUBX253_Flint2*, MUBX520_ITS/26S-82R, MUBX551_ITS/26S-82R and P17/MUBX994_ ITS from the sequencing markers. Amplified specific PCR products of 600, 350, 200, 200, 600, 600 and 900 bp respectively were obtained for these primers. To validate the specific markers, specificity of designed primer sets was further examined against a large sample number (N = 84). The designed primers showed their specific nature and their sensitivity of detection could be as low as 0.3 ng/mL of genomic DNA of the target species. Identification of high KGM content species with samples of different parts of plant and reproducibility testing of the method were successfully carried out. Specific DNA markers developed in this study have a potential to be used as an initial screening tool for economical species. This can contribute to improve the industrial production of KGM flour in Thailand and be used in the breeding programs to maximize KGM production in the country.

Field of Study: Biotechnology
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CONTENTS

Page

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Page

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Page

Page

Page

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

List of Figure

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

List of Table

CHAPTER I
INTRODUCTION

1.1 Research background

Amorphophallus is a perennial, herbaceous plant genus that belongs to the family Araceae. There are over 170 species occurring from West Africa, through subtropical and tropical and South Asia into the tropical portions of the Western Pacific and North Eastern Australia. Many species of this genus are known by the broad common name "Konjac". One of the main commercial products derived from some *Amorphophallus* species is the polysaccharide konjac glucomannan (KGM). KGM is a high molecular weight, water-soluble and neutral polysaccharide (Fang and Wu, 2004; Nishinari, 2000). The molecules are rich in hydroxyl groups that make it easily dissolved in water, leading to high viscosity that forms a thick hydrocolloid even when used in low concentrations (Li *et al.*, 2006). This property makes it one of the most versatile and economically useful hydrocolloids with many industrial applications such as the manufacture of foods, pharmaceuticals and chemicals. (Chua, 2010; Douglas *et al.*, 2005; Luo *et al.*, 2013)

Thailand is both a center of diversity for *Amorphophallus* as well as an emerging producer of economically important *Amorphophallus* species. At present, 58 species have been recorded in Thailand, of which 36 (ca. 65%) are endemic (Boyce *et al.*, 2012). Among the Thai *Amorphophallus*, there are a number of species that have the potential to become highly profitable crops in South East Asia, as they produce significant amounts of KGM. Presently, *A. konjac* is the main species for KGM production in China and Japan. However, cultivation of *A. konjac* encountered with

many cultivation problems such as diseases and this species does not occur in Thailand. Therefore the other *Amorphophallus* native species may be more suitable as a source of KGM in the region. The economic potential of KGM has led to an increased focus on the study of economically significant *Amorphophallus* species (Diao *et al.*, 2014; Gille *et al.*, 2011; Zheng *et al.*, 2013).

Amorphophallus has been studied previously in relation to its morphology (Hetterscheid and Claude, 2012; Hetterscheid, 2006; Hetterscheid and Ittenbach, 1996), palynology (Giordano, 1999; Punekar and Kumaran, 2010) and odor biochemistry (Kite G.C. *et al.*, 1998; Kite and Hetterscheid, 1997). However, the morphological and characters are highly variable therfore it is difficult to identify many species in the vegetative form (Grob *et al.*, 2002; Punekar and Kumaran, 2010). For this reason, a number of molecular markers have been employed to determine relationships and to assess genetic variation in the genus. These include the *LEAFY* (*FLint2*) gene as well as the chloroplast regions *rbcL*, *matK* and *trnL* (Grob *et al.*, 2004; Grob *et al.*, 2002; Sedayu *et al.*, 2010; Wahyudi *et al.*, 2013). However, phylogenetic studies based on these regions do not produce consistent cladograms. This is result from a high level of conflicting signal in the informative characters. Consequently, further variable regions as well as other non-sequencing molecular methods are needed to elucidate the evolutionary history of *Amorphophallus*. This may lead to useful insights into the relationships within the genus, as well as the evolutionary history of important traits such as KGM production. One non-sequencing method that can be valuable in identifying phylogenetically significant groupings of taxa is Randomly Amplified Polymorphic DNA (RAPD) analysis. RAPD can be used for a wide range of applications because of its sensitivity,

simplicity, cost-effectiveness and it does not require sequencing reactions (Ergül *et al.*, 2015; Ke *et al.*, 2015; Williams *et al.*, 1990). Moreover, RAPD segments are generally made up of larger untargeted fragments from throughout the genome that span both coding and non-coding DNA regions (Atienzar and Jha, 2006; Lopes *et al*., 2012). RAPD has been successfully applied for species-level studies in *Amorphophallus*, including work on *A. albus* (Hu *et al.*, 2008), *A. titanum* (Poerba and Yuzammi, 2008) and *A. muelleri* (Poerba and Martanti, 2008).

There are many studies integrated between DNA techniques and phytochemical compositions for investigate quantitative-trait loci, were proved to be useful (El-hawary *et al.*, 2012; Nisar *et al.*, 2015; Xia *et al.*, 2011) since morphological characters alone cannot provide a thorough assessment of those relationships (Liu *et al.*, 2012). Proper integration will lead to the development of comprehensive system for plant characterization that can be conveniently applied at the industry level for quality control of raw material (Kiran *et al.*, 2010). In fact, the association between genetic diversity and KGM content in a broader population of *Amorphophallus* species has not yet been systematically assessed. Therefore, specific DNA markers is proposed to be developed in this study for selecting a right *Amorphophallus* spp that has specific KGM content.

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Chulalongkorn University

1.2 Objectives

In order to achieve specific DNA marker related with KGM content for improving identification efficiency and selection of suitable species for cultivation promotion in Thailand to improve the quality of konjac powder produced in the country, following objectives were set as;

- Determine KGM content in each *Amorphophallus* species in Thailand
- Study the relationship between *Amorphophallus* spp. genetic characteristics, geography and KGM content.
- Develop specific DNA markers of *Amorphophallus* species in Thailand for improvement of the effectiveness of species identification.
- Study relationship between KGM content and specific DNA markers.

1.3 Scope

This research was divided into 3 main parts.

(i) Determination of KGM content from konjac tubers in Thailand.

(ii) Determination of genetic relationship of *Amorphophallus* spp. in Thailand.

- DNA sequencing analysis
- Random amplified polymorphic DNA (RAPD) analysis

(iii) Development of specific DNA markers to differentiate between high-medium and low KGM content group.

- DNA sequencing markers
- SCAR (sequence characterized amplified regions) markers
- Efficiency testing of designed primers.

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CHAPTER II
LITERATURE REVIEW

2.1 Characteristics of genus *Amorphophallus*.

The plant genus *Amorphophallus* belongs to the family *Araceae* and is estimated to around 200 species (Hetterscheid *et al.*, 2012). This genus is a monocotyledon and distributes from the subtropical eastern Himalayas throughout subtropical and tropical Asia into the tropical western pacific and north-eastern Australia. This genus more often found in lime-stone areas and the altitudinal range varies from sea level to ca. 3000 m. The remarkable of this genus is its morphology by solitary leaf with around 2-3 steak in mature plants. One single leaf rises from the tuber, consisting of a vertical petiole and a horizontal leaf-blade (Figure 2.1). The latter is dividing into few or numerous small leaflets (Boyce *et al.*, 2012; Hetterscheid and Ittenbach, 1996).

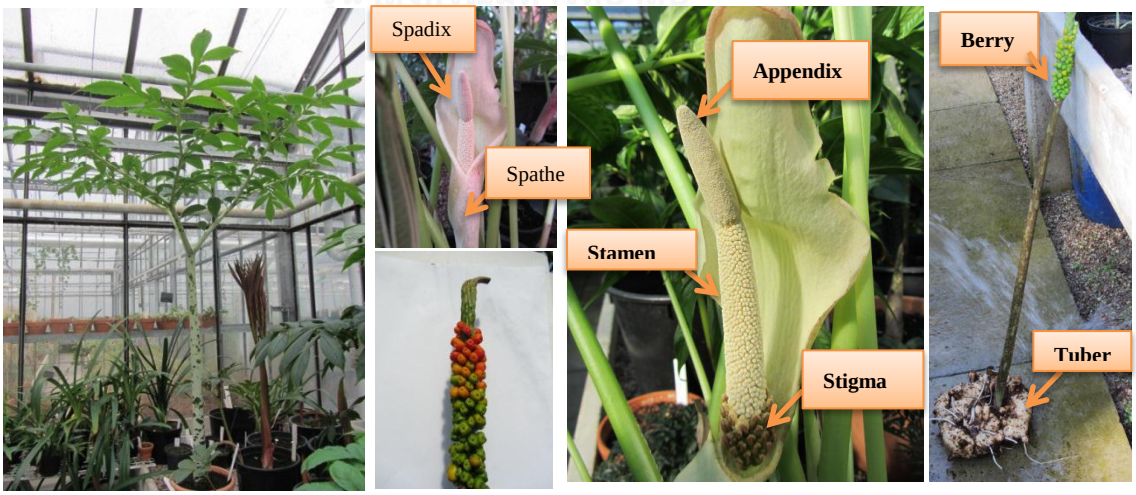

Figure 2.1 Character and inflorescence of *Amorphophallus* species.

Amorphophallus spp. is rich of morphological variation especially in vegetative form. Moreover, this genus is unmatched with any of the other genus in the same family *Araceae* (Hetterscheid and Ittenbach, 1996). Previous studies diagnosed main morphological characteristics of this genus (Boyce *et al.*, 2012; Hetterscheid and Claude, 2012; Hetterscheid and Ittenbach, 1996) as following.

2.1.1 Tuber

A variety of this characteristic is its shape including globose/subglobose-depressed globose-saucer shaped, irregularly elongate like cylindric or vertically elongate and then unbranched or branched, napiform like carrot-shaped and rhizomatous or stoloniferous. The tuber is renewed in each season except for its chains not being renewed every season. Zengze (1990) found that *Amorphophallus* difference from other tuber crops like taro or potato by the string roots with few branches and no destructive intercellular space. Moreover, airway of roots is narrow therefore the roots growth is directly affected by soil moisture.

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2.1.2 Petiole

Varieties of this characteristic include dimension, color, and pattern. This genus has variety of long or short petiole. Mostly of them show smooth petiole while warty to aspirate petiole is rare. Petiole is very thick and usually clearly spotted. The remarkable of this character is a variety of patterns and sheath very short.

2.1.3 Leaves

This genus has compound leaf that usually solitary and rarely paired. The growth of this genus is normally one leaflet per year. The regeneration capacity of leaf is weak therefore once the leaf injury means the loss of photosynthesis organ (Zhang *et al.*, 2010a). Patel and Mehta (1987) found that the leaf shape is different stem from different tuber age. The divisional pattern of leaf shows regular changes with the increasing age of tubers from the first year of seeds propagation. Normally, the leaf shape is steady after four years (Figure **2.2**). Leaves of *Amorphophallus* usually solitary except in seedlings may produce 2-3 leaves.

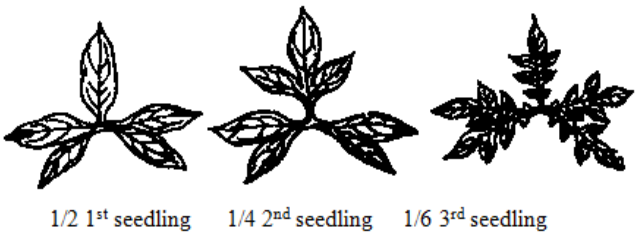

Figure 2.2 Changes of leaf shapes of *Amorphophallus* at the different age. (Zhao, 2010)

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2.1.4 The spathe

Varieties of this characteristic include dimension, color and shape. Spathe base is convolute, rarely open or connate and clearly separated or connected from limb by a constriction. Spathe outside is variously colored, shaped, often cymbiform or campanulate with rarely funnel-shaped. Spathe has boat-shaped for an insect-trap due to when the spathe opens the female flowers are opened and must be pollinated that same day. Spathe is clearly differentiated into tube and blade, sometimes constricted between them whereas convolute tube is rarely connate such as *A. pusillus, A. elliotii*. Inside of spathe's surface is smooth, longitudinally ribbed that

near base verruculose, scabrate or densely covered with scale or hair-like processes or smooth. Blade erect of spathe is spreading with smooth, ribbed or variously undulates or frilled at margins.

2.1.5 Appendix

Varieties of this characteristic include dimension, shape and sculpturing. Appendix is devised as shorter or longer than spathe. Appendix is contiguous with male zone or separated by a constriction, sometimes with large longitudinal folds or irregular deep cracks female zone shorter. Appendix is equal or longer than male zone. Shape of male zone can be found in cylindric, ellipsoid, conoid and obconoid. Male zone usually connects with female and separated by a sterile zone. Male zone may be naked or bear prismatic, subglobose or hair-like sterile flowers. Terminal appendix is usually present or rarely absent and very variable in shape such as conoid or cylindric, globose. Stipitate or basally is found to be narrow and usually smooth with staminode-like structures or entirely covered with staminodes.

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2.1.6 The individual male flower

Male flowers consist of 1, 3, 6 or 8 stamens. Stamens are depressed and has elongate shape. Filaments of male flower can be found as separated or partly or entirely fused within a flower or rarely fused between adjacent flowers. Anthers mostly have 2 pollen sacs with pores apical. Pollen inaperturate is various in shape include globose, elliptic, psilate, striate, verrucate, echinate, areolate, porate, fossulate, reticulate, or scabrous.

2.1.7 The individual female flower

Female flower has one pistil. Ovary is sessile or shortly stipitate with 1 to 4 locule (1 ovule/locule). Style of female flower is clearly separated from ovary. Sometimes, apical projections or branches extend beyond stigma. Whereas, sterile zone is covered with staminodes and rarely partly or entirely naked.

2.1.8 Berry and seed

After successful pollination, the individual female flowers develop into berries that containing the seeds. Berry sometime very large, oval-shaped, one to few-seeded, green initially and followed by orange to red, rarely blue or white. Whereas in seed variety in ellipsoid, testa smooth, thin, embryo large and endosperm absent. The distribution of *Amorphophallus* seed is birds that which follow from the quite strongly and brightly colored berries.

Sedayu *et al.* (2010) studied evolutionary of berry color found that blue, purple, green and yellow berries evolved from red, orange and white berries. In addition, small group of *A. polyanthus* and *A. sumawongii* produced verrucate berries with a very unusual color, which is green in *A. sumawongii* and dirty pinkish-brownish in *A. polyanthus*. This is due to adaptation to survive and suitable to forest floor conditions. It indicated by the type of pollination and type of dispersal (berries without striking, bird-attracting colors and infructescence held close to the soil).

2.1.9 Pollen

Pollen extruded in strands, inaperturate and mostly ellipsoid to ellipsoid-oblong. It occasionally has spherical or subsphaeroidal with medium-sized to large (mean 53 µm., range 34-82 µm.).

Van der Ham *et al.* (1998) divided group of *Amorphophallus* pollen into five groups includes (i) Konjac group, which is supported by same surface type e.g. *A. konjac* (foss to coarsely structure), *A. maxwellii* (foss to psilate); (ii) Longituberosus group, which are striate pollen with different style e.g. *A. aberrans* (areolate), *A. napiger* (psilate) but the relationship in this group still unclear; (iii) Napalensis group, which is grouped by plesiomorphic characters e.g. *A. bulbifer*, *A. muelleri* (Figure 2.3); (iv) Paeoniifolius group, which is supported by the only psilate pollen surface type e.g. *A. paeoniifolius*, *A. prainii*; (v) Yunnanensis group, which is support by striate pollen e. g. *A. corrugatus, A. kachinensis, A. yunnanensis.*

van der Ham *et al.* (2005) studied evolution of ornamentation and ectexine ultrastructure in the pollen of *Amorphophallus.* They divided pollen type into four main groups include (i) African with largely psilate group, (ii) Asian with psilate group, (iii) continental SE Asia with large striate group and (iv) Malesian with striate group. The continental SE Asian striate group lacks in thin psilate ectexines, possesses granules and mostly crowded (Figure 2.3). This group is diverse by the presence and distribution of dark granules. Moreover, continental SE Asia group found to be the main center of diversity of pollen in this genus. This study also indicated that ectexine appearance is not necessarily stabled in *Amorphophallus*.

Furthermore, remarkably diverse pollen ornamentation were found in this genus when compared to other large genera in the same family.

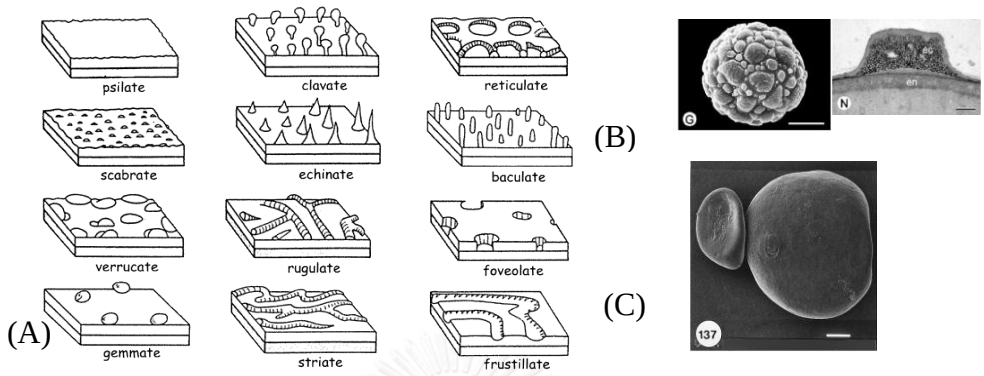

Figure 2.3 (A) Ten main ornamentation types were distinguished within *Amorphophallus*; (B) *A. scutatus* with areolate ectexine with crowded dark granules in middle part and (C) psilate normal pollen grain and sterile grain of *A. bulbifer* (van der Ham *et al.*, 2005; Van der Ham *et al.*, 1998)

2.1.10 Odors

The attractant odors are produced when inflorescence opening. The odor of each species evolved to attract a specific group of pollinators. This genus is notable for having strong and awful inflorescence odors (Willis, 1952). Chemical composition of *Amorphophallus* odors were explored by Kite and Hetterscheid (1997). The result found that the odor has diversified considerably and main composition is dimethyloligosulphide (rotting-meat odor). The other different smells were generally dominated by one or two compound such as isocaproic acid (rancid cheese odor) in *A. elatus*, 4-methoxyphenethyl alcohol (anise-like odor) in *A. albispathus* and isoamyl acetate (banana odor) with ethyl acetate in

A. haematospadix. For carrion-smelling odor, it is often supported by the inflorescence visual with dark brownish or brownish purple color such as *A. paeoniifolius* and *A. konkanensis*. On the other hand, the gaseous-smelling odor is normally appearing in pale color inflorescence such as *A. bulbifer*.

2.2 Taxonomy of the genus *Amorphophallus* (Jaleel *et al*., 2014)

Kingdom **Plantae** - Plants

Division **Magnoliophyta** - Flowering plants

Class **Liliopsida** – Monocotyledons

Sub-Class **Arecidae**

Order **Arales**

Family **Araceae** - Arum Family

Sub-Family **Aroideae**

Tribe **Thomsonieae**

Genus ***Amorphophallus***

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2.3 Life cycle of *Amorphophallus*

This genus is seasonally dormant plant. Dormancy period take place twice time of growing cycle (Figure 2.4). When a leaf drops off after a usual growing season, the tuber will automatically take a resting period. This resting period properly take 3-7 months depending on the species and the underground tuber will be continually growing. Flower and seed-bearing period will develop when plant fully growth, which would be around four growth cycle after that life cycle is completing. The resting period is usually shorter when flowering period already developed.

In Asian species, it declines to develop a leaf after flowering and effective pollination. Therefore, in fruit setting period are never found with leaves. However in some species, when the inflorescence is drop off, a plant may develop a leaf after all. Nevertheless, this behavior cannot be predicted. The inflorescence may replace with leaf in next season or develop alongside with inflorescence (Boyce *et al.*, 2012; Hetterscheid and Ittenbach, 1996).

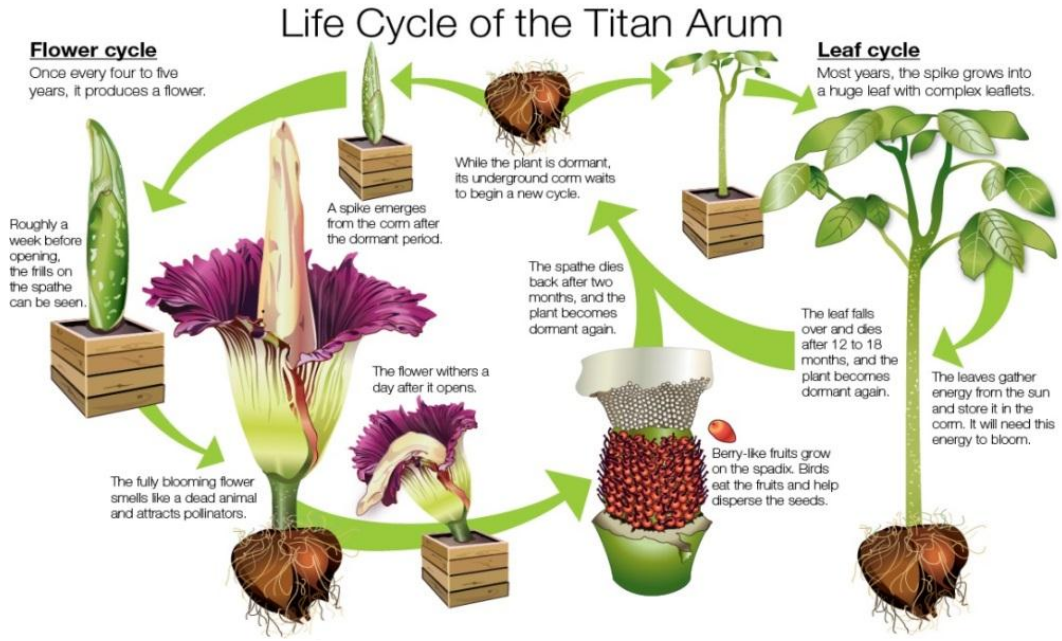

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Figure 2.4 Life cycle of *Amorphophallus titanium* (Chicago Botanic Garden, 2015)

2.4 Distribution of the genus *Amorphophallus*

Hetterscheid and Ittenbach (1996) studied distribution of genus *Amorphophallus* around the world and found that there were 163 species followed with a distribution location (Table 2.1). Their study divided distribution of *Amorphophallus* species into seven regions including African continent, Madagascar, Central and southern India, Northern India/Myanmar/northern Thailand/southern and southeastern China/Laos /northern Vietnam, Central Thailand/southern Cambodia and central Vietnam/eastern China/Taiwan/Japan, Malaysia (northern Kalimantan)/ Indonesia (Sumatra-Java-Nusa

Tenggara)/New Guinea/Northern Australia and Indonesia (Kalimantan)/Celebes Sea/Philippines. By all these seven areas belong to the Old World as Southeast Asia, China, Japan and Africa. From Table 2.1, it showed that genus *Amorphophallus* is mainly distributed in Asia, India, Indochina and China. There is a total of 125 species (76.7%) in Asia, with another 38 species (23.3%) in Madagascar and African continent. The center of genetic origin of *Amorphophallus* was found in Indochina and Southern China. This is because these regions are closely linked and that there are 70 species (43%) of the total of 163 species around the world (Hetterscheid and Ittenbach, 1996; Peiying and Jinfeng, 1986). Moreover, those studies claimed that the original types of this genus can be divided according to the distribution area such as original type from Thailand and Myanmar is *A. prainii*, original type from Thailand and Southern Yunnan is *A. paeoniifolius* and evolutionary types from Southern China and Southeast-Asia is *A. bulbifer*, *A. yuloensis* and *A. muelleri*.

Table 2.1 The distribution area of all genus *Amorphophallus* species

Area	Species number	Percentage of species in each region	Percentage of species in each continental
Thailand	28	China and Indochina : 70 species, 43%	Asia : 125 species, 76.7% Total : 163 species (100%)
China	21		
Vietnam	13		
Myanmar	6		
Laos	2		
India	11	South Asia: 13 species, 8%	
Nepal	1		
Bhutan	1		
Indonesia	18	Southeast Asia:42 species, 25.7%	
Malaysia	15		
Phillipines	9		
African continent	33	Africa and Oceania: 38species, 23.3%	
Madagascar	4		
Oceania(Australia)	1		

Source: Hetterscheid and Ittenbach (1996).

Within this generic distribution this genus shows a very high degree of endemism. Only three species showed a large geographical range include *A. paeoniifolius* is found from Madagascar into Polynesia, *A. muelleri* can be found from Thailand to Sumatra, Java and *A. abyssinicus* has a rather large distribution in Africa (Hetterscheid and Gusman, 2003). Recent active collecting confirmed that closely related species was very often found in neighboring areas (Hetterscheid and Claude, 2012)

Thailand has a species that is rich and complex biodiversity which differs in various parts of the country (Maxwell, 2004; Wikramanayake, 2002). Moreover, Thailand is still one of the bio diverse countries in Southeast Asia that 12,500 higher plant species are found (Parnell, 2000). The reason for the high level of species richness in Thailand is that the country is located on the border between four major biogeographical regions includes the northwest Himalayas, northern China, eastern Indochina, and southern Sundaland. Therefore, the flora is influenced by Indochinese, Indo-Burmese and Malesian elements (Van Welzen *et al.*, 2011). As mention before, Thailand is both a center of diversity for *Amorphophallus* as well as an emerging producer of economically important *Amorphophallus* species. At present, 58 species have been recorded in Thailand, of which 36 (ca. 65%) are endemic (Boyce *et al.*, 2012) are show in Table 2.2.

Table 2.2 *Amorphophallus* species occurring in Thailand.

Species [a]	Distribution in Thailand	Tuber	Berry
A. aberrans *	Northern, South-Western	Elongate	glossy white
A. albispathus *	South-Western, South-Eastern, Peninsular	Napiform, offset	bright red
A. amygdaloides *	South-Western	Globose, offset	not seen
A. asterostigmatus *	Central	Globose, offset	not seen
A. atrorubens *	North- Eastern	Globose, offset	deep blue
*A. atroviridis**	Central	Elongate, branch	white or yellow
A. bangkokensis *	Central	Globose, offset	not seen
A. boyceanus *	Peninsular	Globose, offset	bright red
A. brevispathus *	Central	Elongate, branch	not seen
A. carneus	Peninsular	Globose, offset	not seen
A. cicatricifer *	South-Western	Globose	bright red
*A. cirrifer**	Eastern, South-Western	Elongate	glossy white
A. corrugatus	Northern	Globose, offset	orange
A. cruddasianus	South-Western	Elongate	not seen
A. curvistylis *	South-Western	Globose, offset	not seen
A. echinatus	South-Western	Globose	not seen
A. elatus	Peninsular	Elongate	red
A. elegans	Peninsular	Globose	not seen
A. excentricus	Peninsular	Globose, offset	not seen
*A. fuscus**	Northern	Globose, offset	not seen
A. haematospadix	Peninsular	Globose, offset	not seen
A. harmandii	Northern, North-Eastern, South-Eastern	Elongate, branch	not seen
A. josefbogneri *	South-Western	Elongate, branch	not seen
A. kachinensis	Northern	Globose, offset	orange
A. koratensis	Eastern, Central, South-Eastern	Globose, offset	orange

Source: adapted from Boyce *et al.* (2012).

Table 2.2(2) *Amorphophallus* species occurring in Thailand (continue).

Species [a]	Distribution in Thailand	Tuber	Berry
A. krausei	Northern, North-Eastern, South-Western	Globose, offset	red
A. linearis *	Northern, South-Western, Peninsular	Elongate, branch	not seen
A. longituberosus	Northern, North-Eastern, South-Western, South-Eastern, Peninsular	Elongate, branch	red
A. lunatus *	Northern, South-Western	Elongate	white
A. macrorhizus *	Northern, North-Eastern	Napiform	red
A. maxwellii *	South-Western	Globose	not seen
A. muelleri	Northern, South-Western	Globose	red
A. napiger	North-Eastern, South-Eastern	Elongate, branch	whitish yellow
A. obscurus *	Eastern	Elongate	not seen
A. operculatus *	Peninsular	Elongate, branch	white
A. paeoniifolius	Northern, Eastern, South-Western, Central, Peninsular	Globose, offset	red
*A. polyanthus**	North-Eastern	Elongate, branch	Blackish with whitish spots
A. prainii	North-Eastern, Peninsular	Globose	bright red
A. prolificus *	Central	Globose, offset	not seen
A. putii	Central	Globose, offset	blue
A. pygmaeus *	South-Western, Central	Elongate	white
A. reflexus *	Northern	Elongate	glossy white
A. saraburensis *	Central	Elongate	not seen

Source: adapted from Boyce *et al.* (2012).

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Table 2.2(3) *Amorphophallus* species occurring in Thailand (continue).

Species [a]	Distribution in Thailand	Tuber	Berry
A. saururus *	North-Eastern	Elongate, branch	white
A. scutatus *	North-Eastern, Central	Elongate	red
A. serrulatus *	Northern	Globose	not seen
A. sizemoreae *	Northern	Elongate, branch	white
A. sumawongii *	South-Eastern	napiform, branched	pale green
A. symonianus *	North-Eastern	Globose, offset	not seen
A. thaiensis *	Northern	Elongate, branch	blue
A. tenuispadix *	South-Western, Peninsular	Globose	not seen
A. tenuistylis	South-Western, South-Eastern	Elongate	not seen
A. vogelianus *	Northern	Elongate, branch	white
A. yunnanensis	Northern, North-Eastern	Globose, offset	blue
A. glaucophyllus *	South-Western	Globose	not seen

[a] Species with the one asterisks (*) represent endemic species.

Source: adapted from Boyce *et al.* (2012).

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2.5 *Amorphophallus* species and Konjac glucomannan (KGM) content

Genus *Amorphophallus* have an underground storage tuber in which carbohydrate is the main component. In this genus, some of them are inedible because in tuber tissue is rough and the edible organ has failed formation. In addition, many species contain high amounts of alkaloids and toxic substances such as oxalic acid in fresh tuber. Therefore, it is difficult to remove those substances in the extraction processing result in there are no value as food. Nowadays, there are only around 20 species that are edible (Peiying, 2004). The important *Amorphophallus* species as edible resources are most widely distributed in China, Thailand and Indonesia (Table 2.3).

Table 2.3 The important species of *Amorphophallus* in China, Thailand and Indonesia.

China	Thailand	Indonesia
A. konjac	*A. muelleri*	*A. muelleri*
A. albus	*A. paeoniifolius*	*A. titanum*
A. corrugatus	*A. kachinensis*	*A. variabilis*
A. krausei	*A. krausei*	*A. blumei (Schott)*
A. yuloensis	*A. longituberosus*	*A. decus-silvae*
A. kachinensis	*A. macrorhizus*	*A. campannlatus*
A. bulbifer	*A. corrugatus*	
A. yunnanensis		
A. paeiniifolius		
A. kachinensis		
A. coaetaneus		
A.dunnii		

Source: adapted from Zhao *et al.* (2010).

From Table 2.3, some of this genus is used as economically important crops since those species are rich in KGM that stored in its tuber (Diao *et al.*, 2014). The size of the tuber was increased every season, which may increase its weight up to three times per season (Zhao, 2010). The tuber aged around three-years is suitable for used in konjac flour production. The species that used for cultivation included:

A. konjac is an important crop plant and is also cultivated in Japan and China with generally used in KGM flour production industry. The tuber of this species consists of 49–60% (w/w) water soluble KGM (Li *et al*., 2005). However, *A. konjac* grows in the range temperature between 20 ^{o}C and 25 ^{o}C and not resistant to high temperature in cultivation. The leaf dies quickly when the temperature rises above

25 ^{o}C (Hetterscheid and Ittenbach, 1996). Moreover, this species suffering from poor disease resistance and low coefficient of propagation (Cun *et al.*, 2009).

A. albus is a native species and mainly cultivated as crop in Yunnan, southwest China (Long, 1998). This species produces large subterranean tubers. The tuber of this species consists of KGM around 59.3% (w/w) (Liu *et al*., 2004). However, this species has propagation problem and lack of seed (Hu *et al.*, 2008).

A. bulbifer is a triploid (2n = 39) with is highly disease resistance, multiple seedling production and high coefficient of propagation. This species also tolerate a wide range of temperatures and growing well between 15 and 30 ^{o}C. This species has been successfully domesticated and cultivated in Yunnan, China (Figure 2.5). However, KGM content in this species found at 48-52% which lower than *A. konjac* and *A. muelleri* (Zhang *et al.*, 2010a; Zhang *et al.*, 2009; Zhao, 2010).

A. muelleri is an important wild and cultivated species in Thailand and Java, Indonesia. The property of multiple seedling of this species helps shortening the growth cycle from 3-4 years to 9-10 months (Santosa *et al.*, 2003). Moreover, this species has high disease resistance, resistance to water logging and drought, high propagation coefficient and high KGM content production (72-78%) (Zhang *et al.*, 2010a; Zhao, 2010).

From the above review, it can be indicated that *A. muelleri* and *A. bulbifer* would be exceptional commercial new species which will bring advantages for cultivation due to low-cost production and high-quality konjac flour. However, KGM content does not depend only to species, but also is affected by variety, age, growing region, soil, environment and cultivation management level (Yuanming and Peiying, 1995).

(A)  (B)  (C) 

Figure 2.5 (A) Plantation of *A. bulbifer* in Yunnan, China; (B) bulbil on the leaf and (C) collected tuber.

2.5.1 KGM and their application.

KGM is neutral heteropolysaccharide consists of a linear chain of-1,4-linked D-glucose and D-mannose in a molar ratio of 1:1.6, with side branches through -1,6-glucosyl units. The degree of branching is estimated at approximately 3 branches for every 32 sugar residues. The acetyl groups along the KGM backbone are located, on average, every 9–19 sugar units at the C-6 position. The structure of KGM was showed in Figure 2.6 (Chua *et al.*, 2012).

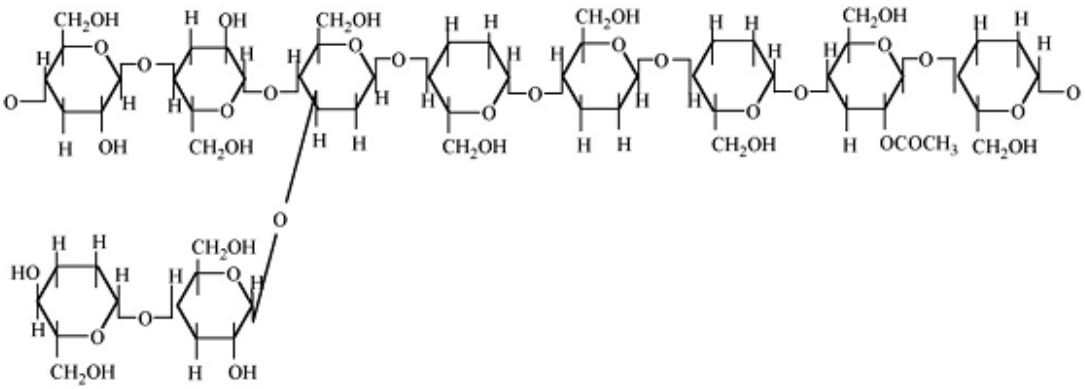

Figure 2.6 Chemical structure of KGM (Chua *et al.*, 2010)

Following KGM structure, it has unique physicochemical properties. The molecular weight of KGM is high, ranges from 200 to 2000 kDa, resulted in KGM solution has the high viscosity with extremely high density (Shiyin and He, 1991). KGM also has highest water holding capacity around 200 times of its original volume because it is rich hydroxyl group molecule. Therefore, KGM is easy to dissolve in water and leads to high viscosity even used at low concentration (Li *et al.*, 2006). KGM can dispensable even in hot or cold water and forms a highly viscous solution in between pH 4.0 and pH 7.0 (Jian *et al.*, 2015; Vanderbeek *et al.*, 2007). Addition of mild alkali to KGM solution, results in the formation of irreversible and thermo-stable gel. The effect of alkali is facilitating deacetylation of the KGM backbone that has a low proportion of acetyl groups. Moreover, KGM is not affected by small intestine human digestive enzymes due to its linkage β-1-4 structure of glucose and mannose. Hence, it contributes as zero calories food (Chua *et al.*, 2010).

KGM is one of the most important and economically hydrocolloids and can be applied in many industries (Table 2.4). Besides, there was a long history of using KGM as food and traditional medicine in China and Japan (Fang and Wu, 2004). KGM is declared as generally recognized as safe (GRAS) thus it is suitable for use in food and pharmaceutical industries (Chua *et al.*, 2010).

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Table 2.4 Application of KGM in main industry

Industry	Application	Reference
Food	Zero calorie food; gelling agent, thickener, food emulsifier; food additive; water-holding; emulsifier and surfactants; flavor encapsulate; fat replacer	(Akesowa and Ekthamasut, 2015; Liu *et al.*, 2012; Yang *et al.*, 2009; ZHAO *et al.*, 2014)
Pharmaceuticals	Diabetic control by absorption of dietary sugar; dietary fiber supplement; weight control; anti-obesity activity; anti-hyperglycemic and hypercholesterolemia activities; prebiotic activity supplement	(Chen *et al.*, 2005; Fang and Wu, 2004; Harijati *et al.*, 2011; Kraemer *et al.*, 2007; Martino *et al.*, 2005)
Chemicals	Film former and emulsifier; drug delivery; bio-adhesive; coating materials; cosmetics	(Chen *et al.*, 2005; Huang *et al.*, 2015; Khan *et al.*, 2015; Liang *et al.*, 2015)
Biotechnology	Materials for encapsulation; immobilization; alternative carbon source; fixation support	(Hong and Qiu, 2008; Li *et al.*, 2011d; Wang *et al.*, 2011; Wen *et al.*, 2008)

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2.6 Plant molecular analysis

Molecular biology method has been used for genetic diversity study. It is based on genetic materials like DNA that could be found in all living organism. There are three types of DNA material included (i) nuclear DNA (ii) chloroplast DNA found in all plants and (iii) mitochondrial DNA in all Eukaryotes. Genetic analysis by polymorphism in DNA level is considered to be a straightaway method to investigate inter- and intra-specific genetic variations of organisms (Egli and Saenger, 2013; Lodish *et al.*, 2000). In present, many molecular methods have been applied for

the genetic diversity analysis such as Random Amplified Polymorphic DNA (RAPD), direct DNA sequencing, etc. Both types of molecular techniques were used to detect and investigate the variation of genus *Amorphophallus* in this thesis.

2.6.1 DNA sequencing analysis

DNA sequencing technique is the most direct way to determine the nucleotide sequences of defined regions. It can provide informative data sets and can be adapted to different levels of discriminatory potential by choosing appropriate target DNA regions (Weising *et al.*, 2005). Sequences with relatively low mutation rates are required for higher level phylogenetic comparisons, while higher mutation rates are needed to discriminate among closely related species (Heinze and Homberg, 2007). In plant, both chloroplast DNA and nuclear sequence data are a versatile tool for plant identification and establishing genetic relationships among plant species such as *matK, trnK, psbA,* 18S *rRNA,* ITS and IGS (Chase *et al.*, 2007; Johnson *et al.*, 2008a). Different sequence loci have been utilized for use at close and distant evolutionary distances (Karp *et al.*, 2012; Nock *et al.*, 2011). In this study, one chloroplast and two nuclear regions were employed.

2.6.1.1 Chloroplast DNA transfer RNA-Leucine and phenylalanine region *(trnL-trnF spacer)*

Chloroplasts region contains highly conserved genes that fundamental to plant life and variable regions which are informative over broad time scales. In non-coding regions of chloroplast DNA, it tends to evolve more rapidly than coding regions. Because the accumulation of insertion and deletions that making

them very useful below the family level (Gielly and Taberlet, 1994). The chloroplast *trnL-trnF* spacer region begins with the *trnL* UAA gene, which includes a group I intron, followed by the spacer that precedes the *trnF* GAA (Figure 2.7). The *trnL* UAA gene is noteworthy for its group I intron (Quandt and Stech, 2005). It is only intron known in the chloroplasts genome. Accordingly, the publication of universal *trnL -trnF* primers make this region has become one of the most heavily used in plant systematics (Huttunen *et al.*, 2013; Stech *et al.*, 2012). Moreover, *trnL-trnF* spacer region might be the one of preferred candidates for species identification (Kress *et al.*, 2009; Shaw *et al.*, 2009). This region used to employ for relative study among *Araceae* and *Lemnaceae*. The result found that *A. glossophyllus* and *A. variabilis* are sister group which are closely related (Rothwell *et al.*, 2004). In the same time, Tam *et al.* (2004) also studied in *trnL-trnF* region for phylogenetic analysis of 118 aroid taxa found that this region was useful for inferring subfamilies and tribal circumscription in *Araceae*.

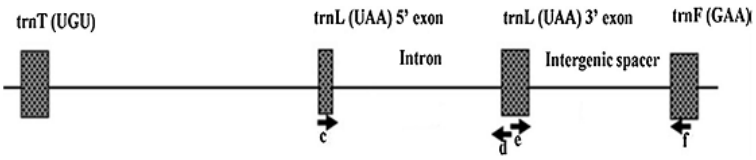

Figure 2.7 Structure of the *trnL–trnF* region with spacer and intron, and the primer sites. (Sarra *et al.*, 2015)

2.6.1.2 Second intron of *FLORICAULA/LEAFY (FLint2)*

FLint2 is a single copy nuclear-encoded homeotic gene in diploid angiosperms (Frohlich and Parker, 2000). There are three exons and two well conserved introns (Figure 2.8). This region is involved in maintaining a transient

phase of indeterminacy that precedes differentiation of lateral derivatives of the apical meristem, flowers, leaves or tendrils (Carmona *et al.*, 2002; Frohlich and Parker, 2000). Since low-copy nuclear genes are informative source of phylogenetic construction in plants, the *Flint2* region is increasingly employed with great success result (Zimmer and Wen., 2013). Previous study showed that *FLint2* region is different in sequence length within one or the same plant species such as 358 until 855 bp in *S. glandulosus* and 637 until 1070 bp in *I. scapigera.* It is indicated that the length polymorphism in *FLint2* became a handy tool for solving problems in plant evolution and systematics (Pankin *et al.*, 2008).

Oh and Potter (2003) used *Flint2* for phylogenetic contraction of Neillia and Stephanandra (*Rosaceae*) found that this region provides more than twice phylogenetically informative characters when compared with the other nuclear data (ITS region) and provide more than ten times when compare with chloroplast DNA data (*trnL-trnF*, *trnD-trnY-trnE-trnT* and *matK-trnK*).

Peng *et al.* (2010) studied phylogenetic tree of *Avena* based on *Flint2* sequences analysis. The result showed that this region is useful by obtain highest percentage of informative characters , overall homoplasy levels, number of well-supported clades and resolution of ingroup relationships.

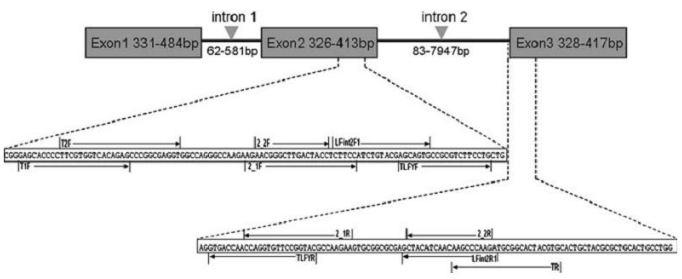

Figure 2.8 Schematic drawing of the *FLORICAULA/LEAFY* gene with amplified region and the location of the primers (Peng *et al*., 2010).

2.6.1.3 Nuclear ribosomal DNA internal transcribed spacer region (nrDNA ITS)

The internal transcribed spacer DNA (ITS) of the nuclear ribosomal cistron beginning with the 3′ end of the 18S DNA, extending through the 5.8S DNA and ending into the 26S DNA, shown in Figure 2.9 (Rogers and Bendich, 1987; Vanderpoorten *et al.*, 2006). ITS region is commonly used as molecular marker for interspecific and intergeneric levels in plants because their rapid concerted evolution. Moreover, this region show higher amount of divergence sequence when compared to flanking coding regions, which could provide better resolution of infraspecies relationships (Chen *et al.*, 2010; Rautenberg *et al.*, 2010). In addition, ITS region is high copy numbers that make them amplifies readily even from older templates and suitable for direct sequencing of PCR products (Gielly *et al.*, 1996)

Low *et al.* (2014) studied phylogenetic tree of Schismatoglottideae (*Araceae*) with nuclear ITS region. The result showed that this region is rich with the GC nucleotide and provides highest informative characters. Consequently, support values in phylogenetic tree were improved. It indicated that this region is useful at a low taxonomic level in *Araceae* group.

Tippery *et al.* (2015) studied *Lemnaceae* family evolution by using nuclear ribosomal data. The result found that ITS region data provide highest supported relationships. In addition, the secondary structure of RNA transcripts for this family was modelled with ITS. The result showed that this region has ability to generate functional and homologous RNA.

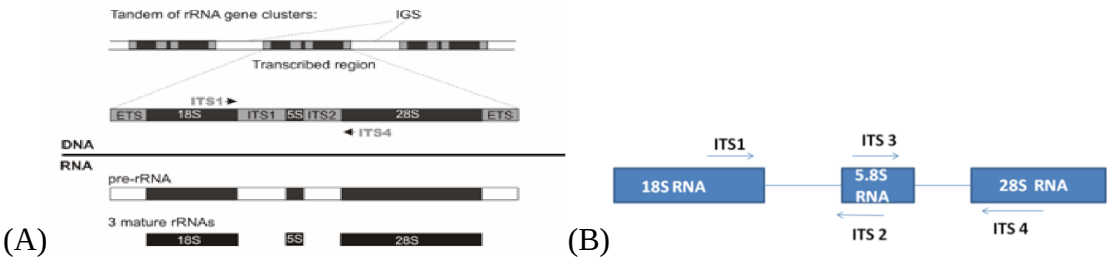

Figure 2.9 (A) Location of ITS region and (B) position of primers for PCR amplification (Shahid *et al.*, 2015).

2.6.2 RAPD (Random Amplified Polymorphic DNA) analysis

RAPD technique uses small single arbitrary oligo-nucleotides primer that hybridizes onto the complementary DNA fragments. When short nucleotides function in pair, they can be used to amplify a set of DNA fragments, shown in Figure 2.10 (Williams *et al.*, 1990). Each of those DNA fragments might be polymorphic between species depending on the genetic distance. DNA polymorphisms come from mutations, deletions and insertions in the genome after speciation (Li *et al.*, 2006). In contrast with DNA sequencing analysis, RAPD distributes throughout the genome, spanning both coding and non-coding DNA regions. Thus, this technique may be more representative of overall genetic patterns and being highly informative for phylogenetic analyses (Atienzar and Jha, 2006; Lopes *et al.*, 2012). RAPD is one of molecular techniques that are most frequently employed due to its sensitivity, simplicity, cost-effectiveness by a large number of samples that can be analyzed quickly and does not require DNA sequence information (Bardakci, 2001; Bussell *et al.*, 2005; Venkatachalam *et al.*, 2008). Moreover, single arbitrary primer allows amplifying of several bands which correspond to several loci. Therefore, the number of primers that can be used is practically unlimited and has potentially covered the

entire genome (Kumar *et al.*, 2013). RAPD is frequently used in genetic population studies to estimate affiliations between closely related plants (Khan *et al.*, 2011b; Kim *et al.*, 2008; Verma *et al.*, 2009).

Venkatachalam *et al.* (2008) used RAPD technique for DNA identification among banana cultivars. The result showed that effective ability of this technique effecting produced clear and reproducible patterns of multiple bands and obtained high percent of polymorphic. RAPD data also showed clear and similar separation between ingroup accession even genotype and fruit characters. Moreover, RAPD provided strong support for their hybrid origins. These observations indicated that RAPD is useful to differentiate two different cultivars for establishing the genetic similarity and distance.

Domyati *et al.* (2011) studied a genetic variation of seven wild medicinal plants in Sinai with comparison molecular techniques including RAPD, ISSR and AFLP. RAPD technique showed the highest level of polymorphism and highest expected heterozygote than other techniques. Therefore, RAPD is able to clarify interspecies populations in plants.

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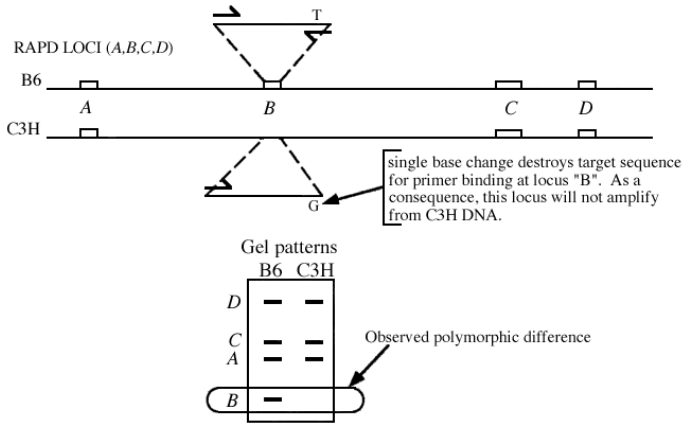

Figure 2.10 Principle of RAPD-PCR technique and arrows indicate primer annealing sites (Silver, 1995).

For increasing discrimination efficiency, long RAPD primers (LP-RAPD) can be optional used. LP-RAPD is sensitive to intra and interspecific genetic variation, high reproducibility and stable than shorter primers. Several LP-RAPD primers were designed for gene-specific PCR resulting in robust and informative RAPD profiles. Previous studies confirmed that LP-RAPD yielded more polymorphic bands than the shorter primers (Finger *et al.*, 2006; Gilling and Holley, 1997; Latif *et al.*, 2008).

2.6.3 Genetic variation of plant in the genus *Amorphophallus*

Researches on genetic variation in genus *Amorphophallus* still limited. However, there are two main techniques that can be employed for the genetic variation study including DNA sequencing and RAPD analysis.

2.6.3.1 Genetic variation by DNA sequencing analysis

Grob *et al.* (2002) peformed phylogenetic study of tribe Thomsonieae (*Araceae*) that comprise two genera of *Amorphophallus* and *Pseudodracontium* with chloroplast *matK* and the *trnL* intron. The result showed that this genus can be divided into five major clades that correspondence with their morphology include (i) monophyly clade of the African species; (ii) second clade of paraphyletic or polyphyletic 13 Asian species with heterogeneous morphology. The unique characteristic for this clade is white color berries; (iii) third clade contains 15 species of six Asian groups and two members of the Longituberosus group. This study demonstrated the close relation between *A. sumawongii* and *A. polyanthus* that share a striking similarity in the berries; (iv) fourth clade is characterized by 12

bp insertion in *matK*. This clade is a highly heterogeneous morphologies except relatively in rhizomatous offsets character; (v) fifth clade compose of *A. longituberosus* and *Pseudodracontium* species indicated that *Amorphophallus* is paraphyletic to *Pseudodracontium*. However, this study concluded that relationship among phylogenetic clades remains unresolved and branched connecting in these clades are poorly supported.

Fei *et al.* (2003) studied mannose-binding lectin gene (*aka*) in *A. konjac*. This study found that the *aka* lectin gene from this species related with other monocot species.

Grob *et al.* (2004) introduced utility of *FLint2* as a tool for phylogeny reconstruction in *Amorphophallus*. The result found that *FLint2* is rather short (143–222 bp) with highly variable and unsaturated in this genus. The *FLint2* phylogeny showed largely congruent with chloroplast regions (*rbcL*, *matK*, and *trnL*). However, in combined analysis found incongruent with chloroplast data in African subclade. In addition, *FLint2* provides four characters to support the relationships among *A. ankarana*, *A. dracontioides*, *A. taurostigma*, and *A. eichleri* while chloroplast data provides only one character. This informed that *FLint2* are clear contributions. However, in this study found that *FLint2* region generate different length of DNA products in *A. napiger* but it fails to relate two *A. napiger* products as sister clade. Moreover, this region has some limitations with unresolved of polytomies clade and lack of ability to provide enough non-conflicting informative characters to produce highly informative phylogeny.

Sedayu *et al.* (2010) constructed a phylogenetic tree by combining of *trn*L, *rbc*L and *LEAFY* in 69 taxa of *Amorphophallus*. The result

showed major clades reflect to biogeographical distribution and some morphological synapomorphies. The result showed a relationship in *A. paeoniifolius* and *A. bangkokensis* in third clade even their considerable vegetative morphological differences. Moreover, this study found small clade of purely Thai-Indochinese species that is grouped in the large Southeast Asia clade. This small clade can not be supported by morphology except elongate tubers. This unique morphology refers to a character of Thai-Indochinese species that absent in all other Southeast Asian species. Morphological evolution was analyzed with molecular based phylogenetic tree. The result found that (i) a nonsessile stigma may have evolved from a sessile one with several reversals; (ii) pollen opening by connective rupturing evolved from pollen opening by pores; (iii) unequally shaped segments of the lamina evolved from equally shaped segments three times in the Asian clades; (iv) simultaneously existing leaf and inflorescences evolved from alternating leaves and inflorescences; (v) blue, purple, green and yellow berries evolved from red/orange and white berry. In blue-berried clade found variable in florescence morphology especially in *A. brevispathus*, *A. coaetaneus*, *A. kiusianus* and *A. yunnanensis*. This result indicated morphology is flexibility in this genus which may due to a strong adaptability to different pollination resources.

Wahyudi *et al.* (2013) studied genetic variability in *A. muelleri* from west and central Java by *trnL* intron. The result found 13 variation sites and 9 informative sites that provide information for create phylogenetic tree. The analysis of each population found in small variation. The highest number of haplotype found in population of Grobogan, central Java due to it is a wide plantation area.

Consequently, *A. muelleri* seeds were possibly obtained from different regions. On the other hands, the least number of haplotype can be found in population of Wonogiri and Karangtengah, central Java. It may due to small and limited cultivation area thus the seeds may come from the same area. Moreover, seeds of *A. muelleri* are apomict formed thus offspring is same identical to the parent. Phylogenetic tree showed that clade of *A. muelleri* can be divided based on their locality. However, the populations in the same location (Cisompet, west Java) are not clustered together. It could suggest that even *A. muelleri* regenerated through vegetative bulbil but the offspring showed differentiate relationship from their parent in *trn*L intron.

2.6.3.2 Genetic variation by RAPD analysis

RAPD technique has been used for investigation of population genetic variation to estimate affiliations between closely related plants. It has been used in several studies in *Amorphophallus* population.

Wenbing *et al.* (2001) studied genetic diversity among wild and cultivated specimens of *A. konjac* by RAPD analysis. The result showed percent of polymorphism at 97.4%. RAPD analysis produced 11 bands and showed polymorphism among cultivation sample. Clustering analysis was carried out by genetic similarity coefficients showed high level among sample (0.97- 1.00). While, genetic similarity between wild and cultivated species was found in medium level (0.48-0.64).

Hu *et al.* (2008) analyzed somaclonal variation of *A. albus* by RAPD analysis. The amplified band ranged from 150 bp to 2 kb with average 7.3 bands per primer. The result showed 20.8% polymorphisms amplify bands from 14

primers. This result refers to high-frequency somaclonal variation of this species. Cluster analysis indicated high similarity coefficients ranged from 0.85 to 1.00 among the plants. This output allowed to classify the plants into distinct groups.

Poerba and Yuzammi (2008) estimated genetic variation of *A. titanum*, from two populations in Sumatra using RAPD analysis. The result showed that RAPD primers can generate 143 scorable bands which 95.84% of them are polymorphic. The range of genetic dissimilarity value among populations was found in low-medium level (0.14-0.59). On the contrast, range of genetic dissimilarity within population was found in high level (0.67-0.77). It indicated that *A. titanum* from Sumatra has genetically diverse among genotypes more than among populations.

Poerba and Martanti (2008) identified genetic variability of six population of *A. muelleri* from east to central Java with RAPD analysis. The results showed that five RAPD primers generated 42 scorable bands of which 69.05% are polymorphic. Size of the bands varied from 300bp to 1500 bp. For clustering analysis, *A. muelleri* accessions were divided into two major clusters, which some of them were grouped based on their populations. The range of individual genetic dissimilarity found in low level (0.02-0.36). Moreover, a highest level of genetic variation was found in Saradan population due to it is conservation in this area whereas a lowest levels of genetic variation was found in Silo-Jember population.

2.7 Molecular markers

Specification of genetic identification and genetic diversity assessment of plant are important in plant breeding program. Traditional identification methods are based on morphological characters such as leaf, inflorescence and fruit (Baker *et al.*, 1998).

However, in some plants morphology characters are highly variable especially in *Amorphophallus.* Therefore it is difficult to identify this genus in vegetative form without an experienced professional taxonomist (Grob *et al.*, 2002). Moreover, morphological characters are influenced by the environmental conditions, tissue specificity and development stage (Dixon *et al.*, 2007b). For these reasons, distinguishing and identification of plant species need a modern method that is more effective, rapid, independent on environmental factors and can be carried out at any stage of the life cycle of plant (Hollingsworth *et al.*, 2009). Among various developed techniques, molecular markers can be a powerful technique more than morphological characters for plant identification (Esa *et al.*, 2006; Terzopoulos *et al.*, 2005).

Molecular markers can be divided into two types include (i) biochemical markers which detect variation at the gene production level such as chemical product, proteins or amino acids; (ii) DNA markers which detect variation at the DNA level such as nucleotide changes by deletion, duplication, inversion and/or insertion (Griffiths, 2000).

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2.7.1 Biochemical markers

Biochemical markers such as volatile chemistry of inflorescence odors were used for discriminate genus *Amorphophallus* (Kite G.C. *et al.*, 1998). However, this marker is limit to classify *Amorphophallus* specimens due to small number of available volatile chemistries. Moreover, only flowering period was applied. Besides biochemical markers need laborious testing, time consuming, requiring large population sizes and space for testing (Khan *et al.*, 2011a; Sharma *et al.*, 2008).

2.7.2 DNA markers

DNA marker techniques, there differed by important features such as genomic abundance, level of polymorphism detected, locus specificity, reproducibility, technical requirements and cost (Table 2.5). Meanwhile, the ideal DNA marker should have criteria as follow: (i) polymorphic and evenly distributed throughout the genome; (ii) provide adequate resolution of genetic differences; (iii) generate multiple, independent and reliable markers; (iv) simple, quick and inexpensive; (v) need small amounts of DNA samples; (vi) link to distinct phenotypes and (vii) no require for information about the genome of those organism (Agarwal *et al*., 2008). Therefore, there are two different techniques namely RAPD and nucleotide sequence that are frequently employed to generate effective DNA marker. Despite RAPD is favorite marker but this marker lack of reproducibility result in less reliable for identification. Therefore, it is a better to improve the reproducibility of RAPD marker by converting RAPD amplicons into Sequence Characterized Amplified Region (SCAR) markers (Kiran *et al.*, 2010).

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2.7.2.1 SCAR marker

SCAR maker is PCR-based monolocus codominant marker that requires the use of two specific primers designed from nucleotide sequence established in cloned RAPD fragment linked to a trait of interest. Specific SCAR sequence primers for PCR amplification may be located at suitable position within or flanking the target RAPD amplicon that used to identify the polymorphism in different species (Figure 2.11). Therefore, this marker generates more reliable result (Dnyaneshwar *et al.*, 2006; Kiran *et al.*, 2010).

Table 2.5 Comparison of various aspects of frequently used molecular marker techniques.

	Abundance	Repro-ducibility	Degree of polymorphism	Locus specificity	Technical requirement	Quantity of DNA required	Major application
RFLP	High	High	Medium	Yes	High	High	Physical mapping
RAPD	High	Low	Medium	No	Low	Low	Gene tagging, Genetic diversity
SSR	Medium	Medium	Medium	No	Medium	Low	Genetic diversity
SSCP	Low	Medium	Low	Yes	Medium	Low	SNP mapping
CAPS	Low	High	Low	Yes	High	Low	Allelic diversity
SCAR	Low	High	Medium	Yes	Medium	Low	Gene tagging, physical mapping
AFLP	High	High	Medium	No	Medium	Medium	Gene tagging

* RFLP restriction fragment length polymorphism, RAPD random amplified polymorphic DNA, SSR simple sequence repeats, SSCP single strand conformational polymorphism, CAPS cleaved amplified polymorphic sequence, SCAR sequence characterized amplified region, AFLP amplified fragment length polymorphism.
Source: adapted from Agarwal *et al.* (2008)

Advantage of SCAR marker is reliable, less sensitive to reaction conditions resulting conduct in any laboratory and fast to screening numerous samples at one time (Agarwal *et al.*, 2008). Moreover, this marker can be carried out using unknown genomic DNA from any developmental stage or any tissue part (Kethidi *et al.*, 2003). In addition, SCAR markers can correlate between DNA identification data and quantity of selected phytochemical data aim to extensive applications in quality control of raw materials as qualitative/quantitative diagnostic tool (Ruzicka *et al.*, 2009).

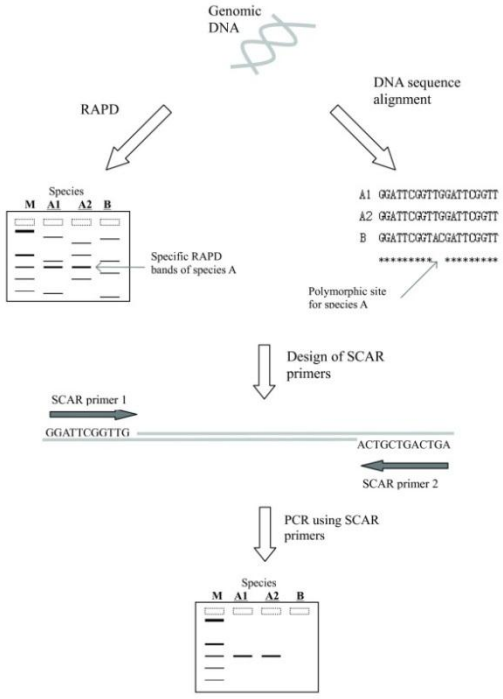


Figure 2.11 Principle of SCAR primers that are designed from RAPD analysis (Shaw *et al.*, 2009).

Marieschi *et al.* (2010) developed SCAR marker for detecting contaminant of commercial Mediterranean oregano aim to speed up the primary screening and allowed the pre-emptive rejection of suspect samples and lower number of pharmacognostic analyses. SCAR markers were developed from three RAPD markers that specific for contaminant plants. The SCAR primers gave specific bands with only 1% sensitivity. Moreover, the relatively short amplicons is suitable for the degraded DNA analysis from dried and stored commercial material.

Rajesh *et al.* (2013) designed SCAR marker to distinguish between tall and dwarf coconut in an early stage. The result showed that primer OPA09 produced a unique band at 260 bp in tall coconut accessions therefore SCAR primers were designed. SCAR marker produced a specific 260-bp amplicon in all tall

accessions, but not in dwarf coconut. Moreover, SCAR marker is utilized for determine the purity of hybrid of dwarf and tall cross seedlings.

2.8 Relevance of plant phytochemical and molecular analysis

DNA analysis with active phytochemical is useful as quantitative-trait loci identification. The study of phylogenetic relationship with plant phytochemical produces effective result than using morphological traits alone. Accordingly, morphological characters can not provide a thorough assessment of genetic diversity since this character is represented by a limited number of loci (Liu *et al.*, 2009; Rayapuram and Baldwin, 2006; Wink, 2003). In present, correlated between DNA markers and qualitative/quantitative variations in phytochemical among closely related species were increasingly studied.

Xia *et al.* (2005) studied chemical components in essential oil with molecular analysis of three species of *Rhizoma Curcuma* (Ezhu) including *C. wenyujin*, *C. phaeocaulis* and *C. kwangsiensis*. The major chemical components found in *Curcumae* species are curdione, curcumol and germacrone. The amount of these chemicals varies in different species or in the same species but grow in different regions. The 5S rDNA spacer markers were generated. The result showed that the phylogenetic result by DNA analysis is similar to the clustering of chemical fingerprints. Moreover, this marker strongly supported *C. wenyujin* and *C. chuanyujin* as two distinct species that can produced highest major constituents in the essential oils of *Rhizoma Curcumae*.

Guo *et al.* (2015) studied quantitative trait of anthocyanin profile in zicaitai (*Brassica rapa*). Seventeen anthocyanin compounds were identified as cyanidin

glycosides in zicaitai. Genetic linkages by 161 insertion/deletion markers, which are candidate genes for control of anthocyanin accumulation in zicaitai, were constructed. The linkage between marker and phenotypic data found that 56.7 % of phenotypic variation. Moreover, those markers showed a positive discrimination between low and high anthocyanin accumulation in zicaitai. It is indicated that study of combining gene marker with anthocyanin accumulation conducted to clarify pigmentation signaling target genes.

The previous studies indicated that proper integration of DNA techniques and phytochemical composition will lead to the development of a comprehensive system of botanical characterization. Moreover, this relation can be conveniently applied at the industry level for quality control of botanical products (Sharma *et al.*, 2008).

In case of *Amorphophallus* species, KGM is valuable product in this species and the content of KGM trend to relate with species. Therefore, it is important to investigate relationship between their genetic, geography and KGM variation on *Amorphophallus* sp. for tagging economic importance genes by creating specific markers. Accordingly, specific markers will be on effective tool for prediction of konjac quality and available tool for selecting suitable *Amorphophallus* species for konjac powder production in the country.

2.9 Multiple sequence alignment method

A sequence alignment is a set of corresponding residues among a collection of nucleotide or amino acid sequences. The sequences in an alignment are assumed to be homologous, which derived from a single common ancestral sequence. Aligned residues are usually interpreted as sharing their evolutionary origin. When a sequence

has no corresponding residue due to an insertion or deletion event, the position is displayed as gap (Yang *et al.*, 2013). Multiple sequence alignment (MSA) is an important step for rapidly construct a reasonable MSA. Moreover, the optimal MSA is successfully obtained once pay attention to suitable algorithmic techniques (Katoh and Toh, 2008). In this study, three optional algorithmic methods were used for generated accuracy and stability MSA.

2.9.1 MAFFT

The MAFFT algorithm has been gaining in popularity in recent years due to its high performance by uses an iterative refinement approach result in fast alignment algorithm (Katoh and Standley, 2014). Moreover, MAFFT were recommended as basis MSA software that should be used (Faircloth, 2015). The MAFFT sequence aligner was originally developed to perform the rapid calculation of large number of sequences MSA. A fast group-to-group alignment algorithm based on Fast Fourier Transform (FFT), which helps to fast calculation for long sequences and the Normalized Similarity matrix, and an approximate distance calculation method (the 6mer method) help to facilitate the rapid calculation. The MAFFT algorithm is employed into two sequential steps (Katoh *et al.*, 2009). MAFFT uses a two-cycle progressive method aiming to achieve a reasonable balance between speed and accuracy (Figure 2.12). In first cycle including (i) the first part is called FFT-NS-1, which involves calculating pairwise distances based on the number of shared k-mers. The objectives of this part are to build an initial phylogenetic tree and progressive alignment. Group-to-group alignment calculations are performed along with a guide tree; (ii) in the second part is called FFT-NS-2, helps to improve

the distance matrix and the guide tree. In the second cycle, consistency-based scoring is employed with iterative refinement. An alignment is divided into two sub alignments and then the two sub alignments are re-aligned again. This process is repeated until no more improvements are made aim to obtain a highest objective score alignment. Finally, refinement method was proposed. The aim of this method is to overcome the progressive method's drawback of incorrectly introduced gap, especially at an early step.

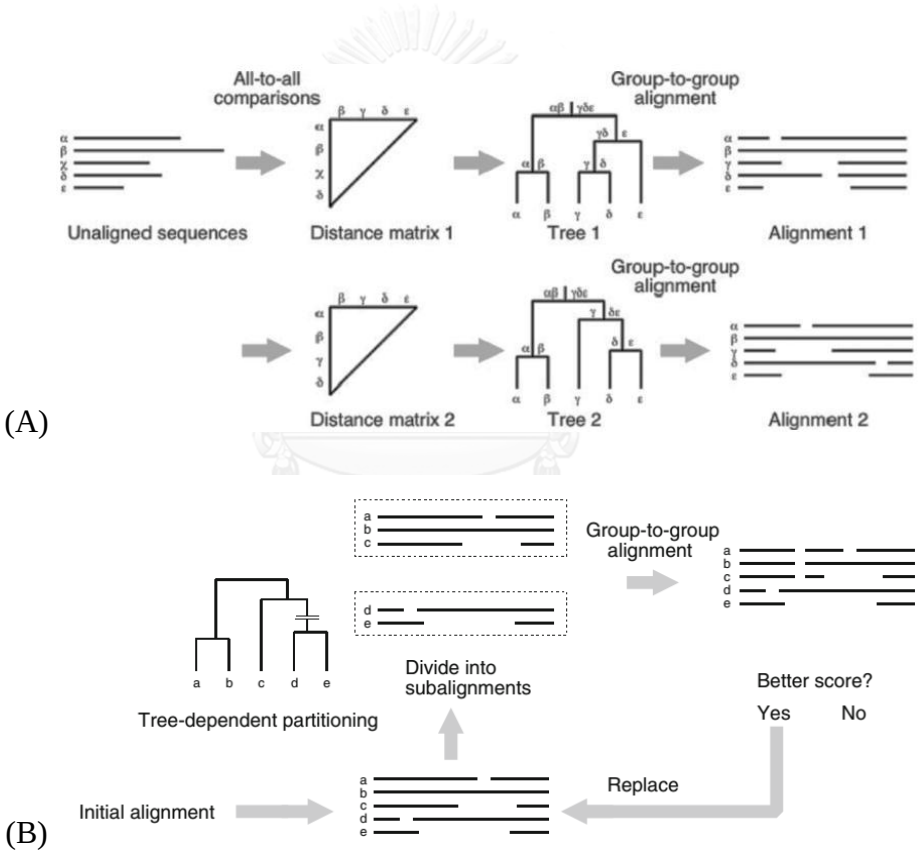


Figure 2.12 Calculation procedures for (A) the progressive options and (B) iterative refinement options in MAFFT (Katoh and Standley, 2014).

Furthermore, MAFFT alignment has several different options depending on different types of input sequences. One of them is L-INS-i option uses as local pairwise alignment with the affine gap cost. This option allows large terminal gaps, which is the most accurate but time consuming (Morgenstern, 2014). In addition, L-INS-i is suitable for sequences that have homology only in partial regions (Katoh and Toh, 2008). Nowadays, several of studies found MAFFT alignment was useful in their research such as evolution of polycomb protein in land plant (Mosquna *et al.*, 2009), diversity of wetland plants (Wang *et al.*, 2011), molecular evolution in *Argonaute* family (Singh *et al.*, 2015).

2.9.2 BMGE (Block Mapping and Gathering with Entropy)

The method was introduced by Criscuolo and Gribaldo (2010). BMGE based on the fact that removing of ambiguously aligned regions with other sources of bias such as highly variable characters will help to improve the overall performance of phylogenetic tree. This software was designed to select regions in a MSA that are suited for phylogenetic tree. For each character, BMGE computes a score closely related to an entropy value (Revuelta *et al.*, 2014). Calculation of these entropy-like scores is weighted with BLOSUM or PAM similarity matrices aim to distinguish among biologically expected and unexpected variability for each aligned character. Sets of contiguous characters with a score above a given threshold are considered as not suited for phylogenetic inference and then removed (Criscuolo and Gribaldo, 2011). BMGE software also employs a novel stationary-based trimming method that allows compositionally heterogeneous characters to be identified and removed. Consequently, BMGE uses the Stuart's χ^2 matched-pairs test of marginal symmetry

that allows assessing the null hypothesis. Two sequences are compositionally homogeneous and iteratively perform character removal or addition steps until the Stuart's test assesses that each pair of sequences presents homogeneous composition. This stationary-based trimming leads to unbiased phylogenetic trees. Moreover, BMGE also aimed to minimize phylogeny reconstruction due to compositional heterogeneity (Kumar *et al.*, 2013). Therefore, BMGE is able to perform biologically applicable trimming on a MSA of DNA, codon or amino acid sequences. For highly diverse dataset, it is important to analyze homology genomic regions due to they may contain a higher probability that associated with functional domains in proteins. Several analyses found that the character trimming performed by BMGE produces datasets leading to accurate trees, especially distantly-related sequences alignments. Therefore, BMGE seems to be a good estimator to select those regions (Batista *et al.*, 2011).

Criscuolo and Gribaldo (2011) studied strong heterogeneous composition in plastid regions (191 genes) within cyanobacteria. The characters were trimmed with the software BMGE. The result found that phylogenetic tree obtains high confidence values for the majority of branches. Moreover, BMGE approach revealed clearly relationships within *Euarchontoglires*.

Abby *et al.* (2014) studied symbiotic interactions with 13 different *Ostreococcustauri* culture in microbial communities. The alignment was selected informative sites by BMGE. The result showed a clear association between T6SS sub-types and their function that is difficult to define from total genomic analyses.

2.9.3 PRANK (Phylogeny-aware alignment algorithm)

This method was first introduced by Löytynoja and Goldman (2010). PRANK is a phylogeny-aware alignment algorithm that has been using to produce excellent alignments for evolutionary phylogeny. Unlike other alignment programs, PRANK makes different in phylogenetic information by distinguishing between alignment gaps. Those gaps caused by insertions and deletions. Afterward, PRANK handles the two types of these events differently (Löytynoja, 2014). By identifying the true evolutionary event, PRANK can handle insertions correctly and avoid penalizing the single event multiple times in later stages of the alignment. Meanwhile in a deletion, the gap needs to be created only once and PRANK removes the flag indicating the gap after the second alignment (Szalkowski, 2012). Therefore, PRANK can distinguish and correctly align near-by insertion and deletions. The classical alignment algorithm considered length differences as deletions and cannot place independent insertions in separate columns with often moves near-by gaps. This action indicates false homologies and resulting in substitutions (Larkin *et al.*, 2007). Whereas, PRANK considers the re-use of a flagged gap as evidence that the gap was created by an insertion. It then changes the flags indicating a pre-existing gap (filled diamond) to ones indicating a permanent insertion (filled square) and does not allow matching of these sites at later alignments. This forces the correct placement of independent insertions into separate alignment columns (Löytynoja, 2014). This phenomenon with evolutionary tree can be showed in Figure 2.13 of five short sequences with two insertions and two deletion events.

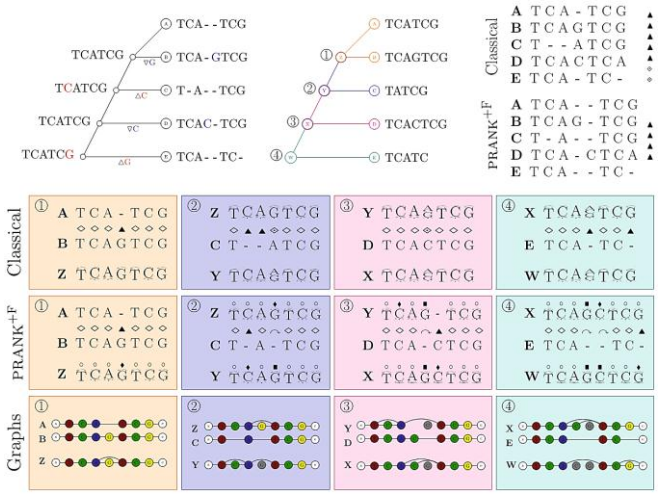


Figure 2.13 The phylogeny-aware algorithm distinguish and correctly alignment by insertion and deletion (Löytynoja, 2014).

PRANK algorithm can compute column-wise reliability scores for the alignment. When a structure model is used, it provides posterior probabilities for the alignment sites evolving under different evolutionary processes. The reliability and probability values are generated by the pair-wise alignments at the different levels of the progressive alignment and associated with the internal nodes of phylogenetic tree. The PRANK alignment provides reliability scores and objective measures to remove less reliably aligned columns from the data. As a part of the alignment process, PRANK algorithm reconstructs the sequence history inferred with ancestral nodes (Löytynoja, 2014; Löytynoja and Goldman, 2010).

Fischer *et al.* (2014) studied largest recurrent gene duplication on adaptation of ten angiosperm genomes. The clusters were aligned using PRANK aim to detect positive selection footprints of LSE genes. They found that this method help to produce most reliable alignment for downstream analysis. Moreover, PRANK provided strong empirical support for the hypothesis that LSE genes are fuel adaptation in angiosperm.

2.10 Phylogenetic tree reconstruction method

Phylogenetic tree or phylogeny is the branch of systematics relating. A phylogeny refers to a genealogical history of a group of organisms and represent of hypothesized ancestor or descendant relationships (Gouy *et al.*, 2010). The phylogeny is visualized by a branching pattern which can be determined by either living or fossilized organism characters (Tamura *et al.*, 2011). Phylogenetic analysis has become an important tool for studying evolutionary in organism. Since the rate of sequence evolution varies with DNA segment. There are many statistical methods that can be used for reconstructing phylogeny from molecular data. Commonly used methods are many classified such as distant method, parsimony method and Bayesian method (Doyle and Gaut, 2000; Yang *et al.*, 2013).

2.10.1 Phylogenetic tree

Phylogenetic tree is a one kind of graph containing nodes connected by lines, usually called braches. External or terminal nodes represent the existing taxa and are often called operational taxonomic units (OTUs) while internal nodes may be call hypothetical taxonomic units or HTUs (Figure 2.14). A particular node can be selected as a rooting or unrooted point of the phylogenetic tree. An unrooted tree shows relationships between organisms but not the pattern of ancestor and descend pathway. Whereas, a rooted phylogeny infers the ancestor and descendant relationship along the direction of each branch (Wilkinson *et al.*, 2007). For phylogeny's branch lengths indicate the similarity or dissimilarity between two species, or the length of time since their species separation (Kress *et al.*, 2009).

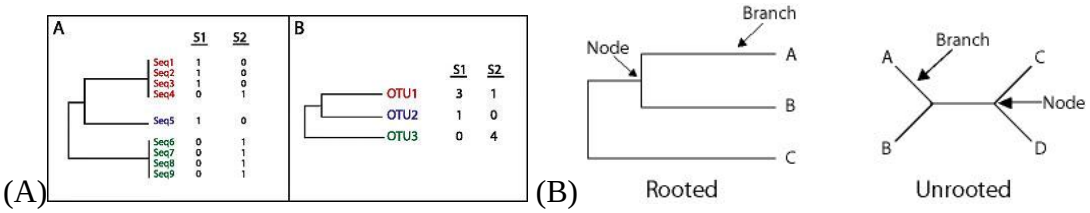


Figure 2.14 (A) Different phylogeny formats represent within same data; (B) rooted and unrooted phylogenetic tree (Lozupone and Knight, 2015; Wilkinson *et al.*, 2007).

2.10.2 Evaluation of DNA fragment patterns

A fingerprint pattern generating from different DNA samples by compared to each other. The preciseness and accuracy of DNA band-scoring are importantly dependent on methodological parameters such as DNA quality, completeness of PCR reaction and electrophoresis conditions (Weising *et al.*, 2005). The DNA banding patterns are scored in term of presence (1) and absence (0) of polymorphic bands in each samples (Ren and Timko, 2001).

2.10.2.1 Similarity index

When the fingerprint patterns have been generated and score bands are assigned to specific positions in all lanes to be compared. Different strategies may be followed to quantify the pairwise similarity of sample that represented in different lanes. Normally, a similarity index is calculated from band-sharing data of each pair of fingerprints (Lynch, 1990). The similarity matrix is used to assess relationships among species with a dendrogram (tree diagram) by cluster analysis. The coefficient formula acquires values between 0 and 1, where 0 means no bands in common and 1 means pattern is identical (Zhang *et al.*, 2011). In this study, Nei's and Li's coefficient (N) was used (equation 1). With principle that Nei and Li

(N) compare the number of bands that shared between individuals. The coefficient of Nei and Li can generate higher weight on positive matches than other coefficient method. Moreover, this method is advantageous due to the band absence can be excluded from analyses and there are no assumptions of Hardy-Weinberg equilibrium (Culley *et al.*, 2007).

$$N = 2a / (a+b) (a+c) \quad \text{(1)}$$

where: a = number of bands present in both individuals.

b = number of bands present in individual A absent in individual B

c = number of bands present in individual B absent in individual A

2.10.3 Tree construction

The main propose of a phylogenetic tree or dendrogram construction is determine relationship and past evolutionary history among individuals, cultivars or populations within species (Swofford *et al.*, 1990). In this study, two commonly methods were used for phylogeny constructions include character-state method like Bayesian method and distance matrix method like Neighbour-joining (NJ).

2.10.3.1 Bayesian Method

Bayesian method is character-state methods that use an optimality criterion like maximum parsimony and maximum likelihood, but do not attempt to search only the single best tree. Bayesian methods also employ the concept of likelihood but search for a set of plausible trees for the data by targeting a probability distribution of trees (Huelsenbeck and Ronquist, 2001). This posterior probability of phylogenetic tree inherently holds an estimate of a confidence of any

evolutionary relationship. Bayesian methods require the prior distribution on the model parameter such as substitution model, branch lengths and tree topology (Nylander *et al.*, 2004). The relative evidence present in the data is used to evaluate how one should update the prior belief (Ronquist *et al.*, 2009; Yang *et al.*, 2013). Posterior probability can obtain by exploring tree space using sampling technique, called Markov chain Monte Carlo (MCMC). MCMC sampling methods starts by simulating a random set of parameters and propose a new state (new set of parameters) by randomly changing the parameter to some extent. In each step, the likelihood ration and prior ratio is calculated for the new state. The ratio r of the posterior probability of the new and current states is then calculated. If $r > 1$, posterior probabilities are moving uphill and the move is always accepted. If $r < 1$, posterior probabilities are moving downhill and the state is rejected (Figure 2.15A) (Mills and Parent, 2014). After an initial convergence to set a probable model solution is called burn-in. This algorithm samples from the posterior probability distribution. The frequency by a particular tree topology is sampled then proportional to its posterior probability. The tree results are usually presented as a consensus tree or maximum posterior tree (Figure 2.15B). Bayesian methods are computer intensive but the support values for the cluster in a tree can be derived from a single MCMC. Bayesian inference provides a natural way of taking phylogenetic uncertainty into account (Ronquist *et al.*, 2009; Yang *et al.*, 2013).

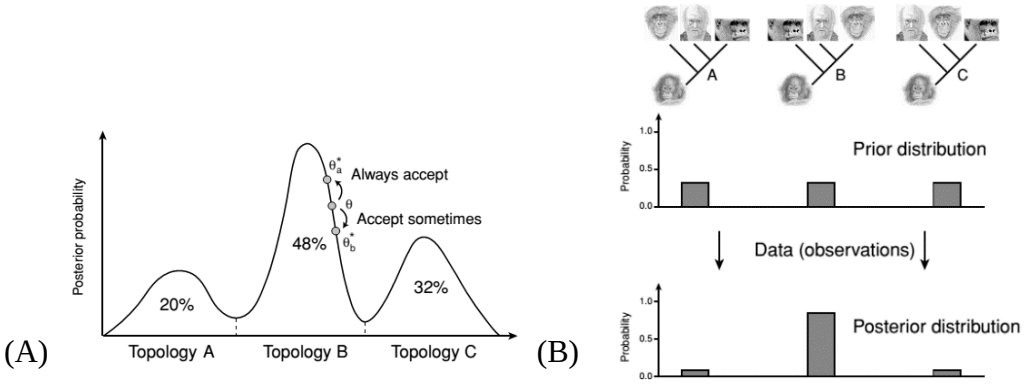
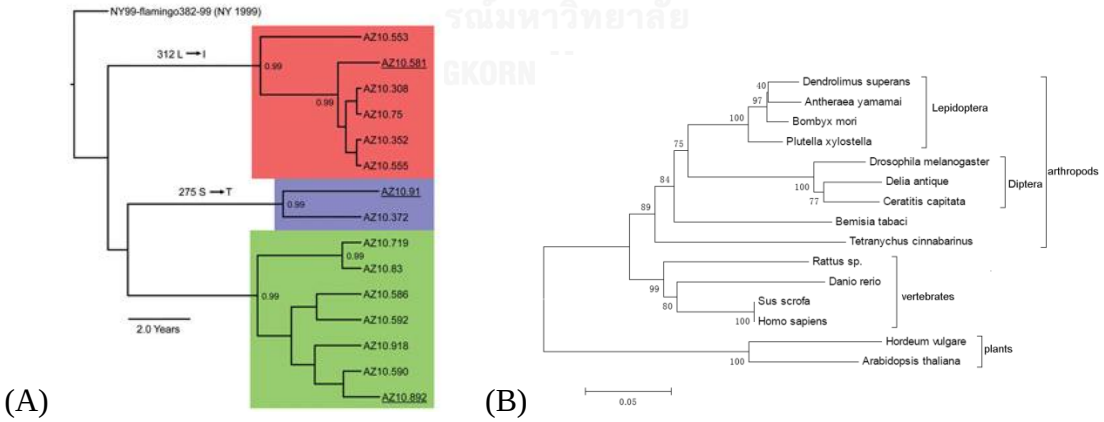


Figure 2.15 A Bayesian phylogenetic analysis with (A) The Markov chain Monte Carlo (MCMC) procedure; (B) the posterior probability focused on consensus after analysis. (Ronquist *et al.*, 2009).

2.10.3.2 Distance matrix

This method is reconstructed a phylogeny by beginning with considering of pairwise distances between data that is the relationships among distance values (Nei and Kumar, 2000). This approach is not particularly base on character because a pairwise distance matrix, rather than changes at individual nucleotide position that is used in the tree building process (Doyle and Gaut, 2000). Distance method is proved to be useful for actual data analysis. In this study, Neighbour-joining or NJ method was used. NJ method constructs a tree by sequentially finding pairs of neighbours, which is the pair of OTUs by a single interior node. This method does not cluster the most closely related OTUs, but rather minimize the length of all internal branches and thus the length of the entire tree. The NJ algorithm starts by assuming a star-like tree that has no internal branches. First, NJ introduces the first internal branch and calculates the length of the resulting tree. The algorithm sequentially connects every possible pair of the OTUs. It finally joins the OUT pair that yields the shortest tree. This process is then repeated again

and again, and always joining two OTUs (neighbours) by introducing the shortest possible internal branch. The length of a branch joining a pair of neighbour to their adjacent node is based on the average distance between the OTUs and all other OTUs, subtracting the average distance of all remaining OTU pairs (Figure 2.16) (Hollingsworth and Ennos, 2004). This method is very suitable with dataset consisting descendants with largely varying rates of evolution. The very fast neighbour-joining algorithm provides a good approximation of the minimum evolution tree therefore NJ method is favorited for distance analyses (Doyle and Gaut, 2000). In addition, the advantages of this method including (i) relatively rapid thus it is suitable for analyzing a large dataset; (ii) calculate the branch length; (iii) allow to correct multiple sequences and (iv) construct only one possible tree. However, some disadvantages can be found such as yield a biased tree under some condition, compress sequence information and depend heavily on the evolutionary model applied (Simonsen *et al.*, 2008).



(A) (B)

Figure 2.16 (A) Bayesian phylogenetic tree (B) NJ dendrogram (Feng *et al.*, 2010; Plante *et al.*, 2014).

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant Materials

Forty-eight accessions of *Amorphophallus* belonging to 37 known species composed of wild (14 species) and cultivars (23 species) were used in the study (Table 3.1). Morphological characters of the wild plants samples were collected and identified by Faculty of Agricultural Technology, Lampang Rajabhat University, Lampang, Thailand and comparison with related literatures. All leaf material of cultivated species except *A.bulbifer*, *A. xiei* were collected from living plant in Hamburg botanical garden (Germany) from the private collection of W. L. A. Hetterscheid and Gothenburg botanical garden (Sweden). *A. bulbifer* and *A. xiei* were collected from researchers' collections in Yunnan, China. All leaf material were subjected to dry by freeze-drying or silica gel and kept at room temperature until needed for experiment. Fresh tubers of the plants were taken only from wild species including *A. bulbifer* and *A. xiei*. They were washed with tap water to remove soil, dirt and other contaminants. Excess water was removed with blotting paper then those tubers were storAed in room temperature before further processing. The voucher specimens of these plants have been deposited at department of biological and environmental sciences, faculty of sciences, Gothenburg University, Sweden.

Table 3.1 Details of *Amorphophallus* samples used in Sequencing and RAPD analysis

Accession	*Amorphophallus* Species [a]	Location	Coding No.	Voucher no.	Habitus	Collection Part	GenBank accession numbers		
							*trn*L-*trn*F	*FLint2*	ITS
1	*A. muelleri* Blume	Northern Thailand, Mae Hong Son	MU-ME01	ME05P1T1	wild	Leaf/tuber	KR534380	KR534482	KR534417
2	*A. muelleri* Blume	Northern Thailand, Tak	MU-TK01	TK04P1T1	wild	Leaf/tuber	KR534379	KR534483	-
3	*A. muelleri* Blume	Northern Thailand, Mae Hong Son	MU-ME02	ME03P1T1	wild	Leaf/tuber	KR534381	KR534481	KR534415
4	*A. muelleri* Blume	Northern Thailand, Tak	MU-TK02	TK08P2T1	wild	Leaf/tuber	KR534378	KR534484	KR534414
5	*A. muelleri* Blume	South-Western Thailand, Kanchanaburi	MU-KC01	KC2010-05GZ	wild	Leaf/tuber	KR534382	KR534480	KR534416
6	*A. muelleri* Blume	South-Western Thailand, Kanchanaburi	MU-KC02	KC2010-02GZ	wild	Leaf/tuber	KR534383	KR534479	KR534418
7	*A. bulbifer* (Roxb.) Bl	China, Yunnan	BU-CN01	CN2010-01GZ	cultivated	Leaf/tuber	KR534405	KR534467	KR534430
8	*A. bulbifer* (Roxb.) Bl	China, Yunnan	BU-CN02	CN2010-02GZ	cultivated	Leaf/tuber	KR534404	-	KR534431
9	*A. xiei* Li & Dao	China, Yunnan	XE-CN01	CNX2010-01GZ	cultivated	Leaf/tuber	KR534366	KR534496	KR534412
10	*A. krausei Engl.*	Northern Thailand, Chiang Mai	KU-CM01	CM10P1T6	wild	Leaf/tuber	KR534395	KR534473	KR534425
11	*A. krausei* Engl.	Northern Thailand, Chiang Mai	KU-CM02	CM19P2T11/2	wild	Leaf/tuber	KR534390	KR534476	KR534420
12	*A. krausei* Engl.	Northern Thailand, Lampoon	KU-CM03	CM05P1T11/2	wild	Leaf/tuber	KR534391	-	KR534421
13	*A. macrorhizus* Craib	Northern Thailand, Lampoon	MC-LO01	LO03P2T5	wild	Leaf/tuber	KR534385	-	KR534445
14	*A. yunnanensis* Engl.	Northern Thailand, Lampoon	YN-LO01	LO03P2T2	wild	Leaf/tuber	KR534364	KR534497	KR534432
15	*A. konjac* K. Koch *	China	KJ-CN01	H.AM. 0251	cultivated	Leaf	KR534392	KR534475	KR534422
16	*A. paeoniifolius* (Dennst.) Nicolson	Northern Thailand, Chiang Rai	PF-CR01	CR01P3T4	wild	Leaf/tuber	KR534374	KR534488	KR534440
17	*A. sumawongi (Bogner) Bogner* **	South-Eastern Thailand, Sa Kaeo	SW-SK01	2010-1662 pG	cultivated	Leaf	KR534370	KR534493	KR534436
18	*A. thaiensis* S.-Y. Hu	Northern Thailand, Chiang Mai	TH-CM01	CM01P5T7	wild	Leaf/tuber	KR534367	KR534495	KR534433
19	*A. longituberosus* Engl. & Gehrm *	Northern Thailand, Tak	LT-TK01	H.AM. 1225	cultivated	Leaf	KR534386	KR534477	KR534446

Table 3.1(2) Details of *Amorphophallus* samples used in Sequencing and RAPD analysis (continue)

Accession	*Amorphophallus* Species [a]	Location	Coding No	Voucher no.	Habitus	Collection Part	GenBank accession numbers		
							*trn*L-*trn*F	*FLint2*	ITS
20	*A. napiger* Gagnepain *	South-Eastern Thailand, Sa Kaeo	NP-PS01	H.AM. 0708	cultivated	Leaf	KR534377	KR534485	KR534443
21	*A. corrugatus* N. E. Br.	Northern Thailand, Lampang	CG-LA01	LA01P1T3	wild	Leaf/tuber	KR534401	KR534470	KR534453
22	*A. tenuistylis* Hett. *	South-Western Thailand, Kanchanaburi	TU-SB01	H.AM. 0771	cultivated	Leaf	KR534368	KR534494	KR534435
23	*A. kachinensis* Engl. & Gehrm.	Northern Thailand, Mae Hong Son	KA-CM01	ME07P1T6	wild	Leaf/tuber	KR534394	-	KR534424
24	*A.tenuispadix* (scuatus)	Eastern Thailand, Chaiyaphum	TP-CP01	CP2010-C5	wild	Leaf/tuber	KR534369	-	KR534434
25	*A. asterostigmatus* Bogner & Hett.	Central Thailand, Saraburi	AS-SB01	JJ003	wild	Leaf/tuber	KR534408	KR534464	KR534457
26	*Pycnospatha arietina*	South-Eastern Thailand, Prachin Buri	EI-PB01	JJEL01	wild	Leaf/tuber	KR534399	-	KR534451
27	*A. pygmaeus* Hett.	South-Eastern Thailand, Chanthaburi	PM-CB01	JJPM01	wild	Leaf/tuber	KR534371	KR534492	KR534437
28	*A. atroviridis* Hett. *	Central Thailand, Saraburi	AV-SB01	H.AM. 0683	cultivated	Leaf	KR534406	KR534466	KR534455
29	*A. putii* Gagnepain *	Central Thailand, Saraburi	PU-SP01	H.AM. 0697	cultivated	Leaf	KR534372	KR534491	KR534438
30	*A. elatus* Ridl. *	Peninsular Thailand, Chumphon	EA-CP01	H.AM. 0853	cultivated	Leaf	KR534400	KR534471	KR534452
31	*A. atrorubens* Hett. & M. Sizemore *	North-Eastern Thailand, Udon Thani	AR-UR01	H.AM 729	cultivated	Leaf	KR534407	KR534465	KR534456
32	*A. harmandii* Engl. & Gehrm. *	North-Eastern Thailand, Udon Thani	HM-WCE01	H.AM. 1498	cultivated	Leaf	KR534396	KR534489	KR534448
33	*A. albispathus* Hett. *	South-Western Thailand, Kanchanaburi	AP-KC01	H.AM. 1451	cultivated	Leaf	KR534410	KR534462	KR534459
34	*A. carneus* Ridl. **	Peninsular Thailand	CN-SH01	2010-2819 pZ	cultivated	Leaf	KR534402	KR534468	KR534454
35	*A. obscurus* Hett. & M. Sizemore *	Eastern Thailand, Ubon Ratchathani	OS-UR01	H.AM. 1546	cultivated	Leaf	KR534376	KR534486	KR534442
36	*A. maxwellii* Hett. *	South-Western Thailand, Kanchanaburi	MW-KC01	H.AM 970	cultivated	Leaf	KR534384	KR534478	KR534444
37	*A. excentricus* Hett. **	Peninsular Thailand, Nakhon Si Thammarat	ET-NT01	2010-2824 pG	cultivated	Leaf	KR534398	KR534472	KR534450
38	*A. fuscus* Hett. **	Northern Thailand	FC-NH01	2010-1652 pZ	cultivated	Leaf	KR534397	-	KR534449

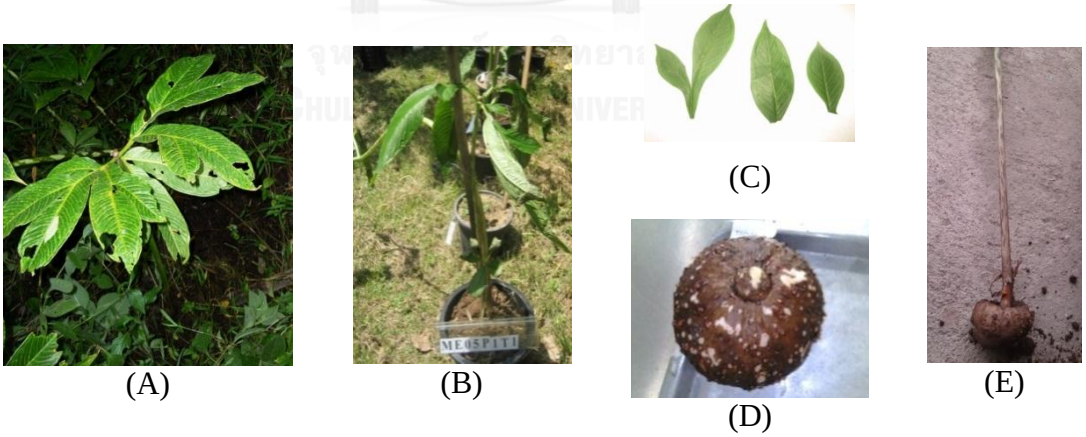
Table 3.1(3) Details of *Amorphophallus* samples used in Sequencing and RAPD analysis (continue)

Accession	*Amorphophallus* Species [a]	Location	Coding No	Voucher no.	Habitus	Collection Part	GenBank accession numbers		
							*trn*L-*trn*F	*FLint2*	ITS
39	*A. latifolius* Hett. & C. Claudel**	Northern Thailand, Chiang Rai	LF-WH01	2012-680 pZ	cultivated	Leaf	KR534388	-	KR534447
40	*A. xiei* Li & Dao	China, Yunnan	XE-CN02	CNX2010-02GZ	cultivated	Leaf/tuber	KR534365	-	KR534413
41	*A. konjac* K. Koch **	China	KJ-CN02	1982-V0424PG	cultivated	Leaf	KR534393	KR534474	KR534423
42	*A. aberrans* Hett. *	Central Thailand	AR-CH01	H.AM. 1225	cultivated	Leaf	KR534411	KR534461	KR534460
43	*A. bulbifer (*Roxb.) Bl *	Northern Thailand, Mae Hong Son	BU-TH01	H.AM.1451	cultivated	Leaf	KR534403	-	KR534429
44	*A. operculatus* **	Peninsular Thailand, Chumphon	OC-NE01	2008-1642 pG	cultivated	Leaf	KR534375	KR534487	KR534441
45	*A. prolificus* Hett. & A. Galloway **	Central Thailand	PL-CH01	2007-G-119	cultivated	Leaf	KR534373	KR534490	KR534439
46	*A. amygdaloides* Hett. & M. Sizemore	Northern Thailand, Tak	AD-KC01	TK08P1T11	wild	Leaf/tuber	KR534409	KR534463	KR534458
47	*A. linearis* Gagne pain *	South-Western Thailand, Kanchanaburi	LE-KC01	CP	cultivated	Leaf	KR534387	-	-
48	*A. kachinensis Engl. & Gehrm.*	Northern Thailand, Chiang Mai	KA-CM02	CM02P1T12	wild	Leaf/tuber	KR534389	KR534469	KR534419
	Anchmanes welwitschii	Sierra Leone	Outgroup1	1993-VO422pW	cultivated	Leaf	KR534363	KR534498	KR534426
Outgroup	*Arisaema fimbriatum*	Peninsula, Thailand	Outgroup2	2009-467pG	cultivated	Leaf	KR534362	-	KR534427
	Gonatopus angustus	Tanzania	Outgroup3	2007-1195CG	cultivated	Leaf	KR534361	KR534499	KR534428

[a] Species with the one asterisks (*) represent plant samples collected from Hamburg Botanical Garden (Germany) and species with double asterisks (**) represent plant samples collected from Gothenburg Botanical Garden (Sweden).

The tubers of wild species (21 accessions) were used for KGM analysis. Leaves of all accessions were used for DNA isolation. Three close relationships with *Amorphophallus* plants including *Pycnospathe arietina, Arisaema fimbriatum* and *Gonatopus angustus*. Those outgroup species were collected from Gothenburg Botanical Garden and were included in the sequencing analysis as outgroup taxa. Whereas, *Pycnospathe arietina* was used in both the sequencing and RAPD analysis as an outgroup.

Additional eighty-seven accessions (Table 3.2) in different species were collected form wild and cultivar for use in determine specificity of designed primers together with related *Araceae* family include *Aglaonema pictum, Anthurium* sp., *Dieffenbachia fournieri, Homalomena pendula* and *Xanthosoma sagitifolium* from *Araceae* collection in Gothenburg botanical garden (Sweden).



(A) (B) (C) (D) (E)

Figure 3.1(A-C) Leaf and (D-E) tuber of collected wild *A. muelleri* (MU-ME01).

(A) (B) (C) (D)

Figure 3.2 Pictures of Herbarium specimens (A) *A. operculatus* (OC-NE01) (B) *A. tenuispadix* (TP-CP01); (C) *A. latifolius* (LF-WH01); (D) *A. sumawongii* (2010-1662pG).

Table 3.2 Details of *Amorphophallus* samples used for determinate specificity of designed primers.

Acces-sion	*Amorphophallus* Species[a]	Code No.	Location	Voucher no.	Habitus
1A	*A. muelleri* Blume	AMU-TK01	Tak (TK)	TK06P1T1	wild
2A	*A. muelleri* Blume	AMU-TK02	Tak	TK03P1T1	wild
3A	*A. muelleri* Blume	AMU-TK03	Tak	TK08P1T1	wild
4A	*A. muelleri* Blume	AMU-TK04	Tak	TK06P2T1	wild
5A	*A. muelleri* Blume	AMU-TK05	Tak	TK05P1T1	wild
6A	*A. muelleri* Blume	AMU-TK06	Tak	T1	cultivated
7A	*A. muelleri* Blume	AMU-TK07	Tak	T2	cultivated
8A	*A. muelleri* Blume	AMU-TK08	Tak	T3	cultivated
9A	*A. muelleri* Blume	AMU-TK09	Tak	T4	cultivated
10A	*A. muelleri* Blume	AMU-TK10	Tak	T5	cultivated
11A	*A. muelleri* Blume	AMU-TK11	Tak	T6	cultivated
12A	*A. muelleri* Blume	AMU-TK12	Tak	T7	cultivated
13A	*A. muelleri* Blume	AMU-TK13	Tak	T8	cultivated
14A	*A. muelleri* Blume	AMU-TK14	Tak	T9	cultivated
15A	*A. muelleri* Blume	AMU-TK15	Tak	T10	cultivated
20A	*A. muelleri* Blume	AMU-KC01	Kanchanaburi (KC)	Kan1	wild
16A	*A. muelleri* Blume	AMU-ME01	Maehongson (ME)	ME04P1T1	wild
17A	*A. muelleri* Blume	AMU-ME02	Maehongson	Mae3	wild
18A	*A. muelleri* Blume	AMU-ME03	Maehongson	Mae1	wild

Table 3.2(2) Details of Amorphophallus samples used for determinate specificity of designed primers (continue).

Acces-sion	*Amorphophallus* Species[a]	Code No.	Location	Voucher no.	Habitus
19A	*A. muelleri* Blume	AMU-ME04	Maehongson	Mae2	wild
21A	*A. muelleri* Blume	AMU-KC02	Kanchanaburi	Kan2	wild
22A	*A. muelleri* Blume	AMU-KC03	Kanchanaburi	Kan3	wild
23A	*A. muelleri* Blume	AMU-KC04	Kanchanaburi	Kan4	wild
24A	*A. muelleri* Blume	AMU-KC05	Kanchanaburi	Kan5	wild
25A	*A. muelleri* Blume	AMU-KC06	Kanchanaburi	Kan6	wild
26A	*A. muelleri* Blume	AMU-KC07	Kanchanaburi	Kan7	wild
27A	*A. muelleri* Blume	AMU-KC08	Kanchanaburi	Kan8	wild
28A	*A. muelleri* Blume	AMU-KC09	Kanchanaburi	T11	cultivated
29A	*A. muelleri* Blume	AMU-KC10	Kanchanaburi	T12	cultivated
30A	*A. muelleri* Blume	AMU-KC11	Growing in China	china1	cultivated
31A	*A. muelleri* Blume	AMU-CN01	Growing in China	china2	cultivated
32A	*A. muelleri* Blume	AMU-CN02	Growing in China	china3	cultivated
33A	*A. muelleri* Blume	AMU-CN03	Growing in China	china4	cultivated
34A	*A. muelleri* Blume	AMU-CN04	Growing in China	china5	cultivated
35A	*A. muelleri* Blume *	AMU-TK16	Tak	H.AM.1539	cultivated
36A	*A. xiei*	AXI-CN01	China	xie1	cultivated
37A	*A. xiei*	AXI-CN02	China	xie2	cultivated
38A	*A. xiei*	AXI-CN03	China	xie3	cultivated
39A	*A. xiei*	AXI-CN04	China	LEG GS	cultivated
40A	*A. bulbifer* (Roxb.) Bl	ABU-CN01	China	bul Guy	cultivated
41A	*A. bulbifer* (Roxb.) Bl *	ABU-CN02	Indian, Nepal	H.AM.1427	cultivated
42A	*A. bulbifer* (Roxb.) Bl **	ABU-CN03	Indian, Nepal	2008-1636pG	cultivated
43A	*A. bulbifer* (Roxb.) Bl **	ABU-CN04	Indian, Bumar	2010-1442pG	cultivated
44A	*A. kachinensis*Engl. &Gehrm.	AKC-CM01	Chiang Mai (CM)	CM09P1T6	wild
45A	*A. kachinensis*Engl. &Gehrm.	AKC-CM02	Chiang Mai	CM05P1T6	wild
46A	*A. corrugatus* N. E. Br.	ACR-LA01	Lampang (LA)	LA01P1T3	wild
47A	*A. corrugatus* N. E. Br.	ACR-HM01	Lampang	2007-G-068	cultivated
48A	*A. krausei* Engl. (=*A. pachystilis*)	AKU-LA01	Lampang	LA04P1T11	wild

Table 3.2(3) Details of *Amorphophallus* samples used for determinate specificity of designed primers (continue).

Acces-sion	*Amorphophallus* Species[a]	Code No.	Location	Voucher no.	Habitus
49A	*A. krausei* Engl.	AKU-CM01	Chiang Mai	CM11P2T11	wild
50A	*A. krausei* Engl.	AKU-NH01	Northern TH	H.AM.040	cultivated
51A	*A. amygdaloides* Hett.& M. Sizemore**	AD-WH01	Western TH (Kanchanaburi)	2010-1650pZ	cultivated
52A	*A. bangkokensis* *	ABK-WT01	Whole TH	H.AM.1343	cultivated
53A	*A. boycei* Hett. *	ABC-WT01	Southern TH	H.AM.515	cultivated
54A	*A. brevispathus* Gagnepain *	ABP-WT01	NakhonRatchasima (NR)	H. AM. 678	cultivated
55A	*A. carneus* Ridl. *	ACN-WT01	Songkhla (SK)	H.AM. 464	cultivated
56A	*A. cicatricifer* Hett. *	ACC-WT01	Kanchanaburi	H.AM. 368	cultivated
57A	*A. cirrifer*Stapf. *	ACF-WT01	Central TH	H. AM. 1647	cultivated
58A	*A. cruddasianus* *	ACD-WT01	Kanchanaburi	H.AM. 967	cultivated
59A	*A. curvistilis*Hett. *	ACV-WT01	Western TH	H. AM.002	cultivated
60A	*A. haematospadix* Hook. *	AHS-WT01	Central to Southern TH	H.AM.1356	cultivated
61A	*A. koratensis* Gagnepain *	AKT-WT01	Sa Kaeo	H.AM. 994	cultivated
62A	*A. lunatus* *	ALA-WT01	NakhonSawan	H.AM.1189	cultivated
63A	*A. polyanthus* Hett.& M. Sizemore *	APY-WT01	Loei (LE)	H.AM. 873	cultivated
64A	*A. prainii* Hook. F *	APN-WT01	Central to Southern TH	H.AM.1685	cultivated
65A	*A. pygmaeus* Hett. *	APM-WT01	Central TH	H.AM.104	cultivated
66A	*A. reflexus* *	ARF-WT01	KamphaengPhet (KP)	H.AM. 1160	cultivated
67A	*A. saraburiensis* Gagnepain *	ASB-WT01	Lopburi (LB)	H. AM. 847	cultivated
68A	*A. saururus* Hett. *	ASR-WT01	Northeastern TH	H. AM. 26	cultivated
69A	*A. scutatus* Hett.& T.C. Chapman *	AST-WT01	Western TH	H.AM.1642	cultivated
70A	*A. sizemorei* Hett. *	ASZ-WT01	NakhonSawan	H. AM. 983	cultivated
71A	*A. symonianus* Hett. *	ASY-WT01	Loei	H.AM.725	cultivated
72A	*A. vogelianus* *	AVG-WT01	Chiang Mai	H.AM. 1173	cultivated
73A	*A. paeoniifolius* (Dennst.) Nicolson *	APF-TK01	Tak	H.AM.64	cultivated

Table 3.2(4) Details of *Amorphophallus* samples used for determinate specificity of designed primers (continue).

Acces-sion	*Amorphophallus* Species[a]	Code No.	Location	Voucher no.	Habitus
74A	*A. paeoniifolius*	APF-LA02	Lampang	LA11P1T4	wild
75A	*A. paeoniifolius*	APF-ME01	Maehongson	ME05P1T4	wild
76A	*A. paeoniifolius*	APF-TK02	Tak	TK03P2T4	wild
77A	*A. macrorhizus* Craib	AMC-LA01	Lampang	LA04P1T5	wild
78A	*A. macrorhizus* Craib	AMC-CM01	Chiang Mai	CM03P1T5	wild
79A	*A. macrorhizus* Craib	AMC-ME01	Maehongson	ME04P1T5	wild
80A	*A. yunnanensis* Engl.	AYN-LA01	Lampang	LA11P2T2	wild
81A	*A. yunnanensis* Engl.	AYN-LO01	Lampoon	LO03P2T2	wild
82A	*A. pygmaeus*	APM-CB01	Chanthaburi	JJPM02	wild
83A	*A. asterostigmatus* Bogner&Hett. *	AAS-SB01	Saraburi	Tus H.AM.791	wild
84A	*A. asterostigmatus* Bogner&Hett.	AAS-SB02	Saraburi	JJAS-003	wild
85A	unknown sample	AUN-SB01	Saraburi	SB012	wild
86A	unknown sample	AUN-CY01	Chaiyaphum	CP002	wild
87A	unknown sample	AUN-CY02	Chaiyaphum	CP003	wild
88A	unknown sample	AUN-CP01	Prachinburi	JJ021	wild

[a]Species with the one asterisks (*) represent plant samples collected from Hamburg Botanical Garden (Germany) and species with the two asterisks(**) represent plant samples collected from Gothenburg Botanical Garden (Sweden).

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(A) (B) (C) (D)

Figure 3.3 Pictures of additional *Amorphophallus* sample including (A) *A. paeoniifolius* (APF-TK01); (B) *A. asterostigmatus* (AAS-SB01); (C); *A. macrorhizus* (AMC-LA01) and (D) *A. pygmaeus* (APM-CB01).

3.1.2 Chemicals

3.1.2.1 Chemicals for determination of KGM content

Name	Company	Country
Crystalloid Phenol (A.R. grade)	Fluka	USA
3,5-Dinitrosalicylic acid (DNS)	Fluka	USA
D- Glucose monohydrate (A.R. grade)	Sigma	USA
95% Ethanol (A.R. grade)	Merck	Germany
Sodium Hydroxide (A.R. grade)	Ajax Finechem	New Zealand
Sodium Potassium Tartrate	Ajax Finechem	New Zealand
Sulfuric Acid (A.R. grade)	Merck	Germany

3.1.2.2 Chemicals for DNA extraction

Name	Company	Country
DNeasy Plant Mini Kit	Qiagen	Germany
Genomic DNA Mini Kit (Plant)	Geneaid	Taiwan
NucleoSpin® Plant II	Macherey-Nagel	Germany

3.1.2.3 Chemicals for PCR reaction

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Name	Company	Country
Bovine Serum Albumin (BSA) 20 mg/ml	New England Biolabs	USA
Deoxynucleotide (dNTP) Solution Mix (10 mM)	New England Biolabs	USA
Oligonucleotide designed primers	Bio Basic Inc.	Canada
G-mix master mix	Qiagen	Germany
GoTaq® Hot Start Polymerase	Promega	USA
MultiScreen® PCRμ96 Filter Plate	Merck Millipore	Germany
RAPD and Sequencing Primer	Eurofins MWG Operon	Germany
Taq DNA Polymerase with ThermoPol® Buffer	New England Biolabs	USA

3.1.2.4 Chemicals for Gel electrophoresis

Name	Company	Country
10X Tris-Borate-EDTA (TBE) Buffer, pH8.3, Ultra Pure Grade	Vivantis	Malaysia
6X Loading Dye	Vivantis	Malaysia
Agarose low EEO	Research Organics	USA
Ethidium Bromide	Bio Basic	USA
GeneRuler DNA Ladder Mix (SM0331)	Thermo Fisher Scientific	Germany
Thermo Scientific™ 50X TAE Buffer (Tris-acetate-EDTA)	Thermo Fisher Scientific	Germany
VC 100bp Plus DNA Ladder	Vivantis	Malaysia

3.1.2.5 Chemicals for PCR Cloning

Name	Company	Country
Ampicillin sodium salt	AppliChem	USA
EcoRIRestriction Endonucleases	Vivantis	Malaysia
Gel/PCR DNA Fragments Extraction Kit	Geneaid	Taiwan
IPTG (isopropyl-beta-D-thiogalactopyranoside)	AppliChem	USA
NEB 5-alpha Competent E. coli	New England Biolabs	USA
Presto™ Mini Plasmid Kit PDH100	Geneaid	Taiwan
QIAGEN PCR Cloning Kit	Qiagen	Germany
Sodium Chloride	Ajax Finechem	New Zealand
Tryptone	Himedia	India
X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactoside)	AppliChem	USA
Yeast Extract	Himedia	India

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3.1.3 Apparatus

Instrument	Model	Company,Country
Autoclave	SS-325	TOMY, Japan
Bio Spectrophotometer	Basic	Eppendroff, Germany
Biological Safety Cabinet	HFSafe-1200	HealForie, China
Biomedical Freezer -40 ^{O}C	CMDF-136	Sanyo, Japan
Bio-Rad My Cycler™ Personal Thermal Cycler	02-242 mycycler-v2	Bio-Rad, USA
Centrifuge	1610	Hettich, Germany
Digital Dry Block Heater	949306	VWR, USA
Electronic UV transilluminator		Syngene, USA
Electrophoresis chamber set	Sub-cell GTwide mini	Bio-Rad, USA
Electrophoresis chamber set	Max Horiz 80-6061-57	GE Bioscience, USA
Freeze dryer	FreeZone6	Labconco, USA
High speed blender	HGBTWT	Waring,USA
High speed microcentrifuge Model	Z36HK	GmbH, Germany
Hot air Oven	FD240	Brinder, Germany
Incubator	LIB101SM	Labtech, Korea
Microwave oven	R.A. 610	Mitsubishi, Japan
MyGenie™ 96 Thermal Block		Bioneer, Korea
Nanodrop 1000Spectrophotometer	ND-1000	Thermo Scientific, USA
Refrigerator	SBC-2DB	Sanyo,Japan
Shaking Incubator	NB205-VN	N-BIOTEK, Korea
Spectrophotometer	4001/4	SpectronicUnicam, USA
T100 thermal cycler		Bio-Rad, USA
Tissue Tissue Lyser II homogenizer	Tissuelyser	Qiagen, USA

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3.2 Methods

3.2.1 Determination of konjac glucomannan (KGM) content

The fresh tuber of wild plant materials (250g) were sliced and dried at 50 ºC for 6-8 h to reduce the moisture content to 8-10% (w.b.). The dried konjac slices were grounded and sifted through 120 mesh sieve to separate starch from glucomannan and other impurities ≥ 125 μm. The resulting crude konjac flour samples were subjected to analysis of KGM content through a 3,5-dinitrosalicilic acid colorimetric assay (DNS) according to a sugar-reduction hydrolysis extraction method (Chua *et al.*, 2012; Liu *et al.*, 2002. ; Zhao, 2010). In order to remove the reducing sugars, 85% ethanol was added to the crude konjac flour samples. This was followed by the extraction of KGM with distilled water and the subsequent hydrolysis of KGM with H_2SO_4. The KGM content was determined by DNS and calculated using equation (2), which determines the absorbency of the KGM hydrolysate at 550 nm wavelength:

$$\text{KGM (\%)} = \frac{\varepsilon T \times 100}{m} \times 100 \qquad (2)$$

In the equation:

ε ----> Molecular weight ratio of Mannose and Glucose in the KGM residues within KGM hydrolysate (Mannose or Glucose molecular weight is 180, molecular weight of residue is 162/180 = 0.9)

T ----> The corresponding glucose weight (mg) according to the absorbency in the standard curve

m ----> The weight of crude konjac flour sample (mg)

Each analysis was performed in triplicate and mean values were calculated. The differences between means were estimated using Duncan's multiple range test with a level of significance of $p < 0.05$ using the SPSS 16.0 software (IBM SPSS, Chicago, IL, USA).

KGM content was used as criterions to divide *Amorphophallus* spp. into three groups including high KGM content (40-70%), medium KGM content (20 – 39%) and low KGM content (3-10%).

3.2.2 Molecular and phylogenetic analyses

3.2.2.1 DNA extraction

All leaf material subjected to dry by freeze-drying or silica gel for long preservation and kept at room temperature. DNA extraction was firstly done by using two optional kits

3.2.2.1.1 DNeasy plant mini kit (Qiagen, catalog# 69104)

Genomic DNA extraction of sequencing and RAPD analysis were performed by using Dneasy Plant Mini Kit to give rapid extraction and high quality extracted DNA. Leaf material of each plant was grounded in QIAGEN TissueLyser II Bead Mill homogenizer to obtain a fine powder. Genomic DNA was isolated according to the manufacturer's protocol. The quantity and quality of the genomic DNA was estimated by Nanodrop 1000 Spectrophotometer. Finally, the extracted DNA was kept at -20 ^{O}C for further use as template in PCR amplification.

3.2.2.1.2 Genomic DNA Mini Kit (Plant) (Geneaid, catalog#GP100)

Genomic DNA extraction was performed by Plant Genomic DNA Mini Kit for specificity designed primers test**.** Dried leaf of each plant was grounded in liquid nitrogen with mortar and pestle to obtain a fine powder. Genomic DNA was isolated from the fine powder according to the manufacturer's protocol. The quantity and quality of the genomic DNA was estimated by BioSpectrophotometer. The extracted genomic DNA was then stored in -20 ^{O}C until used.

3.2.2.2 Sequencing Analysis

To assess the comparative performance of different DNA markers, all forty-eight samples were analyzed with three DNA regions include chloroplast *trnL-trnF* gene, the internal transcribed spacers (ITS) and the second intron of *FLORICAULA/LEAFY* (*FLint2*) region.

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3.2.2.2.1 PCR Sequencing primers

Amplification and sequencing were performed using the primers listed in Table 3.3 by Eurofins MWG Operon, Germany. The chloroplast *trnL-trnF* spacer was amplified and sequenced with universal primers "c" and "f". A section of the internal transcribed spacer (ITS) was amplified using the primer pair P17/26S-82R. The second intron of *LEAFY (FLint2)* was amplified with primers *FLint2* F1 and *FLint2* R1.

Table 3.3 Lists of six PCR sequencing primers used in this study.

Primer name	Direction	Sequence (5´ to 3´)	Reference
primers "c"	forward	CGAAATCGGTAGACGCTACG	Taberlet *et al.* (1991)
primers "f"	reverse	GGGGATAGAGGGACTTGAAC	Taberlet *et al.* (1991)
P17	forward	CTACCGATTGAATGGTCCGGTGAA	Popp and Oxelman (2001)
26S-82R	reverse	TCCCGGTTCGCTCGCCGTTACTA	Popp and Oxelman (2001)
FLint2 F1	forward	CTTCCACCTCTACGACCAGTG	Grob *et al.* (2004)
FLint2 R1	reverse	TCTTGGGCTTGTTGATGTAGC	Grob *et al.* (2004)

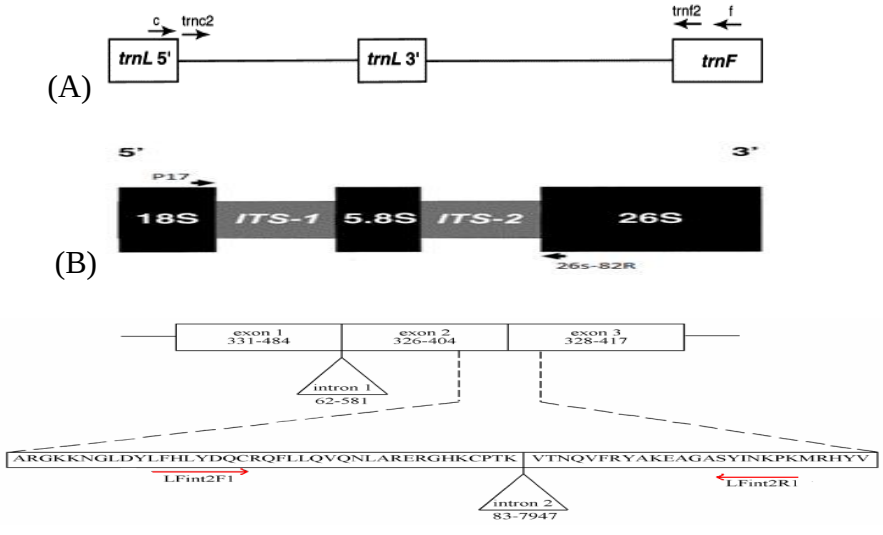

Figure 3.4 Location of PCR sequencing primers on each region (A) *trnL-trnF* gene (B) the internal transcribed spacers ITS) and (C) second intron of *FLORICAULA/LEAFY* (*FLint2*); arrow (→) represent forward primers and arrow (←) represent reverse primers (Grob *et al.*, 2004; Popp and Oxelman, 2001; Taberlet *et al.*, 1991).

3.2.2.2.2 Polymerase Chain Reaction (PCR)

PCR reaction was carried out in different condition for each region was as followed:

The chloroplast gene *trnL-trnF* spacer, the PCR reaction was performed in total volume of 25 μL consisted of 1X PCR buffer, 1.5 mM $MgCl_2$, 0.4 mM dNTP, 1.25 μM forward and reverse primer, 0.8 U GoTaq® Hot Start Polymerase and 30 ng of template DNA. Double stranded DNAs were amplified include an initial denaturation step at 95 ^{O}C for 1 min with 40 cycles of amplification (30s 95 ^{O}C, 1 min 55 ^{O}C, 2 min 72 ^{O}C) with an additional 7 min on 72 ^{O}C on a Bio-Rad My Cycler™ Personal Thermal Cycler.

The internal transcribed spacers (ITS), the PCR reaction was performed in total volume of 25 μL consisted of 12.5 μL G-mix master mix , 1.25 μM forward and reverse primer, 0.8 U μLGoTaq® Hot Start Polymerase and and 30 ng of template DNA. Double stranded DNAs were amplified include an initial denaturation step at 95 ^{O}C for 5 min with 40 cycles of amplification (30 s 95 ^{O}C, 30 s 56 ^{O}C, 30 s 72 ^{O}C) with an additional 7 min on 72 ^{O}C on a Bio-Rad My Cycler™ Personal Thermal Cycler.

The second intron of *FLORICAULA/LEAFY* (*FLint2*),the PCR reaction was performed in total volume of 25 μL consisted of 1X PCR buffer, 1.5 mM $MgCl_2$, 0.4 mMdNTP, 1.25 μM forward and reverse primer, 0.8 U GoTaq® Hot Start Polymerase and 30 ng of template DNA. Double stranded DNAs were amplified with initial denaturation step at 94 ^{O}C for 5 min followed by 35

cycles of amplification (30 s 95 ^{O}C, 30 s 60 ^{O}C, and 30 s 72 ^{O}C) with an additional 7 min on 72 ^{O}C on a Bio-Rad My Cycler™ Personal Thermal Cycler.

PCR products from each region were separated on 1% agarose gels at 100 Volt for 1 h in TAE buffer to indicate the quantity of the fragments.

3.2.2.2.3 DNA sequencing data analysis

PCR product were purified by using Millipore's Multi Screen® PCRμ96 Filter Plate and eluded in 30 μL elution buffer. DNA concentration was estimated by Nanodrop 1000 spectrophotometry. Sequencing for all three genes were performed using the forward and reverse primers by Macrogen (Korea). For sequence analysis, forward and reverse sequence reads were assembled and trimmed were created using GENEIOUS Pro R6 (Version 5.4, Bio matters, New Zealand) (Kearse *et al*., 2012) and manually corrected. Multiple sequences alignments were created by different methods included MAFFT sequence alignment program with L-INS-i algorithm, BMGE Alignment (Block Mapping and Gathering with Entropy) and PRANK multiple aware-alignment program. Character states and indels were coded using SeqState V.1.4.1 (Müller, 2005), under the simple indel coding option (Simmons and Ochotorena, 2000). The best substitution model for each alignment was selected using jModelTest v.2.1.1 (Posada, 2008), under the Bayesian information criterion (BIC) to find the evolutionary model that uses the best-fit model in each of the three regions analyzed. Bayesian inference (BI) analyses of the individual genes was performed using MrBayes v.3.2.1 (Huelsenbeck and Ronquist,

2001), with default prior settings, for ten million MCMC generations with eight chains under the increased temperature of $t = 0.1$.

3.2.2.3 Random Amplified Polymorphic DNA (RAPD) analysis

RAPD-PCR was used to generate banding patterns for the different *Amorphophallus* accessions using arbitrary thirteen RAPD primers, which were selected from previous primer screening test based on their ability to generate DNA bands in all *Amorphophallus* accessions (Table 3.4).

Table 3.4 List of arbitrary primers for RAPD analysis

Primer name	Sequence (5´ to 3´)	Reference
ERIC1R	ATGTAAGCTCCTGGGGATTCAC	Versalovic *et al.* (1994)
ERIC2	AAGTAAGTGACTGGGGTGAGCG	Versalovic *et al.* (1994)
BOXA1R	CTACGGCAAGGCGACGCTGACG	Versalovic *et al.* (1994)
RPO1	AATTTTCAAGCGTCGTGCCA	Watson and Schofield (1985)
AB-04	GGCACGCGTT	Operon, USA
AB-20	CTTCTCGGAC	Operon, USA
AC-09	AGAGCGTACC	Operon, USA
AC-10	AGCAGCGAGG	Operon, USA
AH-18	GGGCTAGTCA	Operon, USA
OPC-02	GTGAGGCGTC	Operon, USA
OPD-04	TCTGGTGAGG	Poerba and Martanti (2008)
OPC-07	CACACTCCAG	Poerba and Martanti (2008)
OPB-17	AGGGAACGAG	Poerba and Martanti (2008)

The RAPD reaction was carried out by amplification of genomic DNA in 15 μL mixture containing 1X Thermopol buffer (2 mM $MgCl_2$), 2% bovine serum albumin (BSA, 10 mg/mL), 1mM dNTPs, 1.25 μL of primer, 0.4 U Taq DNA polymerase and 30 ng template DNA. PCR amplification conditions for long RAPD primers (ERIC1R, ERIC2, BOXA1R, RPO1) were an initial denaturation at 95 ^{O}C for 5 min, followed by 35 cycles of 95 ^{O}C for 30 s, 50 ^{O}C for 30 s, and 65 ^{O}C for 8 min, followed by 65 °C for 5 min. Amplification of decamer primers with initial denaturation at 94 °C for 5 min, followed by 45 cycles of 94 °C for 1 min, 40 ^{O}C for 1 min, and 72 ^{O}C for 2 min, followed by 72 ^{O}C for 4 min. Amplifications were done with MyGenie™ 96 Thermal Block and Bio-Rad My Cycler™ Personal Thermal Cycler for checking reproducibility. PCR products were separated by electrophoresis in a 1.0% (w/v) agarose gel in TBE buffer at 80 V 1.30 h with GeneRuler™ DNA Ladder Mix (SM0331) used for size estimation. The RAPD fragments were photographed using a UV transilluminator and analyzed with a gel documentation system.

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3.2.2.3.1 RAPD data analysis

The RAPD bands were scored as 0 or 1 for the absence or presence of bands, respectively. The presence bands visual detected by GeneTools program (version 3.06.04). After pre-analysis using default setting, a sizing profile of all samples were checked and where necessary manually corrected. Only clear and reproducible bands were scored as 1. The standard DNA marker (GeneRuler™ DNA Ladder Mix, SM0331) was used to assign the size of each RAPD fragment. Polymorphism information content (PIC) was calculated per primer

according to standard procedures (Nagy *et al*., 2012). The RAPD scores obtained were pooled to create a single data matrix and were analyzed for genetic distance using the method described by Nei and Li (1979). Phylogenetic trees were constructed using the RADP data in neighbor joining (NJ) dendrogram using computer based program, GeneTools v3.06.04 (Sysgene, England).

3.2.2.4 Design of specific DNA markers to differentiate between high- medium and low KGM content group.

Genetic data from sequencing or RAPD analysis was correlated with KGM content aiming to determine their relationship and grouping. Any DNA regions or RAPD primers that had ability to separate high or medium KGM content group were chose to generate specific DNA primers.

3.2.2.4.1 Development of sequencing markers

The specific primer sets were designed at target assembled between nucleotide sequences of *A. muelleri*, *A. bulbifer* and *A. xiei* for each region. From nucleotide sequences of *FLORICAULA/LEAFY* (*FLint2*) region, two set of primers were designed include (1) MUBX236_*Flint2* primer set and MUBX253_ *Flint2* primer set. From nucleotide sequences of the internal transcribed spacers (ITS) region, eight set of primer were designed, including (1) HKGM-221ITS primer set, (2) HKGM-281ITS primer set, (3) HKGM-493 ITS primer set, (4) HKGM-548AG primer set, (5) HKGM-548ATG primer, (6) HKGM-787ITS primer set, (7) HKGM-327 ITS primer and (8) HKGM-994ITS primer set. From nucleotide sequences of chloroplast *trnL-trnF gene*, two set of primers were designed nucleotide sequences of only for *A. muelleri* species include (1) MU-300 primer set

and (2) MU-689 reverse primer set. DNA melting point, hairpins and primer-dimers were screened for manual primer design by Primer 3.0 design software through GENEIOUS Pro R8 program (Version 8.0, Bio matters, New Zealand). All designed primers were show in Table 3.5

3.2.2.4.2 Development of SCAR (sequence characterized amplified regions) markers

From RAPD analysis, efficiently marker band was cloned and sequenced to be SCAR marker (Scheef *et al*., 2003). For high-medium KGM content species, specific DNA fragments were found at 600 base pair (bp) by AC-10 primer. After electrophoresis, a 600 bp fragment was excised from the electrophoresed gel. DNA was then eluted from agarose gels using Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan) according to the manufacturer's protocol. The gel-eluted DNA was kept at 4^{o}C until further used.

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3.2.2.4.2.1 Cloning of specific DNA fragment

Specific high-medium KGM content species 600 bp DNA fragment was ligated to pDrive cloning vector (QIAGEN, Germany) in Figure 3.5. The ligation reaction at 10 µl constituting of 1 µl of pDrive cloning, 3µl of the DNA insert vector, 1µl of distilled water and 5 µl of ligation Master Mix. The reaction mixture was incubated at 4 ^{O}C for 2 h before transformed into NEB 5-alpha Competent *E. coli* cell (NEB, USA) by heat shock method at 42 ^{O}C for 30 seconds and immediately placed on ice for 5 minutes. Pipette 950 µl of SOC medium into the mixture and follow by shaking incubated (250 rpm) at 37 ^{O}C for 1 to 2 h. Afterwards, cell suspension was centrifuged and 10-fold dilutions in SOC medium

and spread 50-100 µl on the LB agar plate containing 100 µg/ml of ampicillin, 25µg/ml of IPTG and 40 µg/ml of X-Gal. Selection plates were incubated overnight at 37 ^{O}C. The recombinant clones are selected by using blue-white screening technique with a *lac Z* system following a standard protocol (Maniatis *et al*., 1982), which recombinant clones containing inserted DNA are usually white colony because the disturbing of a *lac Z* gene while those without inserted DNA still have functional a *lac Z* gene to produce β-galactosidase which is detected by X-gal and form blue colony.

3.2.2.4.2.2 Colony PCR and Plasmid Extraction

Colony PCR is a convenient high-throughput method for determining the presence or absence of insert DNA in plasmid constructs. A white colony in each accession was gently picked by a pipette tip and mixed in the mixture. Colony PCR was performed in a 25 µl PCR reaction as previous condition with AC-10 primer. The colony PCR products were electrophoresed through 1 % agarose gel at 100 volt for 1 h. The size of insert was compared with DNA ladder. Only white colony with DNA insertion can be generated PCR product of specific 600 bp fragment were selected to plasmid extraction. The recombinant plasmid DNA was extracted by using Presto™ Mini Plasmid Kit PDH 100 (Geneaid, Taiwan). A single white colony was inoculated into 5 ml of LB medium supplementing with 50 µg/ml of ampicillin and incubated with shaking (250 rpm) at 37 ^{O}C overnight. The culture was transferred into a new 1.5 ml micro centrifuge tube and centrifuged at 16,000 g for 1 minute. The supernatant was carefully removed. Recombinant plasmid

was extracted according the conditions recommended by the manufacturer. Recombinant plasmid DNA was stored at -20 ^{o}C until used.

3.2.2.4.2.3 Detection of recombinant plasmid

The recombinant plasmid DNA was examined by digested with *E*coR I restriction enzyme. The reaction was carried out in a 20 µl reaction volume containing 2µl restriction enzyme buffer, 1 µl of enzyme *E*co RI, 5µl of recombinant plasmid and 5 µl of sterile ultrapure water. The mixture was incubated at 37 ^{O}C for 3 h. After that, followed by inactive enzyme at 65 ^{O}C for 10 minutes. The resulting product was electrophoresed through 1 % agarose gel at 100 volt for 1 h. The size of insert was compared with DNA ladder. Only recombinant clone that showed specific 600 bp DNA fragment were selected to DNA sequencing (Green and Sambrook, 2012).

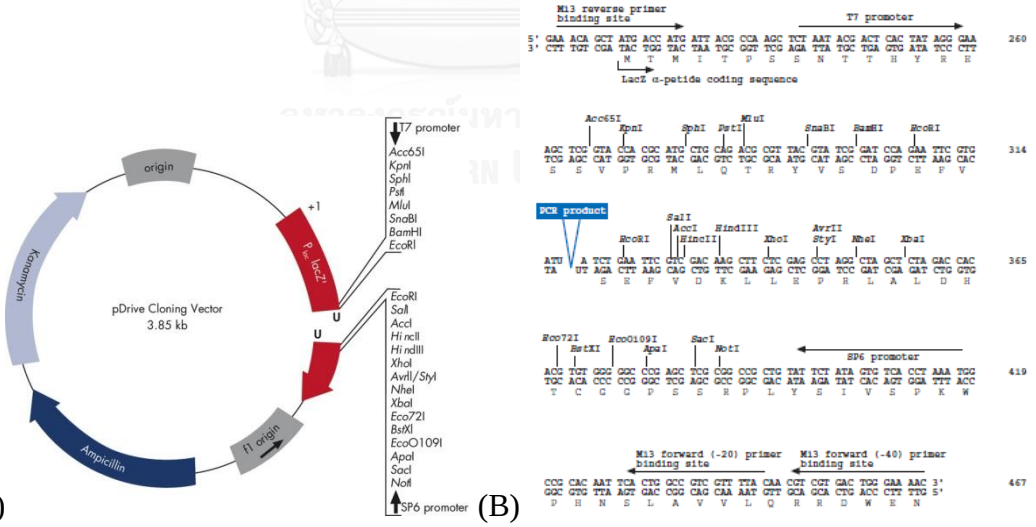

Figure 3.5 pDrive Cloning Vector Map (A) pDriveCloning Vector with U overhangs; (B) DNA sequence of the region surrounding the cloning site.

(QIAGEN®PCR Cloning Handbook , 2010)

3.2.2.4.2.4 DNA sequencing and data analysis

Two recombinant clones for each high and medium KGM content accession (Total 7 species, 17 accessions, 34 clones) were sequenced using T7 promotor forward primer (5'-TAATACGACTCACTATAGGG-3') and SP6 promotor (5'-ATTTAGGTGACACTATAG -3') by Macrogen (Korea). The obtained sequences from each recombinant clone in the same accession were compared to ensure that no overlap DNA fragments in the same size (600 bp). Nucleotide sequences of recombinant clone in each accession were blasted against previously deposited sequences in the GenBank (NCBI) using Blast*N* and Blast*X* (available at http://www.ncbi.nlm.nih.gov). Significant probabilities were considered when the probability (E) value was less than 10^{-4}.

3.2.2.4.2.5 SCAR Primer design

Nucleotide sequences of recombinant clone in each accession were assembled and trimmed were performed using GENEIOUS Pro R8 program and adjusted manually. The alignment was created using MAFFT, with the L-INS-I algorithm. For generate of SCAR primer, two primers were designed at start and end regions of multiple sequence alignment by the Primer 3.0 design software through GENEIOUS Pro R8 program. A pair of primer (KGM-4F forward primer and KGM-595R reverse primer) can generate a 591 bp fragment in representative individuals. Moreover, additional primer set (MUE-129F forward primer and MUE-490R reverse primer) was designed especially for *A. muelleri*, *A. bulbifer* and *A. xiei* from their match conserved regions and generated a 361 bp fragment.

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3.2.2.5 Efficiency testing of designed primers

Fourteen pairs of specific primers were determined their efficacy to distinguish high and medium KGM content species from low KGM content species with PCR condition.

3.2.2.5.1 PCR optimization

Preliminarily tested for PCR optimum condition with 9 accession (MU-ME01, MU-TK01, MU-KC01, BU-CN01, XE-CN01, KA-CM01, KU-CM01, KJ-CN01, CG-LA01) of high or medium KGM content species. PCR reactions were optimized by increasing annealing temperature aim to reduce non-specific amplification products. Annealing temperature was varied into three levels include (1) lower than melting temperature (Tm) for 5 ^{O}C, (2) lower than Tm for 7 ^{O}C and (3) lower than Tm for 10 ^{O}C. The PCR reaction was carried out by amplification of genomic DNA in a 15 μL reaction mixture containing 1X Thermopol buffer (2 mM $MgCl_2$), 2% bovine serum albumin (BSA, 10 mg/mL), 1mM dNTPs, 1.25 μL of primer, 0.1 U Taq DNA polymerase and 30 ng template DNA.

PCR amplification conditions for SCAR primers (Primer No.1 and 2) were an initial denaturation at 95 ^{O}C for 5 min, followed by 35 cycles of 95 ^{O}C for 30 s,varied of annealing temperature (60 ^{O}C, 65 ^{O}C and 70 ^{O}C) for 30 s, and 65 ^{O}C for 8 min, followed by 65 ^{O}C for 5 min.

PCR amplification conditions for designed primers from *FLint2* region (Primer No.3 and 4) were amplified with initial denaturation step at 94^{o}C for 5 min followed by 35 cycles of amplification (30 s 95 ^{O}C, 30 s varied of annealing temperature (55^{o}C, 60 ^{O}C and 65 ^{O}C), and 30 s 72 ^{O}C) with an additional 7 min on 72^{o}C.

Table 3.5 Sequences of fourteen sets of oligonucleotide primers designed from RAPD and Sequencing analysis.

No	Primer Name	Direction	Sequence (5' to 3')	Pro-duct Size (bp)	Tm (^{O}C)	Design from
1	HKGM-4F/	forward	AGCGAGGGTCAGTGGACT	591	69.06	RAPD analysis
	HKGM-595R	reverse	GCAGCGAGGATGTTTTCTTGA		68.88	
2	MUE-129F	forward	GCTGTCTCCTTTGAGCGAGA	361	70.30	RAPD analysis
	MUE-490R	reverse	CCAGAGGAACCTATGCCGTC		72.35	
3	MU-300	forward	CAATACTCGAGAGTCTTTGTAG	300	67.59	*(trnL-trnF)*
	primers "f"	reverse	GGGGATAGAGGGACTTGAAC		60.02	
4	primers "c"	forward	CGAAATCGGTAGACGCTACG	689	64.5	*(trnL-trnF)*
	MU-689	reverse	TAATATTCGACTCTTACTTC		60.05	
5	*FLint2* F1	forward	CTTCCACCTCTACGACCAGTG	236	65.0	(*Flint2*)
	MUBX236_ *Flint2*	reverse	GCTATCCGACGCGCGCCTTG		76.45	
6	*FLint2* F1	forward	CTTCCACCTCTACGACCAGTG	236	65.0	(*FLint2*)
	MUBX253_ *FLin2*	reverse	GTGCATCTATCTAGCTATCCGA		69.45	
7	MUBX-221ITS	forward	AGAGGGCCGCGAAAGT	700	67.5	(ITS)
	26S-82R	reverse	TCCCGGTTCGCTCGCCGTTACTA		76.45	
8	P17	forward	CTACCGATTGAATGGTCCGGTGAA	300	73.88	(ITS)
	MUBX-281ITS	reverse	AACCGGCCGCACCGATGGTG		76.45	
9	MUBX-327ITS	forward	CTCCCCCCGGCAGCGGC	700	77.97	(ITS)
	26S-82R	reverse	TCCCGGTTCGCTCGCCGTTACTA		77.11	
10	MUBX-493ITS	forward	CCGCCACGCGGCGGCGCCG	400	84.82	(ITS)
	26S-82R	reverse	TCCCGGTTCGCTCGCCGTTACTA		77.11	
11	MUBX520_ITS	forward	GGTGACCACGGAACGAAGGAAAG	600	75.33	(ITS)
	26S-82R	reverse	TCCCGGTTCGCTCGCCGTTACTA		77.11	
12	MUBX551_ITS	forward	GTGACCACGGAACGAAGGAAAGGT A	600	75.82	(ITS)
	26S-82R	reverse	TCCCGGTTCGCTCGCCGTTACTA		77.11	
13	MUBX787_ITS	forward	ATTGGCCCACCGTGCGCACAC	450	76.69	(ITS)
	26S-82R	reverse	TCCCGGTTCGCTCGCCGTTACTA		77.11	
14	P17	forward	CTACCGATTGAATGGTCCGGTGAA	900	73.88	(ITS)
	HKGM-994ITS	reverse	TCGCGGCTGGGTTCCTACC		74.03	

PCR amplification conditions for designed primer from ITS region (Primer No.5 - 12), were amplified include an initial denaturation step at 95^{o}C for 5 min with 40 cycles of amplification (30 s 95 ^{O}C, 30 s varied of annealing temperature (60 ^{O}C, 65 ^{O}C and 70 ^{O}C), 30 s 72 ^{O}C) with an additional 7 min on 72 ^{O}C

PCR amplification conditions for designed primer from *trnL-trnF* region (Primer No.13 and 14), were amplified include an initial denaturation step at 95 ^{O}C for 1 min with 40 cycles of amplification (30s 95 ^{O}C, 1 min, varied of annealing temperature (50 ^{O}C, 55 ^{O}C and 60 ^{O}C), 2 min 72^{o}C) with an additional 7 min on 72^{o}C

PCR products were separated by electrophoresis in a 1.0% (w/v) agarose gel in TBE buffer at 100 V 1 h. with GeneRuler™ DNA Ladder Mix (SM0331) used for size estimation. Any Annealing conditions produce clearness of the PCR band were selected for future studied.

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3.2.2.5.2 Specificity testing of designed primers

Fourteen pairs of primers (Table 3.5) were preliminarily tested for specificity with previous *Amorphophallus* set with 48 accessions (Table 3.1). Seven pairs of primers (Primer No.1-2, 5-6, 11-12 and 14) were succeed to specific amplify. Further examined against larger specimens with 87 *Amorphophallus* plant individuals including 31 species and four unknown samples (Table 3.2) and the populations of *Aglaone mapictum*, *Anthurium sp*., *Dieffenbachia fournieri*, *Homalomena pendula* and *Xanthosoma sagitifolium* that belong to *Araceae* family are used as the control (Santosa *et al.*, 2012).

The amplification reactions were performed with optimizes condition as previous investigation. The amplification products were separated by 1 % agarose gels in TBE buffer at 100 V 1 h. Each PCR amplification reaction was repeated three times to ensure reproducibility.

3.2.2.5.3 Repeatability testing of designed primers

After screening of suitable designed primers, seven species of high or medium KGM content species (*A. muelleri*, *A. bulbifer*, *A. xiei*, *A. krausei*, *A. kachienesis*, *A. corrugatus* and *A. konjac*) and two low KGM content species (*A. amylodidas* and *A. yunnanensis*) were selected for re-DNA extraction with Genomic DNA Mini Kit (Plant) (Geneaid, Taiwan) and use optimum PCR amplification with different PCR thermo blocker (T100 Thermal Cycler, BIO-RAD, USA). The amplification products were separated by gel electrophoresis and compared DNA pattern with previous determinate.

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3.2.2.5.4 Sensitivity testing of designed primers

Sensitivity of seven pair of primers were examined against varies different serial dilution of DNA template concentrations of the target DNA template (30, 15, 3, 0.3 ng/ml) of *A. muelleri* (MU-ME01) using the same conditions as were used for the specificity test. The amplification products were analyzed at 1 % agarose gels. The experiment was repeated in three times.

3.2.2.5.5 Detection of specific primers in different tissues of *Amorphophallus*'s plant

Sample of three parts of *A. muelleri* (AMU-TK15 and AMU-KC10) were collected include tuber bud, petiole and dried seed were used for detection the ability of specific primer to amplify DNA in different part of this plant.

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CHAPTER IV

RESULTS AND DISCUSSION

4.1 Determination of konjac glucomannan content

Konjac glucomanan (KGM) content is one of the most important indicators for productivity and quality of Konjac flour. KGM occurs in various levels in the corms of different *Amorphophallus* species. In this study, plant materials were collected from various natural habitats throughout Thailand, and three economical species from China. Since it is important to observe KGM content when the plants are growing in their natural environment, therefore only wild specimens of tubers were selected to analysis. *Amorphophallus* tubers from 24 samples (15 species) and two economical species from China were used in this study. Weights of fresh tubers were varied between 200 – 300 grams. Ages of tubers were controlled by pattern of petiole branching (Budiman and Arisoesilaningsih, 2011). The KGM content of selected samples is shown in Table 4.1 in order of their geographic distribution in each of plant species.

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(A) (B) (C)

Figure 4.1 Picture of (A) tuber of *A. muelleri* (MU-TK01); (B) sliced tuber before drying; (C) dried konjac chip and crude konjac flour.

Table 4.1 Level of KGM content in *Amorphophallus* tubers.

Accession	*Amorphophallus* species	Code No.	Location	Fresh tuber weight (gram)	%KGM
1	*A. muelleri*	MU-ME01	Maehongson	256	68.93^{a} ± 1.98
2	*A. muelleri*	MU-TK01	Tak	270	65.43 ^{ab} ± 1.02
3	*A. muelleri*	MU-ME02	Maehongson	226	66.67 ^{ab} ± 0.53
4	*A. muelleri*	MU-TK02	Tak	234	63.85 ^{ab} ± 0.64
5	*A. muelleri*	MU-KC01	Kanchanaburi	266	62.47^{ab} ± 1.51
6	*A. muelleri*	MU-KC02	Kanchanaburi	223	60.16^{c}± 0.83
7	*A. bulbifer*	BU-CN01	China	201	52.53 ^{d} ±0.90
8	*A. bulbifer*	BU-CN02	China	261	51.83 ^{d} ± 0.66
9	*A.xiei*	XE-CN01	China	205	50.60 ^{d} ± 0.62
10	*A. krausei*	KU-CM01	Chiang Mai	242	55.36^{c} ±1.85
11	*A. krausei*	KU-CM02	Chiang Mai	271	57.76^{c} ±0.54
12	*A. krausei*	KU-CM03	Chiang Mai	260	55.74^{c}±1.30
13	*A. macrorhizus*	MC-LO01	Lampoon	299	6.54^{f} ±0.47
14	*A. yunnanensis*	YN-LO01	Lampoon	260	4.27 ^{f} ±0.06
16	*A. paeoniifolius*	PF-CR01	Chiang Rai	256	1.53^{f} ±0.47
18	*A. thaiensis*	TH-CM01	Chiang Mai	235	2.28^{f} ±0.37
21	*A. corrugatus*	CG-LA01	Lampang	211	35.41^{e} ±1.63
23	*A. kachinensis*	KA-CM01	Chiang Mai	212	51.52 ^{cd} ±0.68
24	*A.tenuispadix*	TP-CP01	Chaiyaphum	253	2.11^{f} ±0.23
25	*A. asterostigmatus*	AS-SB01	Saraburi	237	4.74 ^{f} ±0.30
27	*A. pygmaeus*	PM-CB01	Chanthaburi	256	2.45^{f} ±0.29
40	*A. xiei*	XE-CN02	China	245	48.8 ^{c} ±0.62
46	*A. amygdaloides*	AD-TK01	Tak	240	2.04^{f}±1.25
48	*A. kachinensis*	KA-CM02	Chiang Mai	240	52.14^{c} ±0.75

Different letters indicate significant differences ($p<0.05$) between samples.

The results show that KGM content is in the range from 1.53 to 68.9% of the tuber's dry weight depending on species and their growing location. The highest KGM content was found in *A. muelleri* while *A. kachiensis* and *A. krausei* showed high amount of KGM content and followed by *A. corrugatus*. This finding agrees

with previous studies showing that corms of *A. muelleri* exhibited a higher KGM content than other species of *Amorphophallus* found in Thailand (Akesowan, 1991). Sanguanpong (2002) and Kadprasert (2004) reported a list of species that were used in the KGM production industry in Thailand including *A. muelleri*, and *A. corrugatus*, as well as three undetermined species. However, these studies mention that *A. corrugatus* has a long growth cycle therefore this species has not much favorable in konjac cultivation industry.

In commercial KGM production, *A. konjac* is the main species used in China and Japan. Dormant corms of *A. konjac* contain 49–60% KGM (Chua *et al.*, 2010) which is similar to the KGM level in *A. muelleri* found here (60.16-68.93%) and that of Impaprasert (2013) was found that KGM content in *A. muelleri* exceeded 50%. Nevertheless, *A. konjac* has a limited propagation rate, long growth cycle and problems with soft rot disease and seedling blight (Diao *et al.*, 2014; Zhang and Liu, 2006b; Zhao, 2010). For these reasons, *A. bulbifer* has been cultivated in place of *A. konjac* in Yunnan Province, China, as this species have stronger resistance to disease and has a high propagation coefficient (Zhang *et al.*, 2009). However, this species has a pink and dense flesh that affected to quality of konjac flour (Zhang *et al.*, 2010a). As *A. konjac* does not naturally occur in tropical area like Thailand, native species adapted to the Thai climate that also have a high KGM content may therefore be more suitable for commercial KGM production in the country, as *A. bulbifer* is in southern China. Zhang *et al.* (2010b) compared the content and quality of KGM from *A. konjac, A. bulbifer* and *A. muelleri* found that KGM content is higher in *A. muelleri* (72% ±3.4) than in the commonly grown species in China such as *A. konjac* (62% ±3.3) and lowest in *A. bulbifer* (52% ±3.2). Moreover, based

on the viscosity and transparency of the colloid, the quality of KGM extracted from *A. muelleri* (47,500 mPa.s) is higher than *A. konjac* (32,200 mPa.s) and *A. bulbifer* (11,860 mPa.s). Similar result of Zhao (2010) studied the productivity and quality of KGM extracted from a number of *Amorphophallus* species and found that *A. muelleri* has the highest productivity and quality, follow by *A. konjac* and *A. bulbifer* (Input and output ratio, 10:1/14:1/12:1; KGM content 78%/60%/48%; viscosity 32,000/28,000/24,500 mPa.S respectively). In addition, Impaprasert (2013) found that when using a suitable extraction process, *A. muelleri* produced the highest KGM content at 88.46% and the product had higher viscosity level than commercial products made from *A. konjac* derived KGM. While *A. kachinensis* and *A. krausei* were reported as having been found in China with KGM content 39.8% and 28.5% respectively (Chua *et al.*, 2010).

Among the remaining species, KGM content was low (<10% of dry corm weight) and the results indicate no significant variation in KGM content and growing area among these samples. Pervious study showed that KGM content of *A. paeoniifolius* in Vietnam was 9% (Tien An *et al.*, 2010) which is belong to a low KGM content group as same as in this study. Among many *Amorphophalls* species, it is still limited number of species that rich in KGM content and used for cultivation (Chua *et al.*, 2010; He, 2001; Liu, 2004; Zhao *et al.*, 2010). Because of these reason, it can be assumed that most of *Amorphophallus* species have low KGM content production, only few species that is potential of high KGM content.

In this study, *Amorphophallus* plant can divide into three groups based on the dry weight KGM content of the tubers (Table 4.2).

Table 4.2 Levels of KGM content in *Amorphophallus* tubers.

Level	KGM content (% of total dry weight)	Sample Species
High	40-70	*A. muelleri*, *A. kachinensis*, *A. krausei*, *A. bulbifer*, *A. xiei*
Medium	20-39	*A. corrugatus*
Low	1-19	*A. amygdaloides*, *A. asterostigmatus*, *A. macrorhizus*, *A. paeoniifolius*, *A. pygmaeus*, *A. tenuistylis*, *A. thaiensis*, *A. yunnanensis*

In the high KGM content group have KGM content of more than 40% of the dry corm weight. Like commercial species *A. bulbifer* and agree with the Chinese Ministry of Agriculture for the classification of common konjac flour (KGM content (%) between 60 and 70%) (Liu *et al.*, 2002).

For geographical factor, this factor does have impact on to KGM content variation for accession of *A. muelleri* samples that was significantly different from the sample in Mae Hong Son, Northern Thailand and Kanchanaburi, Western Thailand. In contrast, *A. kachinensis* and *A. krausei* samples from same Northern Thailand do not show significantly different in KGM content levels. KGM content in *A. muelleri* showed significantly different in accession no. 6 (MU-KC02; KGM 60.16 %) from other *A. muelleri* accession. It was collected in local area at Thong Pha Phum district. This maybe transplant tuber that is affect to lower KGM content due to alteration of the ecological environment (Fang and Wu, 2004) whereas accession no.5 (MU-KC01; KGM 62.48%) also from Kanchanaburi was collected in the wild of Thong Pha Phum National Park, which is high nutrient soil and hilly area.

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In addition, highest KGM content found in accession no. 1 (MU-ME01; KGM 68.93%) were collected from Mae Yuam Left Side National Reserve Forest, Mae Sariang district.

Even KGM content can depend on other factors such as location, soil, weather, age of tuber and processing (Fang and Wu, 2004; Zhang and Liu, 2006a; Zhang *et al.*, 2005). Liu (2004) found that the KGM content of *A. konjac* grown in different areas varied slightly (ranging from 58.8% - 52.1%). However, the results also indicate that the species is the main factor determining the productivity and quality of KGM flour, and that the growing conditions are a secondary factor that can later be manipulated for optimal KGM yield. The species identified here as belonging to the high and medium KGM content groups have the potential to become future commercial crops as new raw material resource for konjac flour production. Especially, the reproductive of bulbil-bearing species like *A. muelleri* helps to increase propagation coefficient than corm-propagated alone. Besides this species has high disease resistance, drought tolerance and has high propagation coefficient. Moreover, this species can produce multiple seedlings sequentially during growth, resulting in a high corm yield and shortened growth cycle from the averge 3-4 years down to 9-10 months (Santosa *et al.*, 2003).

The KGM content will be used as additional information along with genetic analysis to characterize *Amorphophallus* species in Thailand.

4.2 Molecular and phylogenetic analyses

4.2.1 DNA extraction

Genomic DNA of each dried leaves of *Amorphophallus* plant was extracted by DNeasy® Plant kit (QIAGEN, catalog # 69104). DNA concentrations were determined by Nanodrop 1000 spectrophotometer measuring the optical density at 260 nm (1 OD 260 unit was equivalent to 30 μg DNA/ml). The ratio of OD260/OD280 of extracted DNA ranged from 1.3 – 2.5 suggesting that some DNA samples were possibly contaminated. Samples showing the ratio much lower than 1.8 was possibly contaminated with residual protein or phenol while the ratio greater than 2.0 may be contaminated with RNA (Sambrook *et al.*, 1989). Since mature leaves were used in this study and *Amorphophallus* leaves can accumulate polysaccharides and secondary compounds during leaf development that would affect reduction of extracted DNA quality (Moreira and Oliveira, 2011). The sample that was not within the optimal range, was re-purified by PowerClean® ProDNA Clean-Up Kit (MOBIO Laboratories, Catalog #12997-50) for a higher quality of extracted genomic DNA. Extracted DNA was stored at -20^{o}C until used.

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4.2.2 DNA sequencing analysis

Forty-eight accessions of *Amorphophallus* belonging to 37 known species composed of wild (14 species) and cultivars (23 species) distribution in each region in Thailand were used in the study and three economical species from China as showed in Table 4.3 and Figure 4.2

Table 4.3 Geographic distribution of *Amorphophallus* species in this study.

Geographic distribution	Amorphophallus species	Total
Northern region	*A. corrugatus, A. kachinensis* *A. krausei, A. longituberosus,* *A. macrorhizus, A. muelleri,* *A. paeoniifolius, A. thaiensis,* *A. yunnanensis, A. bulbifer, A. fuscus*	11 species, 16 samples
Central region	*A. asterostigmatus, A. atroviridis,* *A. cirrifer, A. aberrans, A. prolificus,* *A. napiger, A. tenuistylis, A. putii*	8 species, 8 samples
North- eastern region	*A.opercula*	1 species, 1 samples
South-eastern region	*A. sumawongi, A. pygmaeus*	2 species, 2 samples
Eastern region	*A. tenuispadix, A. atrorubens,* *A. obscurus*	1 species, 1 samples
South-western region	*A. albispathus, A. amygdaloides,* *A. harmandii, A. muelleri, A. linearis,* *A. maxwellii, A.latifolius*	7 species, 7 samples
Southern region	*A. elatus, A. excentricus, A. carneus*	3 species, 3 samples
All region	*A. latifolius*	1 species, 1 samples
China	*A. bulbifer, A. konjac, A. xiei*	3 species, 6 samples

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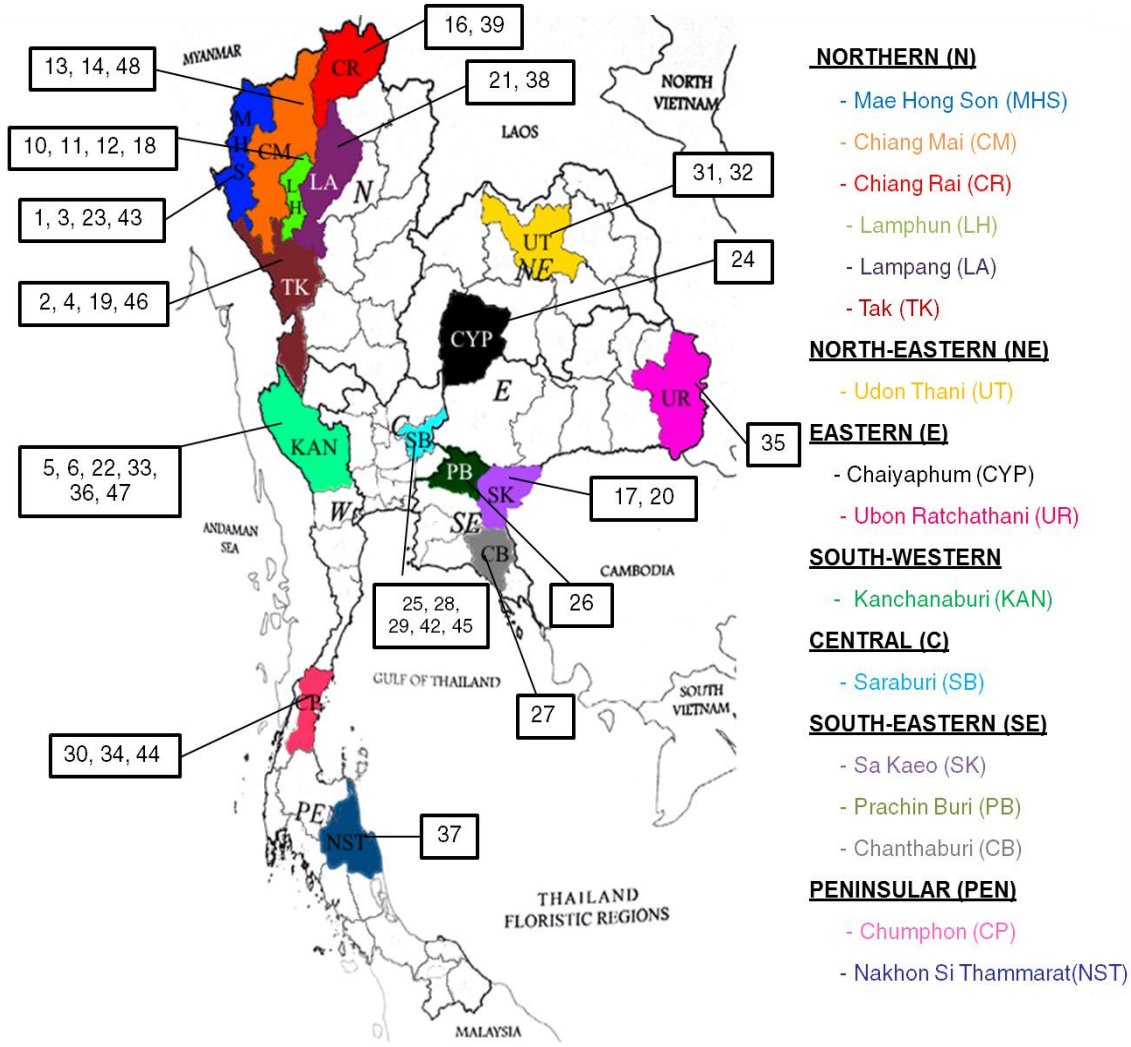

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Figure 4.2 Collection site of *Amorphophallus* spp. with sample accession and abbreviates on the map represent each province. Each province abbreviates that refers to the list is shown on right hand of this figure.

To assess the comparative performance of different DNA markers, each sample was analyzed with three DNA regions include (1) non-coding intergenic spacer DNA regions of *trnL- trnF* spacer in chloroplast (2) internal transcribed spacers (ITS) and (3) the second intron of *FLORICAULA/LEAFY* (*FLint2*) in nuclear

part. Those partial DNA sequences were alignment with MAFFT sequence alignment program with L-INS-i algorithm, which is the most accurate algorithm of MAFFT alignment currently available (Katoh and Toh, 2010; Liu and Warnow, 2014). Since the quality of multiple sequence alignment (MSA) is the main criteria of comparative sequence analysis and the different MSA algorithms for treating gaps can produce different outcomes of phylogenetic inference (Blackburne and Whelan, 2013; Ogden and Rosenberg, 2006). Therefore, in this study same data sets were analyzed with two additional algorithmic parameters program that aimed to examine phylogenetic certainty for quantification of incongruence of phylogenetic signal include:

(1) BMGE Alignment (Block Mapping and Gathering with Entropy), this program remove ambiguously or a too large proportion of gaps in aligned region. This software selects the most suitable region for phylogenetic inference by estimating a variability index for each character. Those indexes are estimated by an entropy-like function weighted by standard BLOSUM similarity matrices (Criscuolo and Gribaldo, 2010). This entropy-based method has been shown to be more accurate than other trimming approaches (Batista *et al.*, 2011).

(2) PRANK multiple aware-alignment program, this program marks the alignment gaps differently depending on their origin in insertion or deletion events lead to treats insertions correctly and avoids over-estimation of the number of deletion events. This determination can be used to provide the inferred ancestral sequences (Löytynoja and Goldman, 2010).

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Phylogenetic tree of the individual DNA region and combined region were constructed by MrBayes v.3.2.1. Bayesian analysis was selected to use in this study because it has a better resolving than the maximum parsimony and maximum likelihood strict consensus tree in previous *Amorphophallus* studied (Sedayu *et al.*, 2010) and more suitable for tackling such branch length variation (Holder and Lewis, 2003; Turner *et al.*, 2013).

4.2.2.1 Character state of individual and combined region.

The character data was analyzed for investigation of statistical quantity and the utility of those regions for resolving phylogenetic relationship. The characteristics and suitable models of nucleotide substitution for each alignments show in Table 4.4.

In addition, other sequencing data of 17 species from Thailand were added only in *FLint2* and ITS region aim to achieve completed sampling set of *Amorphophallus* in Thailand and to determine certainty of phylogenetic tree when new samples were combined. Additional sequencing data was received from collaboration with Hamburg botanical garden, Germany. The character data was analyzed and a suitable model of nucleotide substitution for each alignments show in Table 4.5

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Table 4.4 Value of statistical parameters of individual and combined data matrices with different alignment method.

	trnL-trnF spacer			*FLORICAULA/LEAFY* (*FLint2*)			Internal transcribed spacers (ITS)			Combined data		
	MAFFT	BMGE	PRANK	MAFFT	BMGE	PRANK	MAFFT	BMGE	PRANK	MAFFT	BMGE	PRANK
Models of nucleotide substitution	TPM2uf+I+G	GTR+G+I	HKY + G	TPM1+G	GTR+G+I	HKY+I	TrN+G	TPM2+I+G	HKY+G	TrN+G	TPM2+I+G	HKY+I+G
Number of included positions in matrix	1224	901	1526	372	252	439	1201	796	1660	2699	1950	3413
Length range	835-986	801-901	835-986	177-322	138-253	177-322	805-964	665-796	805-964	1101-2153	886-1494	904-2153
%GC content	32.311	33.157	32.327	50.469	52.630	50.469	67.238	67.2	67.238	48.011	47.058	48.011
Percentage of variable sites (%)	16.83	14.761	8.06	36.828	33.730	25.968	54.397	44.548	26.312	30.641	29.333	17.785
Percentage of informative sites (%)	9.069	9.101	4.718	18.280	16.270	11.845	29.012	38.995	14.641	15.006	14.667	9.2
Number of indel	83	22	115	53	15	70	154	76	236	307	129	454
Percentage of Identification site (%)	57.1	79.1	47.7	30.1	43.7	32.1	20.2	83.3	9.5	24.9	35	20.3
Percentage of Pairwise identify (%)	93.3	97.8	92.5	79.1	89.7	77.8	80.3	83.4	77.9	74.9	76.9	66.6
Percentage of number of clades in ingroup with >0.85 posterior probability	75	80	75	75	70	70	82.6	88.9	86.2	75.6	80	78.4

Table 4.5 Value of statistical parameters of additional *Amorphophallus*'s samples matrices with different alignment method.

	FLORICAULA/LEAFY (*FLint2*)			Internal transcribed spacers (ITS)		
	MAFFT	BMGE	PRANK	MAFFT	BMGE	PRANK
Models of nucleotide substitution	TPM1+G	GTR+G+I	HKY+I	HKY+I+G	HKY+G	GTR+I+G
Number of included positions in matrix	381	252	665	1125	796	1448
Length range	140-323	140-323	140-335	758-994	758-994	758-994
%GC content	50.471	52.886	50.802	66.888	64.692	66.888
Percentage of variable sites (%)	52.752	48.81	22.857	60.089	49.795	14.48
Percentage of informative sites (%)	30.446	30.952	12.481	40.444	43.094	8.137
Number of indel	80	25	133	180	38	320
Percentage of Identification site (%)	41.8	46.5	36.9	38.1	31.5	24
Percentage of Pairwise identify (%)	77.3	88.4	73.6	81.4	86.4	73.3
Number of clades in ingroup with >0.85 posterior probability	76.9	75.7	70	78.9	80.2	62.3

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When considering among individual region and MAFFT alignment, the *trnL-trnF* was the longest with 1,224 bp, whereas *Flint2* was the shortest with 372 bp. The result showed that ITS region has the highest variation and phylogenetic informative, followed by the more conservative *FLint2* and *trnL- trnF* spacer. Both the ITS and *FLint2* regions have a high rate of variable sites due to their non-coding position in the nuclear genome, which has rapid evolutionary rates (Chen *et al.*, 2004), high sequence divergence by biparental inheritance (Wilson, 2003) and intragenic uniformity (Alvarez and Wendel, 2003). Grob *et al.* (2004) found that *FLint2* is relatively short in *Amorphophallus* and highly variable. Moreover, this region yields more informative sites than the chloroplast

genes (*matK*, *rbcL* and the *trnL* intron) investigated in this genus. Similarly, Sedayu *et al.* (2010) also studied the evolution of *Amorphophallus* spp. based on *trn*L, *rbc*L and *FLint2,* and found that *FLint2* had the highest level of variation among these regions. Nevertheless, phylogenetic studies based on these regions do not produce consistent cladograms, due to a high level of conflicting signal in the informative characters. Consequently, there is a need for more nuclear genes for resolving phylogenetic relationship and provide independent gene trees. Therefore, present study showed that additional ITS region had useful to improve discriminate ability due to this region is quite long (805-920 bp) when compare with *FLint2* (170-355 bp) and also preform highest percentage of informative sites. Alike previous studies also showed that the ITS has a higher discriminatory power than *rbcL*, *matK* or *trnH–psbA* region. Moreover, a combination of ITS with any plastid marker was able to discriminate 69.9–79.1% of species, compared with only 49.7% with *rbc*L and *matK* in seed plant (Li *et al.*, 2011a). making this region applicable in genus-level study. Although *trnL*- *trnF* spacer gene presented low informative data but there are suggested that chloroplast data are needed as backbone phylogeny and have ability to amplify templates from old herbarium specimens (Wynns and Lange, 2014). Moreover, this region easy to amplify due to thousands of plastid chromosomes in plant cell with there are single-copy thus liberate of paralogy problem (Alvarez and Wendel, 2003; Palmer, 1987).

With the BMGE alignment, this program is very strict model by trimming of the non-homologous aligned blocks and remains only selecting characters that suited for increase phylogeny resolution (Revuelta *et al.*, 2014). By this reason, BMGE alignment showed shortest character with an average loss sequence character

of 30% for each region and 36% for additional sampling data, lowest indel numbers and reduce percentage of variable sites when compare with MAFFT alignment in every region. As a variable site is a site that contains at least two types of nucleotides and some of them can be informative site (Ward and Holmes, 2007) therefore by reducing number of variable data will reduce percentage of informative data as show in this result. However, percentage of informative site just slightly decreases from MAFFT analysis.

With the PRANK alignment, this program is very relaxing model by using phylogenetic information to treat insertions and deletions as separate evolutionary events (Löytynoja and Goldman, 2010). Resulting in longest character in every regions and additional sampling data with an average increasing sequence character of 27% for each region, increase indel number and reduce both of percentage of variable and informative site when compare with MAFFT alignment. Since PRANK is gap-rich data methods, previous studies also show that the resulting alignment from this program is too long and gappy. However, this error may not be too critical in evolutionary analyses (Collingridge and Kelly, 2012; Löytynoja, 2014). Gaps represent insertion or deletion of the sequence, or calling indels (Strope *et al.*, 2009). When this program creates more alignment gaps, that influence to increase indel number. Besides, reducing of variable site also effect to informative site. The resulted alignments from PRANK may be fragmented by many gaps and may not be as visually symmetrical as the traditional alignments. However, this program can provide correct homology event (Löytynoja and Goldman, 2008).

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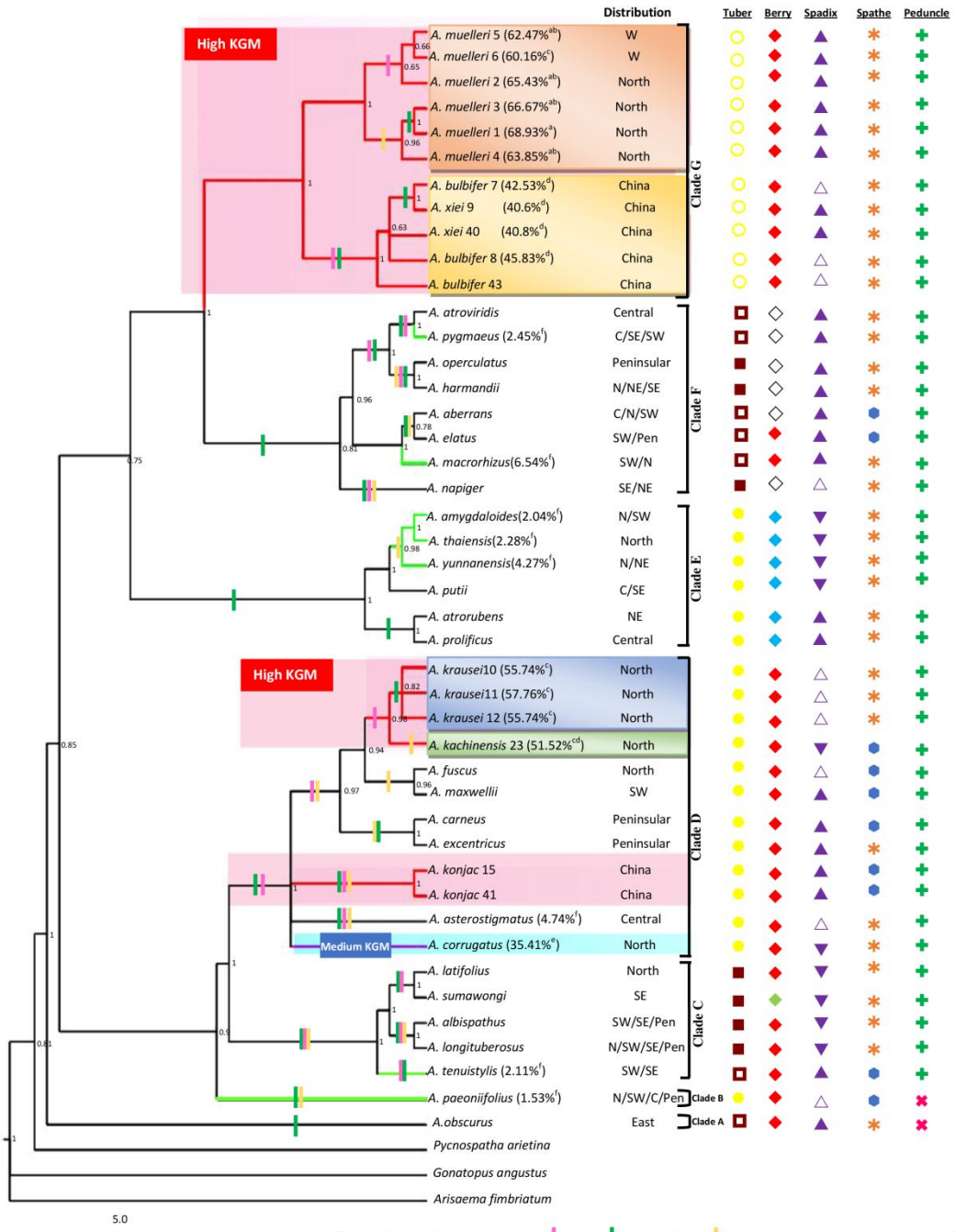
4.2.2.2 *Amorphophallus* phylogeny and morphological variation by clade

4.2.2.2.1 Combined data analysis

The combined molecular analysis was based on the *trnL-trnF* spacer, ITS and *FLint2* that gave an improved resolution among Thai *Amorphophallus* species as compared to earlier studies (Grob *et al.*, 2004; Grob *et al.*, 2002; Sedayu *et al.*, 2010). It shows a number of well-supported clades that are not based on single region data alone. Posterior probability (PP) support in phylogenetic clade were identified followed the work of Antonelli (2008) that can be defined as strong (>0.90), moderate (0.85-0.90), weak (0.75-0.84) or ambiguous (<0.75). Additionally, percentage of number of clades with moderate-strong support PP >0.85 were calculated (Table 4.4). The result showed that each phylogenetic tree has ability to generate percentage of number of clades with PP >0.85 more than 70% in every alignment program. This is indicated that those phylogenetic data relationship are acceptable. The phylogeny that based on the combined data set indicates seven clades (clades A-G) within the *Amorphophallus* in-group by MAFFT analysis (Figures 4.3). Additional phylogenetic tree of BMGE and PRANK alignment were showed in Figures 4.4 and 4.5 respectively. All result from three DNA region analyses are congruent in several parts in morphological characters and some geographical coherence.



Each support DNA region are indicated by different bar : trnL-trnF spacer ( ), ITS ( ) and FLint2 ( ). Number in blanket represent of KGM content with differenced statistic number. Red alphabet represent high glucomannan content group. Geographic distribution (N, North; NE, North-eastern; SW, South-western; C, Central; SE, South-Eastern; Pen, Peninsular), indicated to right of tree. Character of tuber can be divided into four types: globose/no-offset (○), globose with offset (●), elongate/unbranched (■) and elongate with branching (■). Berry color can be divided into four types: red/orange color (◆), white color (◇), blue color (◆) and green color (◆), Length of spadix compare with spathe can be divided into four types: longer (▲), shorter (▼) and equal (△). Color of spathe can be divided into two types: pale color (✱) and dark color (⬟). Peduncle character : long (✚) and short (✖).

Figure 4.3 Combined region phylogenetic tree of *Amorphophallus* spp. in Thailand. Numbers above branches indicate the posterior probability of the clade.

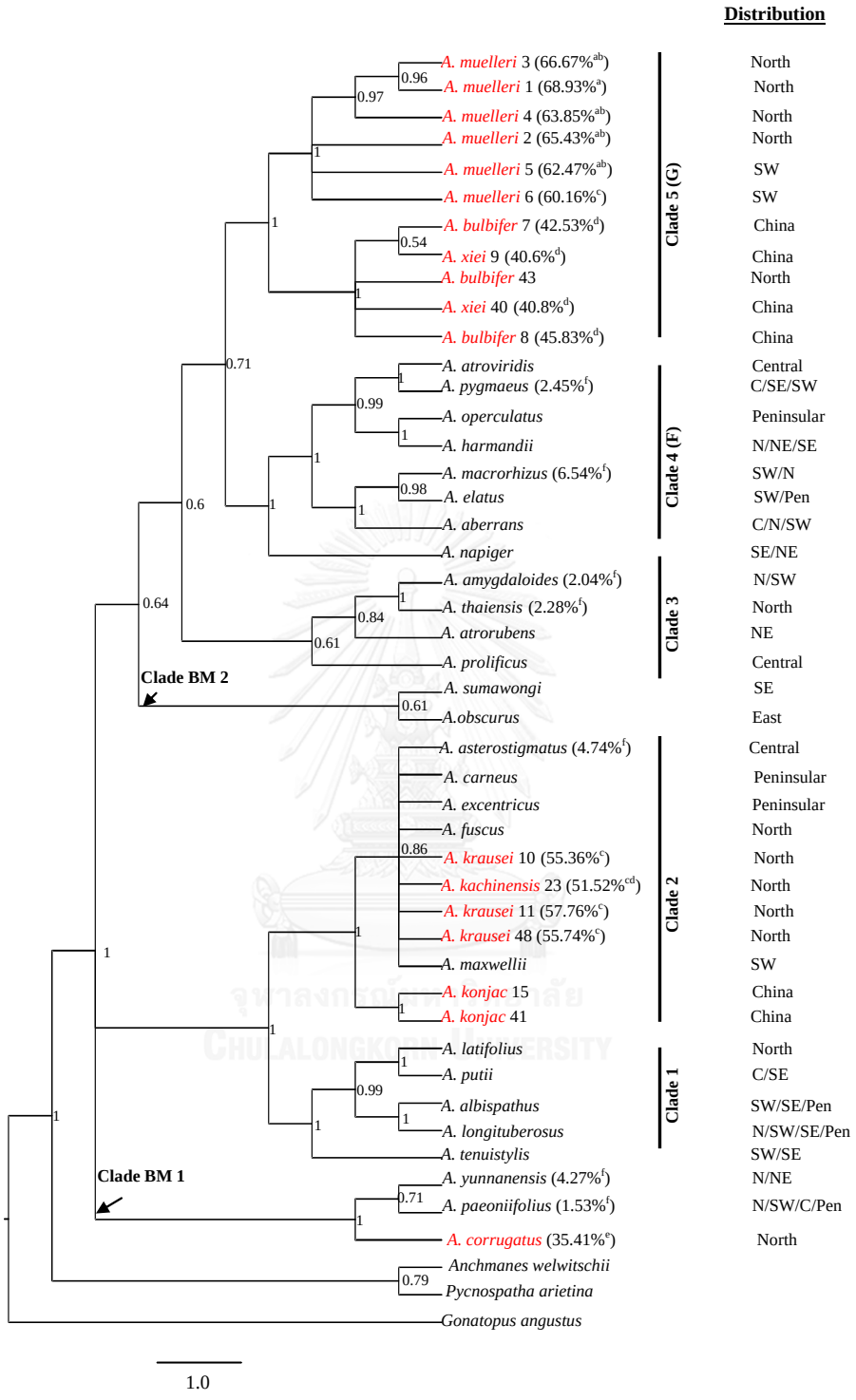


Figure 4.4 Combined region phylogenetic tree with BMGE alignment. Numbers above branches indicate the posterior probability of the clade. Red alphabet represent high KGM group and number in bracket represent of KGM content with significant differences (p<0.05) between samples. Geographic distribution (N, North; NE, North-eastern; SW, South-western; C, Central; SE, South-Eastern and Pen, Peninsular).

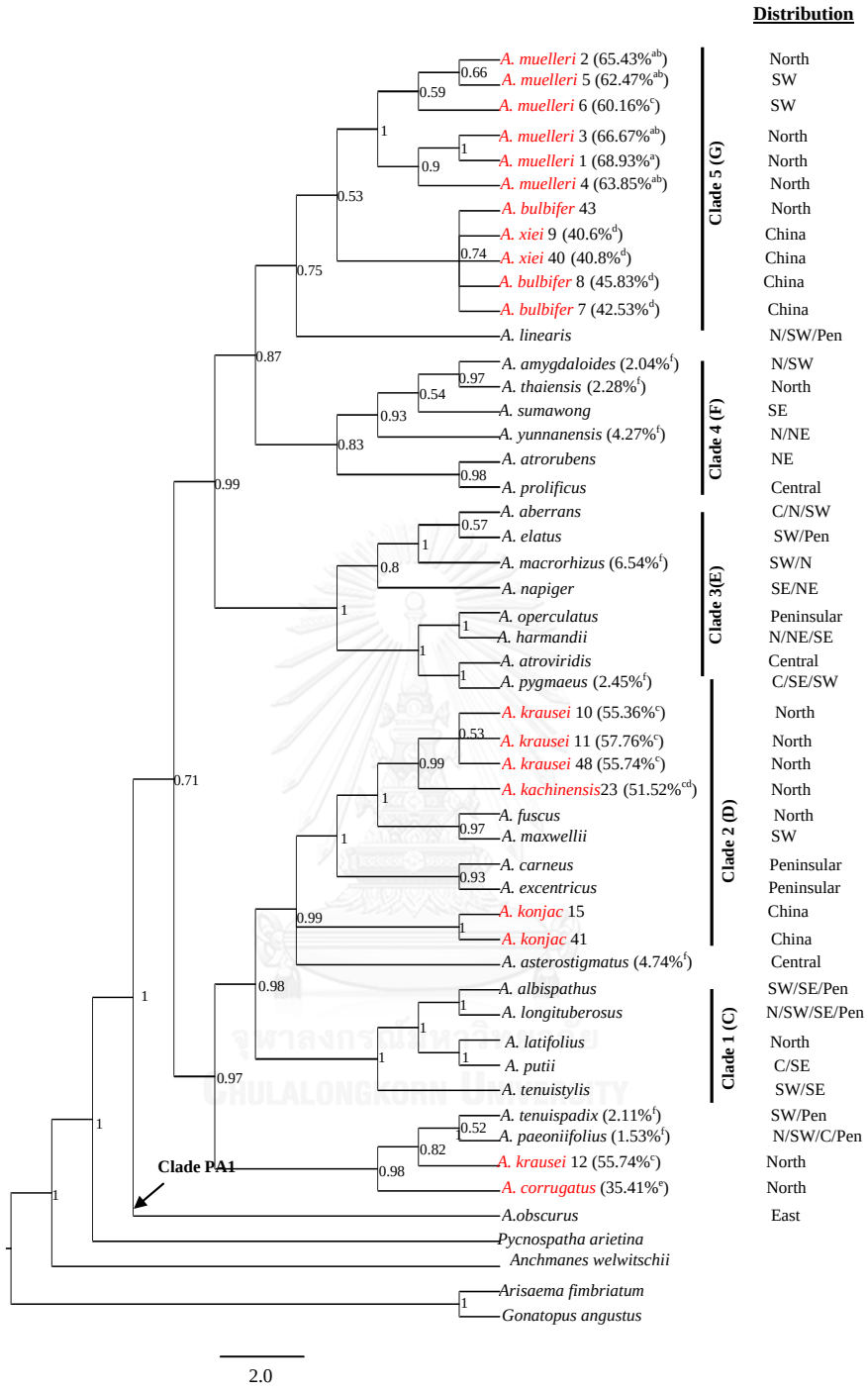


Figure 4.5 Combined region phylogenetic tree with PRANK multiple aware-alignment. Numbers above branches indicate the posterior probability of the clade. Red alphabet represent high KGM group and number in bracket represent of KGM content with significant differences (p<0.05) between samples. Geographic distribution (N, North; NE, North-eastern; SW, South-western; C, Central; SE, South-Eastern and Pen, Peninsular).

The first clade (A) contains only *A. obscurus*, which is certainty as isolated clade with strong support in different alignment program and clearly identify under individual ITS region. This species show smallest inflorescence (around 8 cm high) in Figure 4.6, unique white spotted leaflets and occurs only in the Eastern regions of Thailand.

The second clade (B) contains only *A. paeoniifolius*, which is clearly isolated clade under individual ITS and *FLint*2 region and belongs to low KGM content group. It differs from other species by a unique inflorescence (Figure 4.6) and strongly warty petiole. In this studied, this is only one species in *paeoniifolius* group (Grob *et al.*, 2002). This species can be found all over in Thailand. Similar with previous report that only *A. paeoniifolius*, can be found every region by association with human disturbance and is probably not native to the country (Boyce *et al.*, 2012). This clade is certainty as isolated clade with strong support by MAFFT and PRANK alignment. However, in BMGE alignment, this clade is shared with *A. corrugatus* (PP 1.0) and *A. yunnanensis* (weak support; PP 0.76) that is unrelated morphological unless offset globose tuber.

Those two clades (A and B) show a difference from other clades by their inflorescence peduncle entirely or largely hidden in the soil that seemingly adapted to pollinating and distributing animals living at soil level (Hetterscheid and Claude, 2012).

(A)  (B) 

Figure 4.6 (A) Inflorescence and leaflet of *A. obscurus*; (B) Inflorescence of *A. paeoniifolius*. (The International Aroid Society, 2014)

The third clade (C) consists of five taxa, *A. tenuistylis* was analyzed for KGM content and found belongs to low KGM content group. This clade is identified clearly under all individual DNA regions (*trnL-trnF* spacer (PP 0.98); ITS (PP 0.95); *FLint2* (PP 0.86)) as well as the combined phylogeny, with strong support (PP 1.0). All of them have elongated tuber and most of them are spadix shorter than spathe, exterior spathe and spadix is pale color and red/orange berry except in *A. sumawongi* with wrinkled surface with green color berry. Within this clade, three subclades can be recognized:

(i) first subclade is composed of sister group, *A. latifolius* and *A. sumawongi* that differ by their appendix entirely covered with staminodes (Figure 4.7). However, this sister group in individual *trnL - trnF* and *Flint2* region is changed, *A. sumawongi* is the sister taxon to *A. obscures*, in agreement with previous studies that indicate the species' position in a 'Thai-Indochinese clade' that, despite high phylogenetic signal, is not supported by morphology (Sedayu *et al.*, 2010). However, *A. latifolius* never had been shown in any sample list of previous studied before.

(ii) second subclade is composed of sister group of *A. albispathus* and *A. longituberosus*, they share the general inflorescence morphology, the short, hair-like papillae in the spathe base, the large, helmet-shaped connective with latenal pores and the anise-like scent (Figure 4.8).

(iii) third subclade includes *A. tenuistylis* in isolated position and distinguished by spadix longer than spathe and exterior spathe and spadix is dark color (Figure 4.7).

This clade geography distributes from northern to southern region. When consideration on different alignment program, this clade shows same relation with strong support in every alignment program. Except for *A. sumawongi* that PP value is decease from strong in MAFFT alignment to moderate support in BMGE and PRANK alignment. From previous studies (Grob *et al.*, 2004; Grob *et al.*, 2002; Sedayu *et al.*, 2010) showed that *A. longituberosus* always grouping with Pseudracontium genus, which is their staminodial structure appendix look like *A. latifolius* and *A. sumawongi*.

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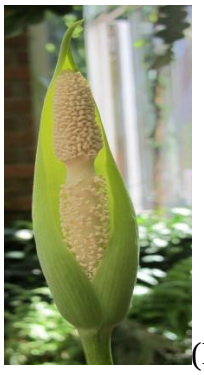
(A)  (B)  (C) 

Figure 4.7 Inflorescence and detail of spadix of (A) *A. latifolius*; (B) *A. sumawongi* and (C) *A. tenuistylis*.
(The International Aroid Society, 2014)

(A)  (B) 

Figure 4.8 Inflorescence and detail of spadix of (A) *A. albispathus* and (B) *A. longituberosus* with tuber. (The International Aroid Society, 2014)

The fourth clade (D) consists of nine taxa (PP 1.0), four of which belong to the medium (*A. corrugatus*) and high KGM content groups (*A. krausei*, *A. kachinensis* and *A. konjac*). This clade is identified clearly under individual ITS region (PP 0.8). Within this clade, there are four subclades:

First subclade contains three sister groups: (i) first sister group comprises of *A. krausei* (3 samples) and *A. kachinensis* with strong support (PP 0.94), they shares enlarge filaments of the lower staminate flowers and come from northern Thailand (Figure 4.9); (ii) second sister group, *A. fuscus* and *A. maxwellii*, share the base dark maroon and purple background spathe (Figure 4.9); (iii) third sister group includes *A. carneus* and *A. excentricus*. There are very similar in morphologies. The mainly difference in *A. carneus* is having smaller offsets, a large/broader appendix with more prominent grooves and the adaxial surface of the leaflets (Figure 4.9). Moreover, both of the species are from southern Thailand.

Second subclade includes *A. konjac* (2 samples), is belonging to high KGM content group and its inflorescence resembles *A. maxwellii* but the latter has much longer styles, larger leaflets, a wide spathe and a thinner appendix (Figure 4.10). Therefore, this species is attributed to this clade.

Third subclade includes *A. asterostigmatus,* with unique character being silvery greyish reddish to greenish with scattered or elliptic dark reddish brown spot petiole. The uppermost staminate of this species is entirely fused, resulting in structure intermediate between staminate flowers and staminodes (Figure 4.10).

Fourth subclade includes *A. corrugatus*, differ by strongly cerebriform appendix and lacking of the staminodes, only the non-staminodial fissures remain (Figure 4.10).

The taxa in this clade occur in a diverse range of localities around Thailand, with the exception of *A. konjac*, which is restricted to China. When consideration on different alignment program, this clade is strong supported in PRANK alignment and PP values of internal node are increased. However, in BMGE alignment, this subclade lose their relationship with ambiguous support (PP 0.5) while *A. fuscus* and *A. maxwellii* sister group still grouped together but with ambiguous support (PP 0.51). Only *A. konjac* subclade still grouped together with a strong support clade. This indicated that clade D is certainty clade with strong support even insertion and deletion event were separated consideration whereas eliminated uncertainty data was affected to their relationship. Grouping of fourth clade (D) also was recognized by Grob *et al.* (2002) by link in morphologically from a unique 12 bp insertion within *mat*K, which resulted in the extra amino acids including valine, tryptophan, tyrosine and leucine.

Furthermore, third clade (C) and fourth clade (D) found to be paraphyly clade that is agreed with previous work that those species were joined in Continental Asia II (Grob *et al.*, 2004; Grob *et al.*, 2002; Sedayu *et al.*, 2010).

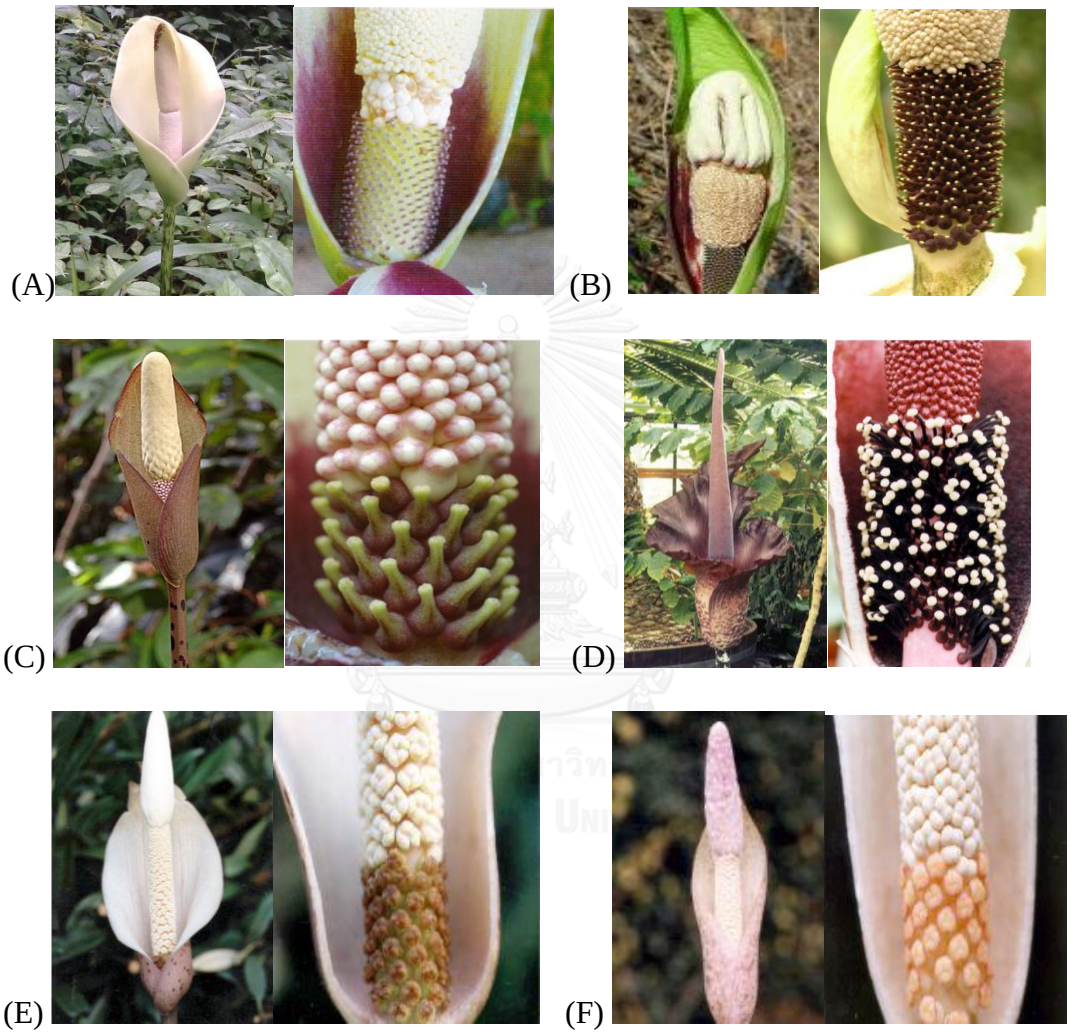


Figure 4.9 Inflorescence and detail of spadix of (A) *A. krausei*; (B) *A. kachinensis*;(C) *A. fuscus*; (D) *A. maxwellii*; (E) *A. carneus* and (F) *A. excentricus*.

(The International Aroid Society, 2014)

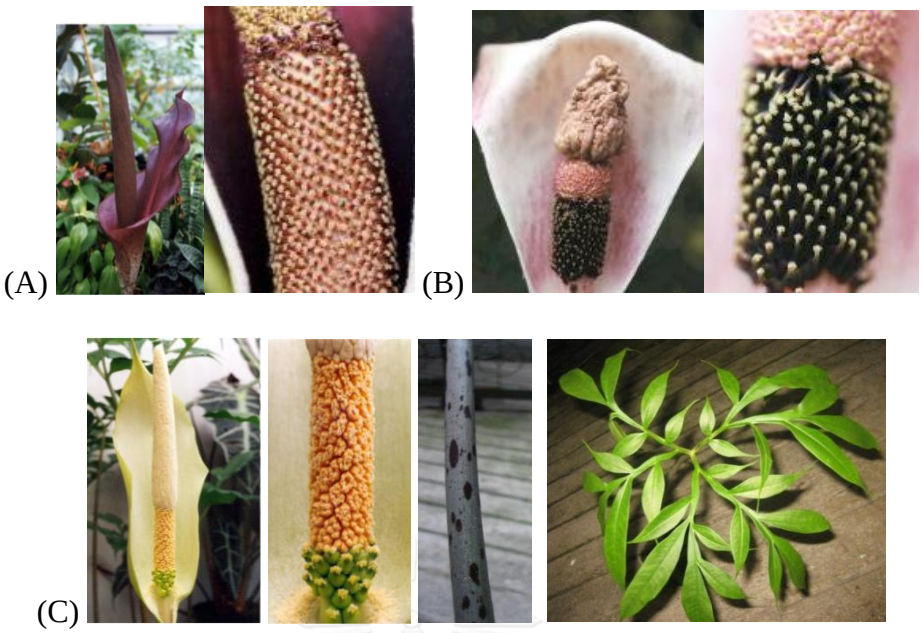


Figure 4.10 Inflorescence and detail of spadix of (A) *A. konjac*; (B) *A. corrugatus* and (C) *A. asterostigmatus*. (The International Aroid Society, 2014)

The fifth clade (E) is made up of six taxa (PP 1.0), three of them (*A. amygdaloides*, *A. thaiensis*, *A. yunnanensis*) contain only levels of KGM content. This clade is clearly identified under individual ITS (PP 0.8) and *FLint2* region (PP 0.92). Blue color berry is the unique morphology for this clade. Additional shared morphologies are globose with offset tuber and pale color of exterior spathe and spadix. Within this clade, two subclades can be recognized.

First subclade composed with *A. amygdaloides*, *A. thaiensis*, *A. yunnanensis* and *A. putii*. They were shared shorter spadix than spathe and their similar florescence. *A. putii* differ by the highly laterally compressed appendix (Figure 4.11).

Second subclade includes *A. atrorubens* and *A. prolificus*, which are separated by longer spadix than spathe inflorescence (Figure 4.12).

This group shares smooth dark greyish or reddish brown petiole and elliptic or elongate acuminate, adaxially slightly glossy green leaflets.

The taxa in this clade are found from northern through central part of Thailand. When consider different alignment program, this clade is strongly supported in PRANK alignment. Again, in BMGE alignment, this clade is lose their relationship between *A. atrorubens* and *A. prolificus* with moderate support (PP 0.87) and *A. yunnanensis* is moved to group with *A. paeoniifolius* but within weak support. Clade E showed agreement with Grob *et al.* (2002) that all samples belong to *yunnanensis* group, with striate pollen, concave spathe, stipitate spadix, striate pollen, blue berries and found in China, Indochina and Thailand.

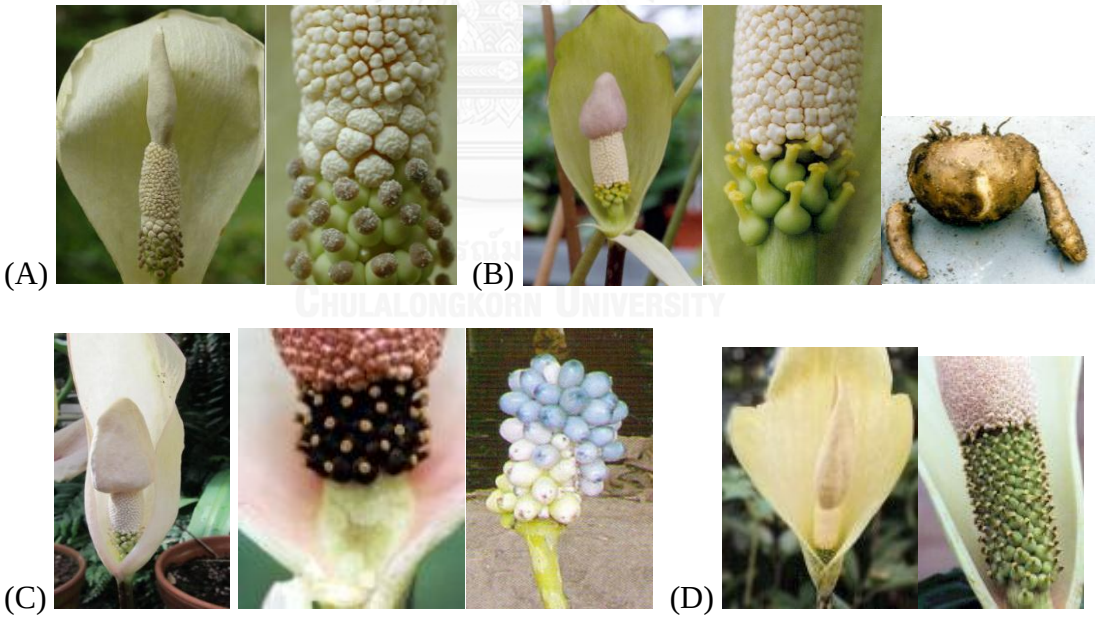


Figure 4.11 Inflorescence and detail of spadix of (A) *A. amygdaloides*; (B) *A. thaiensis* and tuber; (C) *A. yunnanensis* and berry and (D) *A. putii*. (The International Aroid Society, 2014)

(A)  (B) 

Figure 4.12 Inflorescence and detail of spadix of (A) *A. atrorubens* and (B) *A. prolificus* with tuber (The International Aroid Society, 2014).

The sixth clade (F) made up of eight taxa. Two taxa in this clade, *A. pygmaeus* and *A. macrorhizus,* belong to the low KGM content group. The clade is only weakly supported (PP = 0.77) but turn to be strong support in BMGE and PRANK alignment. Those species are characterized by elongated tuber, longer spadix than spathe and unique white color berry except for *A. elatus* and *A. macrorhizus*.

First subclade composed of (i) first sister group includes *A. atroviridis* and *A. pygmaeus,* which shared reddish margin, lower side flushed with purple-red (Figure 4.13); (ii) second sister group for *A. operculatus* and *A. harmandii*, both species differ from other by elongated branching tuber and thin purplish red margins (Figure 4.13).

Second subclade composed of (i) sister group of *A. aberrans* and *A. elatus* differ by dark color in exterior spathe and spadix (Figure 4.14). *A. aberrans* has basal velum interior to the spathe are unique in the genus while *A. elatus* may be looked upon as a small version of *A. macrorhizus*; (ii) isolated taxa of *A. macrorhizus* by hairy appendix, hairs on the petiole, peduncle and produce red fruit (Figure 4.14).

Third subclade belong to *A. napiger* which is distinguished by spadix shorter than spathe, leaf blade have deep rich green with a distinct greyish zone along the midrib, the lax disposition of the pistillate flowers, the stipitate spadix and the oval ovaries (Figure 4.14).

This clade occurs from northern Thailand down into the southern peninsula. When consideration of different alignment program, first subclade show strong support from three alignment program. However, in the second subclade, it is lost their relation in BMGE alignment due to its weak support (PP 0.75) by MAFFT alignment. PP value is decreased to ambiguous support (PP 0.57) in PRANK alignment. Whereas, in BMGE alignment, *A. macrorhizus* shows uncertainty by grouping with *A. elatus* (PP 0.98) instead of *A. aberrans*. It is indicated of uncertainty of this subclade. PP value of *A. napiger* is increased from weak (PP 0.77) to strong (PP 1.0) support. In clade F of this study, *A. pygmaeus* and *A. napiger* shared same clade together, which were also found by Grob *et al.* (2002) and Grob *et al.* (2004). Morphology that supported by this clade is longituberosus-alliance but different from clade C with unique white color berry. This is indicated that the longituberosus group is polyphyletic and apparently, elongated tubers have evolved several times within *Amorphophallus*.

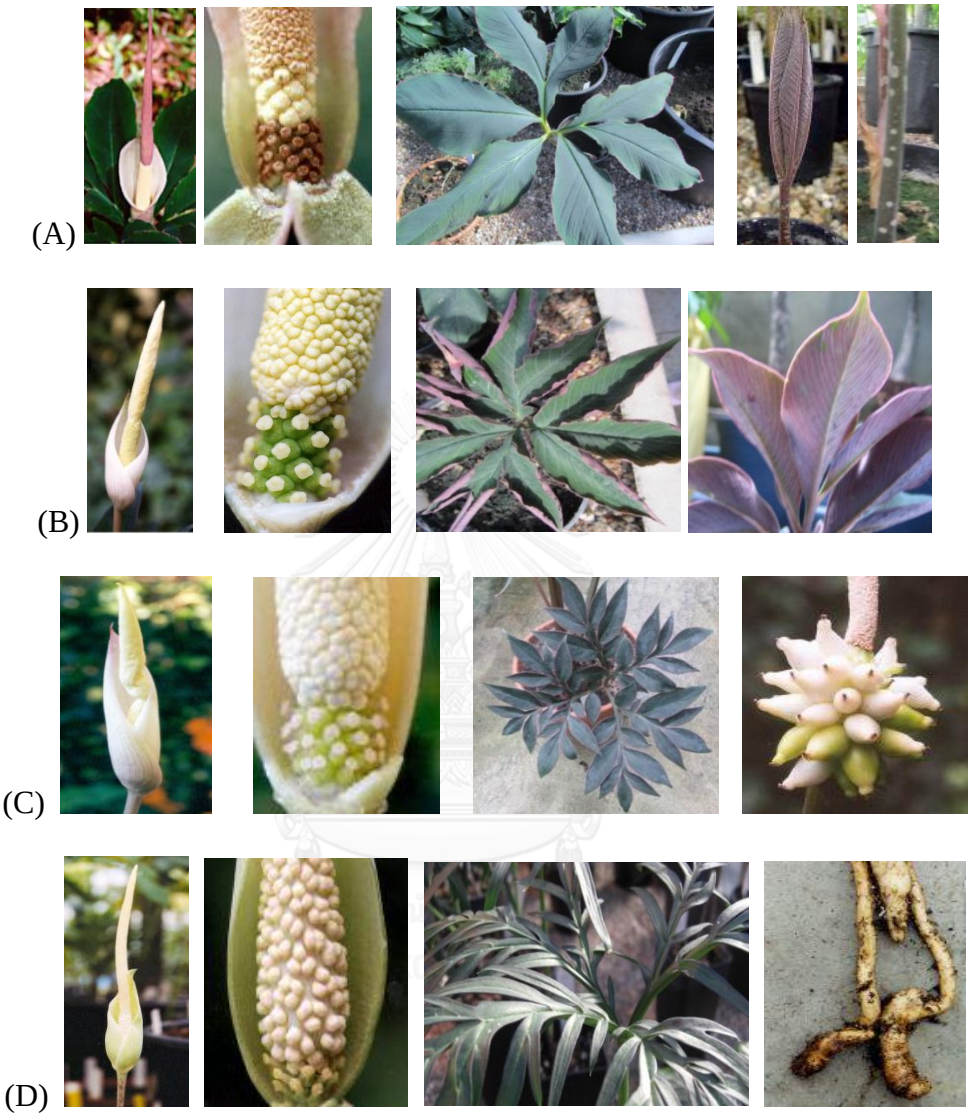


Figure 4.13 Characteristic and inflorescence of (A) *A. atroviridis;* (B) *A. pygmaeus*; (C) *A. operculatus* and (D) *A. harmandii*.

(The International Aroid Society, 2014)

Figure 4.14 Characteristic and inflorescence of (A) *A. aberrans*; (B) *A. elatus*; (C) *A. macrorhizus* and (D) *A. napiger*. (The International Aroid Society, 2014).

The seventh and final clade (G) is a well-supported group (PP 1.0) and clearly identified under all individual DNA regions (*trnL-trnF* spacer (PP 0.97); ITS (PP 0.99); *FLint2* (PP 1.0). However, *trnL- trnF* spacer and *FLint2* can not show internal relationship between those samples. This clade made up of eleven samples belonging to three taxa, all of which were found to have high KGM levels. This clade is characterized by the ability to produce bulbils formed between

leaflets with a reproductive function, globose tuber without offset, pinkish red margin leaflet, exterior spathe and spadix is pale color and the apomictic red berry (Figure 4.15). There are two subclades as followed.

First subclade is only *A. muelleri*, a well-defined geographic distribution. Both of sister clade belong to same province (Kanchanaburi for accession no.5, no.6 and Mae-Hong son for accession no.1, no.2) while samples from Tak province were located in separated subclade this may be due to Tak is connective area between Mae-Hong son and Kanchanaburi.

Second subclade includes *A. bulbifer* and *A. xiei*, which shared similar inflorescence except length of spadix and pink inside tuber. *A. bulbifer* has a spadix equaling or shorter than the spathe and tuber not have the pronounced root-scars and pink inside while *A. muelleri* tuber have yellow inside and rootscars annuliform. *A. bulbifer* and *A. xiei* occurred only in China, while *A. muelleri* is found from northern to western Thailand.

When different alignment program is considering, this clade shows certainty with strong support in *A. muelleri* with three alignments. The relationship in this first subclade is strongly support in PRANK alignment while relationship between both samples from Kanchanaburi is lost, which shows ambiguous support (PP 0.66) in MAFFT alignment before. This is indicated that both samples from Mae Hong son show closer relationship than sample from Kanchanaburi. Despite second subclade of *A. bulbifer* loses their relationship in BMGE alignment but still maintains in isolated clade of *A. bulbifer* no.43 from Mae Hong son in PRANK alignment. It remarkably pointed that *A. bulbifer* which

collected from Thailand have a distance genetic relationship from *A. bulbifer* that collected from China.

(A)

(B)

(C)

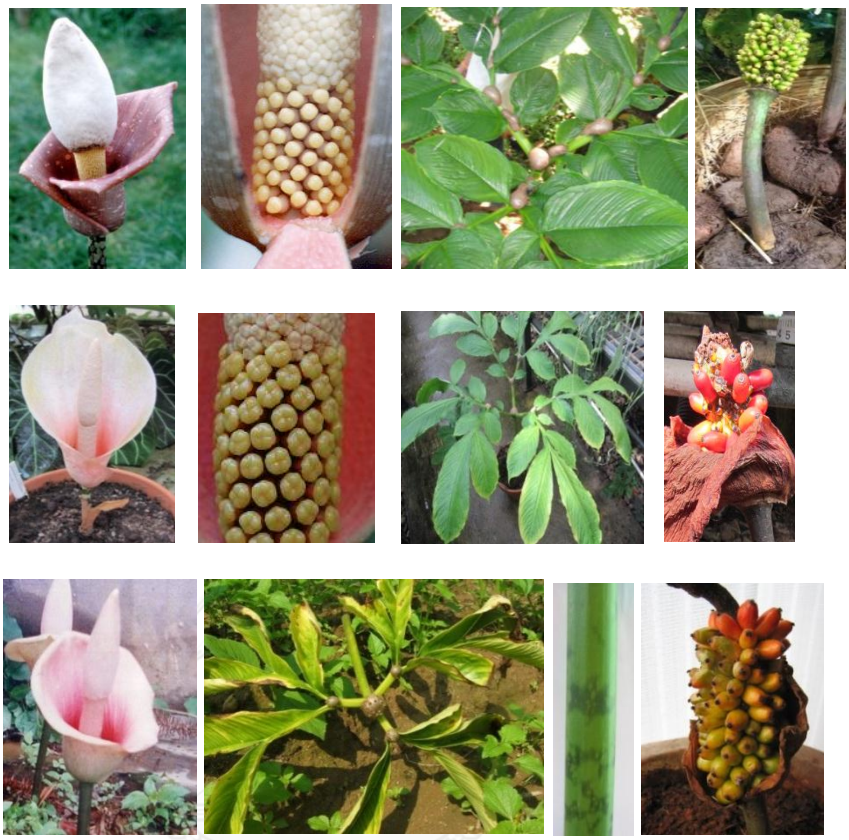


Figure 4.15 Inflorescence and characteristic of (A) *A. muelleri*; (B) *A. bulbifer* and (C) *A. xiei*.

In present study, *A. muelleri*, *A.bulbifer*, *A.xiei* always clustered together with strongly support, with 4-5 bases insertion and substitution along combined sequence, despite their inflorescence morphological differences. Heng and Zhi-Ling (2006) claimed that *A. xiei* formed a close relationship with *A. muelleri* by producing bulbils on leaves and not offset from the tuber. However, the molecular analysis in this present study indicated that *A. xiei* is most closely related to *A. bulbifer* as postulated by Hetterscheid who was mentioned that this species differs only in color from *A. bulbifer* in Flora of China (Li *et al.*, 2010). The exceptional

character of this clade is their ability to produce bulbils on the center of the main branching and on most distal branches, rachises winged distally from the lower main branchings (Boyce *et al.*, 2012).

Hetterscheid and Ittenbach (1996) divided bulbils into three main types namely (i) epiphyllar (e.g. *A. muelleri* and *A. bulbifer*); (ii) intercalary (developing inside rachis e.g. *A. angulatus*, *A. manta*, and *A. sparsiflorus*) and (iii) half-epiphyllar, found only in *A. yuloensis* (Figure 4.16). In Southeast Asia, only four species form bulbils are *A. bulbifer*, *A. muelleri*, *A. yuloensis* and *A. erubescens* (Sugiyama and Santosa, 2008). However, *A. yuloensis* did not show phylogenetic relationship with *A. bulbifer*, *A. muelleri* and *A. xiei* based on *rbc*L and *mat*K maker (Hetterscheid *et al.*, 2012). It is indicated that this clade has unique character to be differentiate from other clade. Moreover, in chromosomal number character indicated that *A. muelleri* and *A. bulbifer* are triploid (2n=39) which is different from among of *Amorphophallus* species that most of them are diploid (2N) such as *A. konjac* (2n=26), *A. paeoniifolius* (2n=28) and *A. prainii* (2n=28). (Chauhan and Brandham, 1984; Zhang *et al.*, 2010a).

Furthermore, fifth until seven clade (E-G) found to be paraphilic clade that is agree with previous work that those species joined in Continental Asia I (Grob *et al.*, 2004; Grob *et al.*, 2002; Sedayu *et al.*, 2010).

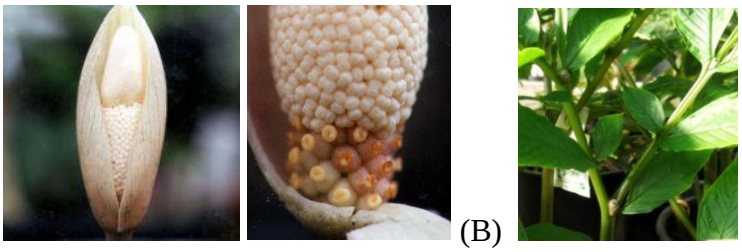
(A)  (B)

Figure 4.16 (A) Inflorescence and (B) bulbil on leaflet of *A. yuloensis*.
(The International Aroid Society, 2014)

4.2.2.2.2 Individual *trnL- trnF* spacer analysis

The *trn*L- *trn*F spacer is one of the most useful markers in plant systematics and still frequently employed (Huttunen *et al.*, 2013; Stech *et al.*, 2012). The characteristics of different alignments were analyzed and shown in Table 4.4. The length of the region ranged from 835 bp (*A. muelleri* no.1) to 986 bp (*A. obscurus*). The *trnL - trnF* spacer showed conserved region by lowest percentage of variation and information site. Phylogenetic trees were reconstructed included MAFFT Alignment (Figure 4.17), BMGE Alignment (Figure 4.18) and PRANK alignment (Figure 4.19)

The result show all of phylogenetic trees composed of seven clades, which are five clades (L1, L2, L4, L5 and L7) showed congruent result among different alignment programs with strong and moderate support. Only two clades (L3 and L6) showed disagreement relationship and is described here.

In MAFFT alignment, clade L3 shows ambiguous support (0.6) among ingroup species and *A. yunnanensis* is isolated subclade with strong support (PP1.0). This species differs from other by blue color berry. However, in BMGE alignment, isolated subclade of *A. yunnanensis* is collapsed and merged with other species with strong support (PP1.0). This isolated relationship of *A. yunnanensis* was lost by removing ambiguously aligned region meanwhile it was also help to increase more strongly support. In PRANK alignment, *A. corrugatus* show separate subclade with strong support (PP1.0) when insertions and deletions were treated differently that show uncertainty of this clade.

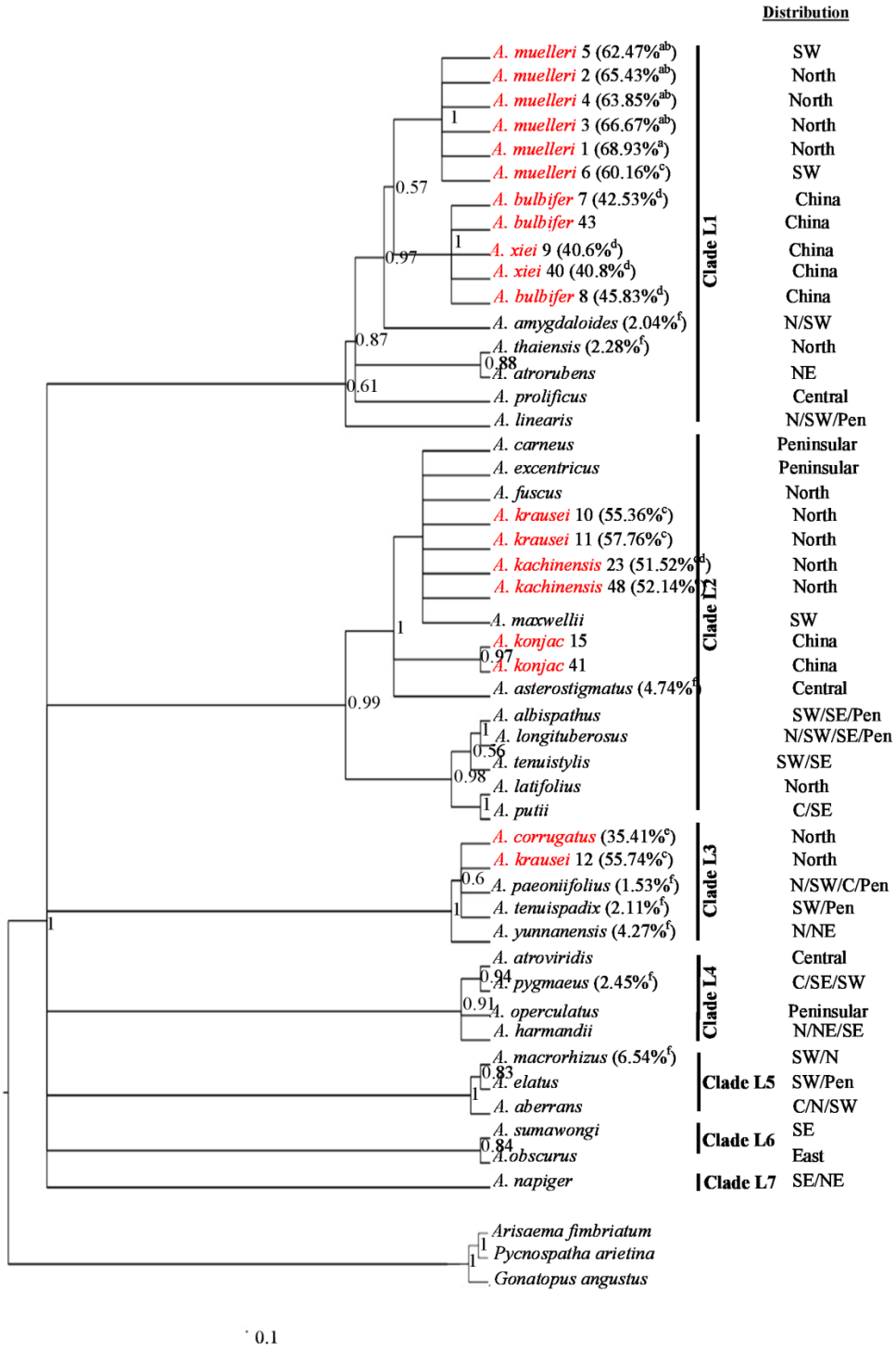


Figure 4.17 Phylogenetic tree of *trn*L-*trn*F spacer with MAFFT alignment. Numbers above branches indicate the posterior probability of the clade. Red alphabet represent high KGM group and number in bracket represent of KGM content with significant differences (p<0.05) between samples. Geographic distribution (N, North; NE, North-eastern; SW, South-western; C, Central; SE, South-Eastern and Pen, Peninsular).

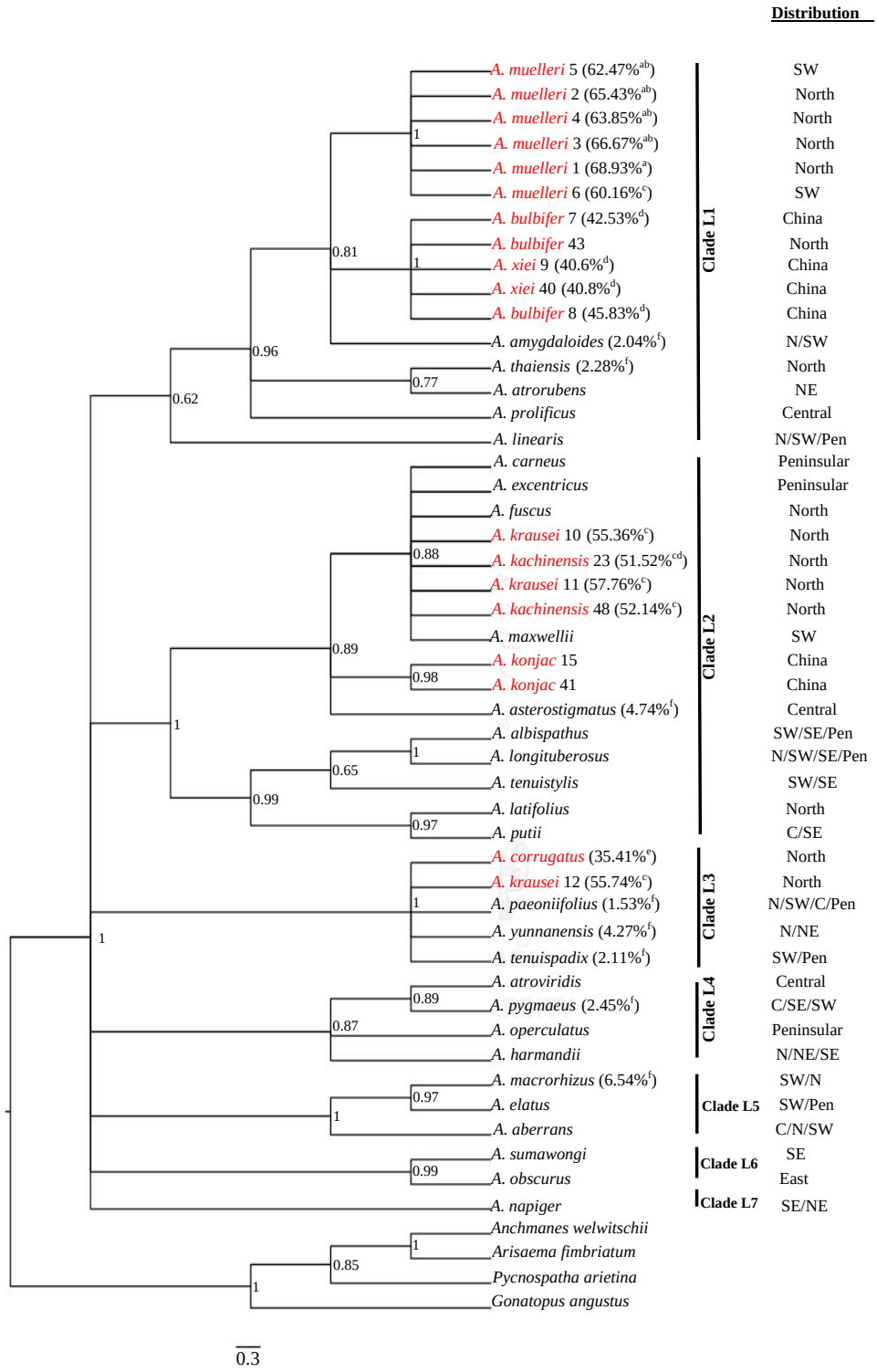


Figure 4.18 Phylogenetic tree of *trn*L-*trn*F spacer with BMGE alignment. Numbers above branches indicate the posterior probability of the clade. Number in bracket represent of KGM content with differenced statistic. Red alphabet represent high KGM content group. Geographic distribution (N, North; NE, North-eastern; SW, South-western; C, Central; SE, South-Eastern and Pen, Peninsular).

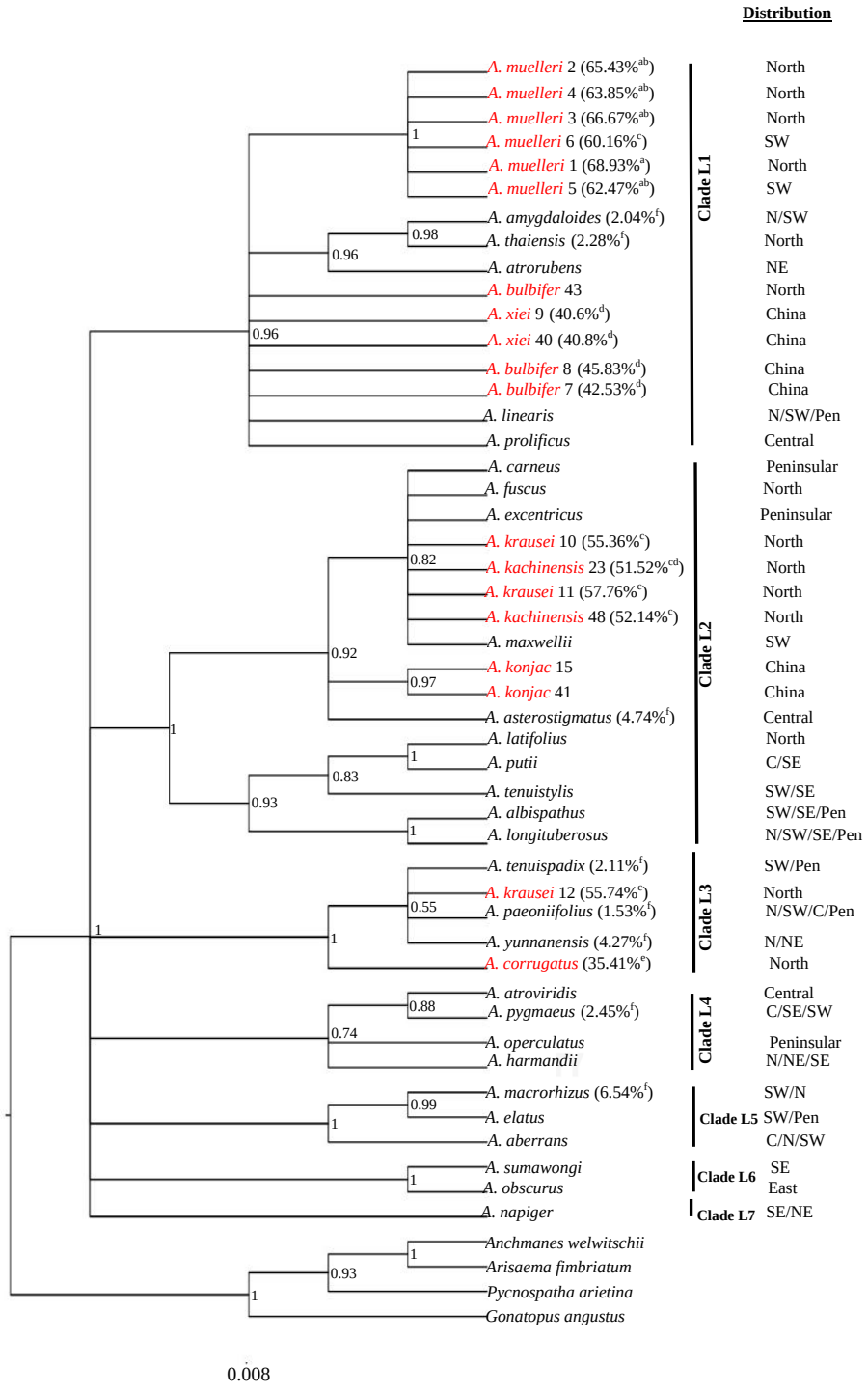


Figure 4.19 Phylogenetic tree of *trn*L-*trn*F spacer with PRANK multiple aware-alignment. Numbers above branches indicate the posterior probability of the clade. Number in bracket represent of KGM content with differenced statistic. Red alphabet represent high KGM content group. Geographic distribution (N, North; NE, North-eastern; SW, South-western; C, Central; SE, South-Eastern and Pen, Peninsular).

For clade L6, there are joined group between two different main morphologies, which are no-offset/offset of globose tuber and red-orange/blue color berry. In MAFFT alignment, sister clade of *A. bulbifer*/*A. xiei* and *A. muelleri* grouped together with ambiguous support (PP 0.57) and clearly isolated from *A. amygdaloides* with strong support (0.97). Whereas, in BMGE alignment, sister clade of *A. bulbifer*/*A. xiei* and *A. muelleri* was lost their relationship and merged with *A. amygdaloides* but within weak support (PP 0.81). In PRANK alignment, Only *A. muelleri* still grouped together while *A. bulbifer* and *A. xiei* were lost their relationship. *A. thaiensis* was moved to grouping with *A. amygdaloides* with strong support (PP 0.98) instead of grouping with *A. atrorubens* with moderate support (PP 0.88), which is similar to combined phylogeny.

The result of this study shows that percentage of number of clades with moderate-strong support PP >0.85 were increased with BMGE alignment when compare with MAFFT and PRANK alignment especially in clade T2, T3, T5 and T6. Whereas, percentage of number of clades with PP>0.85 of PRANK alignment showed similar result with MAFFT alignment. It is pointed that even PRANK alignment that is relaxing model, this alignment still maintained majority of phylogenetic characteristics. As a result of above mentioned grouping, it showed that the *trn*L- *trn*F spacer is highly conserve region since minor changing between phylogenetic trees were found. Nevertheless, this region can not show relationship between accession levels which is agreed with previous studies that relationship of this region was low especially in internal clades such as angiosperms and pleurocarpous mosses (Quandt and Stech, 2004) and Citrus species (Yingzhi *et al.*, 2007). However, it was indicated by several studies that the *trnL-trnF* intergenic

spacer is effective in determining phylogenetic relationships particularly in *Araceae* family like subfamily *Monsteroideae* (Tam *et al.*, 2004) and tribe *Schismatoglottideae* (Sin *et al.*, 2010). In addition, Grob *et al.* (2002) studied phylogenetic of *Amorphophallus* species with the chloroplast gene *matK* and *trnL* intron found that their phylogenetic clade congruence with several parts of this study, especially in clade L2.

4.2.2.2.3 Second intron of *FLORICAULA/LEAFY* (*FLint2*) analysis

The nucleotide sequence from *FLint2* region has been used in many phylogenetic studies with great success, with its introns containing a high level of sequence divergence at the species level (Schlüter *et al.*, 2007; Zimmer and Wen., 2013). The characteristics of different alignments were analyzed and show in Table 4.4 and Table 4.5 in case of additional sampling data set. The length of the region ranged from 177 bp (*A. fuscus*) to 322 bp (*A. operculatus*) with intimidated percentage of variation and information site and lowest indel number. Because of this region is short, making it quite difficult to amplify result in 81% of total sampling species can generated sequencing band. Phylogenetic trees were reconstructed included MAFFT Alignment (Figure 4.20), BMGE Alignment (Figure 4.21) and PRANK alignment (Figure 4.22). For phylogenetic trees of additional samples were reconstructed included MAFFT Alignment (Figure 4.23), BMGE Alignment (Figure 4.24) and PRANK alignment (Figure 4.25).

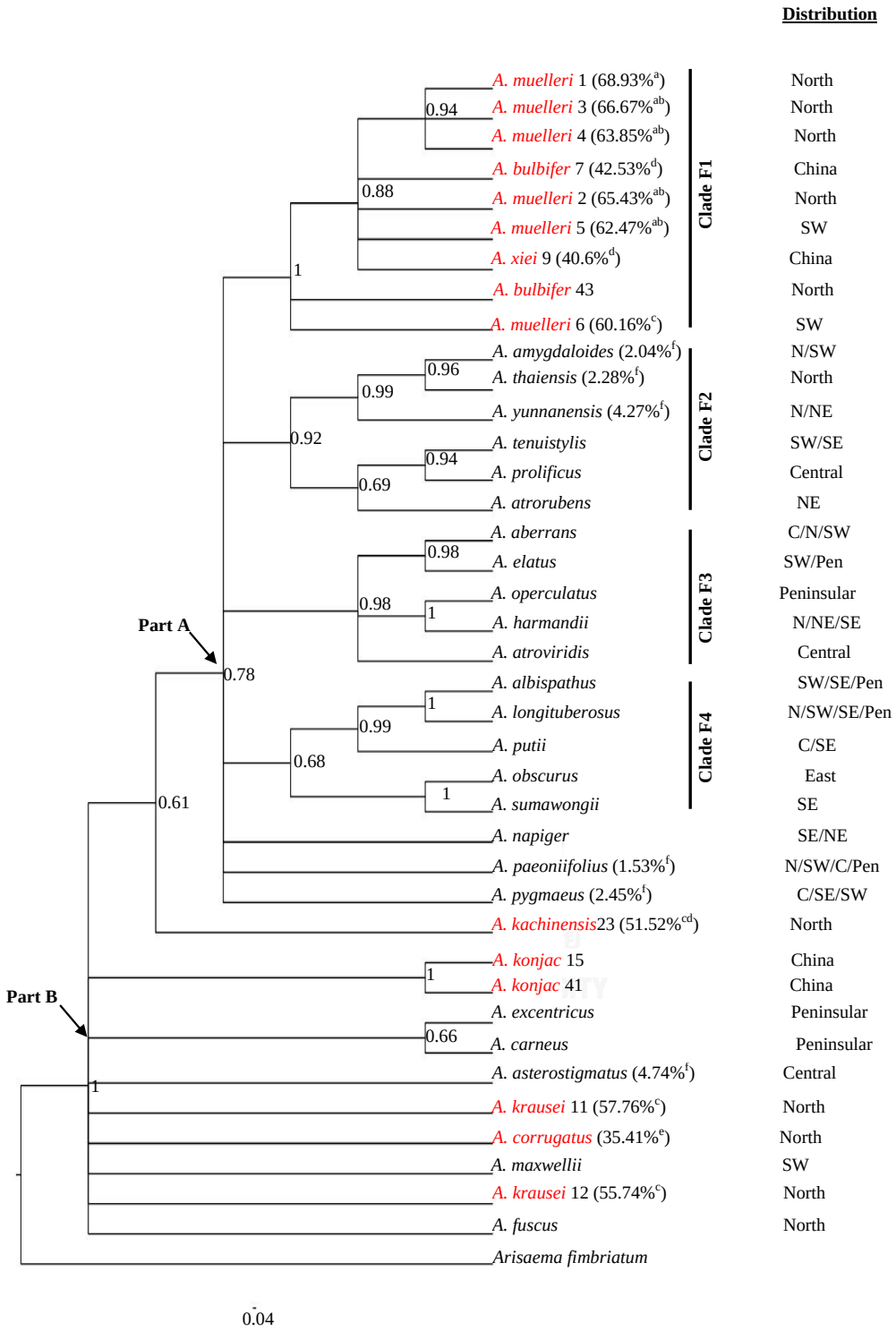


Figure 4.20 Phylogenetic tree of *FLORICAULA/LEAFY (FLint2)* with MAFFT alignment. Numbers above branches indicate the posterior probability of the clade. Number in bracket represent of KGM content with differenced statistic. Red alphabet represent high KGM content group. Geographic distribution (N, North; NE, North-eastern; SW, South-western; C, Central; SE, South-Eastern and Pen, Peninsular).

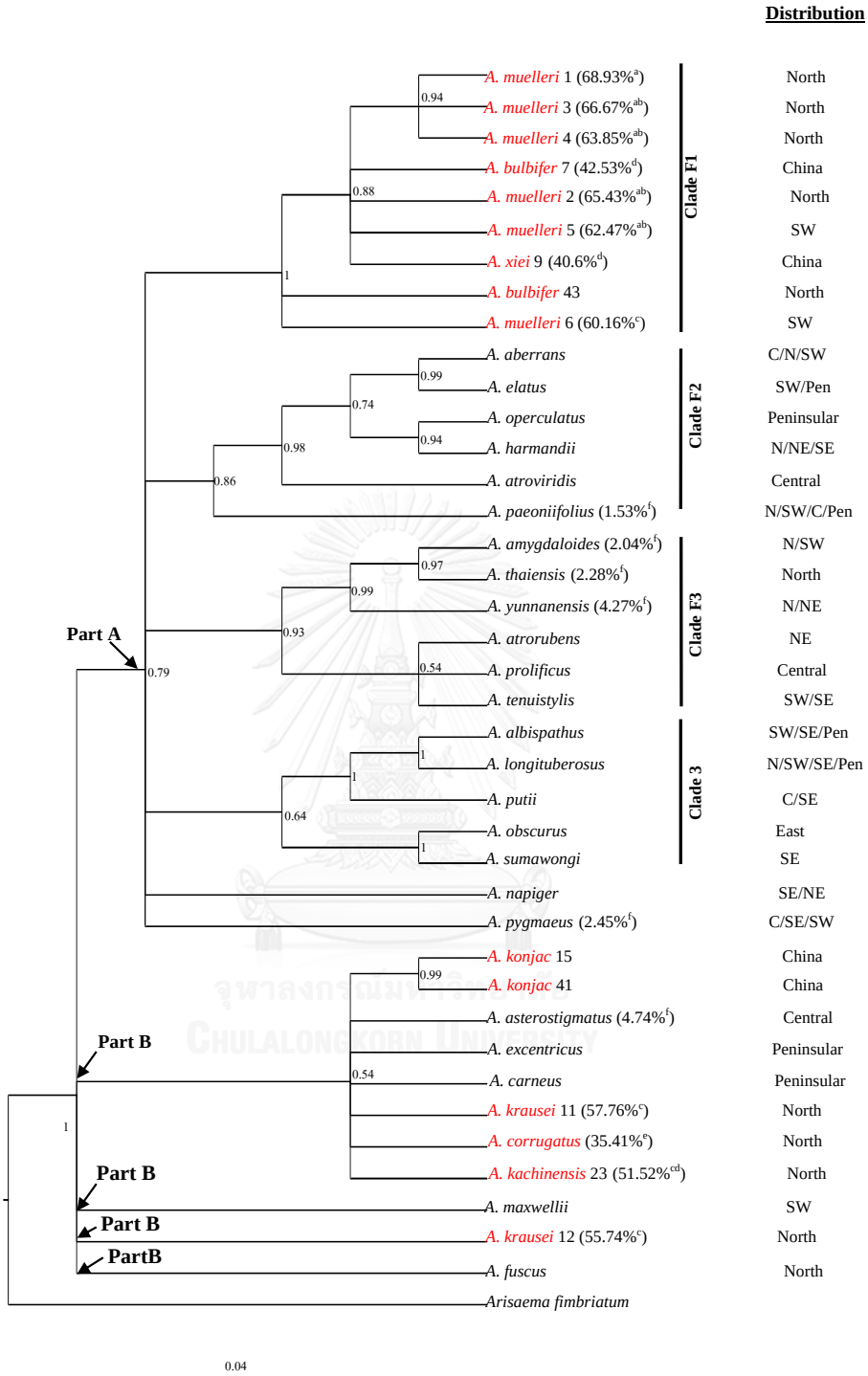


Figure 4.21 Phylogenetic tree of *FLORICAULA/LEAFY (FLint2)* with BMGE alignment. Numbers above branches indicate the posterior probability of the clade. Number in bracket represent of glucomannan content with differenced statistic. Red alphabet represent high glucomannan content group. Geographic distribution (N, North; NE, North-eastern; SW, South-western; C, Central; SE, South-Eastern and Pen, Peninsular).

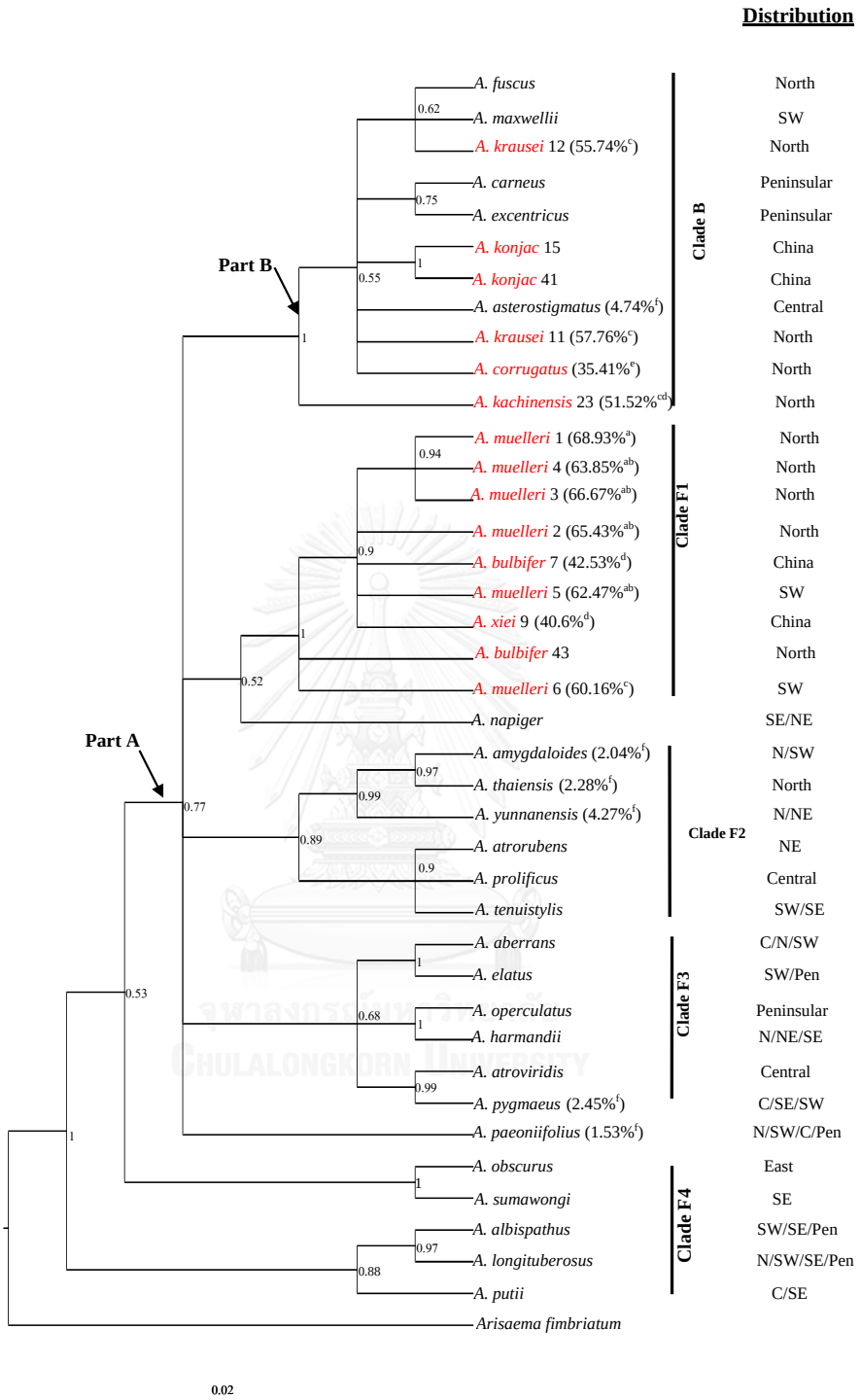


Figure 4.22 Phylogenetic tree of *FLORICAULA/LEAFY (FLint2)* with PRANK multiple aware-alignment. Numbers above branches indicate the posterior probability of the clade. Number in bracket represent of glucomannan content with differenced statistic. Red alphabet represent high glucomannan content group. Geographic distribution (N, North; NE, North-eastern; SW, South-western; C, Central; SE, South-Eastern and Pen, Peninsular).

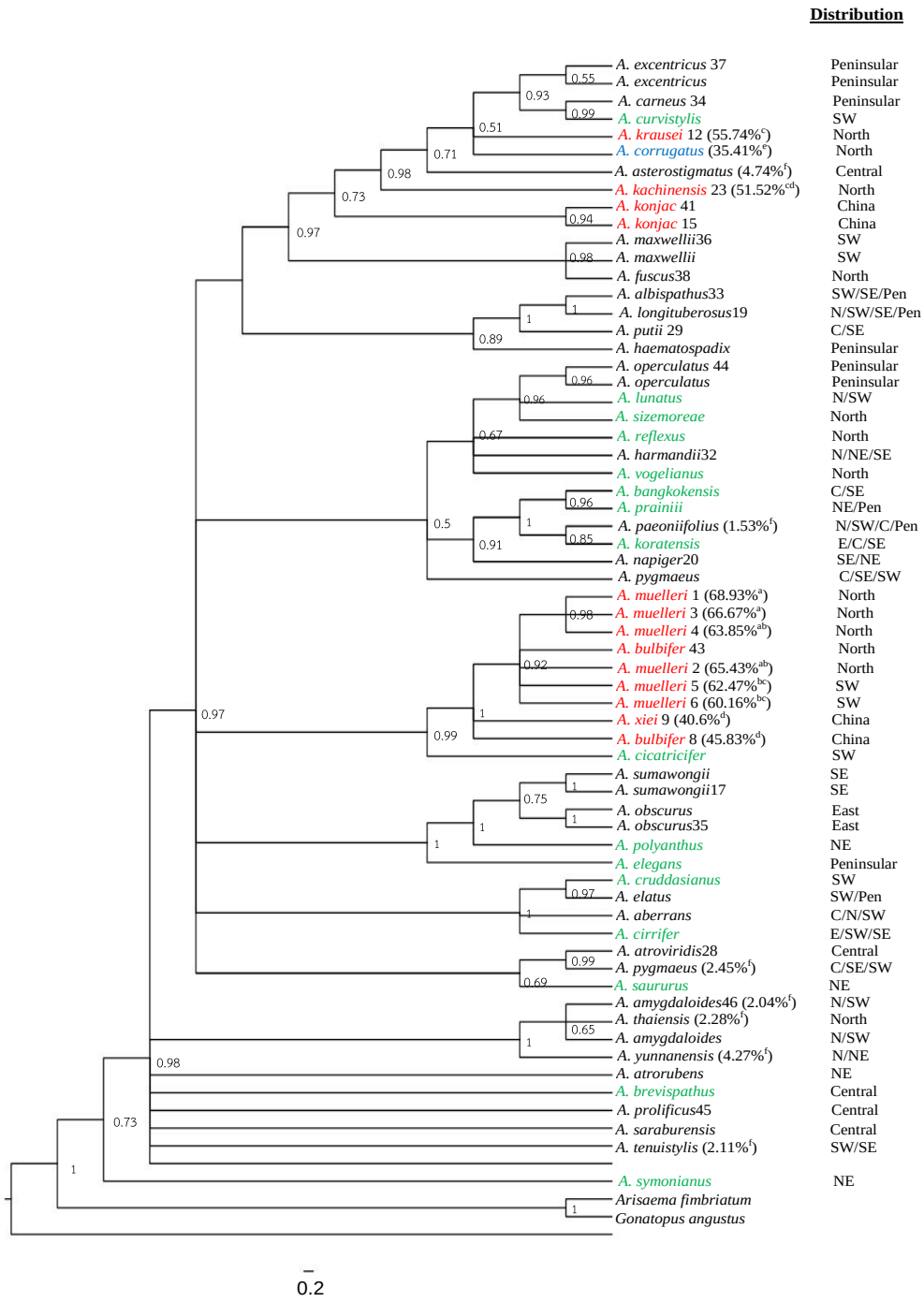


Figure 4.23 Phylogenetic tree of *FLint2* region with additional samples by MAFFT alignment. Numbers above branches indicate the posterior probability of the clade. Green alphabets represent additional samples. Red alphabets represent high KGM content group and number in bracket represent of KGM content with significant differences (p<0.05) between samples. Geographic distribution (N, North; NE, North-eastern; SW, South-western; C, Central; SE, South-Eastern and Pen, Peninsular).

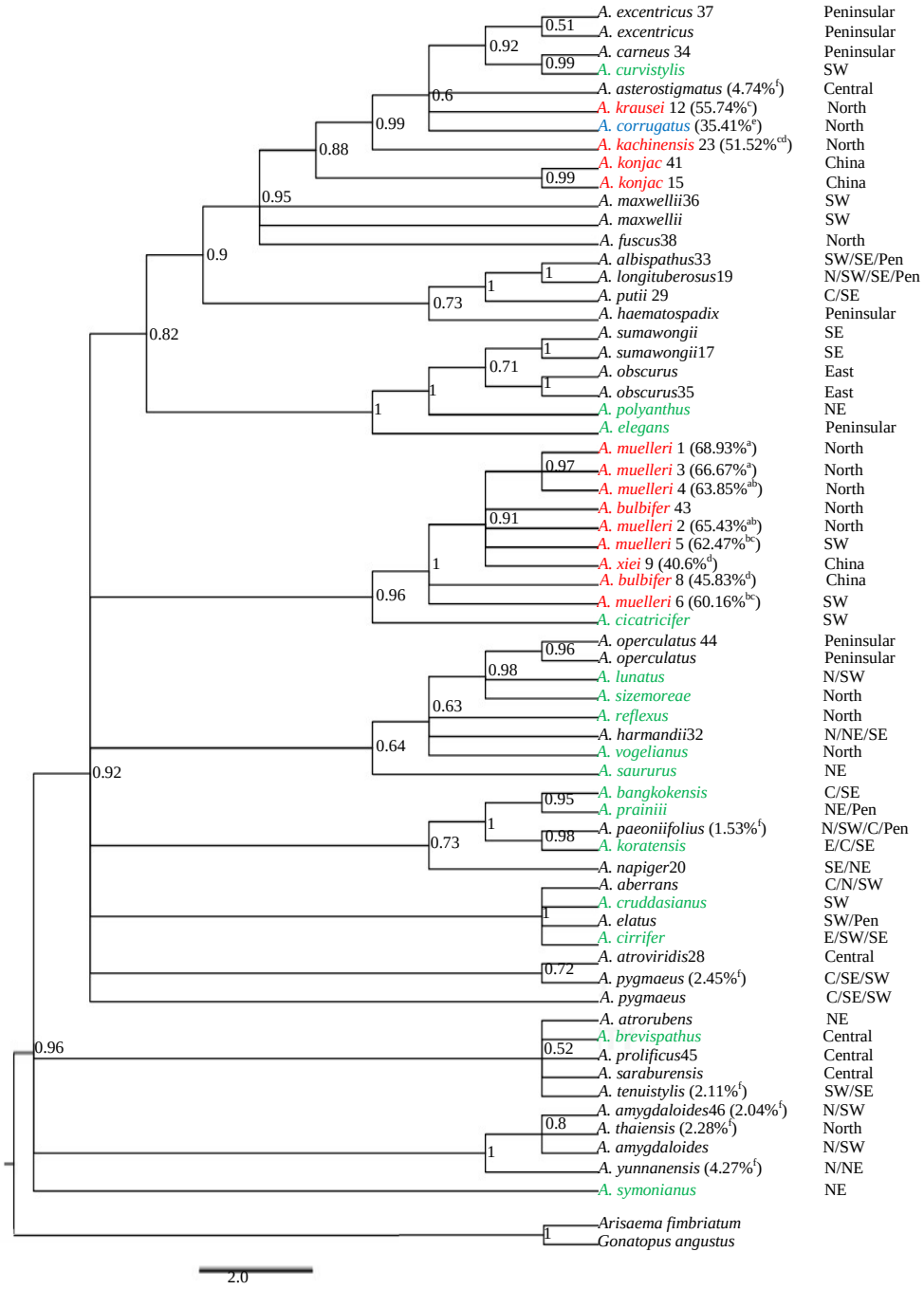


Figure 4.24 Phylogenetic tree of *FLint2* region with additional samples by BMGE alignment. Numbers above branches indicate the posterior probability of the clade. Green alphabets represent additional samples. Red alphabets represent high KGM content group and number in bracket represent of KGM content with significant differences (p<0.05) between samples. Geographic distribution (N, North; NE, North-eastern; SW, South-western; C, Central; SE, South-Eastern and Pen, Peninsular).

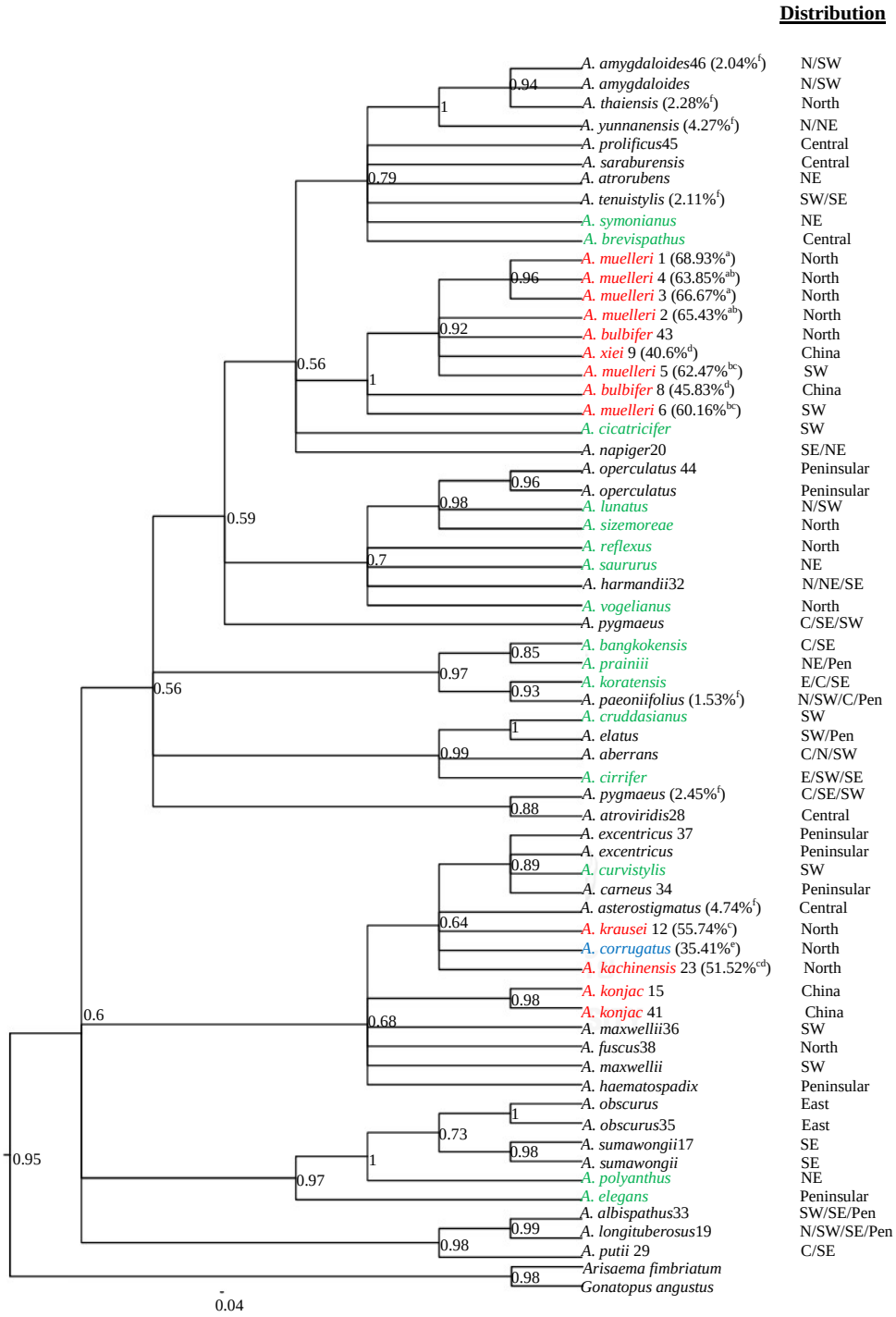


Figure 4.25 Phylogenetic tree of *FLint2* region with additional samples by PRANK alignment. Numbers above branches indicate the posterior probability of the clade. Green alphabets represent additional samples. Red alphabets represent high KGM content group and number in bracket represent of KGM content with significant differences (p<0.05) between samples. Geographic distribution (N, North; NE, North-eastern; SW, South-western; C, Central; SE, South-Eastern and Pen, Peninsular).

The set of phylogenetic trees composed of two main parts in MAFFT alignment. First part (A), composed of seven clades and one isolated clade of *A. kachiensis* while second part (B), composed of eight isolated clade. With different alignment programs, all of those internal relationship clades were changed.

First clade (F1) composed of *A. muelleri*, *A. bulbifer* and *A. xiei* with strong grouping support (PP1.0) in MAFFT and BMGE alignment. In PRANK alignment, *A. napiger* grouped in this clade with ambiguous support (PP 0.52). This species is different by long tuber while other members are globose. In additional samples set found that *A. cicatricifer* link with this clade by assemble 4 based insertions in MAFFT and BMGE alignment while PRANK alignment spited this species out. This species shared morphology with other members by globose tuber, pale color spathe and red color berry but differently recognized by dark reddish brown of petiole and lower leaflets (Figure 4.26). This species is distributed in western region of Thailand.

Second clade (F2), subclade of *A. prolificus* and *A. tenuistylis* was lost their relationship of in BMGE and PRANK alignment like additional samples set in every alignment. It is indicated that this sister clade is uncertainty and may not grouping together. Whereas, other member clades still maintained their strong relationship.

Third clade (F3), subclade of *A. aberrans*/*A. elatus* and *A. operculatus* /*A. harmandii* showed strong grouping support in every alignment. While in BMGE alignment, *A. paeoniifolius* was grouped with this clade in moderate support (PP 0.86). This species is different by globose tuber while other members are long tuber. In the same time, PRANK alignment developed new relationship between

A. atrovirdis and *A. pygmaeus* with strong support (PP 0.99) that similar to combined data. In additional sample set found that this clade was separated into three isolated clades, which *A. elatus* moved to group with *A. cruddasianus* and linked with isolated clade of *A. cirrifer* by sharing of unique dark color of spathe when compared with other species in third clade (Figure 4.26). However, *A. cruddasianus* produces red color berry instead of white color berry that is a main character of this clade. All relationship handled with strong support in every alignment program.

Fourth clade (F4) showed strong grouping support in MAFFT and BMGE alignment. However, in PRANK alignment, this clade was departed by spitting out of subclade of *A. obscurus* and *A. sumawongii* that used to group with other subclades by ambiguous support (PP 0.68). In additional sample set, it is found that this clade can maintain their relationship but lower PP values. Except for *A. carneus* was moved to group with *A. curvistylis* with strong support but color of spathe is different (Figure 4.27).

For second part (B) when removed ambiguous, in BMGE alignment, they collapsed from isolated clade to major clade and still related to each other except *A.maxwelli*. While in PRANK alignment, it is found that all of them are grouping together with isolated clade of *A. kachiensis*. Moreover, this major clade still has presented their relation even additional sample were applied.

When consideration at phylogenetic tree of additional samples, it can be found that they are grouping to suitable clade that correspondence with their morphology like *A. lunatus, A. sizemoreae, A. reflexus, A. vogelianus* which related to clade F3 by long tuber, pale shathe, longer appendix and produce white color berry (Figure 4.27). Moreover, there was a developing of new clades include

A. bangkokensis, *A. prainiii*, *A. paeoniifolius* and *A. koratensis* which all of them are belong to *paeoniifolius* group (Grob *et al.*, 2002).

From the above results, it is indicated that those majority subclades of this sequence still maintain their strong relationship. However, it is difficult to generated relationship between each clade due to this region is short and located in nuclear resulted in high enough variation. With BMGE and PRANK alignment showed advantages by developing internal node relationship between eight isolated clades of second part (B) and revealed true relationship between closed species. However, those programs affected to lower confidence values. Among of three alignments found that PRANK alignment provided congruent relationship between each clade when morphology is the main criteria and this alignment resemble to combined data. It also was pointed out that PRANK alignment program is suitable for short with medium variation sequence due to this program will not produce too much gappy that reduce confidential values.

Figure 4.26 Characteristic and inflorescence of (A) *A. cicatricifer*;
(B) *A. cruddasianus* and (C) *A. cirrifer*
(The International Aroid Society, 2014)

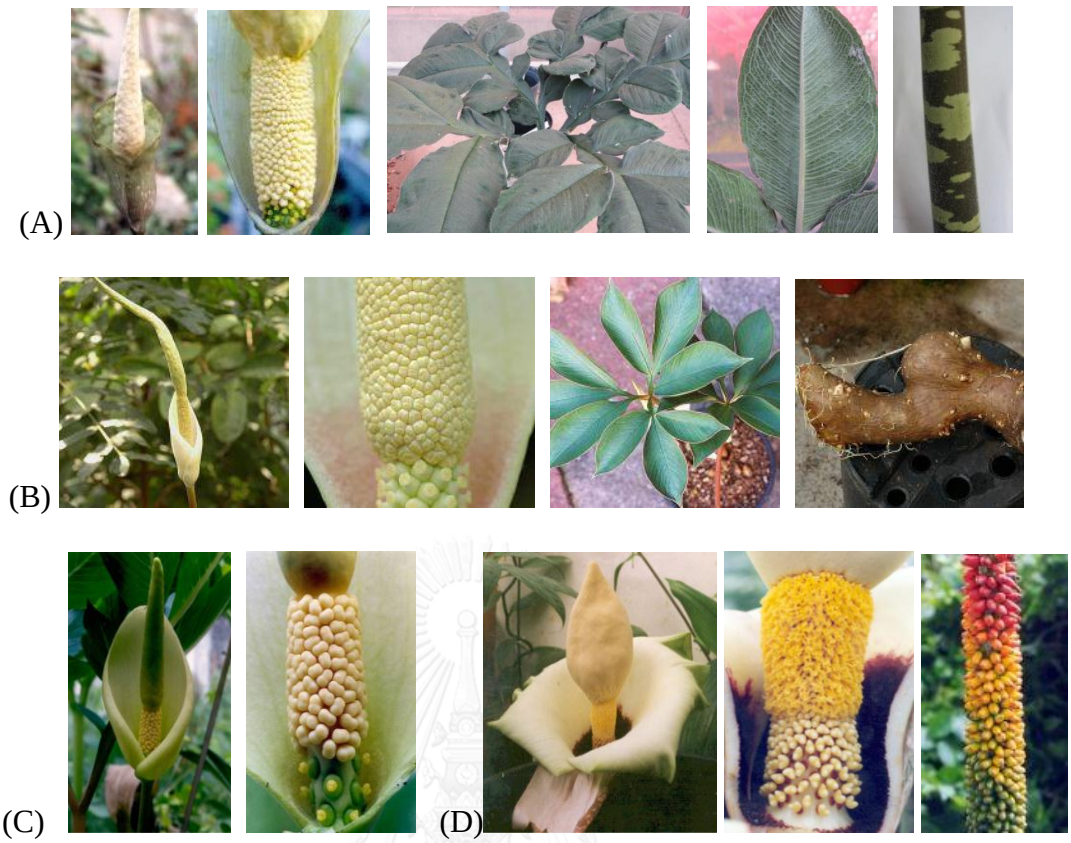


Figure 4.27 Characteristic and inflorescence of (A) *A. curvistylis;* (B) *A. lunatus*; (C) *A. vogelianus* and (D) *A. prainiii.*
(The International Aroid Society, 2014)

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However, phylognentic tree from *Flint2* region alone in this study exhibited low level of sequenced relationship in this genus, resulting in unresolved evolution relationships together which is agreed with previous study by using only this region (Grob *et al.*, 2004). Nevertheless, *Flints2* region has been used in many phylogenetic studies with good success in many plants (Schlüter *et al.*, 2007; Zimmer and Wen., 2013).

4.2.2.2.4 Internal transcribed spacers (ITS) analysis

The nrDNA ITS region has been widely used in sequenced data at the interspecific level in plant phylogenetic studies and extensively applied for phylogeny reconstruction at low taxonomic levels (Baldwin *et al.*, 1995; Yingzhi *et al.*, 2007). The characteristics of different alignments of this region were analyzed and show in Table 4.4 and Table 4.5 with additional sampling data set. The length of the region ranged from 858 bp (*A. sumawongii*) to 944 bp (*A. obscrus*) with highest percentage of variation and information site and indel number. This region tends to be quite variable therefore it is suitable for used in low-level systematic studies. Moreover, it is readily amplifies due to ITS regions occurs in high-copy region, making all sampling species be able to generate sequencing band. Phylogenetic trees that were reconstructed include MAFFT Alignment (Figure 4.28), BMGE Alignment (Figure 4.29) and PRANK alignment (Figure 4.30). For phylogenetic trees of additional samples were reconstructed, include MAFFT Alignment (Figure 4.31), BMGE Alignment (Figure 4.32) and PRANK alignment (Figure 4.33).

The set of phylogenetic tree by in MAFFT alignment composed of four main clades, which are congruent with BMGE alignment while PRANK alignment can improve internal node and increase percentage of number of clades with PP >0.85.

First subclade (I1) revealed relationship between each accession especially in *A. muelleri* with strong support in every alignment. For additional sample was found that only subclade of sample from Mae Hong son was collapsed by BMGE alignment. This may be due to this program removed informative data as ambiguous site.

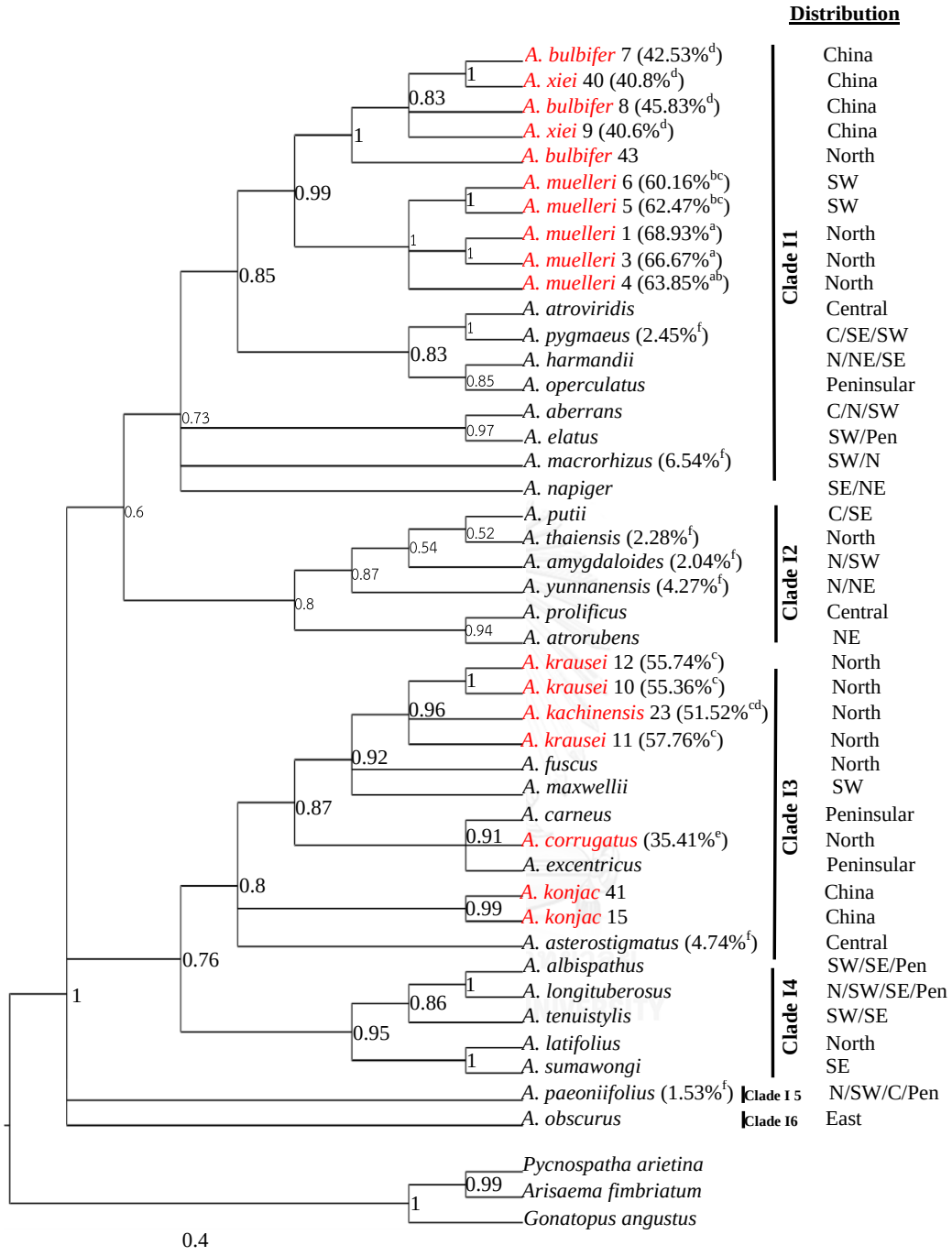

Figure 4.28 Phylogenetic tree of ITS region with MAFFT alignment. Numbers above branches indicate the posterior probability of the clade. Red alphabet represent high KGM content group and number in bracket represent of KGM content with significant differences (p<0.05) between samples. Geographic distribution (N, North; NE, North-eastern; SW, South-western; C, Central; SE, South-Eastern and Pen, Peninsular).

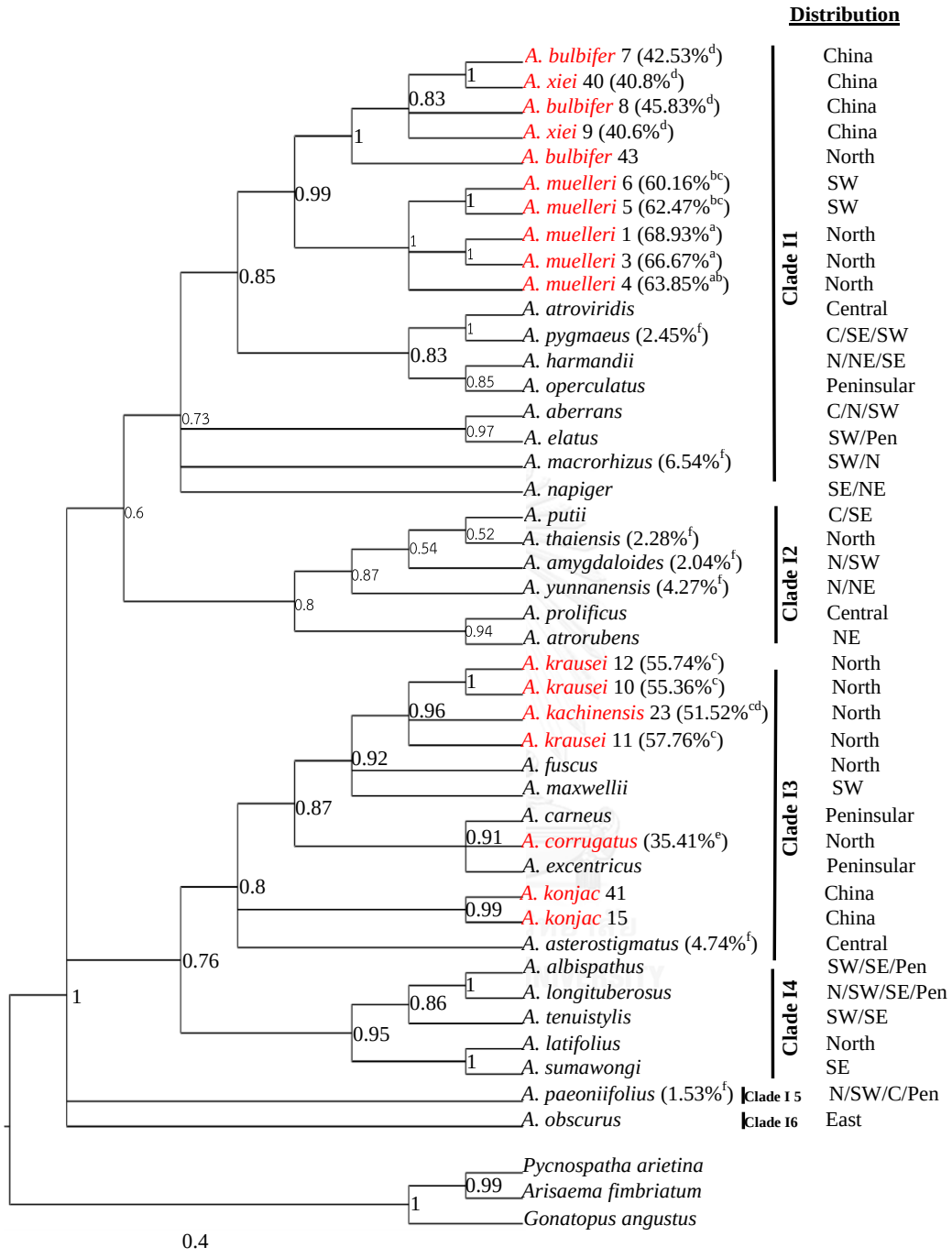

Figure 4.29 Phylogenetic tree of ITS region with BMGE alignment. Numbers above branches indicate the posterior probability of the clade. Red alphabet represent high KGM content group and number in bracket represent of KGM content with significant differences (p<0.05) between samples. Geographic distribution (N, North; NE, North-eastern; SW, South-western; C, Central; SE, South-Eastern and Pen, Peninsular).

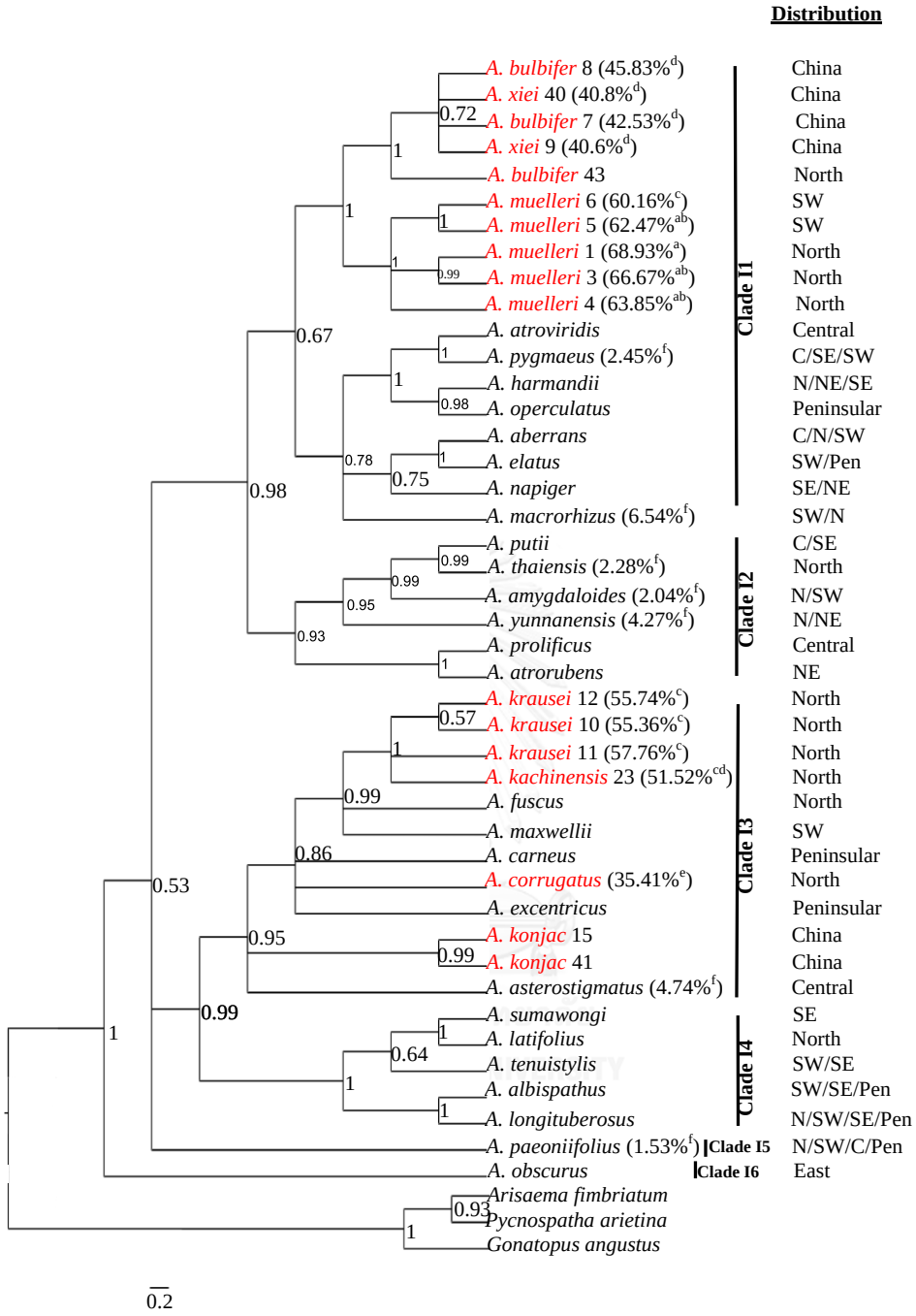

Figure 4.30 Phylogenetic tree of ITS region with PRANK multiple aware-alignment. Numbers above branches indicate the posterior probability of the clade. Red alphabet represent high KGM content group and number in bracket represent of KGM content with significant differences (p<0.05) between samples. Geographic distribution (N, North; NE, North-eastern; SW, South-western; C,Central; SE, South-Eastern and Pen, Peninsular).

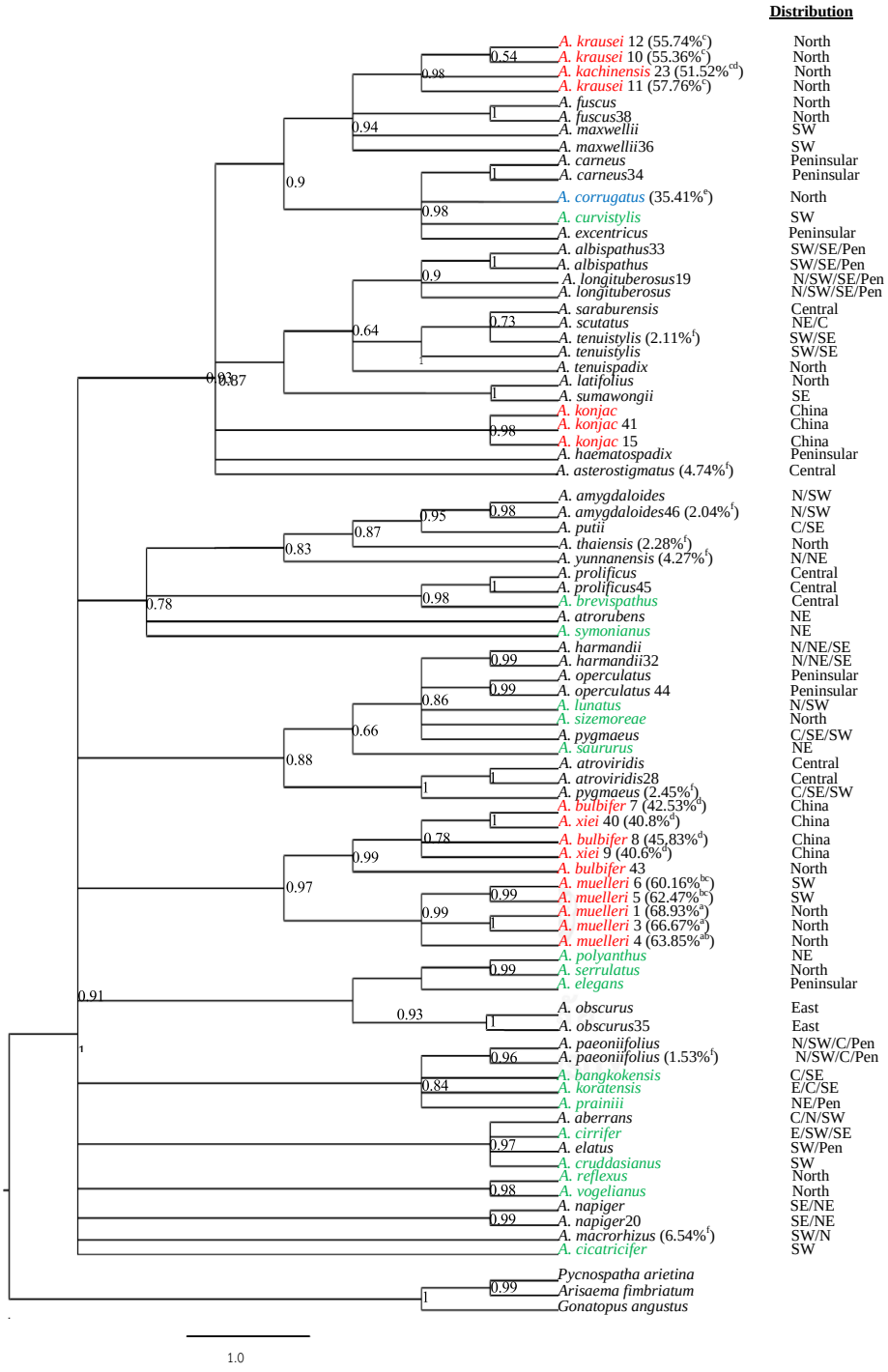

Figure 4.31 Phylogenetic tree of ITS region with additional samples by MAFFT alignment. Numbers above branches indicate the posterior probability of the clade. Green alphabets represent additional samples. Red alphabet represent high KGM content group and number in bracket represent of KGM content with significant differences (p<0.05) between samples. Geographic distribution (N, North; NE, North-eastern; SW, South-western; C, Central; SE, South-Eastern and Pen, Peninsular)

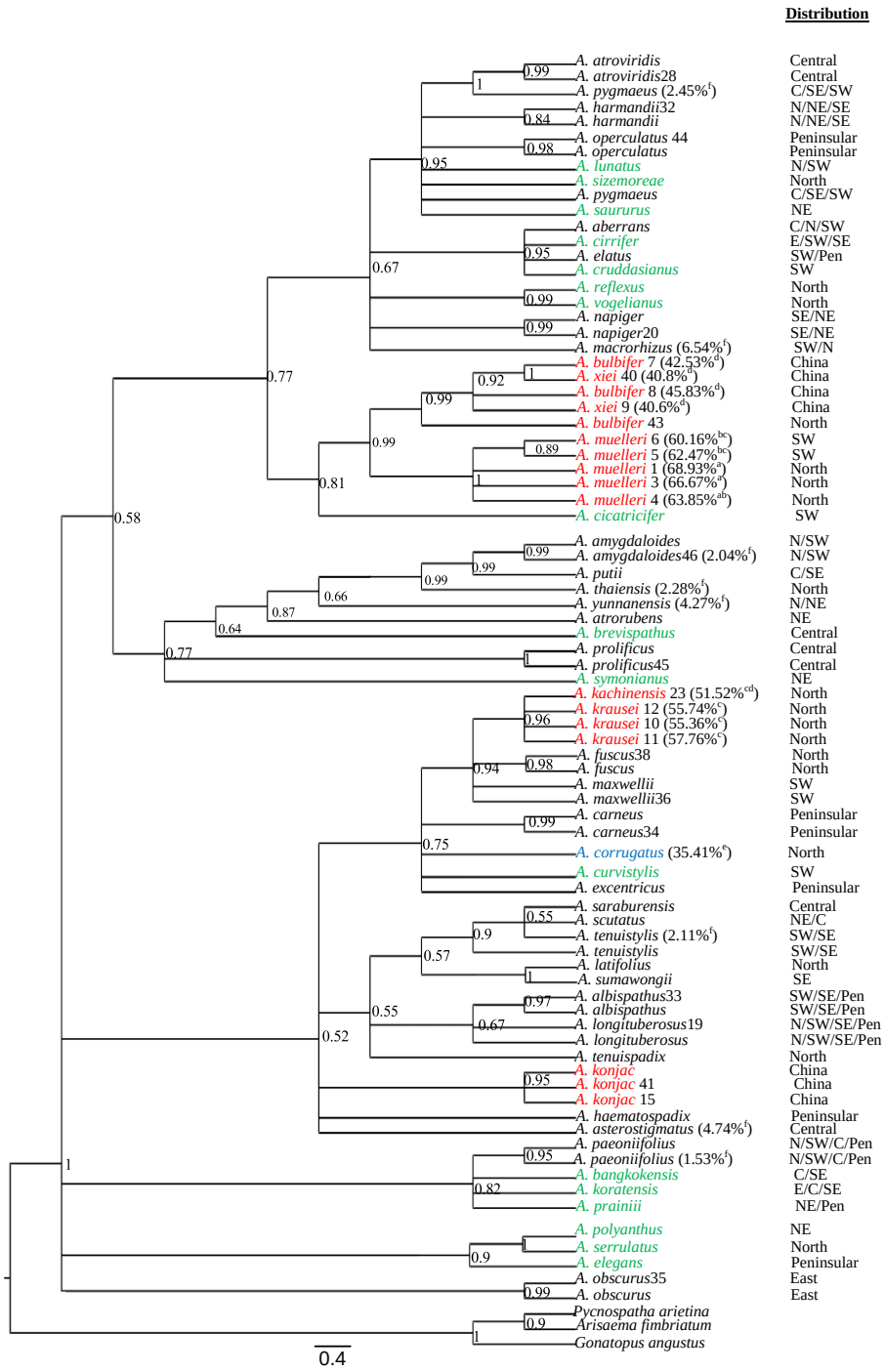

Figure 4.32 Phylogenetic tree of ITS region with additional samples by BMGE alignment. Numbers above branches indicate the posterior probability of the clade. Green alphabets represent additional samples. Red alphabet represent high KGM content group and number in bracket represent of KGM content with significant differences (p<0.05) between samples. Geographic distribution (N, North; NE, North-eastern; SW, South-western; C, Central; SE, South-Eastern and Pen, Peninsular).

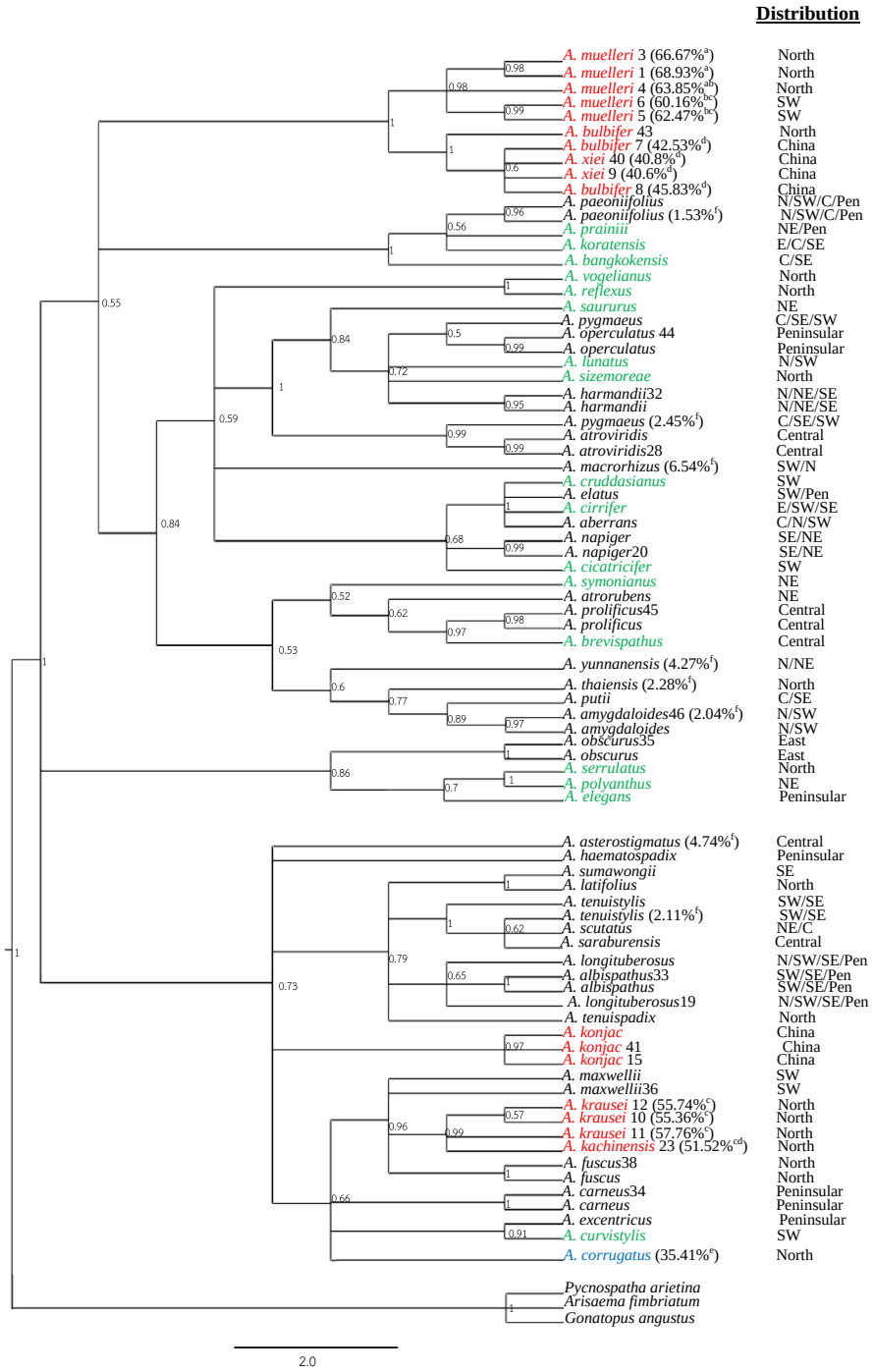

Figure 4.33 Phylogenetic tree of ITS region with additional samples by PRANK alignment. Numbers above branches indicate the posterior probability of the clade. Green alphabets represent additional samples. Red alphabet represent high KGM content group and number in bracket represent of KGM content with significant differences (p<0.05) between samples. Geographic distribution (N, North; NE, North-eastern; SW, South-western; C, Central; SE, South-Eastern and Pen, Peninsular).

Second subclade showed an increased support of sister taxa between *A. harmandii* and *A. operculatus* in BMGE and PRANK alignment. However, in PRANK alignment this subclade was grouped with *A. aberran* from third subclade that correspondence with additional sample data set. Thus, this subclade was certainty but with ambiguous support. In second clade (I2), it was proven that they are strong relationship by increasing of PP values from ambiguous to strong support in PRANK alignment. However, in additional sample set, sister taxa between *A. thaienis* and *A. sumawongii* was broken down. For third and fourth clade (I3), relationship between taxa was still maintained in every alignment.

From the above results, they were indicated that this phylogenetic tree developed high quality of relationship between each clade, even variation of this sequence was high but this region is good enough to discriminate between the most closely related taxa like *A. muelleri*. Therefore, ITS region in this study is suitable for study closely related species in this genus.

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4.2.2.3 Phylogenetic analysis with different alignment methods

4.2.2.3.1 Phylogenetic analysis with BMGE alignment

BMGE alignments were performed and its phylogenetic trees were constructed for *trnL-trnF* region (Figure 4.18), *FLint2* region (Figure 4.21), ITS region (Figure 4.29) and combined region (Figure 4.4) as describe before. When compared percent of PP > 0.85 of each phylogenetic tree with MAFFT alignment. The result found that BMGE alignment help to promote PP value in *trnL-trnF*, ITS and combined region by increasing percentage of number of clades with moderate-strong support PP>0.85 (Table 4.4) when compared with MAFFT and

PRANK alignment. Since BMGE alignment removed uncertainty region, and resulting in an increased homogeneity between the species that caused higher confident values. Moreover, *trnL-trnF* and combined region contained low variation data thus most of informative data was still maintained. On the other hand, ITS region contained high variation site and by trimming of those uninformative site, it will help to provide suitable parts of alignment and more accurate phylogeny. However, with this program, some of the relationships between each species were lost by removing informative region as uncertainty data. Opposite with other region, *Flint2* region's percentage of number of clades PP>0.85 of was decreased. This may be resulted from length of this region is short therefore a removing of ambiguous region will be affected to total sequence and PP value. In addition, branched length with BMGE alignment were shorten when was compared with MAFFT alignment in all phylogeny. When larger sampling data set were applied (samples form whole Thailand) by *FLint2* region (Figure 4.24) and ITS region (Figure 4.32), it was found that percentage of number of clades with PP>0.85 was still increasing with in ITS region but slightly decease in *FLint2* region. From above mentioned results indicated that BMGE program is useful for minimize the bias influenced by uncertain homology region and reveal truly main clade and improving their PP values. Besides this alignment helps to develop some internal relationship clade even it still weak support. On the other hands, this alignment drops off some external relationship clade with weak support. This alignment may not be suitable for shorten sequence but larger sampling set should not be affected to alignment ability.
In previous studies, it showed that the advantages of BMGE program is to manage highly divergent sequence by obtain high confidence values for the majority of

the nodes in cyanobacteria (Criscuolo and Gribaldo, 2011). As well as increasing of the confidence of historic *Euarchontoglires* species tree (Kumar *et al.*, 2013) and increasing of the resolution of aspartic proteinases fungus phylogeny as it helps to guide the structural information by correctly alignment (Revuelta *et al.*, 2014). However, Batista *et al.* (2011) mentioned that this alignment can achieve very robust phylogenetic trees of papillomaviruses but those phylogenies had low statistical support in some internal nodes. Their finding is similar with this study.

4.2.2.3.2 Phylogenetic analysis with PRANK alignment

PRANK alignments were performed and its phylogenetic trees were constructed for *trnL- trnF* region (Figure 4.19), *FLint2* region (Figure 4.22), ITS region (Figure 4.26 **Figure 4.26**) and combined region (Figure 4.5) as was described before. When compared percent of PP > 0.85 with MAFFT alignment. The result found that PRANK helps to improve PP values in ITS and combined data. On the contrary, percent of PP> 0.85 was decrease in *Flint2* region while *trnL-trnF* region showed not difference from MAFFT alignment (Table 4.4). Although PRANK do not increase percent of PP> 0.85 but this alignment help to developed internal relationship between each clades especially in *Flint2* region. Those result came from PRANK algorithm reconstructs ancestral sequences first aim to represent the parents of aligned descendant sequences. Those true ancestors are response for develop of internal nodes of the alignment phylogeny (Russell, 2014). This is indicated that treating insertions and deletions in differently event as PRANK function was useful in *Amorphophallus* species. Moreover, every region still maintained their main phylogenetic characteristics when PRANK alignment was applied. Therefore, it is

clear that phylogeny from each region is true ancestral sequences since PRANK reconstruct by the parental sequences. As a result of above can be showed that the *trnL- trnF* spacer is high conserve region therefore there are minor changing in phylogenetic trees when different alignment method were used. Meanwhile ITS located in nuclear region thus it showed high variation and long sequence length enough to suitable to use PRANK alignment. Whereas *Flint2* region is quite short sequence length therefore it is sensitive to change relation when different alignment was applied. This result agreed with previous studies that PRANK is effective alignment even it is increased sequence length and gappy, such as PRANK alignment revealed African *Rinorea* clades by ancestral state reconstructions (van Velzen *et al.*, 2015) or improved internal relationship in genus *Sibbaldia* from combined data of ITS, *trnL- trnF* spacer and *trn*L intron data (Eriksson *et al.*, 2015).

As a result of above findings that even different alignments with strict program likes BMGE or relaxing program like PRANK were applied. Phylogenetic tree of *Amorphophallus* spp. in this study still maintain their relationship in majority clade with medium-strongly support (PP> 0.85). It is indicated that this evaluation phylogeny is sufficient quality enough to be used as genetic database of *Amorphophallus* spp. in Thailand.

4.2.3 RAPD analysis

RAPD technique was used as a tool for assessing genetic variation and relationship among *Amorphophallus* species. However, RAPD showed some disadvantages in reproducibility and reliability due to low annealing temperature (Agarwal *et al.*, 2008). To overcome this drawback, long RAPD primers (LP-RAPD) and HAT-RAPD (High Annealing Temperature RAPD) technique were employed. LP-RAPD was more reproducible and stable than normal RAPD primers (Fischer *et al.*, 2014; Gilling and Holley, 1997; Nisar *et al.*, 2015). Meanwhile, HAT-RAPD performs at high temperature between 40-46^{o}C with short arbitrary primer aiming to increase the specificity and reproducibility of the annealing conditions (Eimert *et al.*, 2003; Wangspa *et al.*, 2005).

Thirty-five random primers were screened, only thirteen primers (ERIC1R, ERIC2, BOXA1R, RPO1, AB-04, AB-20, AC-09, AC-10, AH-18, OPC-02, OPD-04, OPC-07 and OPB-17) produced clear and reproducible polymorphic bands in all accessions. This technique amplified a total of 269 amplified bands ranging from 150 to 5000 bp with average of 21 scored bands each RAPD markers per primer. All amplified fragments were 100% polymorphic bands. The highest number of RAPD bands (28 bands) was amplified by primer AB-20 (Figure 4.33) while the lowest number of RAPD bands (15 bands) was amplified from ERIC1R. The primers and their amplified band size range are listed in Table 4.6. The discriminating power of each RAPD amplified band was determined by calculate on of the polymorphism information content or PIC value (Nagy *et al.*, 2012). For the primers used in this study, the PIC value ranged from 0.33 (OPD-04) to 0.75 (AB-20) (Table 4.6).

Ten of the thirteen loci were considered to be informative, since they had a PIC value greater than 0.5 (Ramadugu *et al.*, 2015). The PIC value can be used to evaluate the level of gene variation, when PIC>0.5, the locus was of high diversity; when PIC < 0.25, the locus was of low diversity and the locus was of intermediate diversity, when PIC between 0.25 and 0.5 (Botstein *et al.*, 1980).

Table 4.6 RAPD primers with corresponding bands scored and their size range together with polymorphic bands observed in *Amorphophallus* spp.

Primer name	Sequences (5'-3')	Size ranged (bp)	Scored bands	Number of polymorphic bands	Polymorphic Information Content (PIC)	Average Nei and Li's genetic distances
AB-04	GGCACGCGTT	150-2000	23	23	0.56	0.5775
AB-20	CTTCTCGGAC	250-5000	28	28	0.75	0.5225
AC-09	AGAGCGTACC	200-1500	17	17	0.63	0.546
AC-10	AGCAGCGAGG	300-2000	20	20	0.55	0.3925
AH-18	GGGCTAGTCA	200-2000	23	23	0.45	0.514
OPB-17	AGGGAACGAG	100-2000	25	25	0.62	0.5735
OPC-02	GTGAGGCGTC	250-2000	22	22	0.58	0.4715
OPC-07	CACACTCCAG	250-2000	22	22	0.69	0.4615
OPD-04	TCTGGTGAGG	250-3500	25	25	0.32	0.445
BOXA1R	CTACGGCAAGGCG ACGCTGACG	300-3200	20	20	0.44	0.3095
ERIC1R	ATGTAAGCTCCTG GGGATTCAC	300-2500	15	15	0.63	0.301
ERIC2	AAGTAAGTGACTG GGGTGAGCG	250-1600	17	17	0.42	0.305
RPO1	AATTTTCAAGCGT CGTGCCA	300-4000	20	20	0.67	0.3885

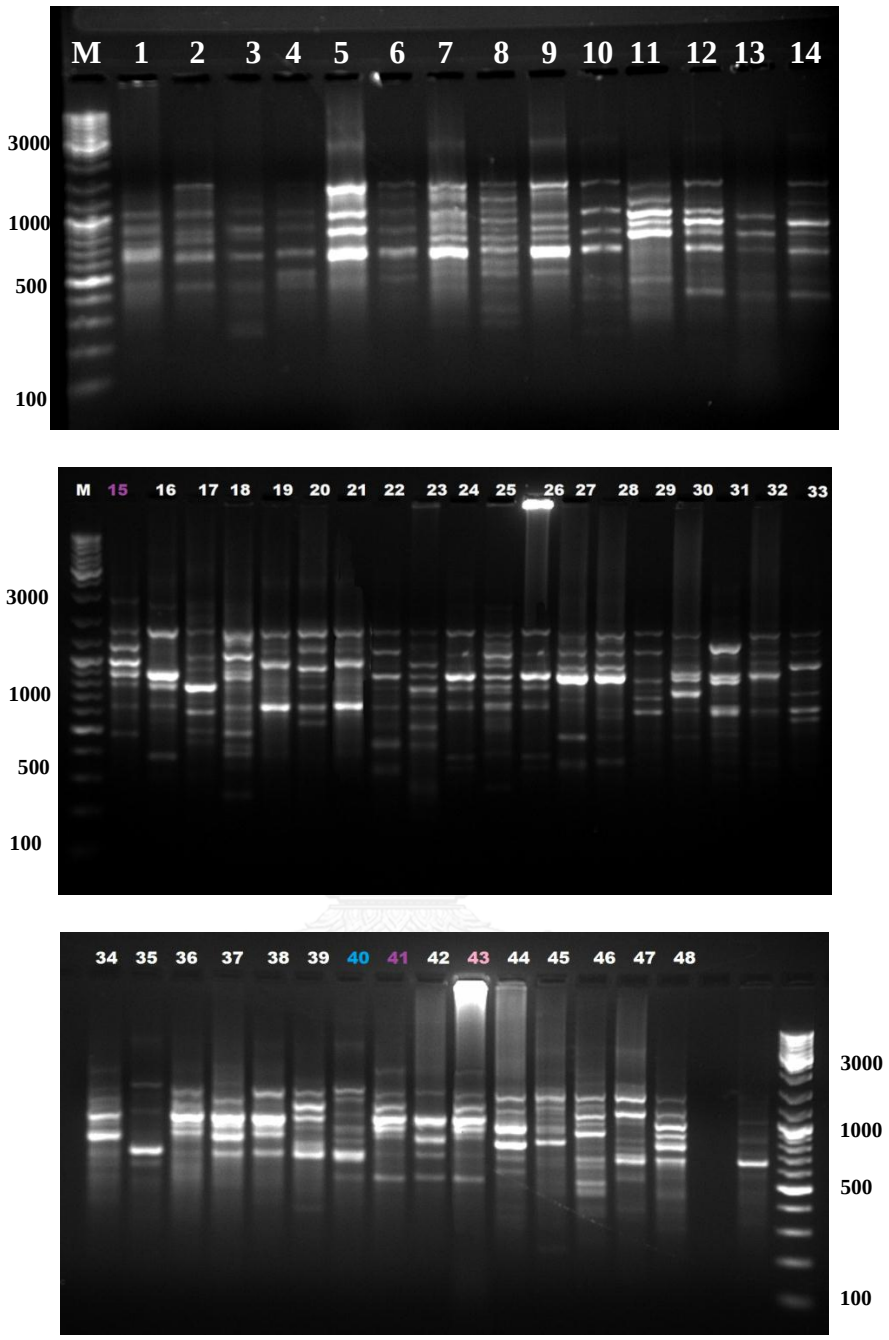


Figure 4.34 RAPD fingerprint of 48 *Amorphophallus* spp. and outgroup plants (No. 26) obtained from the AB-20 primer. Numbers of the plant samples are according to codes used in Table 3.1. M: Gene Ruler 10 kb (size shown in base pair).

The values of pairwise comparisons of Nei and Li 's genetic distance, from all set of primers, among the 48 accession of *Amorphophallus* ranged from 0.075 to 0.949. Comparatively, a highest genetic distance (0.949) was observed between sample no. 1 (*A. muelleri* from northern Thailand) and no. 34 (*A. paeoniifolius* also from northern Thailand) than with other combinations. The lowest genetic distance (0.075) was found in *A. muelleri* from both accessions (no.1 and 3) from Mae Hong Son province in northern Thailand. This result agreed with sequencing analysis that *A. muelleri* in accessions no.1 and no. 3 always clustered together as a sister group in every region with a strong support. When considering the different collected geography in all set of primers, it was found that the genetic distances in the northern region sample was lower (0.1972-0.2216) than other sample from other regions in Thailand (0.3252-0.9323). This may be due to northern original species was isolated by hilly area therefore gene flow was restricted throughout the entire distribution (Ntuli *et al.*, 2015).

A consensus neighbor joining (NJ) tree was generated from the RAPD data (Figure 4.35). All accession clusters observed were correspondence to the DNA sequencing analysis (Figure 4.3). A consensus tree has ability to produce high KGM content clade include *A. muelleri*, *A. bulbifer* and *A. xiei* are grouped in same cluster with strong support (with a bootstrap value of 100%). Whereas *A. kachiensis*, *A. corrugatus* and *A. krusei* are grouped in another separated cluster. This study indicated that *A. muelleri*, *A. bulbifer* and *A. xiei* show a closer relationship, with a minimal genetic distance (0.351) in same cluster whereas *A. paeoniifolius* was the most diverse among the *Amorphophallus* genotypes in this study.

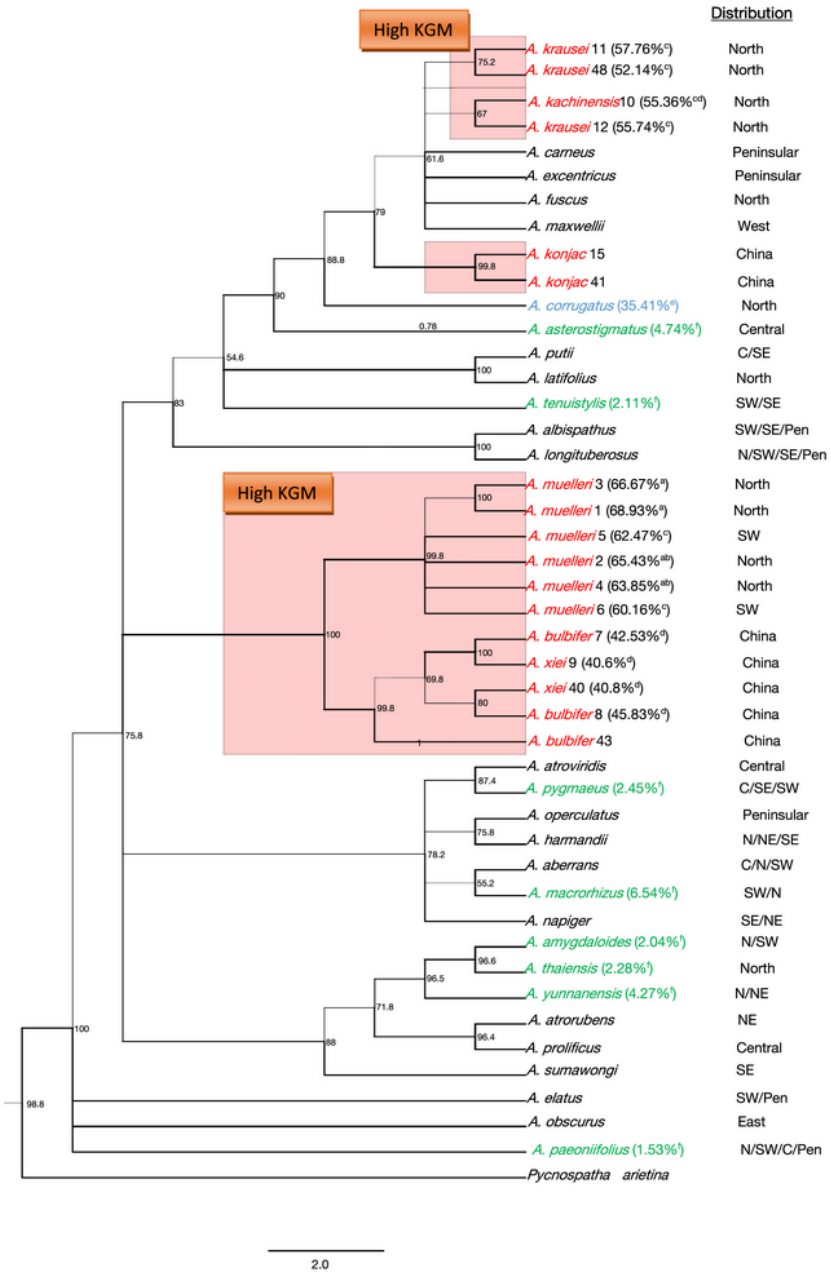


Figure 4. 35 Dendrograms of RAPD profile data of *Amorphophallus* species from Thailand with 13 primers using NJ analysis. Geographic distribution indicated to right of tree (N, North; NE, North-eastern; SW, South-western; C, Central; SE, South-Eastern and Pen, Peninsular). Highlight area is high KGM content group.

The RAPD analysis in this study indicated that all primers can producing a highly polymorphic banding pattern (100%) together with highly genetic distance. This result confirmed the heterogeneity within *Amorphophallus* species. This high genetic variability may come from the occurrence of molecular mutation or recombination in this species. Since, even single base change at the primer annealing site is manifested as precence or absence of RAPD bands. These bands may indicate the occurrence of genetic changes in the genome of species either through the loss or rearrangement of some of their nucleotides. Chromosomal crossing over during meiosis may result in loss of primer attachment pair sites in the offspring leading to novel molecular marker pattern in species (Noormohammadi *et al.*, 2013).

Interestingly, there was a specific band that was only present in high-medium KGM content species. One primer - primer AC-10 – amplified a unique band of 600 bp which that was only presented in the seven high and medium KGM content species, from the total 19 samples (Figure 4.36). The obtained result would be useful information for characterization of high and medium KGM content of *Amorphophallus* plants. Moreover, sequence characterized amplified regions (SCAR) can be further developed to differentiate this plant that have similar vegetative morphological characters. This presence of specific band is consistent with the results of Fahmy *et al.* (2015) that RAPD technique distinguished differences in sesame seed with phenotype (seed coat color and capsule length) and oil content quantitative manner.

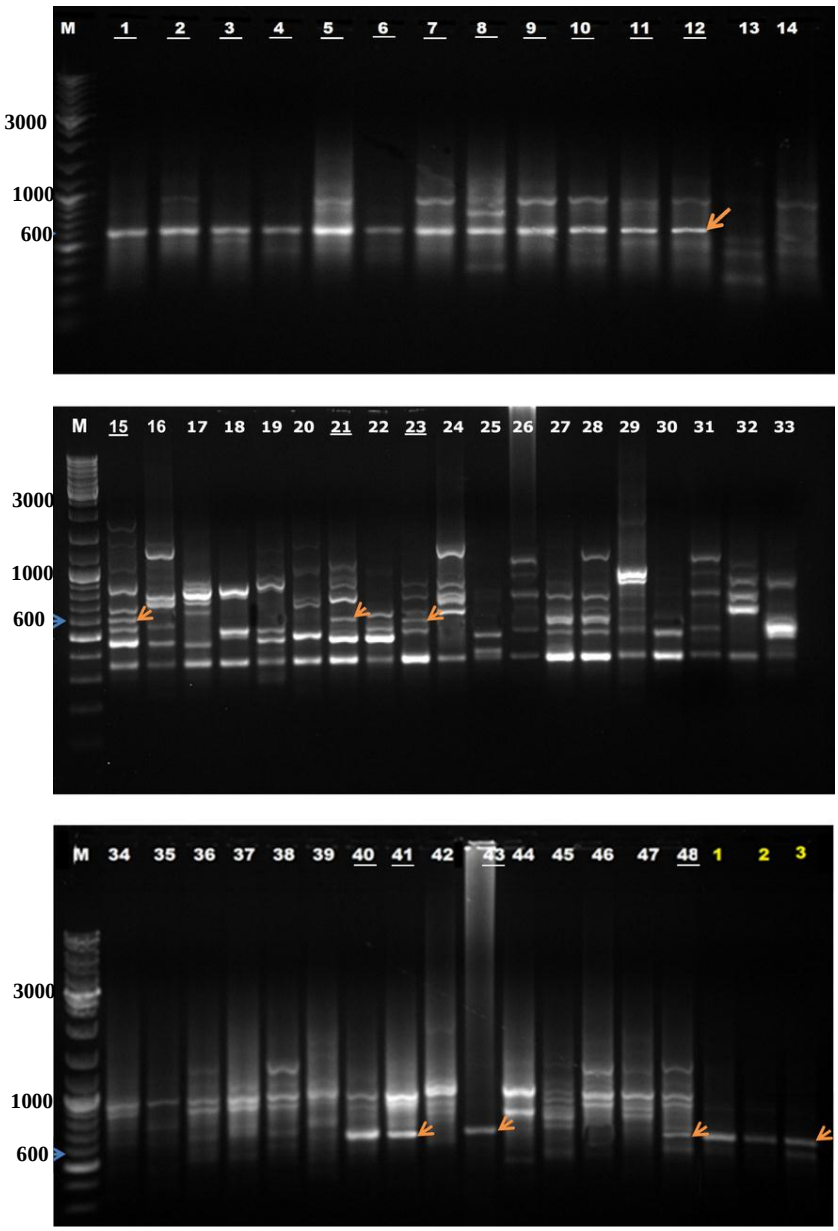


Figure 4.36 RAPD banding pattern of *Amorphophallus* samples using primer AC-10. Arrows indicate the bands that occur only in samples with high and medium KGM content. Numbers of the plant samples are according to codes used in Table 3.1. M: Gene Ruler 10 kb (size shown in base pair).

As different species possessed various levels of genetic variability, therefore RAPD technique is appropriate for revealing genetic variations of this species. The relationships between the RAPD fragments of this species help to explain the nature of *Amorphophallus* variations. Moreover, this technique has the ability to improve the genetic information. It is a rapid way to identify and differentiate potential species by using a meaningful genetic classification screening system instead of laborious and time-consuming biochemical tests (DNS assay for KGM content determination).

4.2.4 Design of specific DNA markers to differentiate between high- medium and low KGM content group.

From the information mention above, genetic data from sequencing or RAPD analysis was correlated with KGM content. The result indicated that the production of KGM can be anticipated based on the species' relationships. Therefore DNA markers can be developed and will give advantage as a tool for economic species identification. DNA sequencing regions or RAPD primers that had ability to separate high or medium KGM content group were chose to generate specific DNA primers.

4.2.4.1 Development of sequencing markers

When KGM content was assessed with genetic data, it was found that samples contained similar KGM content belong to similar clades in the phylogenetic tree. This indicates that KGM content is associated with the species' evolutionary history. High KGM content species are found in clades D and G in the phylogeny inferred here (Figure 4.3), indicating that a high KGM content tuber has probably

evolved at least twice in this genus. Therefore, it is possible that based on the three sequencing regions employed in this study that it would be possible to develop design specific primers for molecular diagnostics of high value species.

Specific primers were manually designed based on specific sequence variation within high KGM content species. Because of those specific regions were detected by eye therefore computer-based design programs were not suitable to apply. Specific primers were designed follow general parameters for PCR primer design (Chun-Lei Xiang, 2012; Dieffenbach *et al.*, 2003; Li *et al.*, 2011c). Unfortunately, for *A. kachiensis, A. corrugatus* and *A. krausei* did not provide informative data for develop specific primer. Therefore, only specific assembled nucleotide sequences of *A. muelleri, A. bulbifer* and *A. xiei* for each region were developed.

In chloroplast *trnL-trnF* spacer, this region found high conserved therefore only *A. muelleri* showed specific nucleotide sequence from low KGM content species. Therefore, two set of primers for *A. muelleri* were designed include (1) MU-300 forward primer with substitution (G/C) at position 300 (Figure 4.37) and (2) MU-689 reverse primer with substitution (T/G) at position 689 (Figure 4.38). The properties of specific designed primers were shown in Table 4.7. In addition, the result from this region showed specific nucleotide 21 bases of *A. kachiensis, A. corrugatus* and *A. krausei* at position 221. However, this position is a repeated set thus it is not suitable to develop specific primer (Figure 4.39).

Figure 4.37 Specific character of *A. muelleri* at position 300based on *trnL-trnF* spacer in *Amorphophallus.* Arrow indicates specific MU-300 forward primer annealing site. Asterisks (*) indicate that all samples provide nucleotide identity.

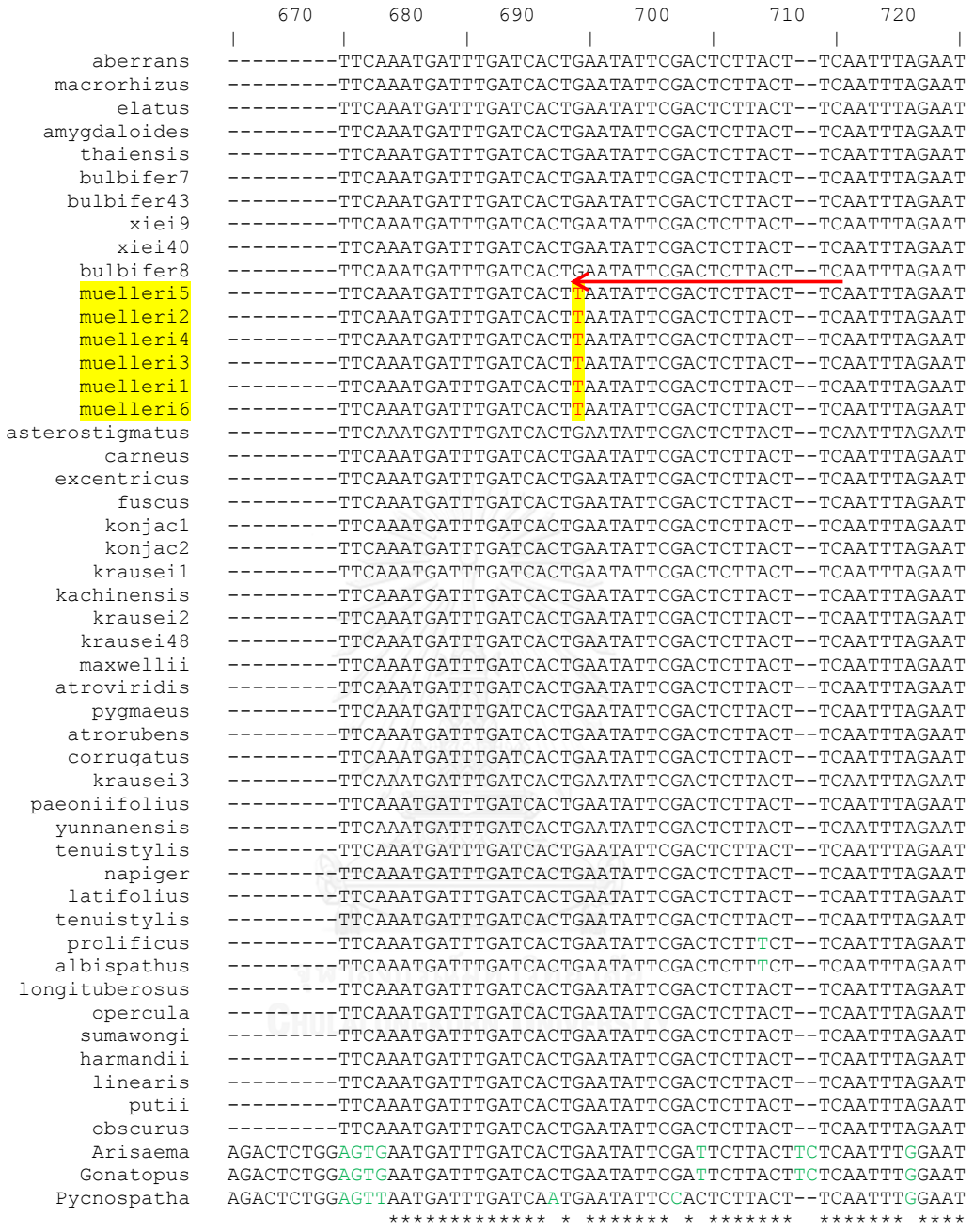


Figure 4.38 Specific character of *A. muelleri* at position 689 based on *trnL-trnF* spacer in *Amorphophallus.* Arrow indicates specific MU-689 reverse primer annealing site. Asterisks (*) indicate that all samples provide nucleotide identity.

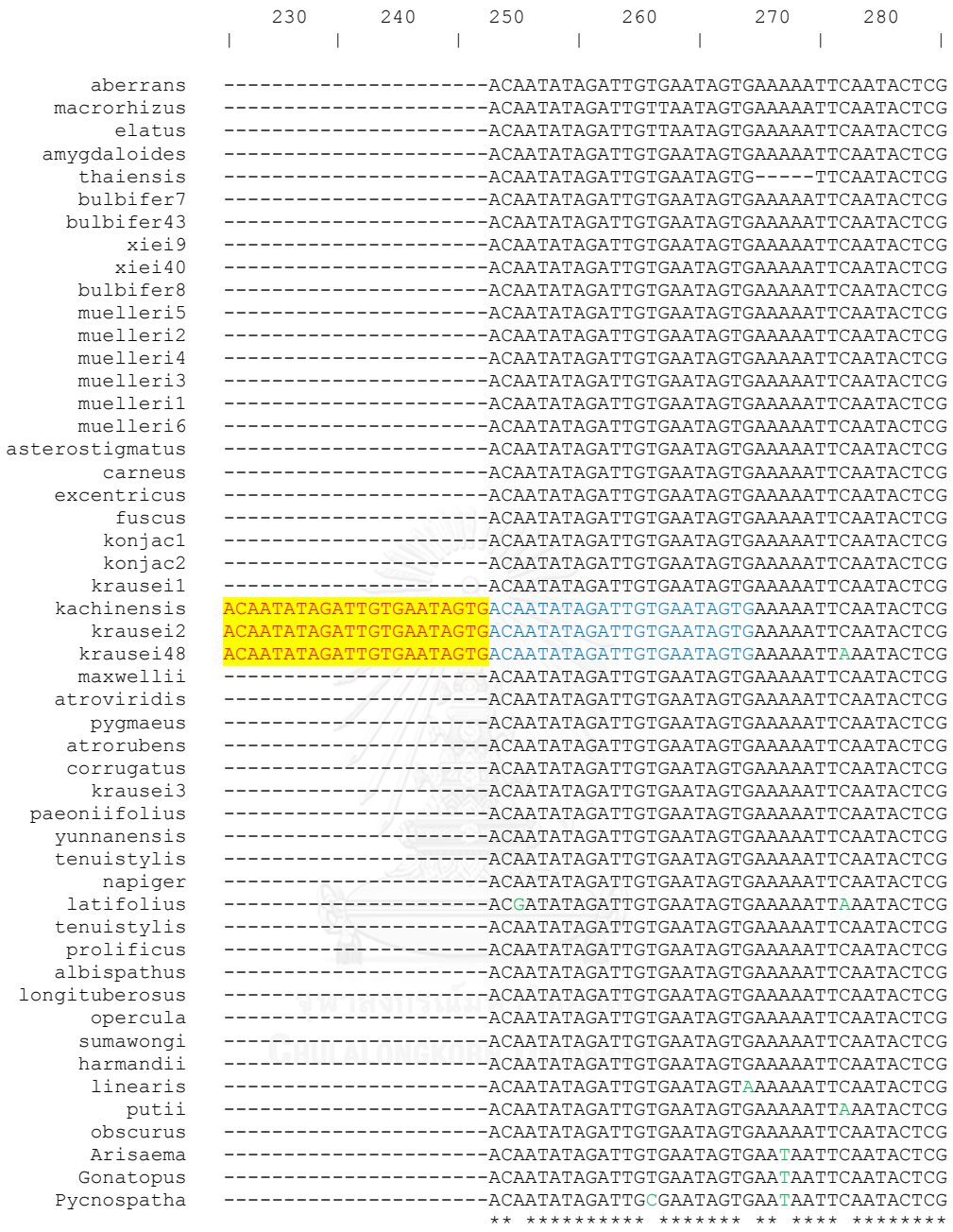


Figure 4.39 Character matrix based on *trnL-trnF* spacer of *Amorphophallus*. Highlight area is specific nucleotide character of *A. kachiensis*, *A. corrugatus* and *A. krausei* at position 221 as repeated 21 bases set. Asterisks (*) indicate that all samples provide nucleotide identity.

In nuclear region of *FLint2* in additional samples data set (total 54 species), there found the specific nucleotide sequences of *A. muelleri*, *A. bulbifer* and *A. xiei*. Because of *FLint2* quite short therefore only two set of primers were designed. First primer set is MUBX236_ *FLint*2 reverse primer with substitution (G/C) at position 236 (Figure 4.40). Second primer set is MUBX253_ *FLint*2 reverse primer with substitution (T/A) at position 253 (Figure 4.40). The properties of specific designed primers from ITS region were shown in Table 4.7.

For the internal transcribed spacers (ITS) region of additional samples data set, there found the specific nucleotide sequences of *A. muelleri*, *A. bulbifer* and *A. xiei*. Because of ITS region is highly variation and long length therefore seven primer sets were designed including (1) MUBX222_ITS forward primer with insertion (A) at position 222 and substitution (T) at position 241 (Figure 4.41), (2) MUBX281 ITS reverse primer with insertion (CCA) at position 281 (Figure 4.42), (3) MUBX327 ITS forward primer with three substitution at position 237 (C); position 349 (A) and position 404 (C) (Figure 4.43), (4) MUBX-493 ITS forward primer with two substitution at position 493 (C/A) and position 519 (CG) (Figure 4.44), (5) MUBX520_ITS forward primer with two substitution at position 420 (G/C) and position 548 (AG/GT) (Figure 4.45), (6) MUBX551_ITS forward primer with insertion (GTA) at position 551 (Figure 4.45), (7) MUBX795_ITS forward primer with substitution (A/C) at position 795 (Figure 4.46), and (8) MUBX974_ITS reverse primer with two substitution at position 974 (G) and position 976 (T) (Figure 4.47). The properties of specific designed primers from ITS region were shown in Table 4.7.

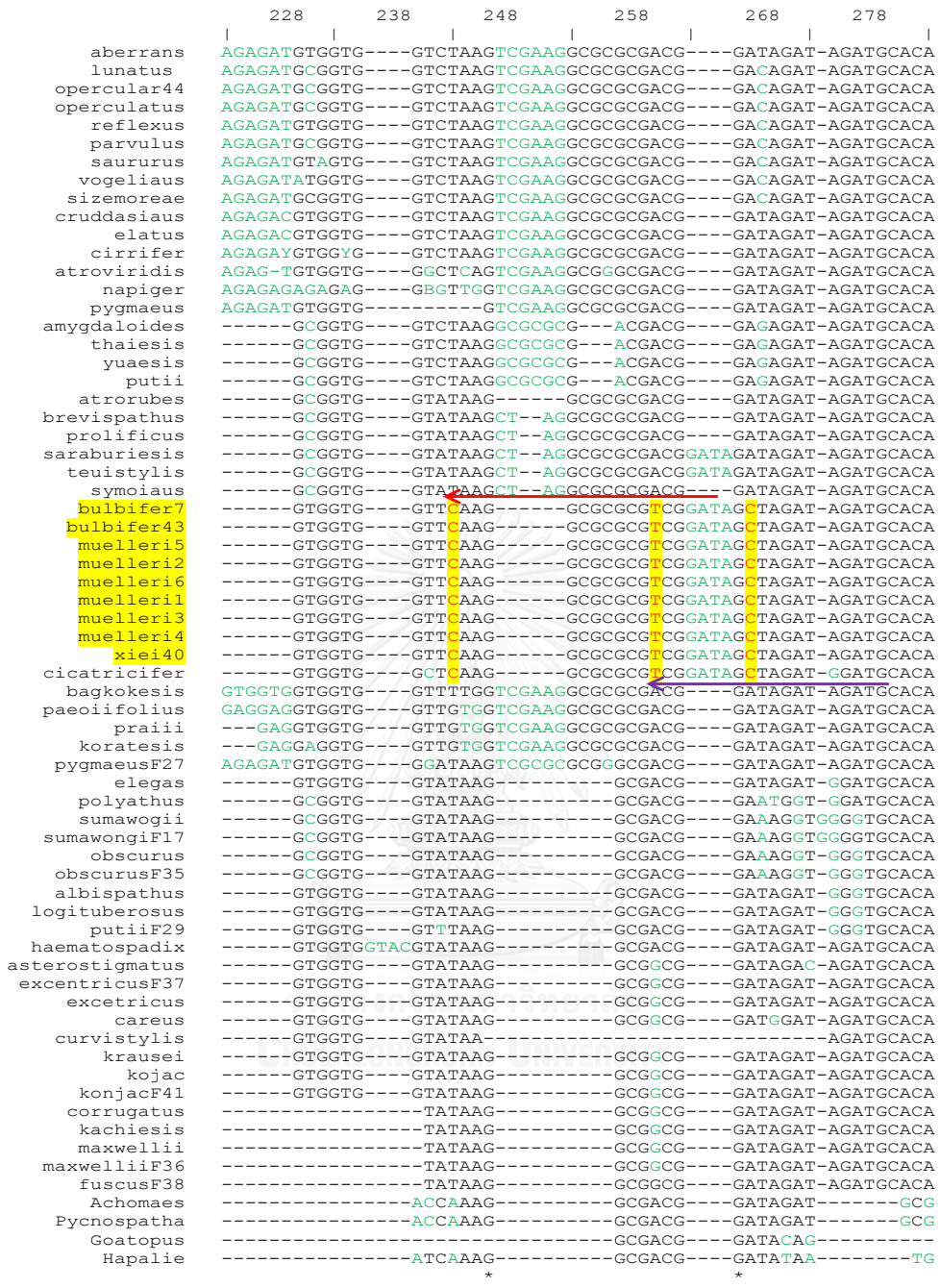


Figure 4.40 Specific character of *A. muelleri*, *A. bulbifer and A. xiei* based on *Flint2* region in *Amorphophallus.* Arrows indicate specific annealing site of MUBX236_ *FLint*2 reverse primer (red) and MUBX253_ *FLint*2 reverse primer. Asterisks (*) indicate that all samples provide nucleotide identity.

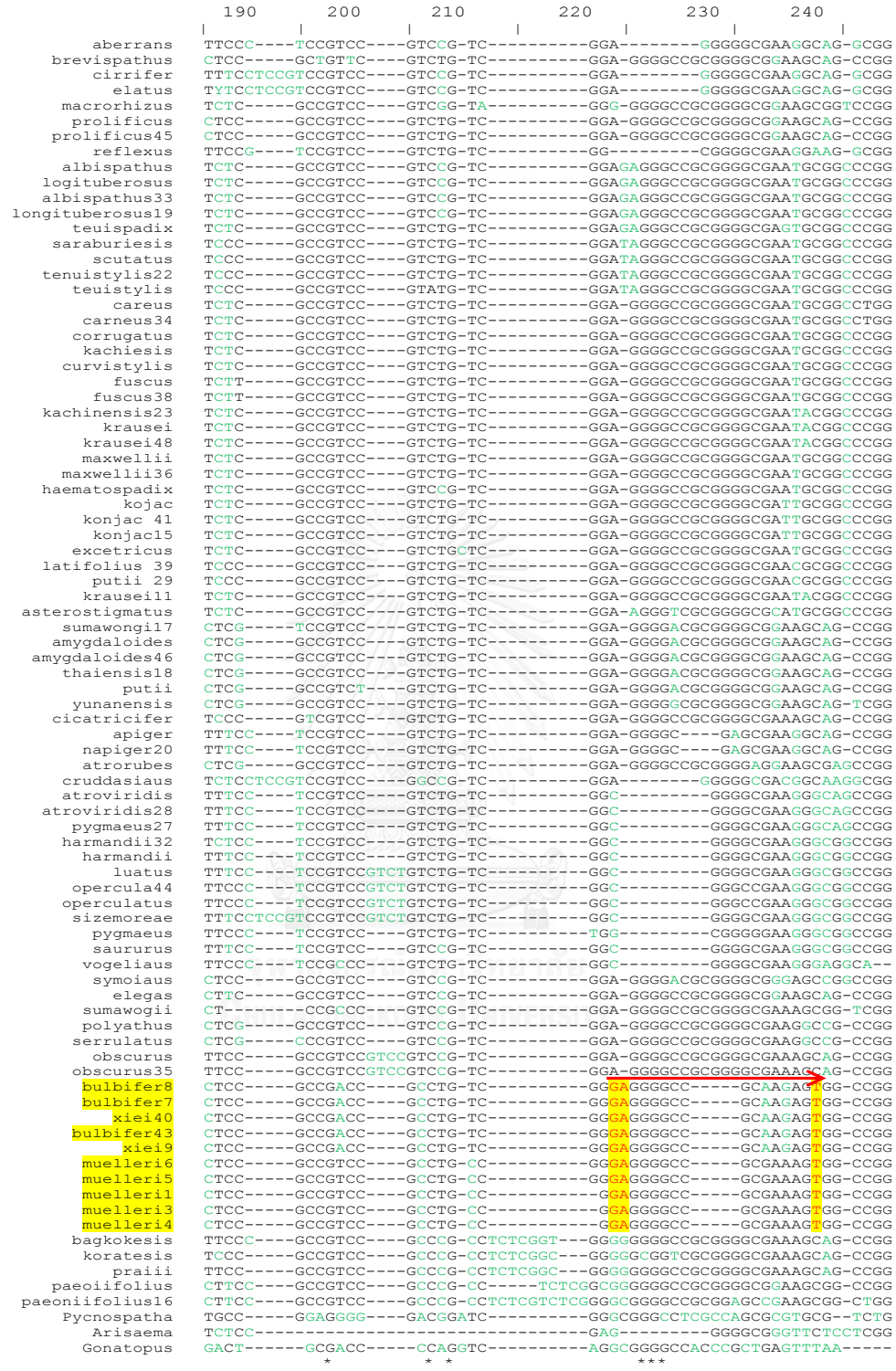


Figure4.41 Specific character of *A. muelleri*, *A. bulbifer and A. xiei* of MUBX222_ITS forward primer based on ITS in *Amorphophallus.* Arrow indicates specific annealing site. Asterisks (*) indicate that all samples provide nucleotide identity.

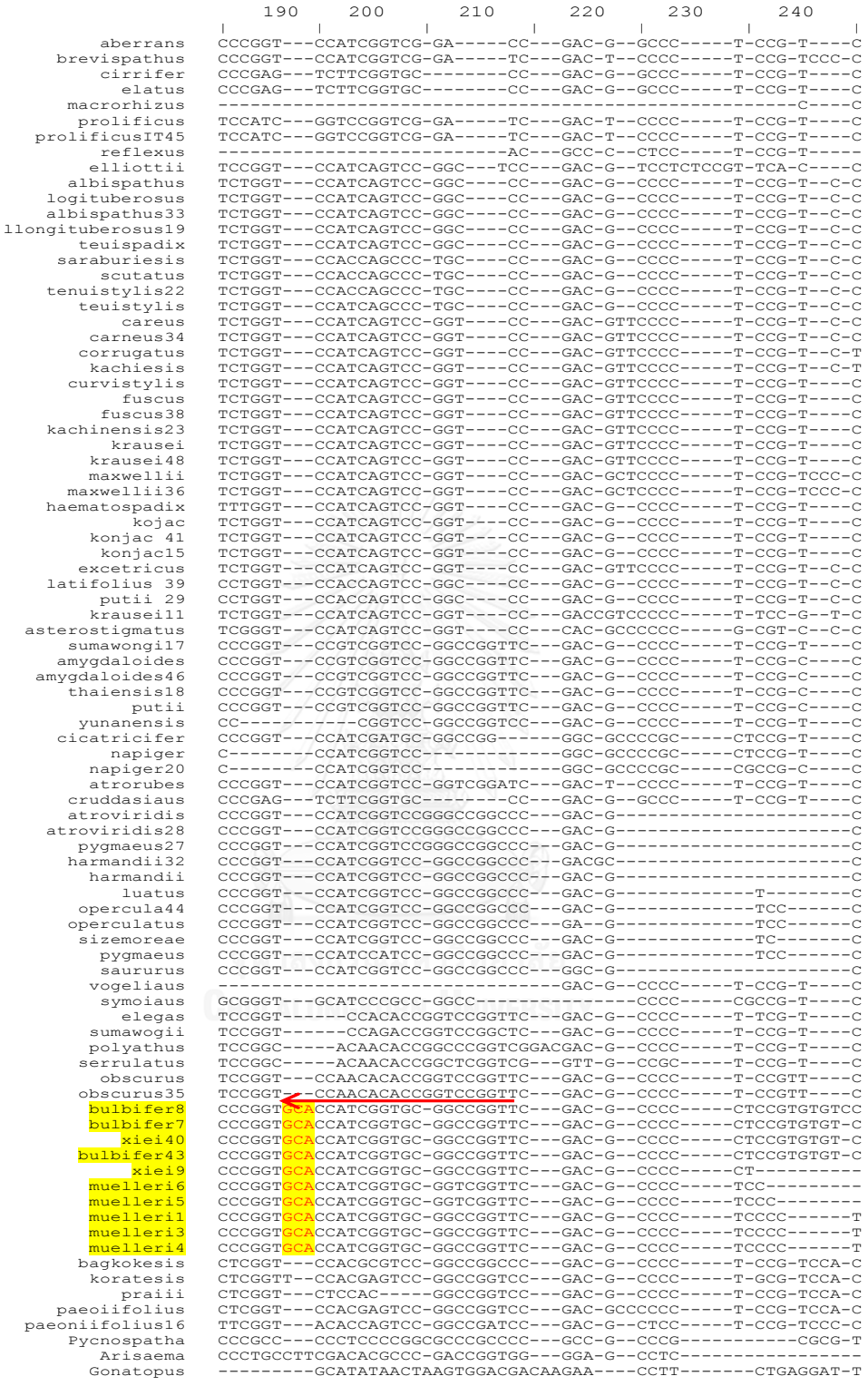


Figure 4.42 Specific character of *A. muelleri, A. bulbifer and A. xiei* of MUBX281_ITS reverse primer based on ITS in *Amorphophallus.* Arrow indicates specific annealing site.

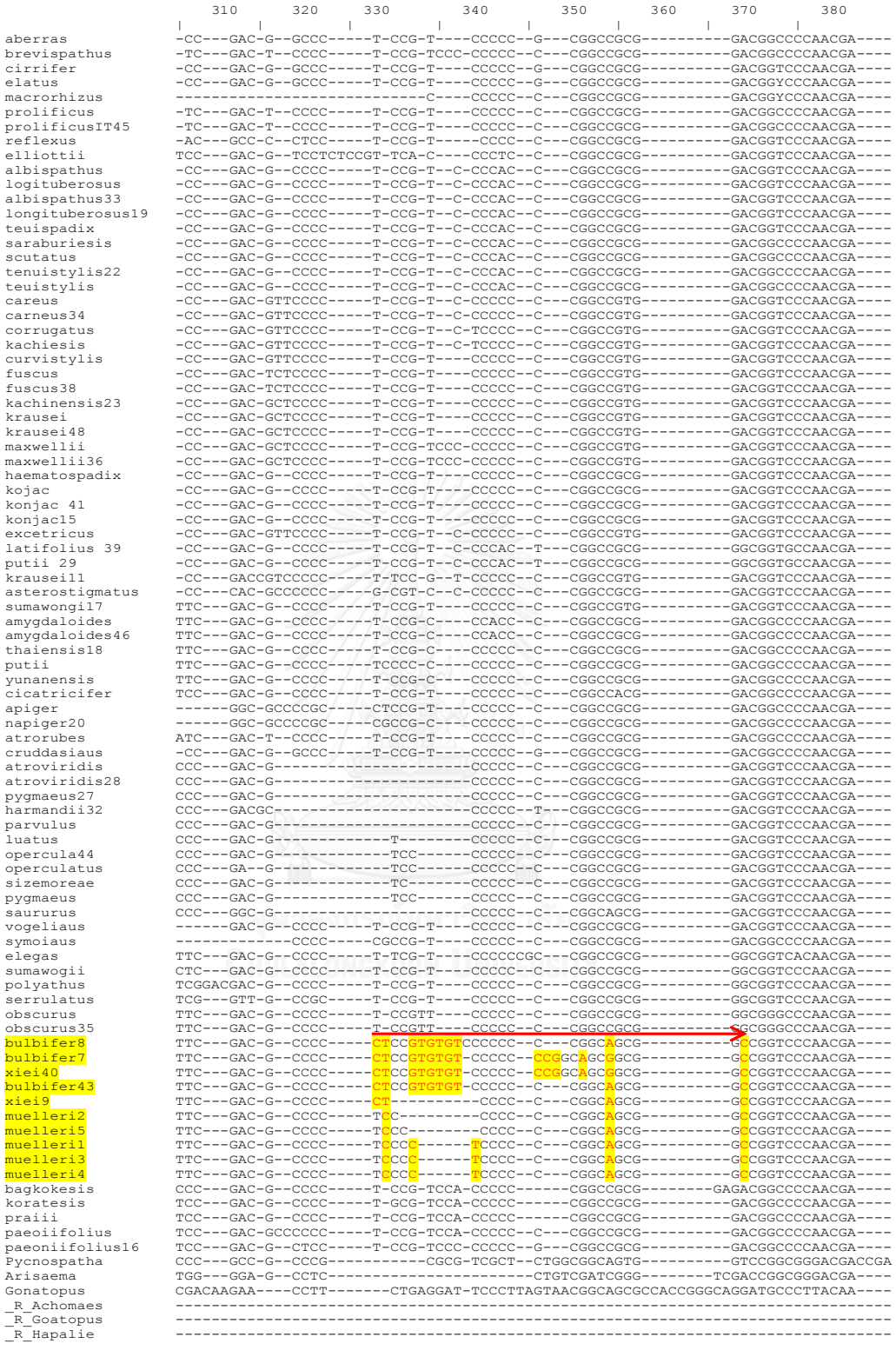


Figure 4.43 Specific character of *A. muelleri, A. bulbifer and A. xiei* of MUBX327_ITS forward primer based on ITS in *Amorphophallus*. Arrow indicates specific annealing site.

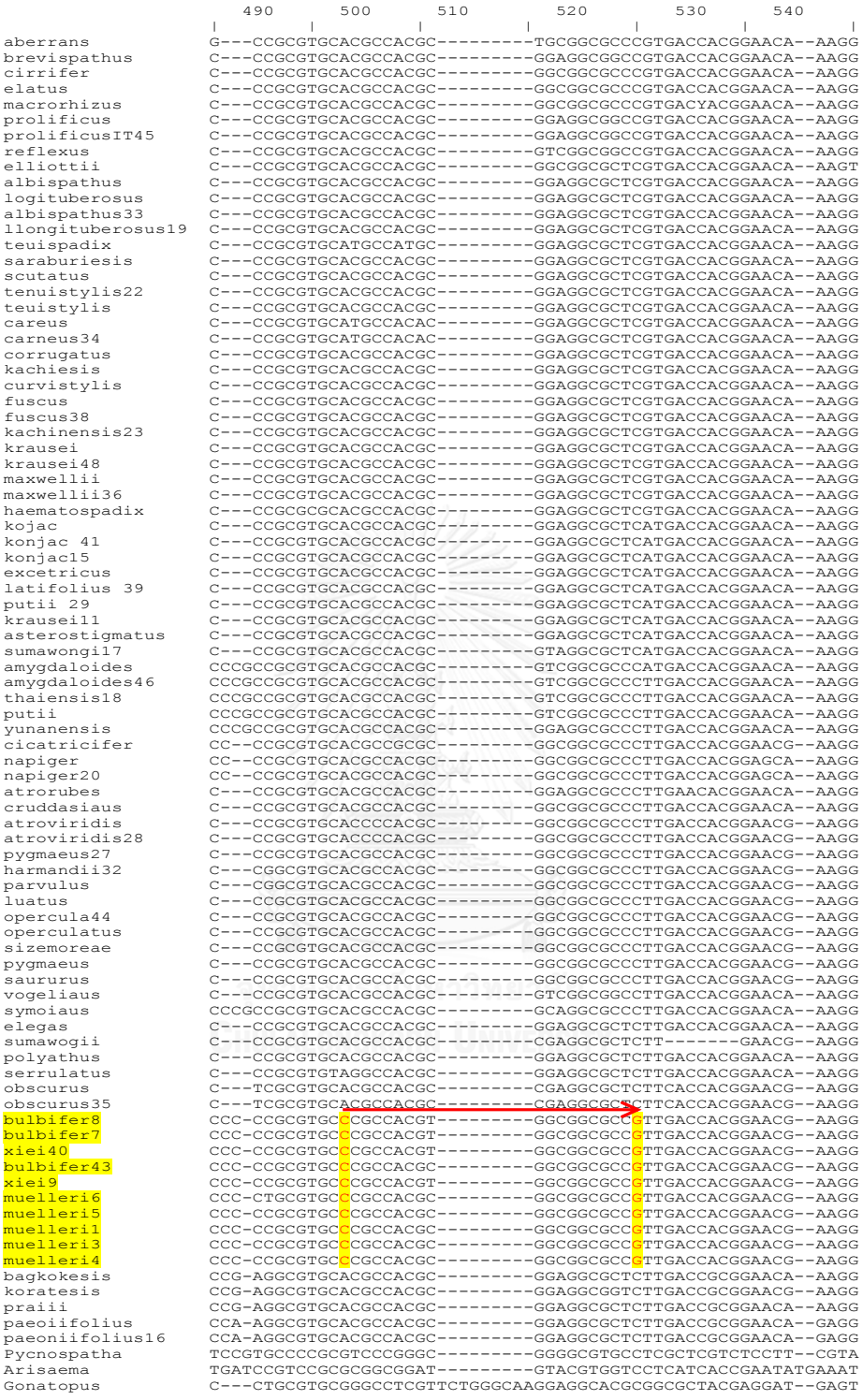


Figure 4.44 Specific character of *A. muelleri*, *A. bulbifer and A. xiei* of MUBX493_ITS forward primer based on ITS in *Amorphophallus.* Arrow indicates specific annealing site.

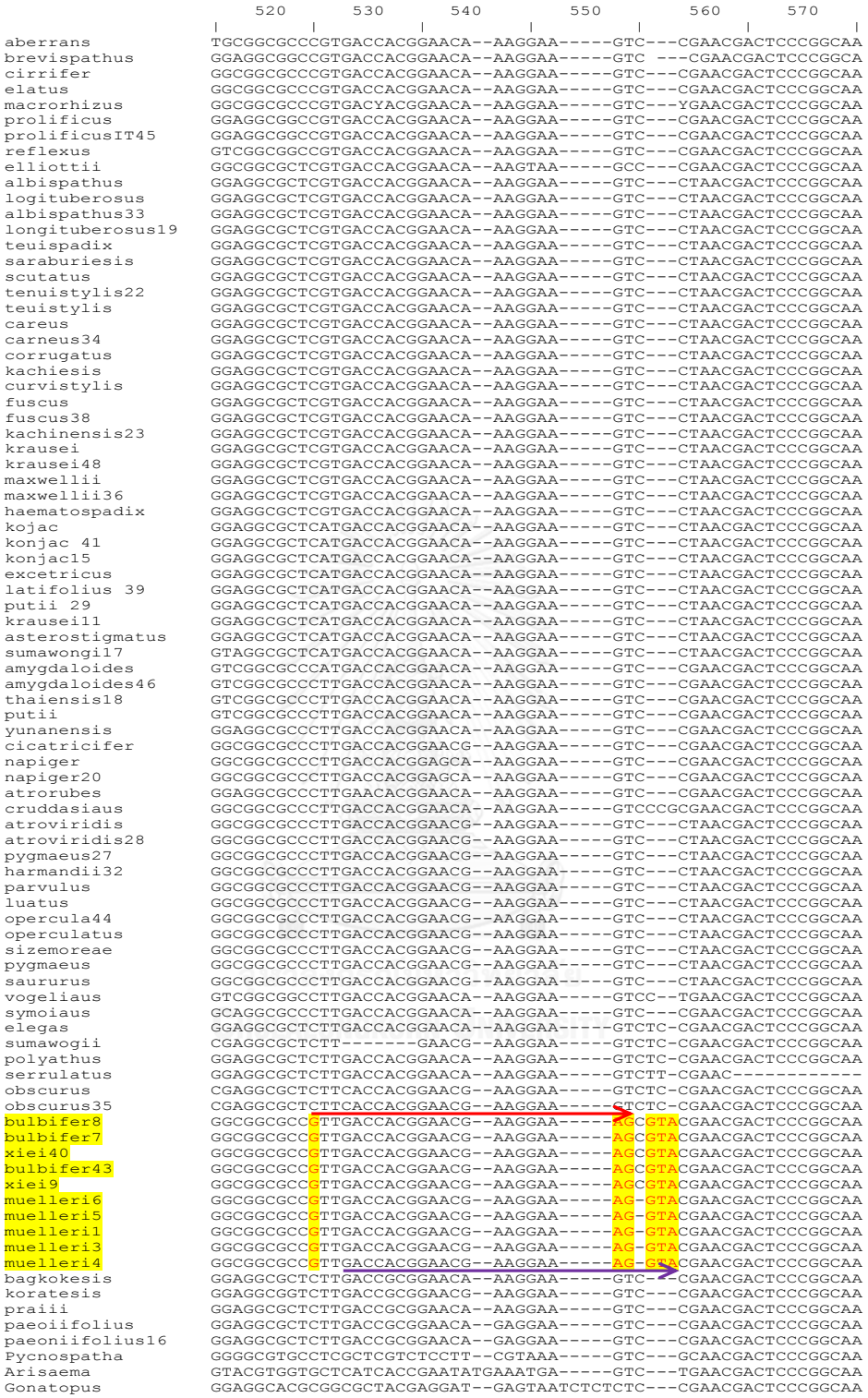


Figure 4.45 Specific character of *A. muelleri*, *A. bulbifer and A. xiei* of MUBX520_ITS forward primer (red) and MUBX551_ITS forward primer (purple) based on ITS in *Amorphophallus.* Arrow indicates specific annealing site.

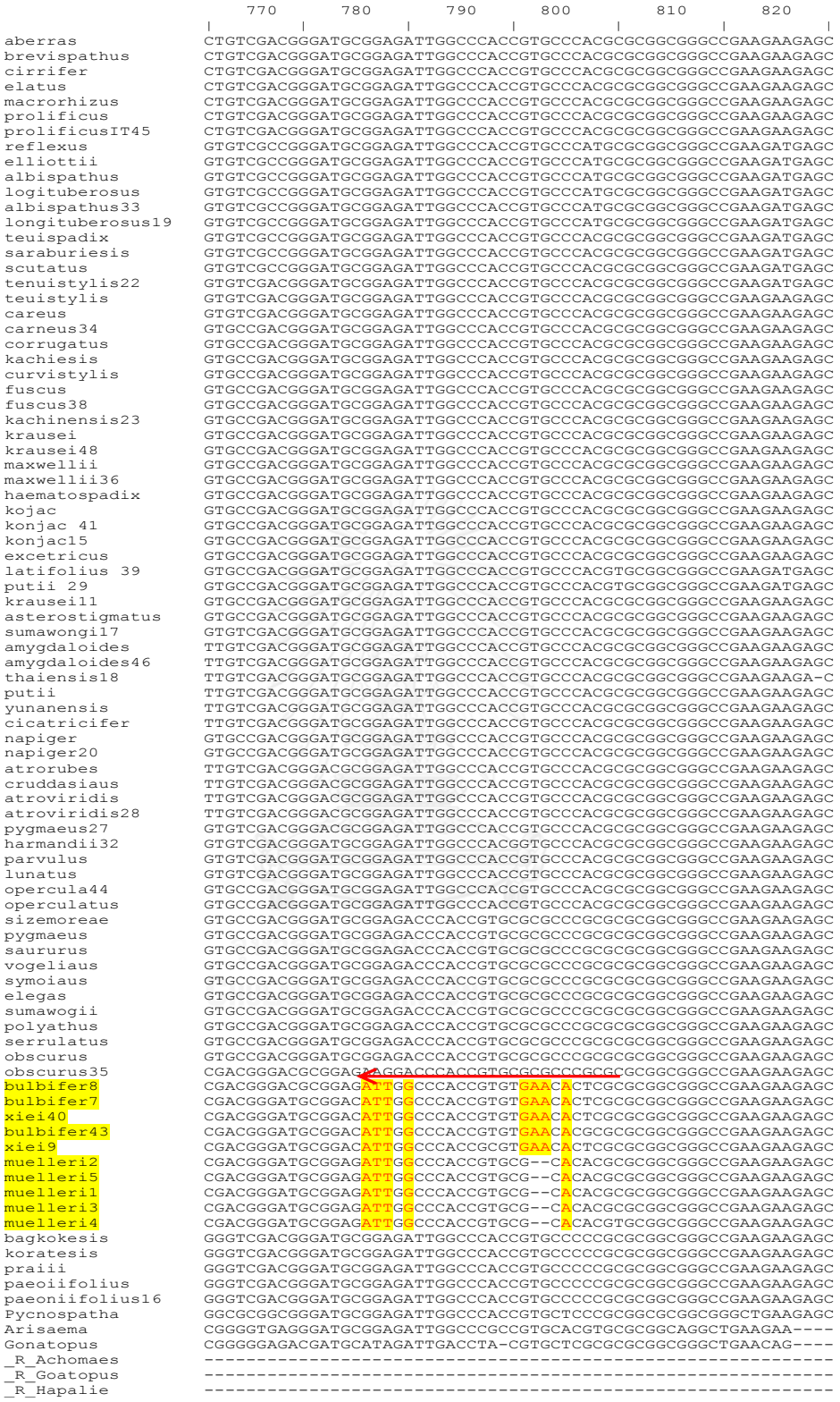


Figure 4.46 Specific character of *A. muelleri*, *A. bulbifer and A. xiei* of MUBX787_ITS forward primer based on ITS in *Amorphophallus*. Arrow indicates specific annealing site.

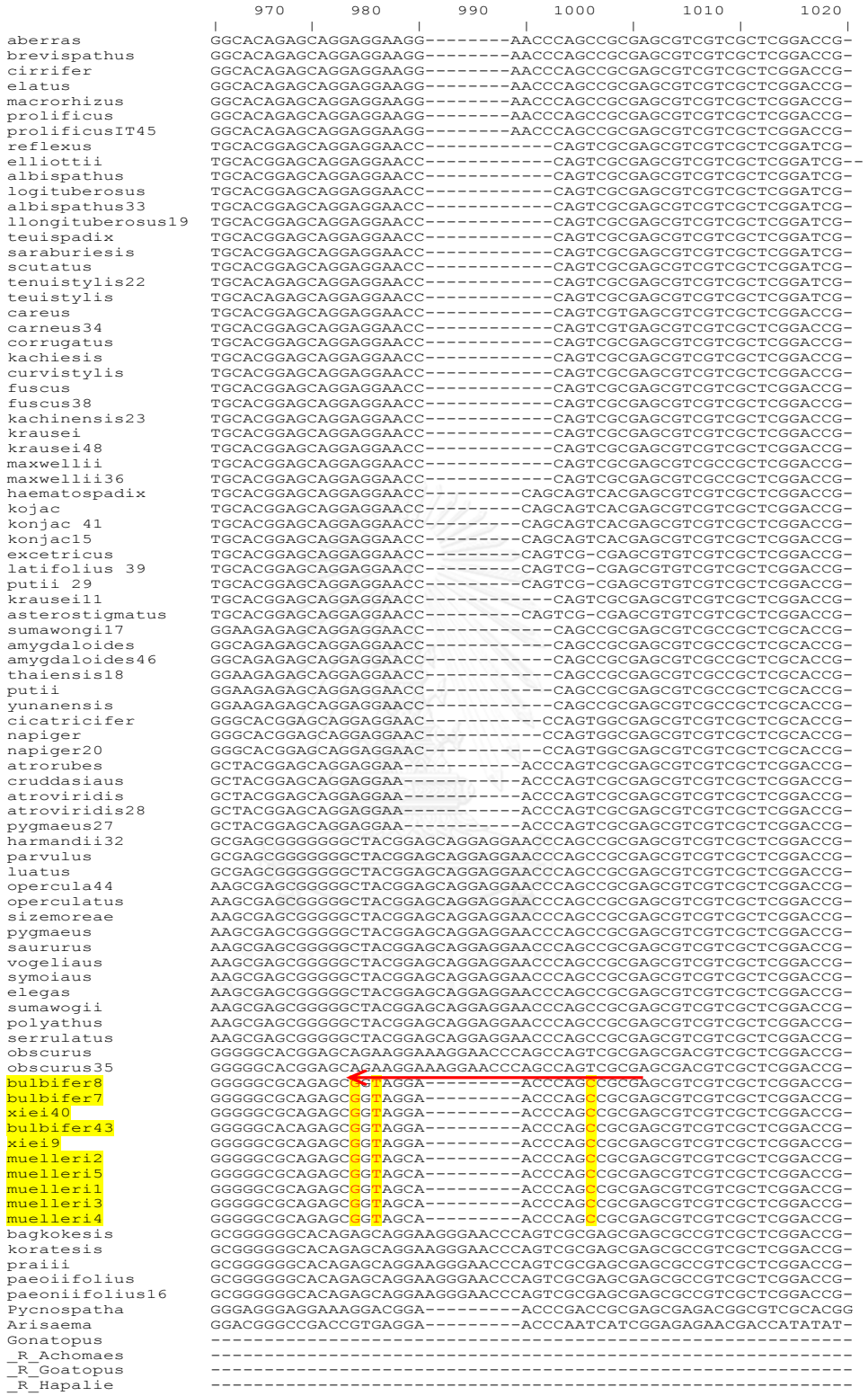


Figure 4. 47 Specific character of *A. muelleri, A. bulbifer and A. xiei* of MUBX994_ITS reverse primer based on ITS in *Amorphophallus.* Arrow indicates specific annealing site.

Table 4.7 The properties of specific designed primers of high KGM content group based on DNA sequencing analysis.

Primer Name	Direction	Sequence (5'-3')	Primer length (bp)	Melting Temperature (T_m)	GC content (%)	Hairpin	Self-dimer	Expected size of amplicons (bp)	Blast N results (E value)
MU-300	forward	CAATACTCGAGAGTCTTTGTAG	22	51.3	40.91	-1.07	-9.96	300	No similarity
MU-689	reverse	TAATATTCGACTCTTACTTC	20	53.3	47.6	1.54	-4.8	689	*A. muelleri* (*TrnL*) (1.1)
MUBX236_*Flint2*	reverse	GCTATCCGACGCGCGCCTTG	20	63.8	64	No	-3.07	181	*A. muelleri* (1.1)
MUBX253_*Flint2*	reverse	GTGCATCTATCTAGCTATCCGA	22	56.3	45.5	No	-8.26	190	*A. muelleri* (1.1)
MUBX222_ITS	forward	AGAGGGCCGCGAAAGT	16	66.6	68.8	No	-4.3	700	*A. konjac* (GDP-D-mannose pyrophos phorylase mRNA; 9 bp) (26)
MUBX281_ITS	reverse	AACCGGCCGCACCGATGGTG	21	76.9	71.4	-3.12	-6.5	213	No similarity
MUBX327_ITS	forward	CTCCCCCCGGCAGCGGC	17	78	88.2	-2.29	-9.75	700	*A. konjac* (putative sucrose synthase mRNA; 8 bp) (30)
MUBX493_ITS	forward	CCGCCACGCGGCGGCGCCG	19	79.2	84.7	-2.9	-1.4	400	*A. konjac* (GDP-D-mannose pyrophos phorylase mRNA; 9 bp) (8.6)
MUBX520_ITS	forward	GGTGACCACGGAACGAAGGAAAG	23	71.5	56.5	No	-1.6	600	*A. konjac* (GDP-glucose pyrophosphorylase mRNA;9 bp) (13)
MUBX551_ITS	forward	GTGACCACGGAACGAAGGAAAGGTA	25	71.5	52	1.6	-1.6	600	*A. konjac* (GDP-glucose pyrophosphorylase mRNA; 11 bp) (0.97)
MUBX787_ITS	forward	ATTGGCCCACCGTGCGCACAC	21	78.2	66.7	-1.4	-1.8	450	*A.bulbifer* (5S ribosomal RNA genes; 9 base) (11)
MUBX994_ ITS	reverse	TCGCGGCTGGGTTCCTACC	19	71.7	68.4	-1.6	-4.3	900	*A. konjac* (GDP-glucose pyrophosphorylase mRNA; 9 bp) (8.6)

From Table 4.7, all designed primers show optimal efficiency properties. Length of designed primer is appropriate with optimal length between 16 and 24 bases that tend to be sequenced specific (Dieffenbach *et al.*, 2003). Melting temperature (T_m) is the temperature at which 50% of the DNA duplex dissociates to become single stranded and can be determined by the primer length and base composition (Hahn, 2013). T_m of designed primer is ranging in from 55°C- 75°C aiming to sequenced specific with annealing temperature. However designed primers from ITS region trend to have higher T_m (75- 80°C) that is recommended for amplifying high GC content targets (Chang *et al.*, 2009). In this study, variation of T_m between forward and reverse primers were less than 5°C to prevent low specificity and poor amplification (Siragusa and Carimi, 2009). A pair of primer in each designed primer is refered to their original sequencing primer in Table 3.3. Most of designed primers have reasonable GC content which is ranging between 40-60%. They provided a sufficient thermal window for efficient annealing. Hairpin is formed via intra-molecular interactions which negatively affect to primer-template binding and leading to poor or no PCR amplification (Dieffenbach *et al.*, 2003). In this study, all of designed primers have acceptable ΔG of hairpin form that should be more than -3 kcal/mol for 3′end hairpin and four of the designed primers did not form hairpin. Self-dimer (homo-dimer) is formed by inter-molecular interactions between two identical primers which have acceptable ΔG more than -5 kcal/mol for 3'end (Chang *et al*., 2009). In this study, eight of total designed primers are acceptable value. The length of PCR product from a designed primer should be in the range of 150-1000 bp as it can be screened on agarose gel rapidly in PCR amplification (Siragusa and Carimi, 2009). BLASTing of designed primers with NCBI sequence

database was done in order to avoid amplify with non-targeted homologous regions. The result showed that MU-300 and MUBX281_ITS primer have no similarity with any sequencing database. While designed primers in *FLint2* and *trnL- trnF* region showed highly similarity nucleotide sequence with *A. muelleri* at 100% identification. Remarkably, blasted result revealed a part of similarity nucleotide sequence in six designed primer from ITS region with GDP-D-mannose pyrophosphorylase and GDP-glucose pyrophosphorylase which are responsible for KGM biosynthetic pathway in Figure 4.48 (Diao *et al.*, 2014; Gille *et al.*, 2011). In addition, the significant matching parameter is Expect value (E). The lower the E-value means the more significant the match is. However, in short identical alignments primer has relatively high E value. This is because the calculation of the E value takes into account of the length of the query sequence and shorter sequences have higher probability of occurring in the database purely by chance from long sequences (Johnson *et al.*, 2008b).

The above result indicated that twelve designed primers from DNA sequencing analysis have suitable properties that lead to a high potential specific primer for discriminating high KGM content group from low KGM content group. The high KGM species are found in two separated clades in sequencing data therefore those designed primers can be developed for individual species detection (*A. muelleri*) or a group of *A. muelleri*, *A. bulbifer* and *A. xiei*. Therefore, those designed primers can not detect all member of high KGM content group in one step.

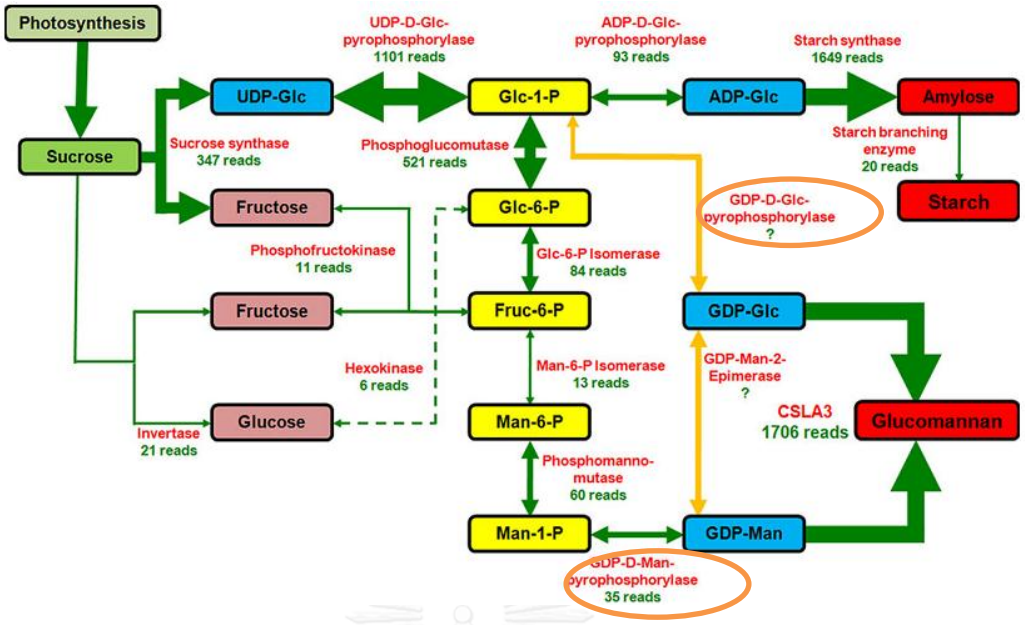


Figure 4.48 Proposed pathways of KGM biosynthesis. Orange circles indicate two enzymes that blasted similarity with designed primer in this study (Gille *et al.*, 2011).

4.2.4.2 Development of SCAR markers

RAPD analysis is more suitable for identifying high-medium KGM content species since primer AC-10 had ability to produce a unique 600 bp band which occur exclusively in all high and medium KGM content species. To develop a rapid and specific method, RAPD marker was converted to a SCAR marker which is more consistent and general specific results than RAPD marker in order to screen and validate of *Amorphophallus* plant.

4.2.4.2.1 Cloning and detection of recombinant plasmid

Unique RAPD fragment at 600 bp from 19 accessions (7 species) was excised from the electrophoresed gel and cloned using a TA cloning approach with pDrive cloning vector (Figure 3.5). Transformation of ligated vector into NEB 5-alpha Competent *E. coli* cell by heat shock method. The recombinant clones were selected by using blue-white screening technique (Figure 4.49). It was found that white colonies were observed in each sample less than 20 colonies. This may be due to low efficacy to heat-shock transformation (Brooks and Ericson, 2010). Afterward, recombinant clones were checked for presence or absence of insert DNA in plasmid constructs by colony PCR method with AC-10 primer. The result showed that most of white colonies in each accession generated PCR product of specific 600 bp fragment (Figure 4.50). Those recombinant clones were selected to plasmid extraction. The recombinant plasmids were double checked by digesting with a restriction enzyme *E*coR I to separate cloning vector and specific band. A product with expected size of approximately 600 bp was observed (Figure 4.51). Only recombinant plasmids that showed specific DNA fragment were selected to DNA sequencing. Finally, two recombinant plasmids for each high and medium KGM content accession were selected and sequenced for forward and backward directions. Total, 38 recombinant clones were sequenced. The result showed that the sequences of both recombinant clones were compared for each accession and no differences were observed. This result indicated that the specific RAPD fragment represented single migrating fragment that has no overlap DNA fragments in the same size (600 bp).

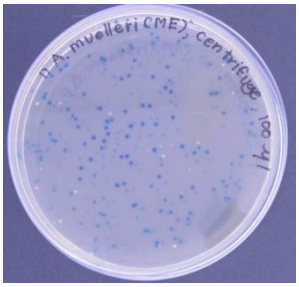
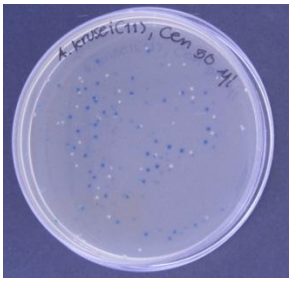
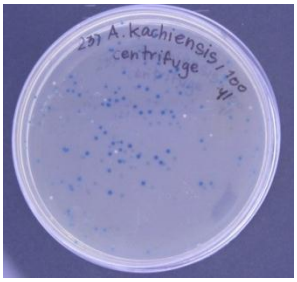
(A)  (B)  (C) 

Figure 4.49 The recombinant clones as white colony of (A) *A. muelleri*, (B) *A. krausei* and (C) *A. kachiensis* by using blue-white screening technique.

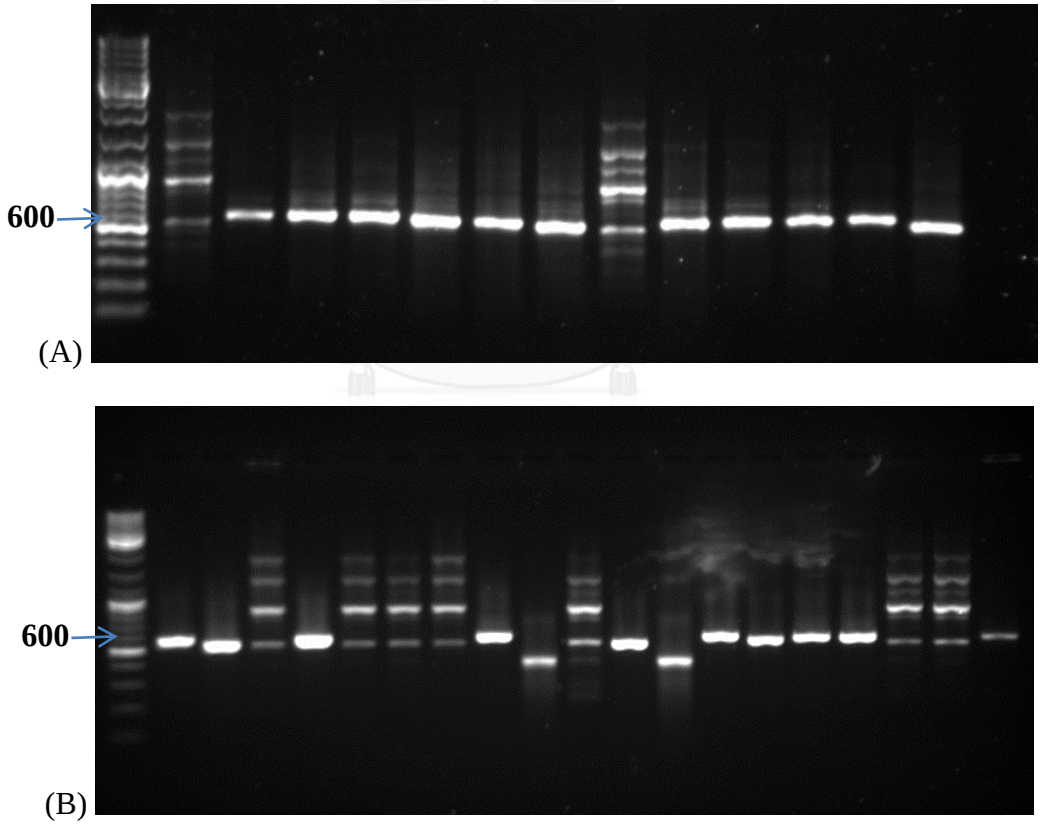


Figure 4.50 Recombinant clones with DNA insertion show specific 600 bp PCR product by Colony PCR of (A) *A. muelleri*, (B) *A. krausei*.

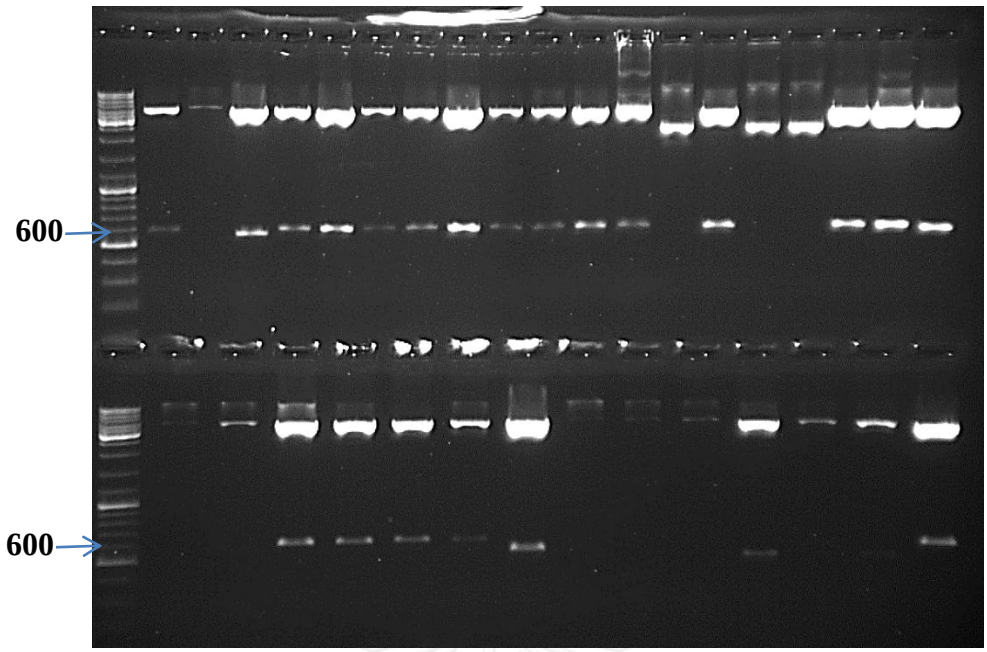


Figure 4.51 Detection of recombinant plasmids with *E*coR I restriction enzyme. Recombinant plasmids with DNA inserted show a specific 600 bp fragment.

4.2.4.2.2 SCAR Primer design

All of nucleotide sequences of total 19 clones are homology searched using BLASTing with the NCBI GenBank Database. The result found that those sequences did not reveal significant similarity (E values > 10^{-4}) with any sequence in the GenBank database. Therefore, it was regarded as an unknown sequence. Except for the amplified fragment of *A. konjac* showed 62% blast similarity to starch synthase III precursor which is related with regulatory functions in KGM synthesis (Diao *et al.*, 2014). This suggests that this marker might be related to some KGM synthesis process of *Amorphophallus* species. However, KGM pathway is not fully understand yet (Diao *et al.*, 2014). The alignment of nucleotide sequences of the recombinant clone was created and SCAR primer was developed. Two primers were designed at a begining and end regions of multiple sequence alignment by the Primer

3.0 design software. A pair of primer for high-medium KGM content is HKGM-4F forward primer and HKGM-595R reverse primer. This SCAR primer consists of the original bases of the RAPD primer plus internal sequence of RAPD marker. Because of the sequences of the primers that were designed from internal sequences are less polymorphic than primer that includes initial RAPD primer sequences (Kiran *et al.*, 2010). The reason why the HKGM-4F/HKGM-595R primer pair can be amplified in all high-medium KGM content may be due to their genetic similarities and the genome constitutions are diverse enough to create distinct RAPD banding patterns which allow species to be differentiated (Scheef *et al.*, 2003). Moreover, additional primer set was designed especially for *A. muelleri*, *A. bulbifer* and *A. xiei* from their conserved regions matching and produced a 361 bp fragment (MUE-129F forward primer and MUE-490R reverse primer). The properties of SCAR primers were shown in Table 4.8.

TAGCAGCGAGGGTCAGTGGACTCGAACCAAAGCAAAAGAAAGAGAGAAGACTCTGCCATGAAAAGAAAGTCATAC

CTCAGGACCATTATAAAAGGAGGAGAGCGGCCAATACATGTAGGCTGTCTCCTTTGAGCGAGAGGCCCCCTCTTTG

ACAAGCCAGCATATGTCTCCAATGGTCTTGCTCTGTTCCACTAAAGCCGCCAGGTCATGATGGCCATAGAAGTCGAG

GCAGTGGAACAGGCCCAGATGATCCATCGCCCAGCTGTCAGCATAATAGAATGATATAAACAACGGCTTGTCCAAG

ATGCTGGCTATAGGACCTTCAGCCGATAAATTATGAATGTTCAGCAGCACAAAAATCCATGAAGTCTGCCGATAATAT

GTCTGGGTCCGGTCTTCGCGCCGCACAAATGCCCGGTACGGGCGAAATCTGTCTGACGGACCCCTACATGCGGCA

ACAGACGGCATAGGTTCCTCTGGGGGAAACGGATACTGCTGCGCCTCCAGACGGCTACTCCCTATGTTGCTCAGAT

GCTTCAAAGTCTTCAAGAAAACATCCTCGCTGCTA

Figure 4.52 Nucleotide sequences of *A. muelleri* (No1, MU-ME01). Sequences and positions of the forward primer and reverse primer are underlined.

Table 4.8 The properties of SCAR primers based on RAPD analysis.

Primer Name	Direction	Sequence (5'-3')	Primer length (bp)	Melting Temper-ature (T_m)	GC content (%)	Hairpin	Self-dimer	Expected size of amplicons (bp)
HKGM-4F	forward	AGCGAGGGTC AGTGGACTC	19	69.06	63.2	No	0.00	600
HKGM-595R	reverse	GCAGCGAGGAT GTTTTCTTG	21	68.88	47.6	No	0.00	
MUE-129F	forward	AGCTGTCTCCTT TGAGCGAG	20	70.30	55	No	0.00	350
MUE-490R	reverse	CCAGAGGAACC TATGCCGTC	20	72.35	60	No	9.7	

4.2.5 Efficiency testing of designed primers

Efficiency of amplification referred to how close a primer pair is able to amplify a product to the theoretical optimum number of product for each PCR cycle (Dieffenbach *et al.*, 2003). Therefore, specific primers that could produce a fragment to be used for identifying high-medium KGM content taxa, PCR fragments from the 14 primers (12 primers from DNA sequencing marker and 2 primers from RAPD marker) were screened with presence or absence for each primer against the KGM content group at specific base pair. Moreover, this testing aims to generate reproducible DNA fragment of high-medium KGM content species with good and clarity PCR product. Meanwhile it helps to reduce the occurrence of non-specific amplifications in the negative control reactions.

4.2.5.1 PCR optimization

In general, PCR amplification cycle has three steps include DNA templates melting, primer annealing and extension. The important factor that lead to increased specificity in effective PCR amplifications is a suitable primer annealing temperature (An *et al.*, 2013; Li *et al.*, 2011b). Higher annealing temperature were selected for further study because higher annealing temperature help to increase specific amplification and reduce non-specific amplification products (Srivastava *et al.*, 2012). PCR reactions were optimized with annealing temperature which is determined by the T_m of both primers. Mostly annealing temperature is 5 °C lower than T_m (Chang *et al.*, 2009; Marieschi *et al.*, 2011). In this study, annealing temperature of each primer pair was varied into three levels include (1) lower than T_m for 5 ^{O}C , (2) lower than T_m for 7 ^{O}C and (3) lower than T_m for 10 ^{O}C to determine the optimal annealing temperature (Table 4.9). PCR conditions of specific primer reactions were the same as sequencing or RAPD reactions that described before, except annealing temperature. The primer pairs were first screened the optimal annealing temperature on sample set of high or medium KGM content species included *A. muelleri* (No 1-6), *A. bulbifer* (No. 7, 8 and 43), *A. xiei* (No. 9 and 40), *A. krausei* (No. 10-12), *A. corrugatus* (No 21), *A. kachiensis* (No. 23 and 48) and *A. konjac* (No.18 and 41). The result showed that all of designed primer can be amplified at optimum temperature that was lower than T_m at 5 ^{O}C which is correspondence with previous studies (Anil *et al.*, 2014; Hazarika and Neog, 2014; Masuzaki *et al.*, 2008). Moreover, most of annealing temperature that is more than 65 °C prevents formation of the secondary structures of primer leading to success amplifications (Li *et al.*, 2011c).

Table 4.9 Annealing temperatures used at specific designed primer.

No	Primer Name		Annealing temperature			Expected PCR Product (pb)
		Melting temperature (T_m)	Lower than T_m (5 ^{O}C)	Lower than T_m (7 ^{O}C)	Lower than T_m (10 ^{O}C)	
1	HKGM-4F/ HKGM-595R	70.06/ 68.88 (70)	65	63	60	600
2	MUE-129F/ MUE-490R	70.30/72.35 (70)	65	63	60	350
3	MU-300/ primers "f"	67.59/ 60.02 (60)	55	53	50	300
4	primers "c"/MU-689	64.5/ 60.05 (60)	55	53	50	690
5	*FLint2* F1/ MUBX236_*Flint2*	65.0/76.45 (65)	60	58	55	200
6	*FLint2* F1/ MUBX253_*Flint2*	65.0/69.45(65)	60	58	55	200
7	MUBX222_ITS/ 26S-82R	67.5/76.45 (65)	60	58	55	700
8	P17/MUBX281_ITS	73.88/76.45(75)	70	68	65	300
9	MUBX327_ITS/ 26S-82R	77.97/77.11(75)	70	68	65	700
10	MUBX493_ITS/ 26S-82R	84.82/77.11 (75)	70	68	65	400
11	MUBX520_ITS/ 26S-82R	75.33/77.11 (75)	70	68	65	600
12	MUBX551_ITS/ 26S-82R	75.82/77.11 (75)	70	68	65	600
13	MUBX787_ITS/ 26S-82R	76.69/77.11(75)	70	68	65	450
14	P17/MUBX994_ ITS	73.88/74.03 (70)	65	63	60	900

Success rate of specific band that was amplified with designed primer set was about 78.57% (11 primer sets of total 14 primer sets) at optimal annealing temperature. Successful designed primer sets included two SCAR primer

sets (HKGM-4F/ HKGM-595R and MUE-129F/MUE-490R), one set of *trnL-trnF* marker (primers "c"/MU-689), two sets of *Flint2* marker (*FLint2* F1/ MUBX236_ *Flint2* and *FLint2* F1/ MUBX253_*Flint2*) and six sets of ITS marker (MUBX327_ ITS/26S-82R, MUBX493_ITS/26S-82R , MUBX520_ITS/26S-82R, MUBX551_ITS/ 26S-82R, MUBX787_ITS/26S-82R, P17/MUBX994_ ITS). When the annealing temperature was reduced (lower 10 ^{O}C), those primers still generate specific DNA band without any non-specific band. This result confirmed the specificity of those designed primers for high or medium KGM content species. However, some of designed primers were failing. The failure of amplifications was observed for multiple bands found in two primer sets of ITS markers (MUBX222_ITS/26S-82R and P17/ MUBX281_ITS). While the failure of amplifications without amplify band found in MU-300/primers f set which showed high value of hairpin structure. The failure amplification come from loss of polymorphism resulting in the same band was amplified in all sample tested, the amplification of multiple bands of similar or varied sizes or no bands were amplified (Polashock and Vorsa, 2002). Moreover, the result of multiple bands may be due to amplification of variable repeated regions or alternate alleles and the case of no amplification may suggests that the primer set was not functioned under the optimal PCR conditions. This is also include primer characteristics like hairpin formation that could also be important factors (Polashock and Vorsa, 2002). The ineffective amplifications can be due to the primers with lower T_m and asynchronous primer annealing resulted in non-specific amplifications with low yield products (Li *et al.*, 2011c). Those false amplifications reduced the potential of the marker for accurate authentication (Kumar *et al.*, 2014). Therefore, the three unsuccessful designed primers were not be further characterized.

4.2.5.2 Specific testing of designed primers

Specificity is the frequency of a mispriming event occurs in amplification. Primers with poor specificity tend to produce PCR products with unrelated and undesirable fragments when they are visualized with gel electrophoresis (Dieffenbach *et al.*, 2003). Specific primer was observed by presence or absence of correct exactly amplification band for each species (Theerakulpisut *et al.*, 2008; Zhao and Wu, 2012). Eleven pairs of primers were tested for specificity with the same *Amorphophallus* set (48 accessions). This analyze was done to ensure that amplification of the band with correct molecular weight.

The result showed that single unique bands were correctly amplified for high or medium KGM content species by seven pairs of designed primer with exhibited no amplification in any of low KGM content, including two SCAR primer sets (HKGM-4F/ HKGM-595R and MUE-129F/MUE-490R), two sets of *Flint2* marker (*FLint2* F1/ MUBX236_*Flint2* and *FLint2* F1/ MUBX253_*Flint2*) and three sets of ITS marker (MUBX520_ITS/26S-82R, MUBX551_ITS/26S-82R, P17/MUBX994_ ITS). All of specific DNA band pattern of each primer was showed in Figure 4.53-4.59. However, some of designed primers lose their polymorphism by produced non-specific band with low KGM species especially from designed primer from *trnL-trnF* region. This non-specific result agreed with Yang *et al.* (2001) that developed molecular marker based on *trnL- trnF* sequences in medicinal *Rheum* spp. found that this region is very high degree of homology and only one nucleotide differed from the adulterants. Resulting in this primer still produced a faint or smearing band in several adulterants. Moreover, designed primers were loss of their

polymorphism maybe due to primer mismatches specificity binding region when convert to specific primer, those binding region loss their DNA variation or primer amplifying sequence from multiple site in the genome (Paran and Michelmore, 1993; Polashock and Vorsa, 2002; Zhao and Wu, 2012). Liu *et al.* (2012) studied allele-specific PCR primer design method found that primers generated from substitution types A/C (T/G) had the low detection efficiency because TG and CA matches had weak destabilization strength. The primers including these specific matches at 3'end were easier to make non-specific amplification. This result corresponded with non-specific four designed primer in this study that 3'end of those primers were A/C or T/G substitution types. This is interesting to note that a single base difference in the primer has resulted in completely different amplicons targeting and different in priming sites (Mulpuri *et al.*, 2013).

Further studied, seven specific primer sets were specificity tested with larger specimens of high or medium and low KGM contents (84 samples) with outgroup species (5 species) for to avoid mismatching primer. In addition, this testing help to confirm accurate and specific property of designed primer.

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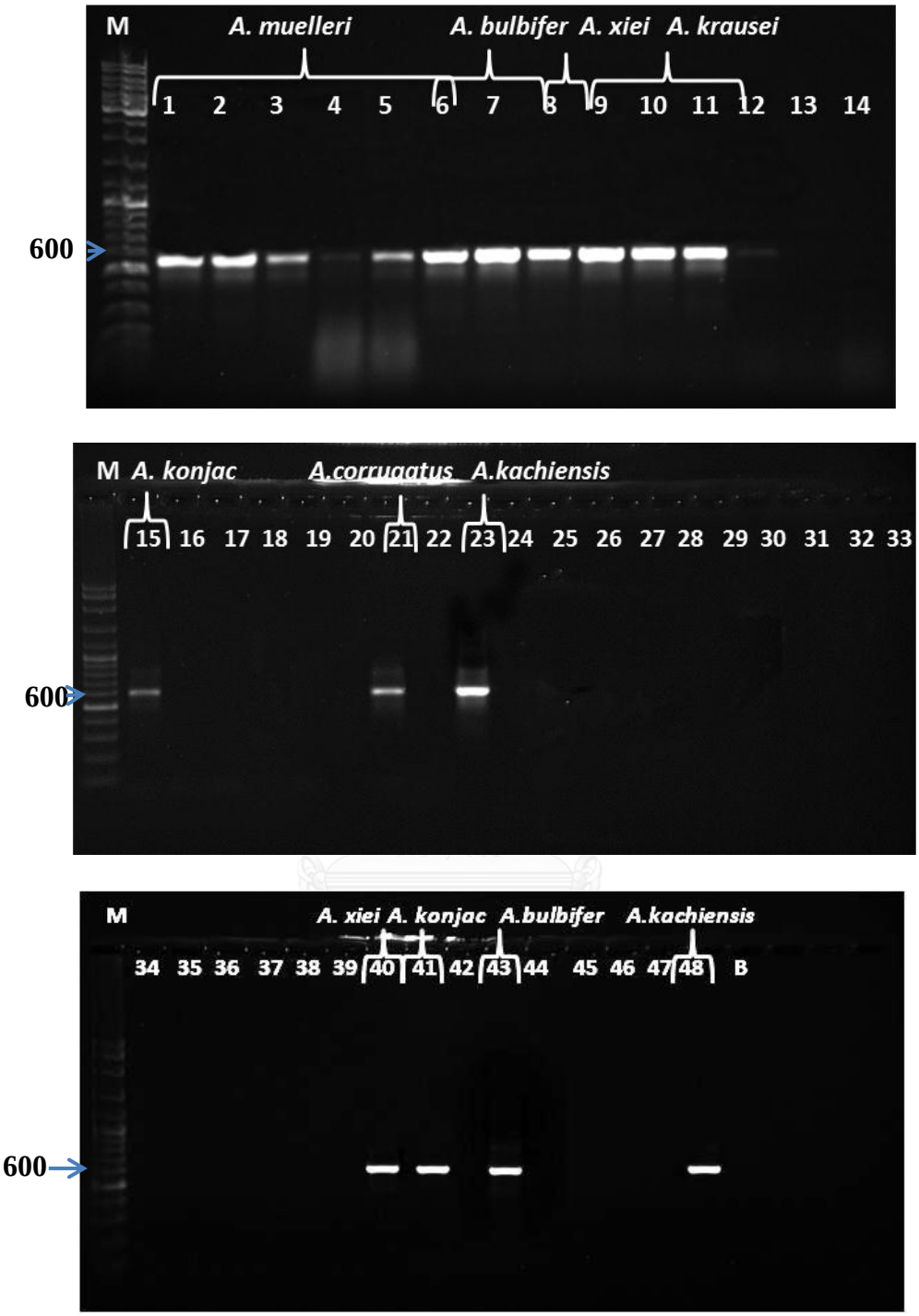

Figure 4.53 Specific DNA band pattern of all high KGM content species characterized by amplification with HKGM-4F/ HKGM-595R primer set.

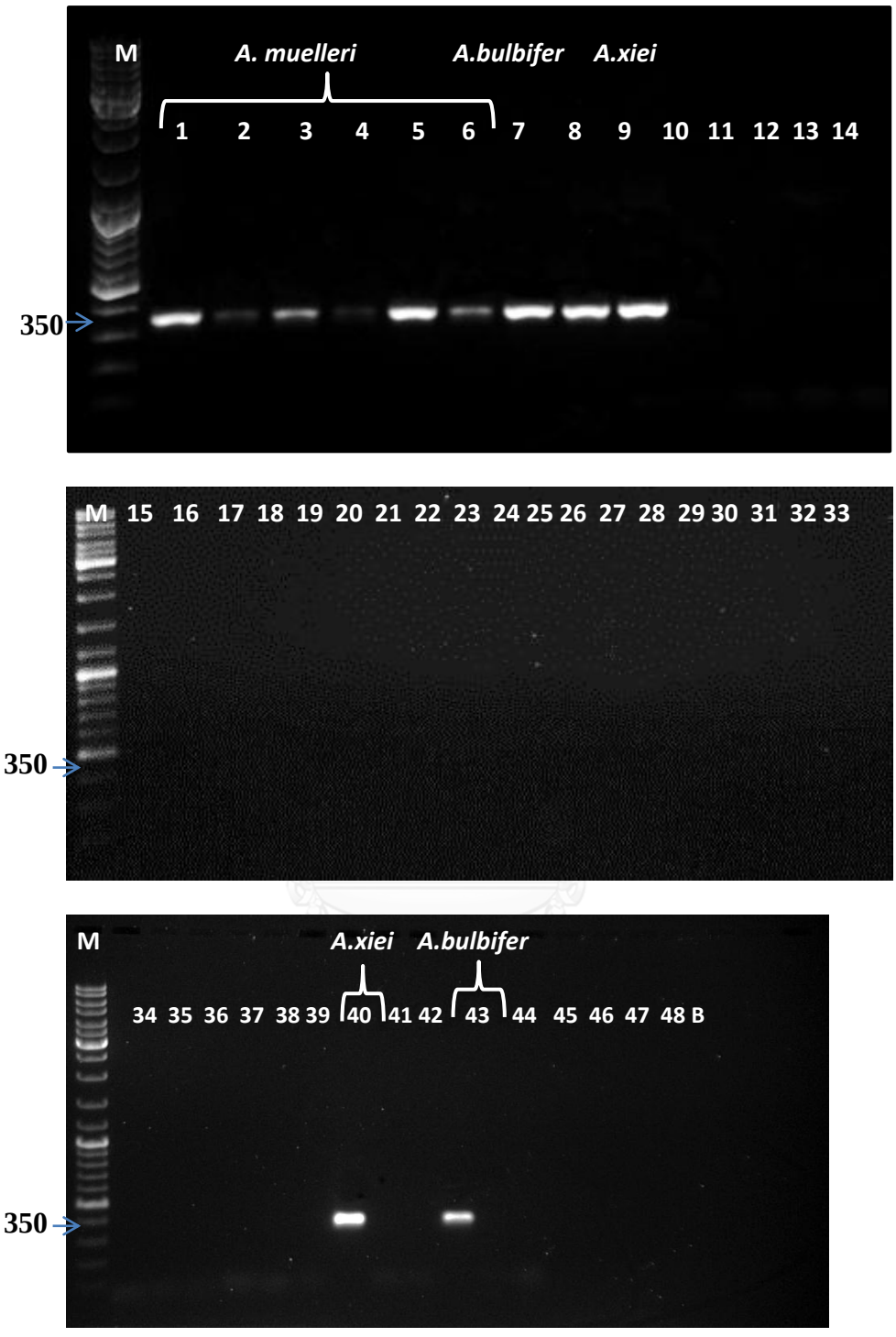

Figure 4.54 Specific DNA band pattern of *A. muelleri, A.bulbifer, A.xiei* characterized by amplification with MUE-129F/MUE-490R primer set.

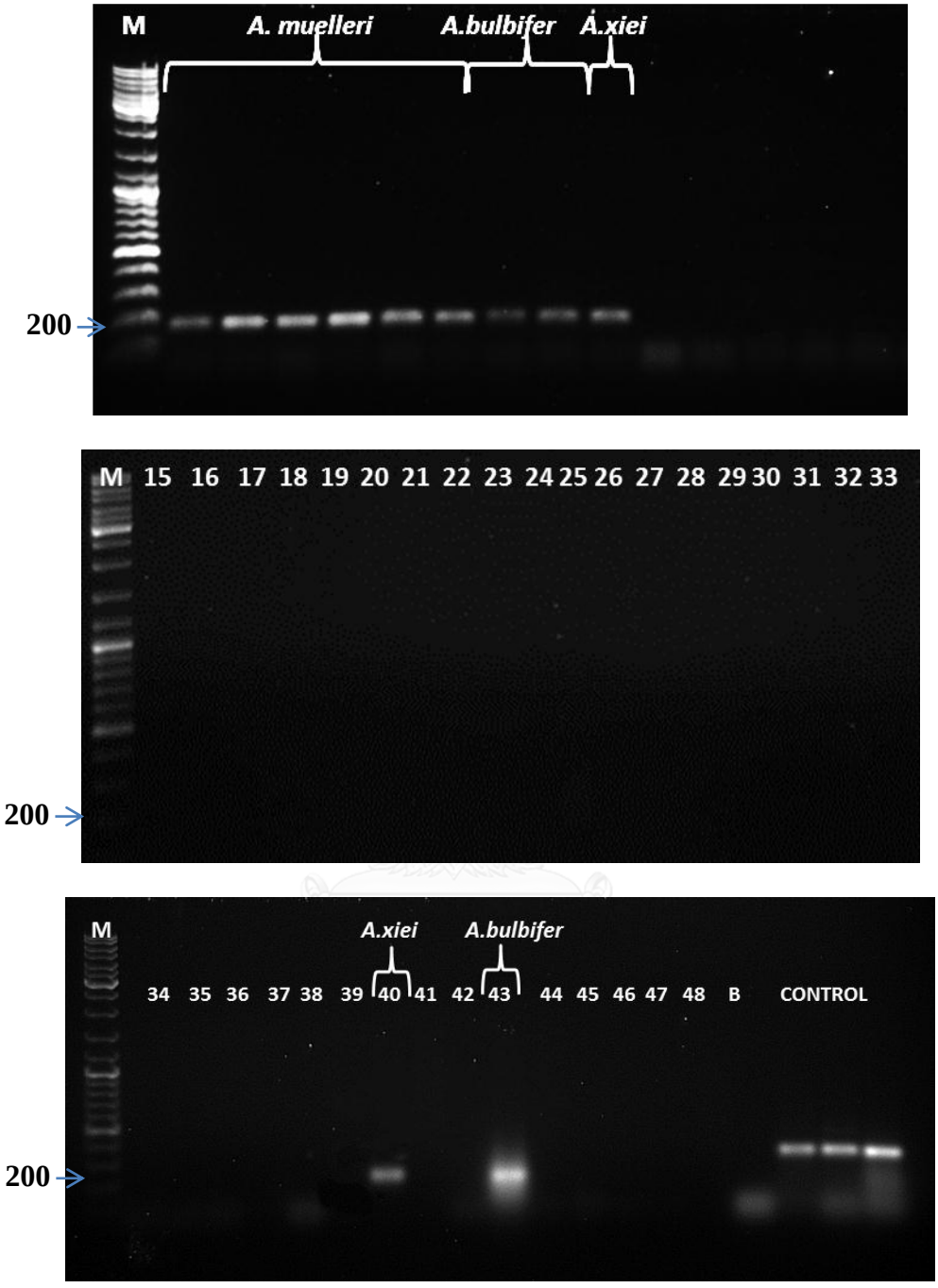

Figure 4.55 Specific DNA band pattern of *A. muelleri, A.bulbifer, A.xiei* characterized by amplification with *FLint2* F1/ MUBX236_*Flint2* primer set.

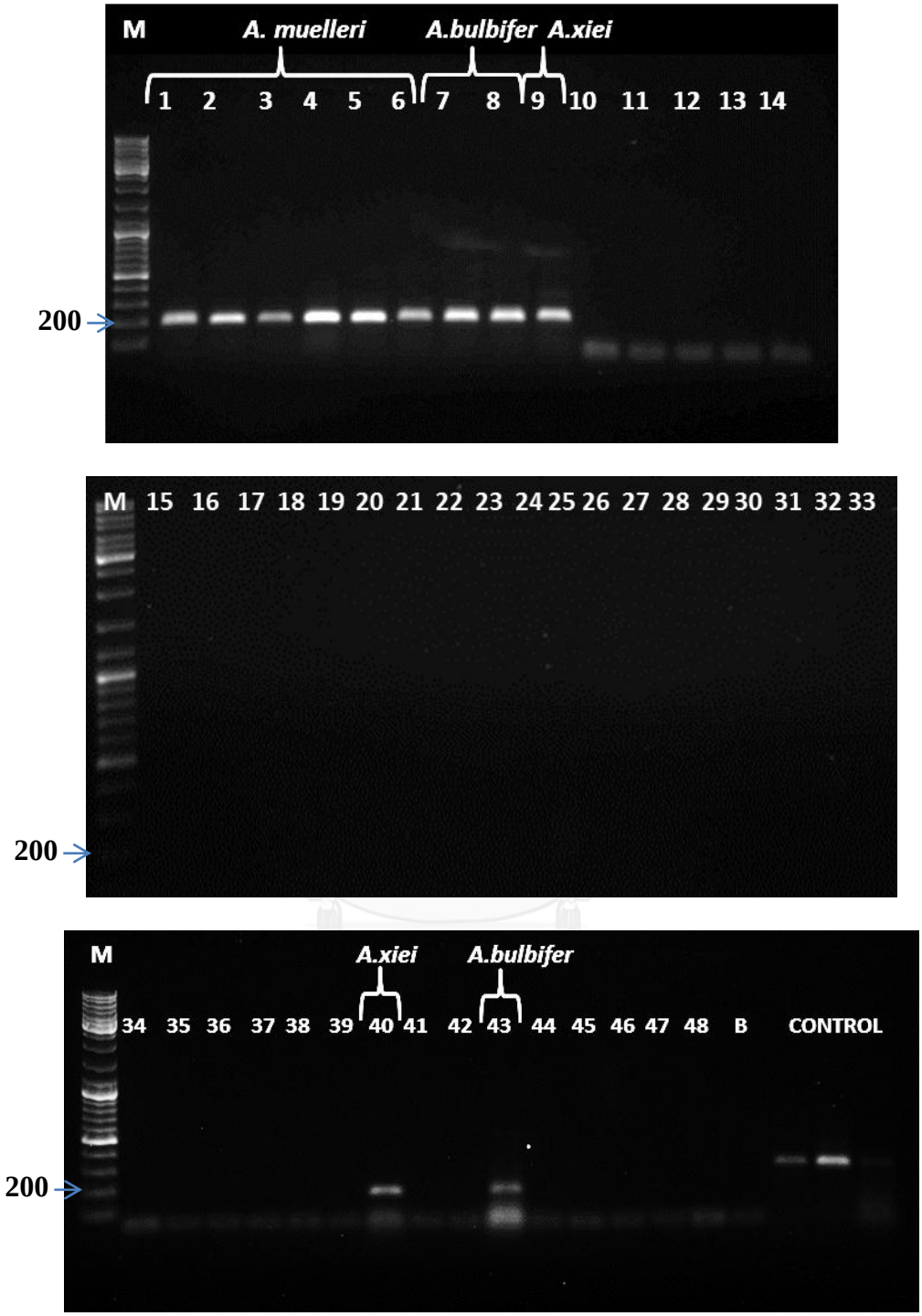

Figure 4.56 Specific DNA band pattern of *A. muelleri, A.bulbifer, A.xiei* characterized obtained by amplification with *FLint2* F1/ MUBX253_ *Flint2* primer set.

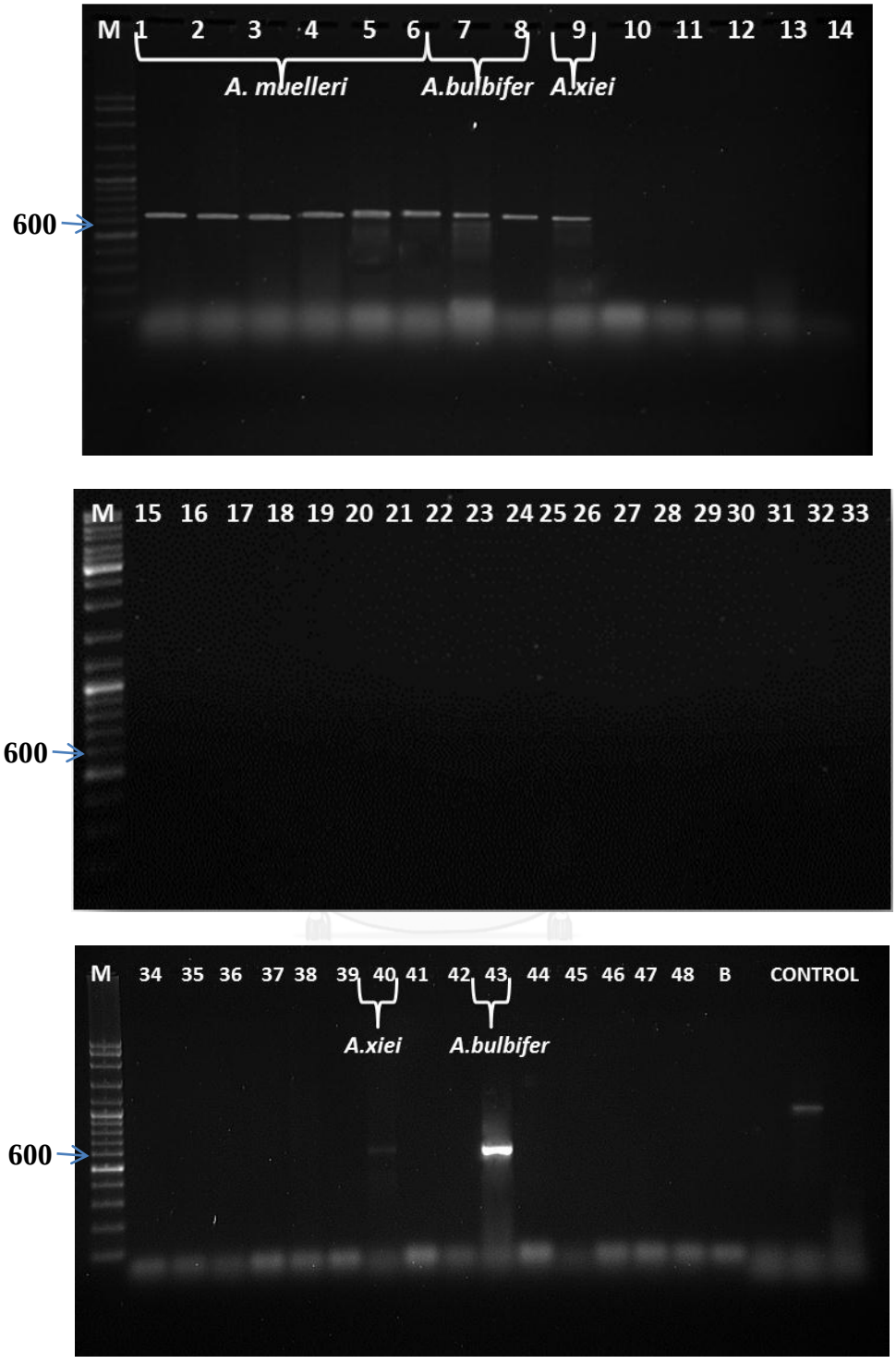

Figure 4.57 Specific DNA band pattern of *A. muelleri*, *A.bulbifer*, *A.xiei* characterized by amplification with MUBX520_ITS/26S-82R primer set.

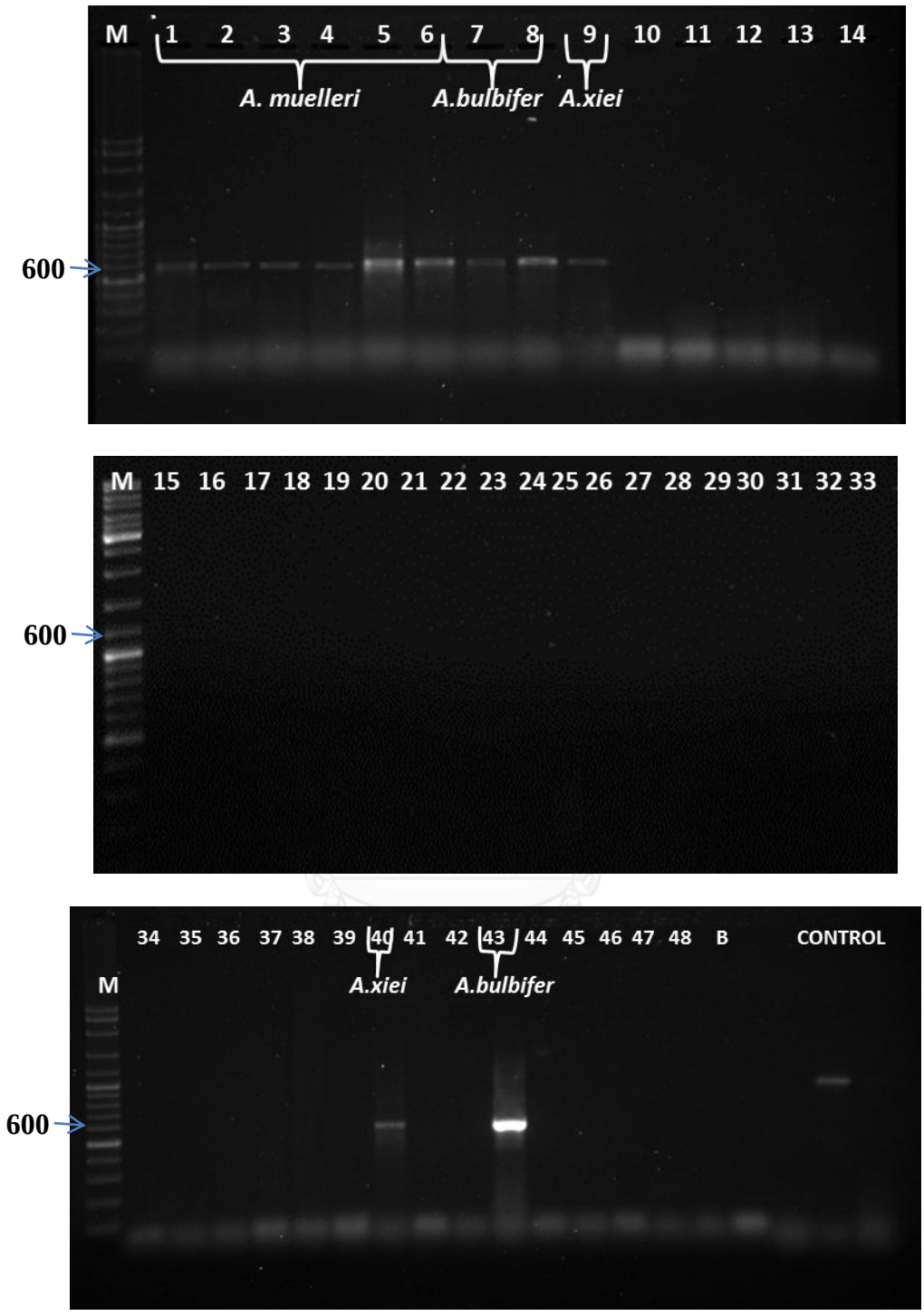

Figure 4.58 Specific DNA band pattern of *A. muelleri, A.bulbifer, A.xieii* characterized by amplification with MUBX551_ITS/26S-82R primer set.

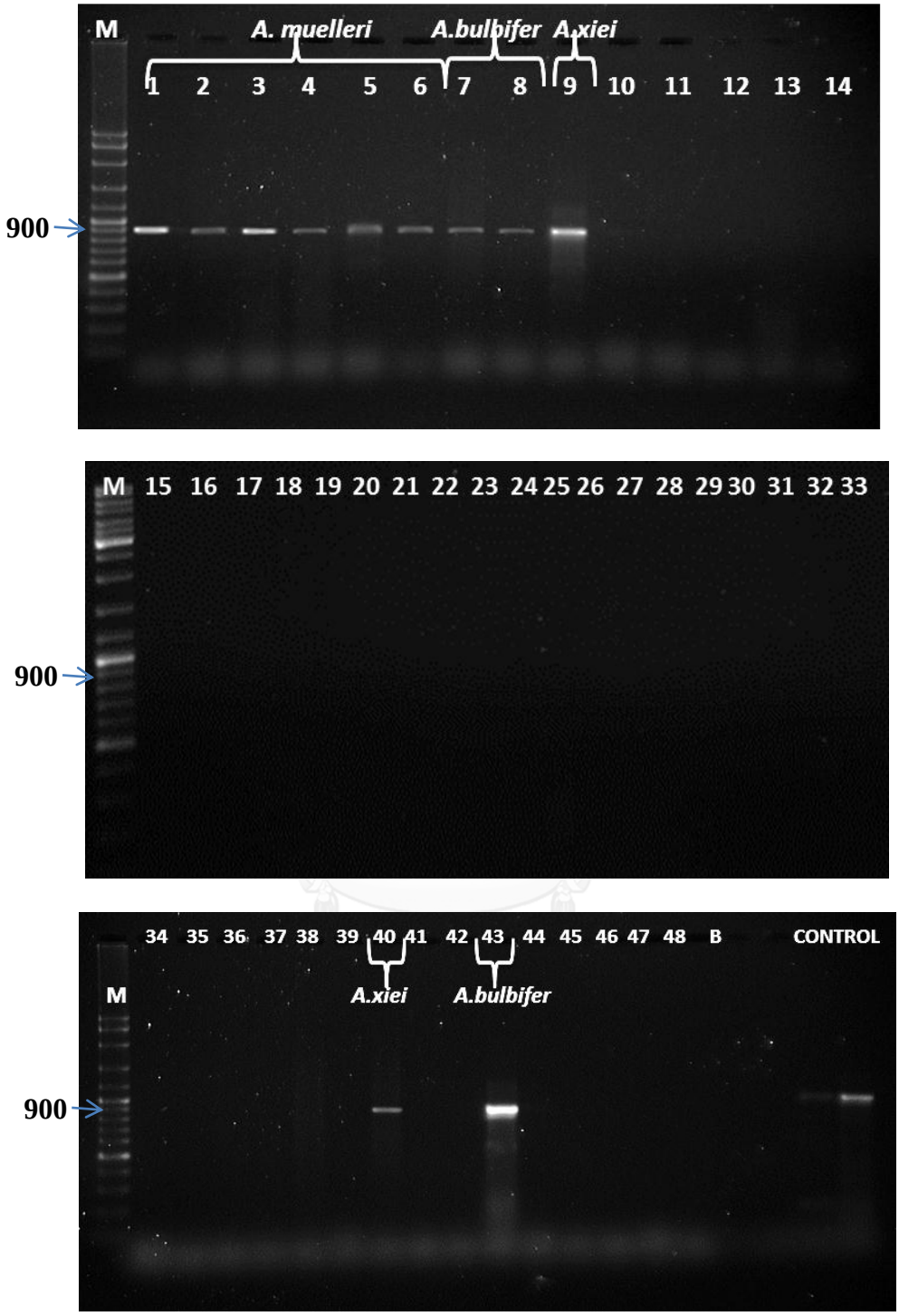

Figure 4.59 Specific DNA band pattern of *A. muelleri*, *A.bulbifer*, *A.xieii* characterized by amplification with P17/MUBX994_ ITS primer set.

Further examination of specific primers against larger specimens (84 *Amorphophallus* plant individuals) includes *A. muelleri* (35 samples), *A. bulbifer* (4 samples), *A. xiei* (4 samples), *A. krausei* (3 samples), *A. kachiensis* (2 samples), *A. corrugatus* (2 samples) and low KGM content species (34 samples). Fifty samples of additional specimens representing high or medium KGM content species. The result showed that all designed primer can produce single, distinct and clearly identified band only in high or medium KGM content species with expected size (Figure 4.60-Figure 4.63). In contrast, those specific primer pairs did not amplify low KGM content species. Throughout all of additional samples, the result did not show any false positives (amplified bands with incorrectly identifying a species). Moreover, the specificity test for those primer pairs allowed amplifying specific fragments from different geographical population. This is indicated that our designed primers are specific to high KGM content trait but not to the geographic region. Therefore, it can be suggested that those primers possessed high effectiveness towards a large number of representatives of high KGM content species. The sampling of multiple species for testing the markers will allow double-checking for identifications and delimitations of studied species (China Plant *et al.*, 2011).

Moreover, additional outgroup control testing were examined aiming to ensure the reliability of the designed primers (Siragusa and Carimi, 2009; Zhao and Wu, 2012) . The target samples of outgroup control that belong to *Araceae* family are *Aglaone mapictum*, *Anthurium sp.*, *Dieffenbachia fournieri*, *Homalomena pendula* and *Xanthosoma sagittifolium* (Figure 4. 64) (Santosa *et al.*, 2007).

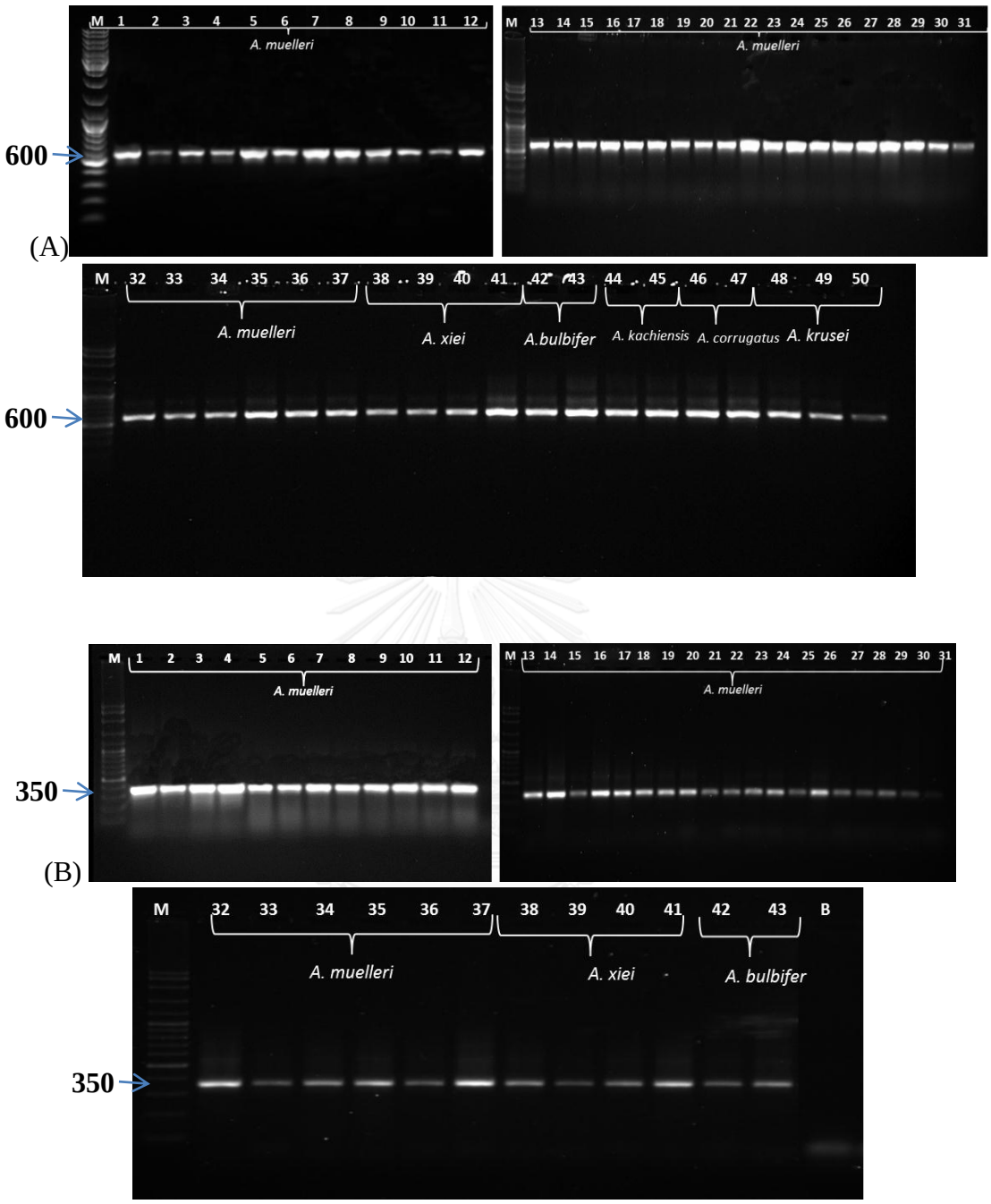

Figure 4.60 Amplification results of two SCAR markers including (A) HKGM-4F/ HKGM-595R and (B) MUE-129F/MUE-490R in additional dataset of high or medium KGM content species. Alphabet "B" refers to negative control.

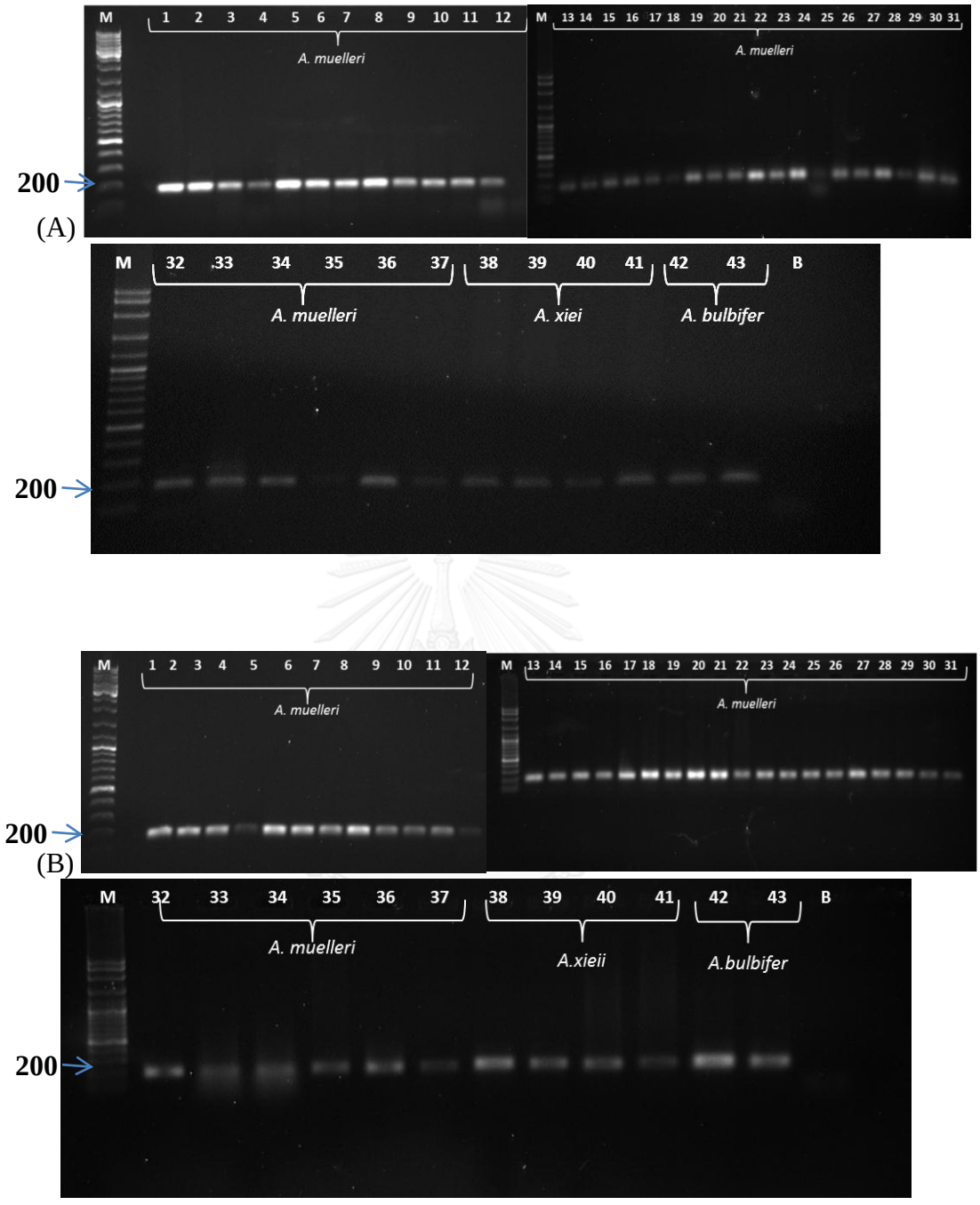

Figure 4.61 Amplification results of (A) *FLint2* F1/ MUBX236_*Flint2* and (B) *FLint2* F1/ MUBX253_*Flint2* in additional dataset of high KGM content species. Alphabet "B" refers to negative control.

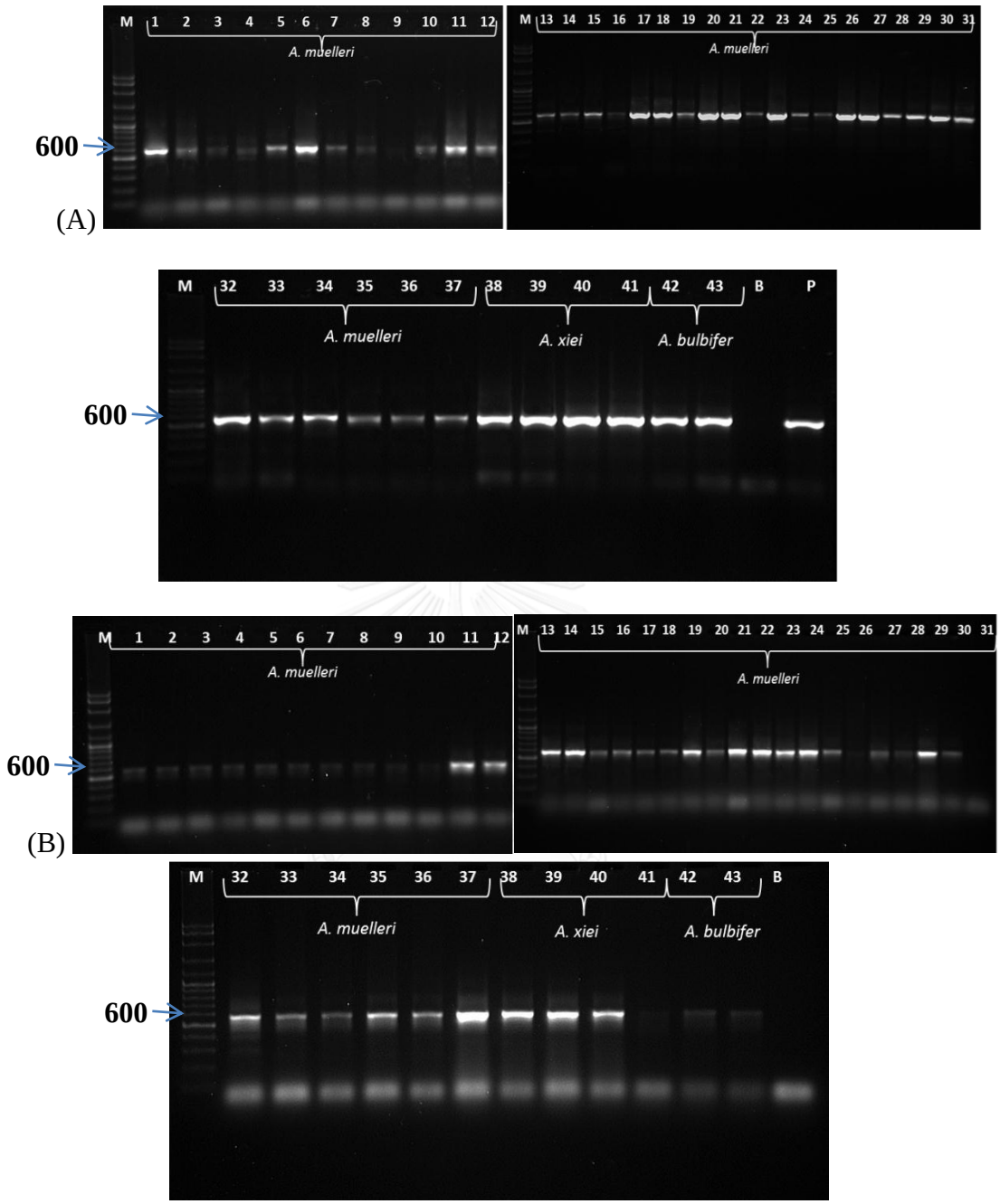

Figure 4.62 Amplification results of (A) MUBX520_ITS/26S-82R and (B) MUBX551_ITS/26S-82R in additional dataset of high KGM content species. Alphabet "P" refers to positive control. Alphabet "B" refers to negative control.

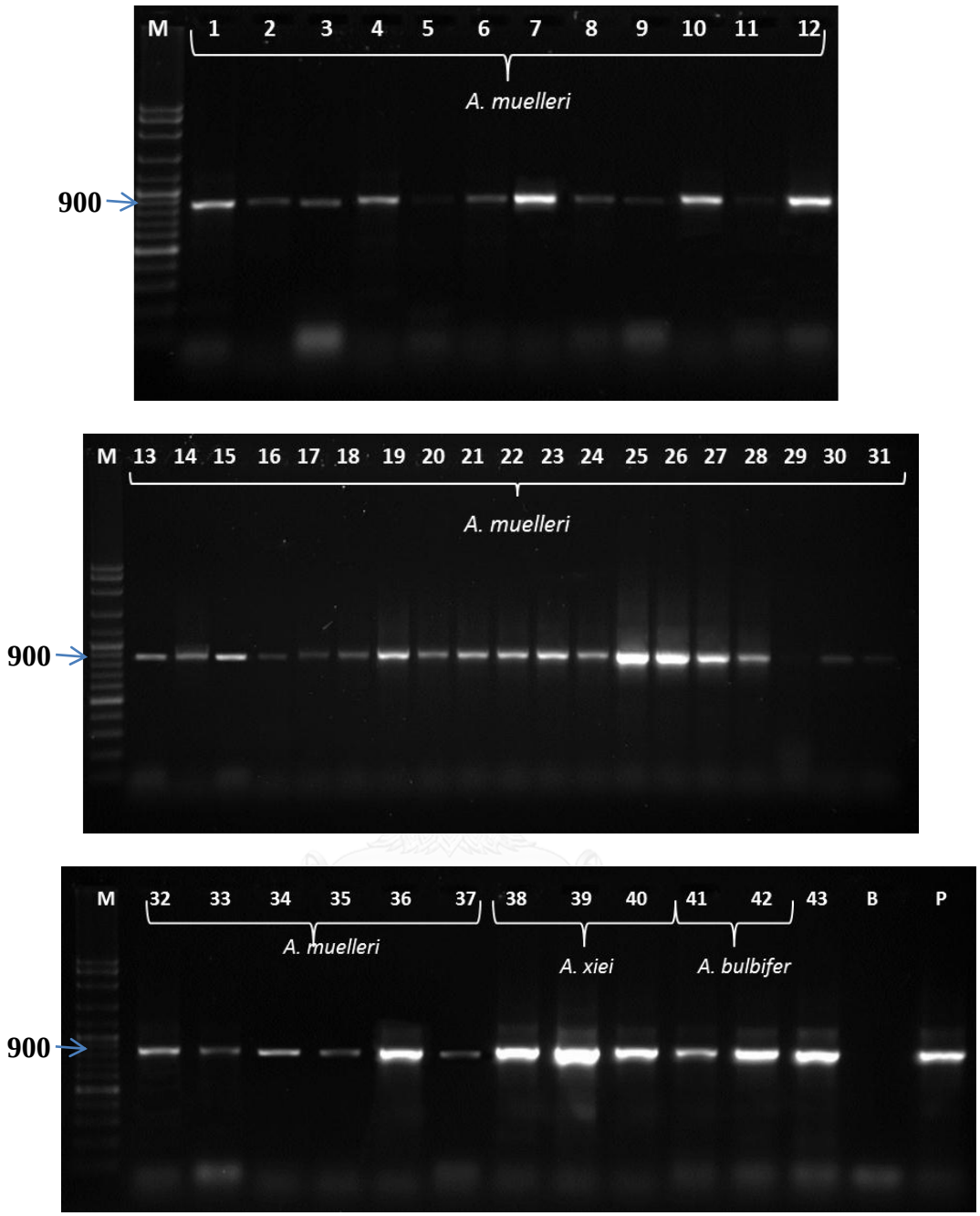

Figure 4.63 Amplification result of P17/MUBX994_ ITS in additional dataset of high KGM content species. Alphabet "B" refers to negative control.

(A)  (B)  (C) 

(D)  (E) 

Figure 4.64 Characters of (A) *Aglaone mapictum*; (B) *Anthurium sp.*; (C) *Dieffenbachia fournieri*; (D) *Homalomena pendula* and (E) *Xanthosoma sagittifolium*

The result from outgroup specificity testing showed that seven designed primers were highly efficiency since none of the outgroup DNA gave any visible PCR product. From all results of specific testing, it is now clear that high KGM content specific primer sets based on RAPD and DNA sequence polymorphism in this study are reliable amplification in high KGM content of *Amorphophallus* species without any influence from geographical or population difference. The confirmation of these specific primers in different genetic species suggested that these markers can be exploited in a wide range of genetic background and a prerequisite for breeding. Especially, in SCAR marker that was successful to amplify all of high or medium KGM content species with highly specification. SCAR markers contain high-copy number and disperse around genomic sequences within the amplified region. Thus, this technique is valuable in large-scale and locus-specific applications (Kiran *et al.*, 2010). The outcome from this study was corresponded with several studies that developed SCAR marker and they found that it gave highly efficiently output for

discrimination and authentication of specific organism such as plant, animal, insect even in virus (Boltz *et al.*, 2010; Dnyaneshwar *et al.*, 2006; Klinbunga *et al.*, 2010; Navarro *et al.*, 2010).

4.2.5.3 Repeatability testing of specific primers

After screening of the suitable designed primers, 12 plant accession (7 species) of high or medium KGM content species with 2 plant sample accession of low KGM content (*A. amylodidas*, *A. yunnanenis*) were re-DNA extraction with Genomic DNA Mini Kit (Plant) (Geneaid, Taiwan). Moreover, PCR reaction was performed with different PCR thermo blocker (T100 Thermal Cycler, BIO-RAD). Savva *et al.* (2000) reported that the repeatability of the assay should be confirmed by repeating the PCR condition using different genomic template extraction in case of genomic template are varied. The result showed that the repeatability of seven designed primers were stable (Figure 4.64-4.65). These results clearly indicated that major influential factors like variation of DNA caused by difference in extraction or different PCR machine did not affect the ability of designed primer in this study. Therefore, this designed primer can be used widely in different laboratories regardless of external factors.

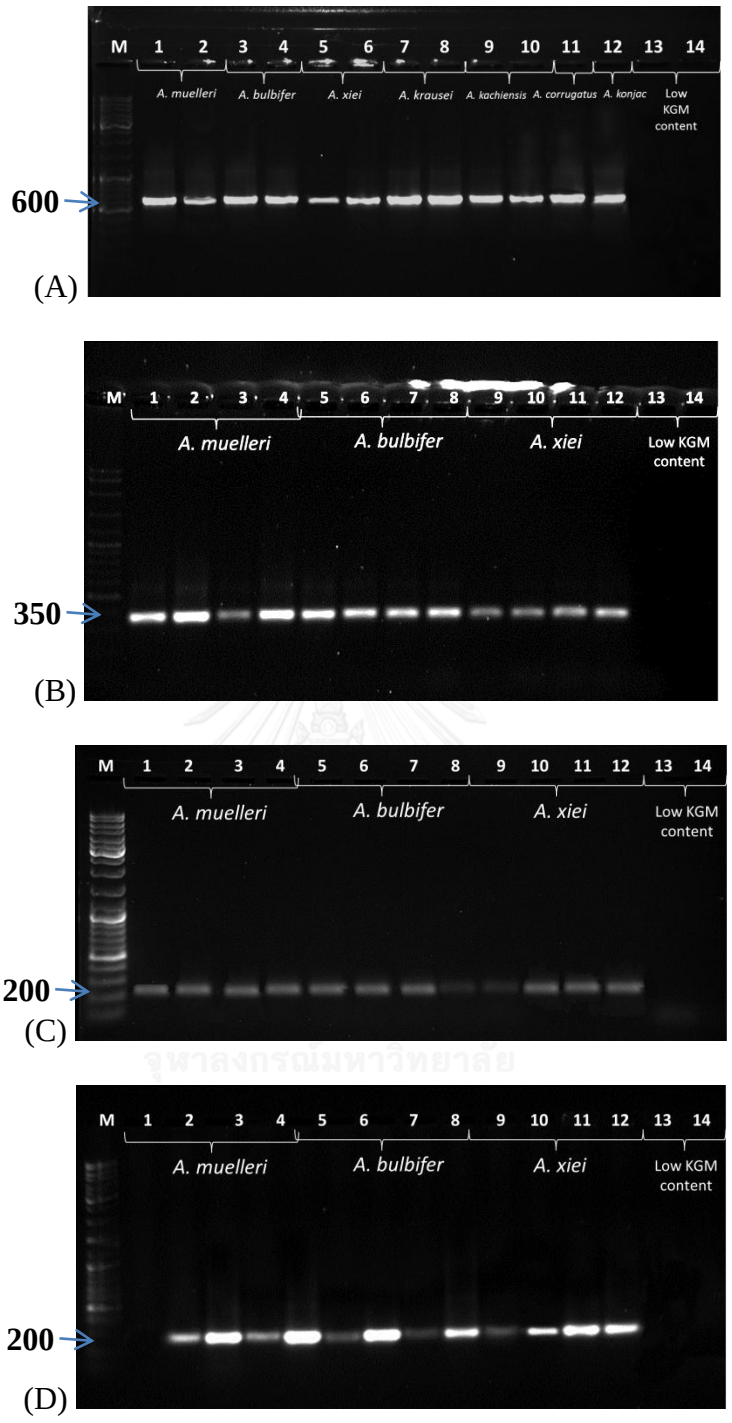

Figure 4.65 Amplification result in reproducibility testing with different genomic DNA extraction kit and PCR machine of (A) HKGM-4F/ HKGM-595R, (B) MUE-129F/MUE-490R, (C) *FLint2* F1/ MUBX236_ *Flint2*, (D) *FLint2* F1/ MUBX253_*Flint2*

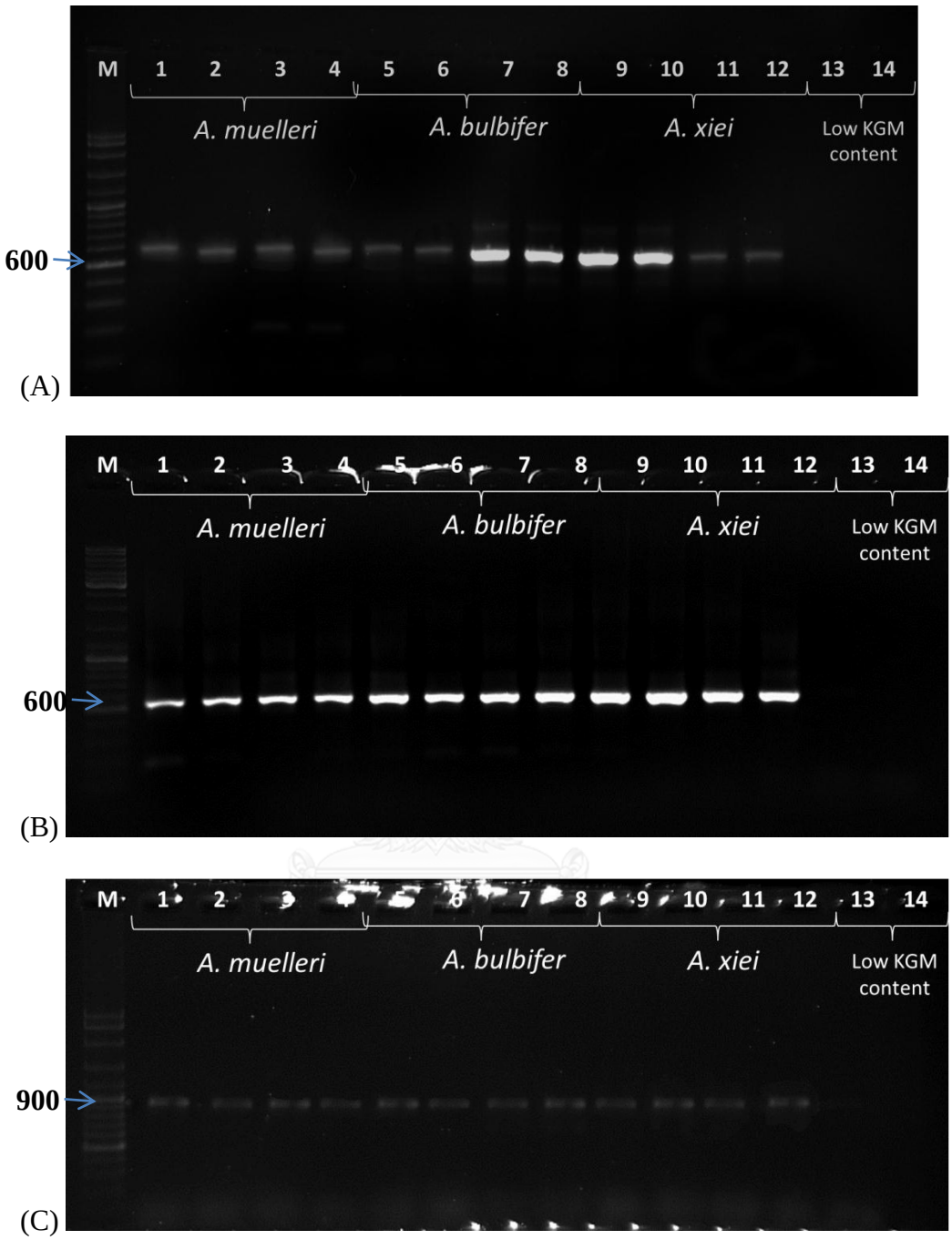

Figure 4.66 Amplification result in reproducibility testing with different genomic DNA extraction kit and PCR machine of (A) MUBX520_ITS/26S-82R, (B) MUBX551_ITS/26S-82R and (C) P17/MUBX994_ ITS

4.2.5.4 Sensitivity testing of specific primers

The DNA concentration is crucial point in the production of reproducible PCR product. Not only to ensure the largest number of amplified bands but also to confirm the fidelity of the PCR condition. Therefore, different template concentrations at least twofold should be detected for efficiency of primer as sensitivity testing (Dnyaneshwar *et al.*, 2006; Zhao and Wu, 2012). Sensitivity of specific primers developed under this study was examined. A serial dilution of DNA template concentrations from *A. muelleri* (No1, MU-KC01) was tested by PCR aiming to determine the minimum quantity of DNA detected. The DNA concentration of *A. muelleri* was adjusted to 30 ng/ml. After that, DNA template concentrations were prepared as 15 (5:5), 3 (1:100) and 0.3 (1:100) ng/ml. The result showed that developed seven specific primer pairs are highly sensitive by producing positive band in every serial dilution concentration (Figure 4.66). Meanwhile PCR band in agarose gel became clearer when increase in DNA concentration except for specific primers from *Flint2* region that can maintain their intensity PCR band in lowest concentration. This analysis revealed that specific primers in this study are robust and highly efficient that even at low DNA concentration. This advantage can be applied to detect the presence of small amount of testing sample. Likewise in many previous studies, Theerakulpisut *et al.* (2008) detected sensitivity of NSS-A/NSS-S specific markers and found that those primers were able to detect the presence of as little as 5 mg of *Cuscuta* species in the mixture of stem powders. Meanwhile Zhao and Wu (2012) found that selected SCAR markers allowed a detection of 1% of the contaminant of *Origanum* in commercial product.

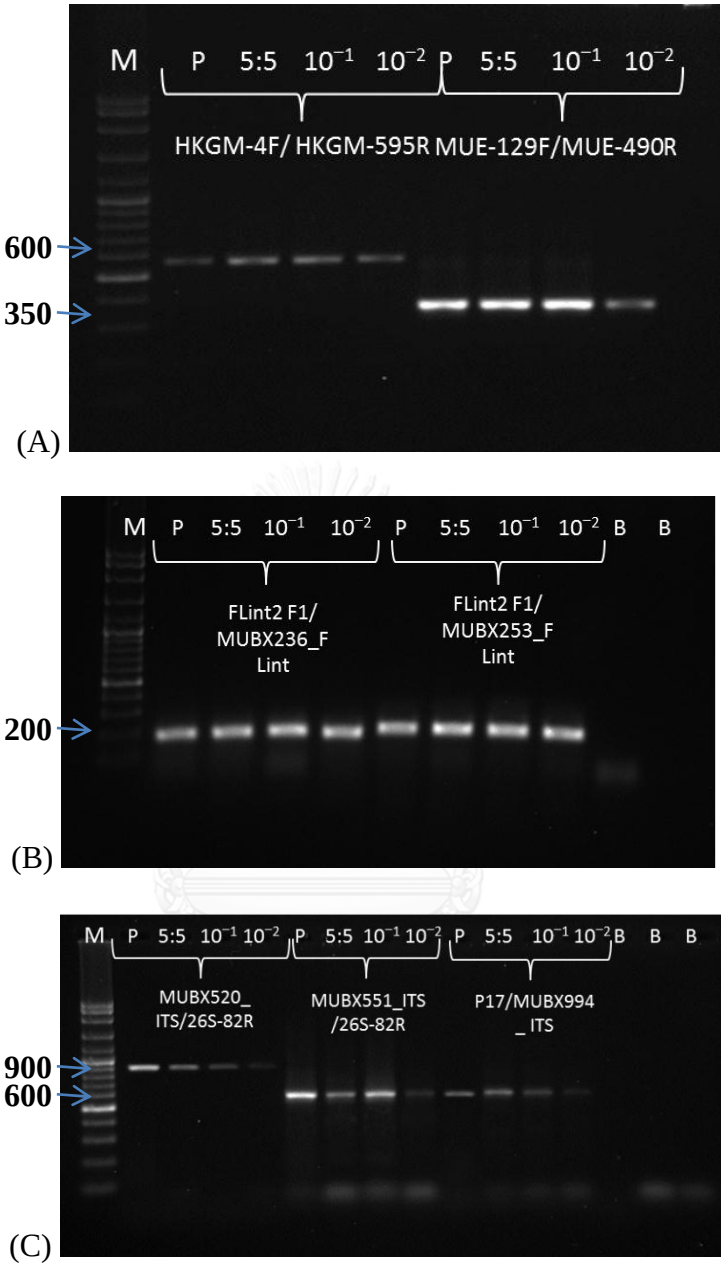

Figure 4.67 Amplification result obtained from seven specific primers (A) HKGM-4F/ HKGM-595R and MUE-129F/MUE-490R; (B) *FLint2* F1/ MUBX236_ *Flint2* and *FLint2* F1/ MUBX253_*Flint2* and (C) P17/MUBX994_ ITS, MUBX520 _ITS /26S-82R and MUBX551_ITS/26S-82R on serial dilution of *A. muelleri*. "P" refers to initial DNA concentration at 30 ng/ml. Blank use as negative control by distilled water.

4.2.5.5 Detection of specific primers in different tissues of *Amorphophallus*'s plant

Since different tissue of plant can contain impurity or other macromolecules that may inhibit PCR reaction (Adinolfi *et al.*, 2007). Therefore, using different tissues of plant to determine stability of specific primer is important. Moreover, this testing determines ability of specific primer to amplify sample in different part of this plant. Sample of three parts of *A. muelleri* (AMU-TK15 and AMU-KC10) were used in this testing, includes tuber bud, petiole and dried berry (Figure 4.67). The result showed that all specific primer produced clear and discriminatory PCR fragment in every plant tissue (Figure 4.68). This finding pointed that specific primers in this study are stable enough for applying with any part of *Amorphophallus* plant and easy to use when there is limited access to parts of plant sample. Especially, in Konjac processing industry that only tuber sample can be reached. Moreover, this advantage of specific primer can overcome tissue specificity problem in morphological characters (Dixon *et al.*, 2007a; Kuras and Korbin, 2004)

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(A)  (B)  (C) 

Figure 4.68 Different part of *A. muelleri* includes (A) tuber bud, (B) petiole and (C) dried berry.

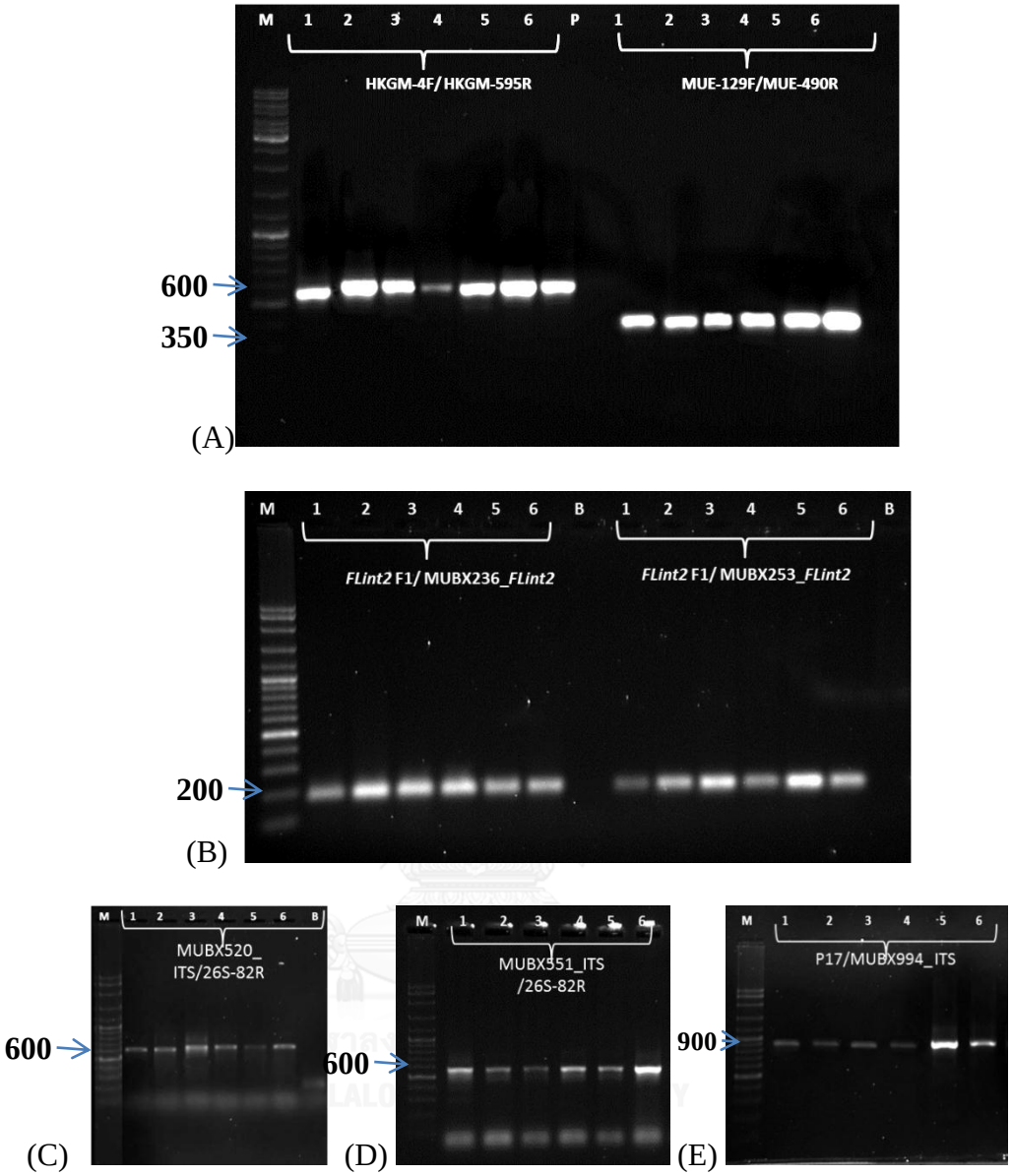

Figure 4.69 Amplification result obtained from seven specific primers (A) HKGM-4F/ HKGM-595R and MUE-129F/MUE-490R; (B) *FLint2* F1/ MUBX236_ *Flint2* and *FLint2* F1/ MUBX253_*Flint2* and (C) P17/MUBX994_ ITS; (D) MUBX520 _ITS /26S-82R and (E) MUBX551_ITS/26S-82R on different plant tissue of *A. muelleri*.

As specific primer identification is a PCR-based procedure that yields a unique band of known size and allows a species to be identified directly after gel electrophoresis (Arif *et al.*, 2010; Chua *et al.*, 2010; Liu *et al.*, 2012). A result from validation testing are clearly indicated that specific primer sets in this study are useful for identifying large numbers of high-medium KGM content species in HKGM-4F/ HKGM-595R primer set and especially for high KGM content species (*A. muelleri*, *A. bulbifer* and *A. xiei*) in MUE-129F/MUE-490R, two sets of *Flint2* marker (*FLint2* F1/ MUBX236_*Flint2*, *FLint2* F1/ MUBX253_*Flint2*) and three sets of ITS marker (MUBX520_ITS/26S-82R, MUBX551_ITS/26S-82R, P17/MUBX994_ ITS) in a single-step PCR. Moreover, highly sensitivity and easy to amplify different parts of plant sample of this specific primers are practical when morphological identification cannot be conducted when lacking of whole specimens and a small amount of DNA is available. It provide an option of using parts of plant to perform DNA extraction and PCR amplification and leaving the main parts of plant for morphological verification (Yang *et al.*, 2013). Therefore, those specific primers can be used for initial screening of high-medium KGM content species or high KGM content species from unknown *Amorphophallus* plant which taken from natural habitat without sequencing requirement. Moreover, this specific primer with single-step PCR assay is rapid and cost-effective method. It is also suitable for raw material quality control process. Our studied results showed the similar finding with several previous studies in develop molecular markers that linked to a trait of interesting genes, for example developed SCAR markers linked to drought tolerant gene in sugarcane genotypes (Srivastava *et al.*, 2012), RAPD-SCAR marker associated with tall-type palm trait in coconut

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(Rajesh *et al.*, 2013), SCoT primer from start codon targeted gene for discriminated edible and toxic accessions of *Jatropha curcas* (Mulpuri *et al.*, 2013). On the other hands, those specific markers can be applied for discrimination or authentication of purity product. Adinolfi *et al.* (2007) had developed specific primer to differentiate *Echinacea purpurea* from *E. angustifolia* and *E. pallida*. These different species are commonly confused and probably used indifferently for the same therapeutic purposes. Marieschi *et al.* (2011) had developed SCAR marker for the identification commercial Mediterranean oregano from adulterant.

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CHAPTER V

CONCLUSIONS

The amount of Konjac glucomannan which was found in *Amorphophallus* tubers (14 species) ranged from 1.53 to 68.9% dry weight and was mainly dependent on the identity of species. The highest KGM content was found in *A. muelleri* (60.16-68.93%) followed by *A. krausei* (55.74-57.76%), *A. kachinensis* (55.36%), *A. bulbifer* (51.83 -52.53%), *A. xiei* (50.60 %) and *A. corrugatus* (35.41%) respectively. Among the remaining species, KGM content was low (<10% of dry weight). *Amorphophallus* spp. in this study can be divided into three categories according to their KGM content including high (40-70%), medium (20-39%) and low (<20%) .

The phylogeny from 48 *Amorphophallus* specimens from Thailand based on nucleotide region sequences of chloroplast *trnL-trnF* spacer, ITS and *Flint2* is in agreement with morphological characteristics. Among DNA regions, the ITS region has highest variation and is phylogenetically informative. Comparison between different alignment programs including MAFFT, BMGE and PRANK produced a constant genetic database in majority clade of *Amorphophallus* relationship. Genetic distance which is based on RAPD data pointed out that genetic relationship within this genus is high and is in congruence with sequence data.

Phylogenetic trees from both molecular analyses (DNA sequencing and RAPD) indicated congruity between the evolutionary history and KGM content. It could be confirmed that KGM content varies significantly between species. DNA sequencing analysis demonstrated that high KGM content species have evolved at least twice in the genus within a unique separate clade of *A. muelleri*, *A. bulbifer* and

A. xiei. RAPD that was performed by the primer AC-10 produced a unique 600-bp band in all high to medium KGM species.

Molecular markers specific to identify *Amorphophallus* species that produce high levels of KGM were developed. Based on the three sequencing regions (*trnL-trnF* spacer, ITS and *Flint2*) employed in this study, twelve specific primers were developed only for the group of *A. muelleri*, *A. bulbifer* and *A. xiei*. In RAPD analysis, a unique 600 bp was used to develop specific SCAR markers, one primer set for high to medium KGM content species and another one for group of *A. muelleri*, *A. bulbifer* and *A. xiei*. In specificity testing, seven specific primers were designed. They show a high efficiency in amplifying specific DNA fragmenst in large sampling groups and can distinguish high from low KGM content *Amorphophallus* species or other member of *Araceae* family. The lowest concentration of DNA template that can be detected is 0.3 ng/mL. This amount indicates a high sensitivity of specific designed primer in this study. Reproducibility and stability testing showed that seven pairs of specific designed primers could maintain their ability to amplify specific band regardless of external factors, such as different DNA extraction methods, different PCR equipment or different parts of plant.

Hence, the new seven highly specific primer pairs were proven to be useful. They constitute a rapid and costeffective method to identify high KGM content species and can be applied for a large-scale screening of *Amorphophallus* species. This can be applied for identification of the species that produce the highest KGM level found in Thailand and has a high potential to identify the most appropriate species for commercial KGM flour production. The use of specific DNA markers to determine the KGM content in these species is an important contribution towards the

further development of industrial-scale production of KGM in Thailand.

Recommendations for further research

From all of the result and summary of this study, specific markers for detecting high KGM content species could be successfully developed. This opens the way to expand the application of these specific markers by combining them with multiplex PCR. Additionally, even more specific markers for high to medium KGM content should be developed for custom use and to increase the reliability of the results.

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APPENDIX

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APPENDIX A

KGM analysis procedures

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A.1 Determination of KGM content by 3,5-dinitrosalicylic acid (DNS) colorimetry measurement.

The outcome of reaction is D-glucose and D-mannose which are reductive monosaccharide from KGM extracted by DNS acid hydrolysis. The reducing sugar (D-glucose and D-mannose) can be reworded into amino acid compounds with 3,5-dinitrosalicylic (DNS) acid subjected to the alkaline and heating treatment. Somewhat, there is a correlation between the amount of reducing sugar and the color intensity of reactive solution, and the KGM content of the konjac flour sample can be terminated by colorimetric measurement.

A.1.1 Preparation of 3,5-dinitrosalicylic acid (DNS) reagent

Solution A: phenol 6.9 g is dissolved into 10% NaOH solution (15.2 mL), after that that solution is dilutes to 69 mL with Deionized (DI) water and $NaHSO_3$ 6.9 g is added in final step.

Solution B: 225 g of sodium potassium tartrate ($KNaC_2O_6H_4$) is dissolved into 10% NaOH (300 mL), then 1% of 3,5- DNS (880 mL) is added in final step.

Followed by combined the solutions A and B together. This mixture solution is stored in a brown bottle aim to prevent the light at room temperature. This mixture solution should be use after 7-10 days and can be keeping within 2 weeks.

A.1.2 Construction of standard D-glucose and D-mannose calibration curves

For preparation of a 1.0 mg/mL glucose standard solution, 0.1 g of glucose (drying in the 105 ^{O}C to constant weight before use) is dissolved in DI water until reach a constant volume of 100 mL. The D-Glucose stock solution at concentration of

1 mg/mL is then pipetted to 0.40, 0.80, 1.20, 1.60 and 2.00 mL into each 50 mL volumetric flasks and using DI water as a blank solution. After that DI water is added to the volume of 2.00 mL with 1.50 mL of 3,5-DNS solution to each flask. Follow by heating each mixture solution for 5 min in a boiling water bath and cool down to room temperature and be diluted with DI water into 50 mL volumetric flask. Absorbance of solution is measured at 550 nm and then plot of the measurement value of absorbance encounter with the glucose content (mg) that constructed. A D-mannose standard curve was constructed by using the same procedure that described above. The experiment can be done in three replicates time.

A.1.3 Preparation of KGM sample

Konjac flour sample (0.2 g) is added to 85% ethanol (50 mL) and mixed by a magnetically stir in a 50 ^{O}C water bath for 30 minute. To remove soluble sugar, the precipitated KGM is collected by filtration via filter paper (No.1). After that the KGM flour is taking to dry at 60 ^{O}C or place upon a boiling water bath to remove the residual ethanol.

A.1.4 Hydrolysis of KGM sample

Konjac flour (from section A.1.3) is hydrolyzed with 3 M H_2SO_4 (10 mL) in boiling water bath for 90 minute and then allowed to cool down until room temperature. The KGM solution is neutralized with NaOH solution and then adjusts with DI water to volume 50 mL therefore this solution can be called as the KGM hydrolysate. This KGM hydrolysate is centrifuged at 3000 rpm for 10 min aim to get a supernatant solution. The experiment can be done in three replicates.

A.1.5 Colorimetric reaction assay

The KGM hydrolysate supernatant at volume 0.5 mL placed in 50 mL volumetric flask and then add 3,5-DNS reagent (1.50 mL) of to make the colorimetric reaction. The mixture is heated in a boiling water bath for 5 minute and leaves it cool down to room temperature. After that the mixture is diluted to 50 mL in the same volumetric flask with DI water. The reagent blank make by using DI water instead of KGM hydrolysate with the same color reaction. Absorbance is measured at 550 nm immediately and the glucose content is determined from the standard curve. The KGM content is determined follow the evaluation of equation (A1) and (A2),

$$\%\text{glucose} = \frac{\text{glucose content}}{\text{Net weight of sample}} \times 100 \qquad \text{(A1)}$$

$$\%\text{KGM} = \%\text{glucose} \times \varepsilon \qquad \text{(A2)}$$

Where:

ε = Molecular weight ratio of mannose and glucose in the glucomannan residues with in KGM hydrolysate (The molecular weight of mannose or glucose is 180, the molecular weight of residue is 162, giving 162/180 = 0.9).

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Table A- 1 The absorbance of standard glucose solution at different concentrations.

Glucose concentration (mg/mL)	Absorbance at wavelength 550 nm			
	Replication			Average
	1	2	3	
0.0000	0.000	0.000	0.000	0.000
0.0083	0.080	0.084	0.082	0.082
0.0166	0.177	0.179	0.182	0.179
0.0249	0.267	0.272	0.265	0.268
0.0332	0.350	0.349	0.350	0.350
0.0415	0.430	0.415	0.423	0.423

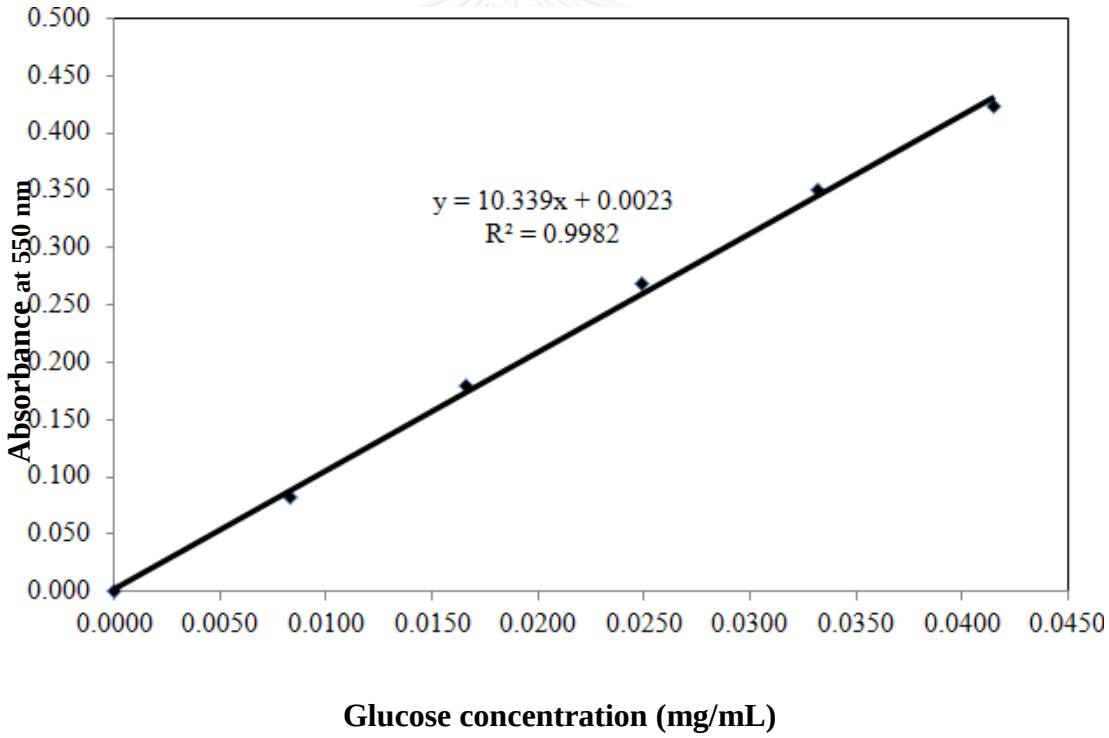

Figure A- 1 Standard curve of glucose for glucomannan analysis.

Table A- 2 The absorbance of standard mannose solution at different concentration.

Mannose concentration (mg/mL)	Absorbance at wavelength 550 nm			
	Replication			Average
	1	2	3	
0.000	0.000	0.000	0.000	0.000
0.008	0.093	0.092	0.095	0.093
0.016	0.195	0.188	0.193	0.192
0.024	0.287	0.282	0.285	0.285
0.033	0.382	0.366	0.375	0.374
0.041	0.458	0.462	0.465	0.462

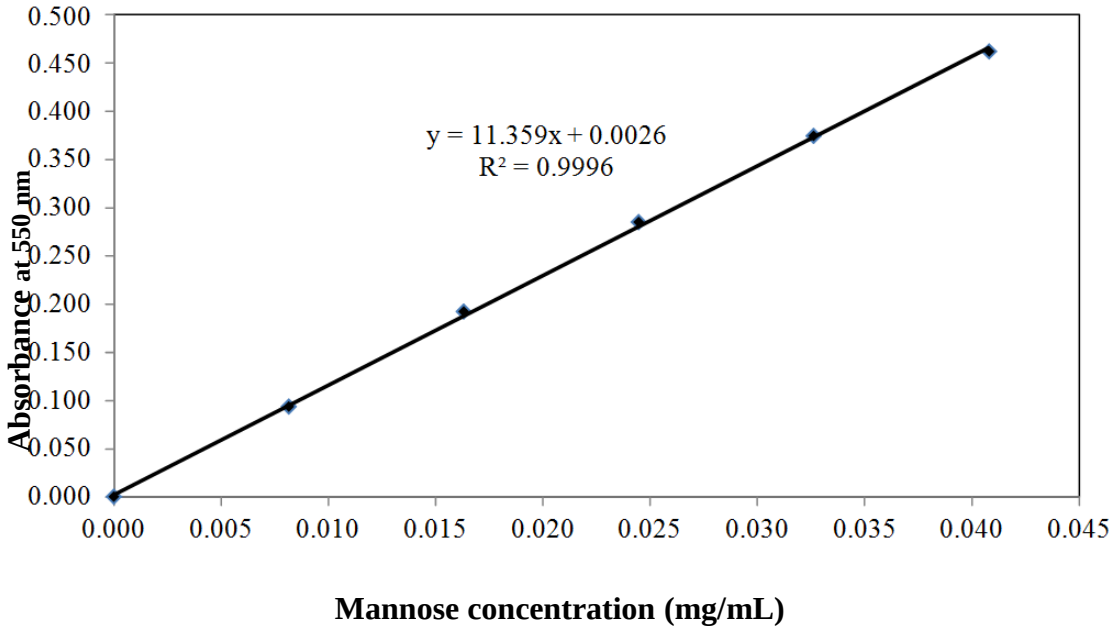

Figure A- 2 Standard curve of mannose for glucomannan analysis.

Table A- 3 Calculating of KGM content by 3,5- DNS colorimeter measurement**.**

sample	**Weight of sample (mg)**	**Net weight of sample (mg/mL)**	**Absorbance at 550 nm**	**glucose (mg)**	**%glucose**	**% KGM**
KGM flour	200	0.04028	0.399	0.038369	95.26	85.73

From calibration curve:

$$y = 10.339x + 0.0023$$
$$x = y - 0.0023/ 10.339$$

y = absorbance at 550 nm (0.399); x= glucose content (mg)

$$x = (0.399-0.0023)/10.339$$

$$= 0.038369$$

From $$\%glucose = \frac{\text{glucose content}}{\text{Net weight of sample}} \times 100$$

$$\%glucose = 0.038369/0.04028 \times 100$$

$$= 95.26$$

$$\% KGM = \% glucose \times \varepsilon$$

$$= 95.26 \times 0.9$$

$$= 85.7$$

In the equation:

ε = Molecular weight ratio of mannose and glucose in the glucomannan residues with in KGM hydrolysate. (The molecular weight of mannose or glucose is 180, the molecular weight of residue is 162, giving 162/180 = 0.9)

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APPENDIX B

RAPD Scoring DNA band

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Table B- 1 DNA fragments result was generated from AB-04 primer with 48 accession of *Amorphophallus* spp. The RAPD bands were scored as 0 or 1 for the absence or presence of bands.

Number	Band	Amorphophallus accession																																													
of band	Size	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
1	1500	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	1400	0	1	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0
3	1300	0	0	0	0	0	0	0	1	1	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0
4	1200	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	1	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
5	1100	0	1	1	1	0	1	1	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	1	0	0	0	1	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
6	1031	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	1	1	1	0	0	0	0	0	1	1	1	0	0	0	0	0	1	1	0	0	0	0	0	1	1	0	1	1
7	950	0	1	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0
8	900	0	0	1	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	1	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
9	850	0	1	1	1	0	1	0	1	1	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
10	800	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	1	1	0	1	0	1	0	0	1	0	0	1	1	0	0	0	0	0	1
11	750	0	0	1	0	0	1	0	0	1	0	0	0	1	1	0	1	1	0	0	0	0	1	0	1	0	0	1	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	1	0	1	0
12	700	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	1	0	1	0	1	1	0	0	0	0	0	1	1	1	0	1	0	0	1	1	1	0	0	1	0	0	1	0	0	1	1
13	650	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	1	0	0	0	1	0	0	1	0	0	1	1
14	600	0	1	1	1	0	1	1	0	1	1	1	1	1	0	0	1	1	0	1	1	0	1	0	0	1	0	0	1	0	1	1	1	1	0	0	1	1	1	1	0	0	0	0	1	1	1	0	0
15	550	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	1	1	1	1	1	0	1	1	1	0	0	1	0	0	1	0	0	1	1	0	1	1	0	1	0	0	1	1
16	500	0	0	0	0	0	1	0	1	0	0	1	0	1	0	1	1	1	0	1	1	0	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	1	0	0	0	0	1	1
17	450	0	0	0	1	0	1	0	0	0	1	0	1	0	1	0	0	0	1	1	0	0	0	0	1	1	0	0	1	1	0	1	0	1	1	1	0	0	0	1	0	0	1	0	1	0	0	0	1
18	400	0	0	0	1	0	0	0	1	0	1	1	0	1	0	1	0	1	1	1	1	1	0	0	0	0	0	1	1	1	0	0	0	1	1	0	1	1	1	0	0	1	0	0	1	0	0	0	0
19	350	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	1	0	0	0	0	1	1	1	1	0	0	0	1	1	1	0	1	1	0	1	1	1	1	0	1	0	0	1	0	0	0	1
20	300	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	1	0	1	1	1	0	1	0	1	1	1	1	0	1	1	0	0	1	1	1	1	0	0	1	0	1	0	0	0	1
21	250	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	1	1	0	1	1	0	1	0	0	0	1	0	1	1	1	0	0	0	0	0	1	0	0	0	1
22	200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0
23	150	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0

Table B- 2 DNA fragments result was generated from AB-20 primer with 48 accession of *Amorphophallus* spp. The RAPD bands were scored as 0 or 1 for the absence or presence of bands.

Number of	DNA Band	*Amorphophallus* accessions																																														
DNA band	Size	1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
1	8000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	6000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	5000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
4	4000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0
5	3500	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	3000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	2500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0
8	2000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	1	0	0	0	0	1	1	0
9	1500	0	0	1	0	0	0	1	0	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	1	1	1	1	0	1	1	0	0	0
10	1400	0	0	0	0	0	1	0	1	0	0	0	1	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	1	1	1
11	1300	0	0	0	0	0	1	1	1	0	1	0	0	0	0	0	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	1	0	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	0	0
12	1200	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	0	0	1	1	1	1
13	1100	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	0	1	1	1	1	0	1	1	0	1	1	0	0	1	0	1	0	0	1	0	1	0	0	1	1	1	1	1
14	1031	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0	1	0	0	1	1	0	1	0	0	0	0	0	1	1	0	1	0	1	0	1	0	1	1	0	1	0	0	1	0	0
15	950	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	1	0	1	0	1	1	1	0	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1
16	900	1	1	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	1	0
17	850	0	0	1	0	0	1	1	0	1	1	0	0	0	0	0	1	1	0	1	0	1	0	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0
18	800	0	0	1	1	0	0	0	0	0	0	1	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	0	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1
19	750	1	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	0	1	1	0	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0
20	700	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	1	0	1	0	0	0	1	1	1	1	1	0	1	0	0	0	0	0	0	0	1	1	1	1	1	1
21	650	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	1	0
22	600	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	0	1	1	1	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1
23	550	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	1	0	1	0	0	0	0	1	0	1	0	0	0	0	1	1	1	0	0	0	0	1	0
24	500	0	0	0	0	1	0	1	0	1	1	0	0	0	0	0	0	1	1	0	0	1	0	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0
25	450	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1
26	400	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	1	0	0	0	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0
27	350	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	1	0	1	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
28	300	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
29	250	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0

Table B- 3 DNA fragments result was generated from AC-09 primer with 48 accession of *Amorphophallus* spp. The RAPD bands were scored as 0 or 1 for the absence or presence of bands.

Number of	DNA	Amorphophallus accession																																													
DNA band	band size	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
1	1500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0
2	1300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
3	1200	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	1	0	0	0
4	1100	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	1	0	0
5	1031	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	1	0
6	850	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	0	1	1	0	0	0	0	0	0	0
7	800	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1
8	750	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	1	1	1	0	0	0	0	0
9	700	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	0	1	0	0	1	0	0	0	1	0	1	0	0	0	0	1	0	1	0
10	650	0	0	0	1	0	1	0	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	0	0
11	600	1	1	1	0	1	0	1	0	1	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	1	1	0	0	0	0	0	1	0
12	550	0	1	1	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
13	500	1	0	1	1	1	1	0	1	0	1	0	0	0	1	0	0	1	1	1	1	0	1	0	1	1	0	0	0	1	0	0	0	0	1	1	1	1	1	1	1	0	1	1	1	0	0	1	1
14	450	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
15	400	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1	1	1	1	1	0	1	1
16	250	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	1	1	0	1	0
17	200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0

Table B-4 DNA fragments result was generated from AC-10 primer with 48 accession of *Amorphophallus* spp. The RAPD bands were scored as 0 or 1 for the absence or presence of bands.

Number of	DNA	Amorphophallus accession																																													
DNA band	band size	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
1	1500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
2	1400	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	1	1
3	1300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
4	1200	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	1	0	0	1	1	1	1	0	1	1	0	1	1	1	1	1
5	1100	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	1	1	1	1	0	1	0	1	1	1	0	1	1	1	0	0	1	1	0	1	1	0	1	0	0	1	1	0	1
6	1031	0	1	0	0	1	0	0	1	0	0	0	0	0	0	1	0	1	1	1	0	0	0	0	0	1	1	0	0	0	1	1	0	0	1	1	0	1	1	0	1	1	1	0	0	1	1	1	1
7	950	0	0	0	0	0	0	1	0	1	1	1	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
8	900	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	1	0
9	850	0	1	0	0	1	0	0	0	1	1	1	1	0	1	1	0	0	0	0	1	1	0	0	0	0	0	1	0	0	1	1	1	1	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0
10	800	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	1	0	1	0	1	1	0	0	1	0	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0
11	750	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	1	0	1	1	0	0	1	0	1	0	1	1	0	1
12	700	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1	0	1	0	0	0	0	0	1	0	0	1	0	1	1	0	1	1	0	0	0	0	0	0	0	0
13	600	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	1
14	550	0	1	1	1	1	1	1	1	1	1	0	1	1	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0
15	500	1	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16	450	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
17	400	1	0	0	0	0	1	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
18	350	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
19	300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
20	250	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	1	0	0	1	1	1	1	0	1	1	0	1	1	1	1	1

Table B- 5 DNA fragments result was generated from AH-18 primer with 48 accession of *Amorphophallus* spp. The RAPD bands were scored as 0 or 1 for the absence or presence of bands.

Number of	DNA Band	Amorphophallus accessions																																													
DNA band	Size	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
1	2000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
2	1500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0	1	0	0
3	1400	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
4	1300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	1
5	1200	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	1	0	1	0	1	0	0	0	1	0	1	0	1	1	0
6	1100	0	1	1	1	1	1	1	1	1	0	1	1	0	1	0	0	0	1	1	0	0	0	0	1	0	0	1	0	0	1	0	1	0	0	1	0	1	0	0	0	1	0	0	1	1	1	1	0
7	1031	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	1	0	1	1	1	0	0	0	0	0	1	1	1	1	1	0	1	0	1	0	0
8	950	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	1	1	0	0	0	1	1	0	1	0	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0
9	900	0	1	0	0	0	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1
10	850	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	1	1	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
11	800	0	0	0	0	0	0	0	1	0	0	1	1	0	0	1	1	1	0	1	0	0	0	0	0	0	0	0	1	0	1	1	1	1	0	0	0	0	0	0	1	1	1	0	1	1	0	0	1
12	750	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0
13	700	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	1	0	0	1	1	1	0	0	0	1	0	1	1	1	1	0	1	1	0	1	0	1	0	0	0	0	0	0	0
14	650	0	1	0	0	1	0	1	0	0	0	0	0	1	1	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
15	600	1	0	0	0	0	0	0	1	1	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	1	1	1	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1
16	550	0	1	0	0	1	0	1	0	0	0	0	1	1	0	0	1	1	0	0	0	0	0	0	1	1	0	0	0	0	1	1	1	0	0	1	0	1	0	0	0	0	1	0	1	0	0	1	0
17	500	1	0	0	1	0	1	0	0	0	0	0	0	0	1	1	0	1	0	1	0	0	0	1	1	1	0	1	1	1	0	0	0	1	0	0	1	0	1	1	1	1	0	0	0	0	0	0	0
18	450	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1	0	1	1	0	1	1	0	0	0	0	0	0	1	0	1	1	0	0	1	0	0	1	0	0	0	0	1	0	0	0
19	400	0	0	0	0	0	1	1	0	1	1	1	1	1	1	1	0	1	0	1	0	0	1	1	0	0	0	1	1	1	0	1	0	0	0	0	1	1	1	0	1	1	1	0	0	1	1	0	1
20	350	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	1	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
21	300	1	1	1	1	1	0	1	0	1	1	0	0	1	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
22	250	0	0	0	1	0	1	1	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
23	200	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table B- 6 DNA fragments result was generated from OPC-02 primer with 48 accession of *Amorphophallus* spp. The RAPD bands were scored as 0 or 1 for the absence or presence of bands.

Number of	DNA Band	Amorphophallus accessions																																															
DNA band	Size	1	1	2	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
1	2000	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
2	1500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1	0
3	1400	0	0	1	0	0	0	1	1	1	0	0	1	0	0	0	0	0	1	0	1	0	0	1	0	0	1	0	0	0	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	0	1	0	0	1	1
4	1300	0	0	0	0	0	0	0	1	0	1	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	1	0	0	0	1	0	1
5	1200	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	0	1	0	0	1	0	0	1	0	1	1	1	0	1
6	1100	0	0	0	0	0	0	1	0	0	1	1	1	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	0	1	1	1	1	0	1	1	0	0	1	0	0	0
7	1031	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	1	0	1	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
8	950	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0
9	900	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
10	850	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	1	0	0	0	1	1	0	1	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0	1	1	0	0	0	1	0	1
11	800	0	0	0	1	1	1	1	1	1	1	1	0	0	1	0	0	0	0	0	0	1	1	1	0	1	1	0	0	0	0	1	1	0	0	0	1	0	1	1	0	0	1	1	0	0	1	1	0	1	1
12	750	0	1	1	1	0	0	1	0	0	1	0	0	1	1	1	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0
13	700	1	1	0	0	0	0	0	0	1	1	0	0	0	0	0	1	1	0	0	0	1	0	1	1	0	1	0	0	0	1	1	0	1	0	1	1	0	0	1	1	0	0	1	1	0	1	0	0	0	1
14	650	0	0	1	0	0	0	1	1	0	1	0	1	1	0	0	1	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
15	600	1	0	1	0	0	0	0	1	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	1	0	1	1	0	0	1
16	550	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0	1	1	0	0	1	0	0	1	1	0	0	1	1	0	0	0	1	0	0	1	0	0	0	0	1	0	1	1	0
17	500	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	1	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1
18	450	0	1	0	0	0	1	1	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	1	0	0	1	0
19	400	1	1	1	1	1	0	0	1	1	1	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
20	350	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
21	300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
22	250	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table B- 7 DNA fragments result was generated from OPD-04 primer with 48 accession of *Amorphophallus* spp. The RAPD bands were scored as 0 or 1 for the absence or presence of bands.

Number of	DNA Band	Amorphophallus accessions																																												
DNA band	Size	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
1	3500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	3000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	2500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
4	2000	0	0	0	0	1	0	1	1	1	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	1	0	0	0	0
5	1500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	0	0	0	0	0	0	0	0
6	1400	0	0	0	0	1	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0
7	1300	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	1200	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	1	0	0	1	0	0	1	0	0	0	0	1	0	0
9	1100	0	0	0	1	0	0	0	1	0	1	0	0	0	0	1	0	0	1	1	1	1	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	1	1	1	1	1
10	1031	1	1	1	0	1	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	1	1	0	0	0	0	1	0	1	1	1	0	1	1	0	0	1	0	1	0	0
11	950	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0
12	900	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	1	0	0	0	1	0	0	0	0	1	0	1	0	0	0	1	1	0	1
13	850	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	1	0	1	0	1	1	1	1	1	0	0	0	0	1	0	0	0	0
14	800	0	0	1	1	1	1	1	1	0	1	0	0	0	1	1	1	1	0	0	0	0	0	0	1	0	1	0	0	0	1	1	0	1	0	0	1	0	0	0	1	1	0	1	0	0	1	0
15	750	1	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	1	0	0	0	0	0	1	0	1	0	1	1	1	0	0	0	0	1	0	1	1
16	700	0	0	0	1	0	1	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	0	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1
17	650	1	1	1	0	0	0	0	0	0	0	0	1	1	0	1	1	1	0	1	1	0	0	1	0	0	1	1	0	1	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
18	600	0	0	0	0	0	0	0	1	0	1	1	1	0	1	0	1	0	1	1	0	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	0	0	1	0	1	0	1
19	550	0	0	0	0	1	1	1	0	1	0	0	0	0	0	1	0	0	0	1	0	1	1	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0
20	500	1	1	0	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0	1	0	0	0	1	1	1	0	0	1	0	1	0	1	1	1	1	1	1	0	0	0	1	0	0	1	1	0	1
21	450	1	1	1	1	1	1	1	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0
22	400	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	1	1	1	1	0	1	0	0	0	0	0	0	0	1
23	350	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	1	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
24	300	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	1	1	0	0	0	1	1	1	1	1	1	0	1	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0
25	250	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table B- 8 DNA fragments result was generated from OPC-07 primer with 48 accession of *Amorphophallus* spp. The RAPD bands were scored as 0 or 1 for the absence or presence of bands.

Number of	DNA Band	Amorphophallus accessions																																												
DNA band	Size	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
1	2000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
2	1500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0
3	1400	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0
4	1300	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	1	1	0	0	0	0	0	1	0	0	0	0
5	1200	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	1	0	1	0	0	1	0	0	1	0
6	1100	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	0	0	0	0	1	1	1	0	1	0	1	1	1	1	1	1	0	1	0	0	0	1	1	0	1	1	0	1	1	1	1	1
7	1031	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	1
8	950	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	1	0	0	0	0	0	1	0
9	900	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	1	0	1	0	0	1	0	1	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1
10	850	0	1	0	0	0	1	0	0	0	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0
11	800	0	1	0	0	0	0	1	1	1	0	1	1	1	0	0	1	1	0	0	1	0	0	1	1	1	1	1	1	1	1	1	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0
12	750	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	1	1	0	1	0	1	1	0	0	1
13	700	1	1	1	0	0	1	1	1	1	0	0	0	0	1	1	1	0	0	0	1	1	0	0	0	0	1	1	1	0	1	0	1	1	0	0	1	1	0	0	1	1	0	1	0	0	1	0
14	650	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0
15	600	0	0	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	0	0	0	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1	0
16	550	1	1	0	0	1	0	1	0	0	1	0	0	0	1	1	1	0	0	1	0	0	1	0	0	0	0	0	0	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	0	0	1
17	500	1	1	1	1	0	1	0	1	1	1	1	1	0	0	0	0	0	1	1	1	1	1	1	0	1	1	1	1	1	0	0	1	1	0	1	1	1	0	1	0	1	1	1	0	0	1	1
18	450	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	0	1	0	1	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	1
19	400	1	1	1	1	0	1	0	1	1	1	1	1	0	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0	0	0	1	0	1	0	0	1	0
20	350	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	1	1	0	0	1
21	300	1	1	1	1	0	1	0	1	1	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	0	1	1	1	0	0	1	0	0	0
22	250	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0

Table B- 9 DNA fragments result was generated from OPB-17 primer with 48 accession of *Amorphophallus* spp. The RAPD bands were scored as 0 or 1 for the absence or presence of bands.

Number of	DNA Band	Amorphophallus accessions																																													
DNA band	Size	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
1	2000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
2	1500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	1400	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	1300	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1	1	0	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	1200	0	0	0	0	0	0	0	1	1	1	1	0	0	0	1	0	0	1	1	0	1	1	1	0	0	0	1	1	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0
6	1100	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	1	0	0	0	1	0	0	1	1	0	1	1	0	0	0	0	0	0	0	1	0	0	1	0	1	1	0	1	0	0	1	1
7	1031	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0
8	950	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	900	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
10	850	1	0	0	0	1	0	1	1	1	0	1	1	0	0	0	0	0	0	0	0	1	0	0	1	1	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1
11	800	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	1	0	0	0	1	0	0	1	0	0	0	0	1	0	0	1	0	1	1	0	0	1	1	0	0	0	0	1	0	0	11	0	0
12	750	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	1	1	1	0	0	0	0	0	0	1	0	1	0	1
13	700	1	0	0	0	1	1	1	1	1	1	1	1	0	0	1	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	1	0	0	0	1	1	0	1	0	1	1	1	0	1	0	0	1	1
14	650	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	1	0	1	0	1	0	0	0	0	0
15	600	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	1	1	0	0	1	1	1	0	0	1	0	0	1	1	1	1	1
16	550	1	0	0	0	0	0	1	0	1	1	1	1	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	1	1	0	1	0	1	1	1
17	500	0	0	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0	0	1	1	1	0	0	1	1	1	1	1	0	1	1	0	1
18	450	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1	1	0	1	1	0	0	1	0	1	1	1
19	400	1	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0	1	1	0	0	1	0	0	1	1	0	1	1
20	350	0	1	1	1	1	1	1	1	0	0	0	1	0	1	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1	0	1	1	0	0	0	0	1	1
21	300	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0	1	0	1	1	1	1	0	0	1
22	250	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0	0	1	0	1	0	1
23	200	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	1	0	0	1	0	0
24	150	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
25	100	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0

Table B- 10 DNA fragments result was generated from ERIC1R primer with 48 accession of *Amorphophallus* spp. The RAPD bands were scored as 0 or 1 for the absence or presence of bands.

Number of	DNA Band	Amorphophallus accessions																																													
DNA band	Size	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
1	3000	0	0	1	0	0	0	0	0	0	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	2000	0	0	0	0	0	0	1	1	0	1	0	0	0	1	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1
3	1500	0	0	1	1	1	1	0	0	1	0	1	1	1	0	1	0	1	1	1	0	1	1	1	0	1	0	0	1	1	1	1	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0
4	1400	0	0	0	1	1	1	1	0	1	1	1	1	1	0	1	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1	0	0	0	0	1	0	1	0	0	1	1	1	0	0	0	0
5	1300	0	0	1	0	0	0	1	0	0	1	1	0	1	0	0	1	0	1	1	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1
6	1200	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	0	0	1	1	0	0	1	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	1	1	1
7	1000	0	0	0	1	0	0	1	0	0	0	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	1	1	0	1	0	0	1	0	0	0	0	0	0	0
8	900	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	800	0	0	0	1	0	0	1	0	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	0	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
10	700	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	1	1	0	1	0	0	1		1	1	0	1	0	1	1	0	1	1	1	0	0	0	0	0	0	0	0
11	600	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	0	0	0	1	0	0
12	500	0	0	0	1	0	1	0	0	0	1	1	1	1	0	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	0	0	0	0	0
13	400	0	0	0	1	0	1	1	0	1	0	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	1	1	1	1	0	1
14	300	0	0	0	1	0	1	0	0	0	0	1		1	0	0	1	1	0	0	0	1	1	1	0	1	1	1	0	0	1	1	1	0	0	1	1	0	1	1	0	0	1	1	0	0	1	0	0
15	200	0	0	0	1	0	1	1	0	1	0	1	1	1	0	1	1	1	1	1	0	1	1	1	0	1	0	0	1	1	1	1	0	0	0	1	1	1	0	1	1	0	1	1	0	0	1	0	1

Table B- 11 DNA fragments result was generated from ERIC2 primer with 48 accession of *Amorphophallus* spp. The RAPD bands were scored as 0 or 1 for the absence or presence of bands.

Number of DNA band	DNA Band Size	Amorphophallus accessions																																													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
1	1400	1	1	1	1	1	1		1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0	0	0
2	1300	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	0	0	0	0
3	1200	0	1	1	1	1	0	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0
4	1100	0	0	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	0	0	0	0	0
5	1031	0	0	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
6	950	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
7	900	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	1	0	0	1	1	1	0	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	1
8	850	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	1
9	800	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
10	750	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1
11	700	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1	0	1	0	0	0	0	0	1	0	0	0	1	1	1	0	0	0	0	1	0	0	0	0	0	0	0
12	600	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	1
13	500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	0	0	0	1	1	1	1	1	1	1	1	0	1	0	0	1	1
14	400	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	1	0	1	1	1	0	0	0	1	0	0	0	1	1	1	0	0	0	0	0	1	0
15	350	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
16	300	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	0	0	0	1	0	0
17	250	0	0	0	1	0	1	0	0	0	1	1	1	1	0	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	0	0	0	0	0

Table B- 12 DNA fragments result was generated from BOXA1R primer with 48 accession of *Amorphophallus* spp. The RAPD bands were scored as 0 or 1 for the absence or presence of bands.

Number of DNA band	DNA Band Size	Amorphophallus accessions																																													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
1	1400	0	0	0	1	1	1	0		1	1	1	1	0	0	1	0	1	1	0	0	0	0	0	0	0	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	1300	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	1	0	1	1	0	0	1	1	1	0	0	0	0	0	0	1	1	1	0	0	1	0	0	0	0	0	0
3	1200	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0
4	1100	0	1	0	1	1	1	0	0	1	0	1	1	0	1	1	1	1	1	0	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0
5	1031	0	1	0	1	1	1	0	0	1	0	1	1	0	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0	0	0	0	0	0	0	1	1
6	950	0	1	0	1	1	1	1	0	1	1	1	1	0	0	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0
7	900	0	1	0	1		1	0	0	1	0	1	1	1	0	1	1	0	0	0	1	1	1	0	1	1	1	1	0	0	0	0	1	0	1	1	0	0	1	0	0	1	0	0	0	0	0	0	0
8	850	0	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	0	0	1	0	0	1	0	1	1	0	1	1	1	1	1	0	1
9	800	0	1	0	0	0	0	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	1	1	1	0	0	1	1	1	1	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0
10	750	1	1	1	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	0	1	1	1	0	0	0	0	1	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0
11	700	0	1	0	1	0	1	0	0	0	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1	1	0	0	1	1	1	1	0	0	0	0	0	1	0	1	0	0	1	1	1	1	1	0	1
12	650	0	1	0	1	0	1	0	0	0	0	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	1	1	1	0	0	1	1	1	1	1	0	1
13	600	0	0	0	1	0	1	1	0	1	0	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	1	1	1	1	0	1
14	550	0	0	0	1	0	1	0	0	0	0	1		1	0	0	1	1	0	0	0	1	1	1	0	1	1	1	0	0	1	1	1	0	0	1	1	0	1	1	0	0	1	1	0	0	1	0	0
15	500	0	0	0	1	0	1	1	0	1	0	1	1	1	0	1	1	1	1	1	0	1	1	1	0	1	0	0	1	1	1	1	0	0	0	1	1	1	0	1	1	0	1	1	0	0	1	0	1
16	450	0	0	1	0	1	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	0	0	0	1	0	0	0
17	400	0	0	1	0	1	0	0	0	1	1	1	1	0	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0
18	350	0	0	1	0	1	1	0	1	0	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1
19	300	0	0	1	0	1	0	0	0	0	1		1	0	0	1	1	0	0	0	1	1	1	0	1	1	1	0	0	1	1	1	0	0	1	1	0	1	1	0	0	1	1	0	0	1	0	0	1
20	250	0	0	1	0	1	1	0	1	0	1	1	1	0	1	1	1	1	1	0	1	1	1	0	1	0	0	1	1	1	1	0	0	0	1	1	1	0	1	1	0	1	1	0	0	1	0	1	1

Table B- 13 DNA fragments result was generated from RPO1 primer with 48 accession of *Amorphophallus* spp. The RAPD bands were scored as 0 or 1 for the absence or presence of bands.

Number of DNA band	DNA Band Size	Amorphophallus accessions																																													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
1	1400	0	0	0	0	0	0	0	0	1	0	1	1	1	0	1	0	0	1	1	0	1	1	0	0	0	1	1	1	1	1	1	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0
2	1300	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
3	1200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	1100	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
5	1031	0	0	0	1	1	1	1	1	0	0	1	1	1	0	0	1	0	0	0	0	1	1	0	1	0	1	1	0	0	0	0	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0
6	950	0	0	1	0	0	0	0	0	0	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	900	0	0	0	0	0	0	1	1	0	1	0	0	0	1	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1
8	850	0	0	1	1	1	1	0	0	1	0	1	1	1	0	1	0	1	1	1	0	1	1	1	0	1	0	0	1	1	1	1	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0
9	800	0	0	0	1	1	1	1	0	1	1	1	1	1	0	1	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1	0	0	0	0	1	0	1	0	0	1	1	1	0	0	0	0
10	750	0	0	1	0	0	0	1	0	0	1	1	0	1	0	0	1	0	1	1	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1
11	700	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	0	0	1	1	0	0	1	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	1	1	1
12	650	0	0	0	1	0	0	1	0	0	0	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	1	1	0	1	0	0	1	0	0	0	0	0	0	0
13	600	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	550	0	0	0	1	0	0	1	0	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	0	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
15	500	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	1	1	0	1	0	0	1		1	1	0	1	0	1	1	0	1	1	1	0	0	0	0	0	0	0	0
16	450	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	0	0	0	1	0	0
17	400	0	0	0	1	0	1	0	0	0	1	1	1	1	0	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	0	0	0	0	0
18	350	0	0	0	1	0	1	1	0	1	0	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	1	1	1	1	0	1
19	300	0	0	0	1	0	1	0	0	0	0	1		1	0	0	1	1	0	0	0	1	1	1	0	1	1	1	0	0	1	1	1	0	0	1	1	0	1	1	0	0	1	1	0	0	1	0	0
20	250	0	0	0	1	0	1	1	0	1	0	1	1	1	0	1	1	1	1	1	0	1	1	1	0	1	0	0	1	1	1	1	0	0	0	1	1	1	0	1	1	0	1	1	0	0	1	0	1

Table B- 14 Genetic distance among 48 acession of *Amorphophallus* spp. from all set of primers.

Sample	S1	s2	s3	s4	s5	s6	s7	s8	s9	s10	s11	s12	s13	s14	s15	s16
s1	0	0.25127	0.075	0.51515	0.75	0.65714	0.53846	0.65	0.55556	0.67857	0.625	0.625	0.57447	0.61538	0.73333	0.57692
s2	0.2513	0	0.46154	0.45946	0.72727	0.64103	0.53333	0.59091	0.45	0.56667	0.53846	0.5	0.4902	0.53571	0.63265	0.46429
s3	0.075	0.46154	0	0.28302	0.2	0.38182	0.6087	0.26667	0.46429	0.21053	0.14706	0.26471	0.19403	0.25	0.35385	0.25
s4	0.5152	0.45946	0.28302	0	0.37778	0.55	0.41935	0.28889	0.36585	0.40984	0.32075	0.50943	0.34615	0.47368	0.4	0.40351
s5	0.75	0.72727	0.2	0.37778	0	0.53191	0.63158	0.34615	0.54167	0.29412	0.3	0.43333	0.32203	0.4375	0.36842	0.34375
s6	0.6571	0.64103	0.38182	0.55	0.53191	0	0.57576	0.40426	0.44186	0.46032	0.41818	0.41818	0.48148	0.38983	0.5	0.49153
s7	0.5385	0.53333	0.6087	0.41935	0.63158	0.57576	0	0.52632	0.35294	0.62963	0.56522	0.6087	0.55556	0.56	0.5814	0.64
s8	0.65	0.59091	0.26667	0.28889	0.34615	0.40426	0.52632	0	0.33333	0.23529	0.16667	0.33333	0.11864	0.375	0.19298	0.28125
s9	0.5556	0.45	0.46429	0.36585	0.54167	0.44186	0.35294	0.33333	0	0.40625	0.35714	0.39286	0.30909	0.43333	0.39623	0.33333
s10	0.6786	0.56667	0.21053	0.40984	0.29412	0.46032	0.62963	0.23529	0.40625	0	0.10526	0.13158	0.14667	0.2	0.26027	0.15
s11	0.625	0.53846	0.14706	0.32075	0.3	0.41818	0.56522	0.16667	0.35714	0.10526	0	0.14706	0.07463	0.25	0.29231	0.16667
s12	0.625	0.5	0.26471	0.50943	0.43333	0.41818	0.6087	0.33333	0.39286	0.13158	0.14706	0	0.22388	0.25	0.38462	0.19444
s13	0.5745	0.4902	0.19403	0.34615	0.32203	0.48148	0.55556	0.11864	0.30909	0.14667	0.07463	0.22388	0	0.26761	0.25	0.15493
s14	0.6154	0.53571	0.25	0.47368	0.4375	0.38983	0.56	0.375	0.43333	0.2	0.25	0.25	0.26761	0	0.3913	0.26316
s15	0.7333	0.63265	0.35385	0.4	0.36842	0.5	0.5814	0.19298	0.39623	0.26027	0.29231	0.38462	0.25	0.3913	0	0.30435
s16	0.5769	0.46429	0.25	0.40351	0.34375	0.49153	0.64	0.28125	0.33333	0.15	0.16667	0.19444	0.15493	0.26316	0.30435	0
s17	0.4872	0.34884	0.45763	0.59091	0.52941	0.47826	0.56757	0.4902	0.40426	0.46269	0.49153	0.45763	0.44828	0.52381	0.53571	0.39683
s18	0.5455	0.5	0.40625	0.46939	0.5	0.60784	0.47619	0.35714	0.38462	0.30556	0.28125	0.34375	0.2381	0.29412	0.34426	0.29412
s19	0.7826	0.68	0.30303	0.45098	0.27586	0.54717	0.63636	0.34483	0.51852	0.24324	0.27273	0.33333	0.26154	0.4	0.30159	0.37143
s20	0.6333	0.5625	0.275	0.47692	0.36111	0.43284	0.58621	0.33333	0.44118	0.18182	0.25	0.25	0.24051	0.2381	0.32468	0.21429
s21	0.4894	0.37255	0.31343	0.46154	0.49153	0.37037	0.55556	0.35593	0.30909	0.28	0.31343	0.22388	0.30303	0.29577	0.375	0.23944
s22	0.5472	0.50877	0.28767	0.44828	0.35385	0.53333	0.56863	0.35385	0.40984	0.23457	0.23288	0.28767	0.22222	0.24675	0.31429	0.22078
s23	0.8333	0.69231	0.32353	0.54717	0.33333	0.52727	0.69565	0.36667	0.5	0.26316	0.32353	0.35294	0.31343	0.33333	0.32308	0.30556
s24	0.7895	0.71429	0.34483	0.48837	0.36	0.46667	0.61111	0.4	0.52174	0.39394	0.41379	0.48276	0.4386	0.3871	0.49091	0.48387
s25	0.6	0.55102	0.35385	0.52	0.4386	0.5	0.62791	0.4386	0.58491	0.36986	0.41538	0.41538	0.4375	0.30435	0.54839	0.42029
s26	0.9487	0.81395	0.38983	0.54545	0.37255	0.52174	0.72973	0.33333	0.48936	0.31343	0.35593	0.38983	0.31034	0.46032	0.39286	0.30159
s27	0.8571	0.73913	0.35484	0.48936	0.33333	0.5102	0.65	0.2963	0.44	0.34286	0.32258	0.41935	0.27869	0.42424	0.35593	0.30303
s28	0.8696	0.72	0.36364	0.52941	0.34483	0.50943	0.68182	0.34483	0.44444	0.2973	0.33333	0.39394	0.29231	0.4	0.30159	0.31429
s29	0.8537	0.73333	0.34426	0.52174	0.32075	0.5	0.64103	0.32075	0.46939	0.30435	0.34426	0.37705	0.3	0.38462	0.37931	0.35385
s30	0.8125	0.72222	0.34615	0.56757	0.40909	0.4359	0.6	0.5	0.55	0.46667	0.46154	0.42308	0.52941	0.46429	0.55102	0.53571
s31	0.6444	0.59184	0.32308	0.6	0.47368	0.5	0.67442	0.50877	0.58491	0.31507	0.38462	0.35385	0.4375	0.27536	0.45161	0.36232
s32	0.6667	0.65217	0.35484	0.57447	0.40741	0.55102	0.65	0.37037	0.48	0.34286	0.32258	0.35484	0.27869	0.33333	0.42373	0.21212
s33	0.6667	0.57692	0.32353	0.54717	0.46667	0.45455	0.65217	0.43333	0.53571	0.28947	0.32353	0.29412	0.37313	0.25	0.47692	0.36111
s34	0.9498	0.76744	0.42373	0.63636	0.45098	0.52174	0.78378	0.4902	0.65957	0.43284	0.49153	0.45763	0.44828	0.42857	0.46429	0.39683
s35	0.6279	0.61702	0.52381	0.70833	0.56364	0.52	0.65854	0.52727	0.4902	0.43662	0.49206	0.42857	0.45161	0.43284	0.53333	0.37313
s36	0.5385	0.44186	0.42373	0.59091	0.60784	0.47826	0.56757	0.45098	0.44681	0.46269	0.45763	0.42373	0.44828	0.39683	0.53571	0.52381
s37	0.5217	0.48	0.24242	0.4902	0.37931	0.4717	0.63636	0.41379	0.44444	0.32432	0.33333	0.30303	0.32308	0.25714	0.42857	0.25714
s38	0.4884	0.44681	0.30159	0.54167	0.41818	0.44	0.5122	0.38182	0.33333	0.29577	0.30159	0.2381	0.25806	0.28358	0.43333	0.28358
s39	0.6111	0.6	0.57143	0.80488	0.66667	0.62791	0.82353	0.66667	0.63636	0.53125	0.57143	0.46429	0.52727	0.5	0.66038	0.46667
s40	0.84	0.7931	0.64444	0.86667	0.62162	0.625	0.82609	0.72973	0.69697	0.69811	0.68889	0.64444	0.68182	0.71429	0.71429	0.67347
s41	0.5556	0.55	0.46429	0.80488	0.58333	0.48837	0.82353	0.66667	0.63636	0.5	0.53571	0.39286	0.52727	0.43333	0.69811	0.4
s42	0.5484	0.54286	0.68627	0.83333	0.81395	0.68421	0.7931	0.76744	0.69231	0.66102	0.68627	0.60784	0.68	0.56364	0.75	0.56364
s43	0.7143	0.69231	0.6	0.75	0.61702	0.61905	0.87879	0.65957	0.67442	0.5873	0.63636	0.6	0.59259	0.59322	0.65385	0.59322
s44	0.6744	0.65957	0.46032	0.79167	0.52727	0.6	0.85366	0.63636	0.72549	0.46479	0.52381	0.52381	0.48387	0.40299	0.56667	0.40299
s45	0.72	0.66667	0.4	0.74545	0.41935	0.57895	0.875	0.58065	0.72414	0.35897	0.48571	0.45714	0.44928	0.40541	0.52239	0.37838
s46	0.5385	0.44186	0.42373	0.59091	0.60784	0.47826	0.56757	0.45098	0.44681	0.46269	0.45763	0.42373	0.44828	0.39683	0.53571	0.52381
s47	0.8571	0.73913	0.35484	0.48936	0.33333	0.5102	0.65	0.2963	0.44	0.34286	0.32258	0.41935	0.27869	0.42424	0.35593	0.30303
s48	0.3968	0.29412	0.37143	0.21429	0.23944	0.22078	0.30556	0.48387	0.42029	0.30159	0.30303	0.31429	0.35385	0.53571	0.36232	0.21212

Table B- 14 Genetic distance among 48 acession of *Amorphophallus* spp. from all set of primers (continue).

Sample	s17	s18	s19	s20	s21	s22	s23	s24	s25	s26	s27	s28	s29	s30	s31	s32
s1	0.48718	0.54545	0.78261	0.63333	0.48936	0.54717	0.83333	0.78947	0.6	0.94872	0.85714	0.86957	0.85366	0.8125	0.64444	0.66667
s2	0.34884	0.5	0.68	0.5625	0.37255	0.50877	0.69231	0.71429	0.55102	0.81395	0.73913	0.72	0.73333	0.72222	0.59184	0.65217
s3	0.45763	0.40625	0.30303	0.275	0.31343	0.28767	0.32353	0.34483	0.35385	0.38983	0.35484	0.36364	0.34426	0.34615	0.32308	0.35484
s4	0.59091	0.46939	0.45098	0.47692	0.46154	0.44828	0.54717	0.48837	0.52	0.54545	0.48936	0.52941	0.52174	0.56757	0.6	0.57447
s5	0.52941	0.5	0.27586	0.36111	0.49153	0.35385	0.33333	0.36	0.4386	0.37255	0.33333	0.34483	0.32075	0.40909	0.47368	0.40741
s6	0.47826	0.60784	0.54717	0.43284	0.37037	0.53333	0.52727	0.46667	0.5	0.52174	0.5102	0.50943	0.5	0.4359	0.5	0.55102
s7	0.56757	0.47619	0.63636	0.58621	0.55556	0.56863	0.69565	0.61111	0.62791	0.72973	0.65	0.68182	0.64103	0.6	0.67442	0.65
s8	0.4902	0.35714	0.34483	0.33333	0.35593	0.35385	0.36667	0.4	0.4386	0.33333	0.2963	0.34483	0.32075	0.5	0.50877	0.37037
s9	0.40426	0.38462	0.51852	0.44118	0.30909	0.40984	0.5	0.52174	0.58491	0.48936	0.44	0.44444	0.46939	0.55	0.58491	0.48
s10	0.46269	0.30556	0.24324	0.18182	0.28	0.23457	0.26316	0.39394	0.36986	0.31343	0.34286	0.2973	0.30435	0.46667	0.31507	0.34286
s11	0.49153	0.28125	0.27273	0.25	0.31343	0.23288	0.32353	0.41379	0.41538	0.35593	0.32258	0.33333	0.34426	0.46154	0.38462	0.32258
s12	0.45763	0.34375	0.33333	0.25	0.22388	0.28767	0.35294	0.48276	0.41538	0.38983	0.41935	0.39394	0.37705	0.42308	0.35385	0.35484
s13	0.44828	0.2381	0.26154	0.24051	0.30303	0.22222	0.31343	0.4386	0.4375	0.31034	0.27869	0.29231	0.3	0.52941	0.4375	0.27869
s14	0.52381	0.29412	0.4	0.2381	0.29577	0.24675	0.33333	0.3871	0.30435	0.46032	0.42424	0.4	0.38462	0.46429	0.27536	0.33333
s15	0.53571	0.34426	0.30159	0.32468	0.375	0.31429	0.32308	0.49091	0.54839	0.39286	0.35593	0.30159	0.37931	0.55102	0.45161	0.42373
s16	0.39683	0.29412	0.37143	0.21429	0.23944	0.22078	0.30556	0.48387	0.42029	0.30159	0.30303	0.31429	0.35385	0.53571	0.36232	0.21212
s17	0	0.63636	0.47368	0.43662	0.24138	0.53125	0.45763	0.55102	0.53571	0.48	0.4717	0.40351	0.42308	0.53488	0.53571	0.50943
s18	0.63636	0	0.3871	0.26316	0.39683	0.15942	0.375	0.55556	0.47541	0.52727	0.51724	0.48387	0.50877	0.66667	0.37705	0.34483
s19	0.47368	0.3871	0	0.20513	0.38462	0.32394	0.24242	0.39286	0.39683	0.36842	0.33333	0.21875	0.25424	0.48	0.36508	0.4
s20	0.43662	0.26316	0.20513	0	0.26582	0.17647	0.225	0.42857	0.32468	0.32394	0.32432	0.25641	0.28767	0.5	0.27273	0.27027
s21	0.24138	0.39683	0.38462	0.26582	0	0.33333	0.31343	0.47368	0.375	0.41379	0.44262	0.35385	0.36667	0.45098	0.34375	0.37705
s22	0.53125	0.15942	0.32394	0.17647	0.33333	0	0.31507	0.49206	0.34286	0.4375	0.43284	0.40845	0.45455	0.54386	0.34286	0.28358
s23	0.45763	0.375	0.24242	0.225	0.31343	0.31507	0	0.37931	0.38462	0.22034	0.19355	0.18182	0.18033	0.38462	0.29231	0.29032
s24	0.55102	0.55556	0.39286	0.42857	0.47368	0.49206	0.37931	0	0.23636	0.38776	0.30769	0.28571	0.29412	0.2381	0.30909	0.38462
s25	0.53571	0.47541	0.39683	0.32468	0.375	0.34286	0.38462	0.23636	0	0.5	0.42373	0.39683	0.37931	0.38776	0.29032	0.38983
s26	0.48	0.52727	0.36842	0.32394	0.41379	0.4375	0.22034	0.38776	0.5	0	0.13208	0.15789	0.15385	0.44186	0.39286	0.24528
s27	0.4717	0.51724	0.33333	0.32432	0.44262	0.43284	0.19355	0.30769	0.42373	0.13208	0	0.1	0.09091	0.43478	0.38983	0.21429
s28	0.40351	0.48387	0.21875	0.25641	0.35385	0.40845	0.18182	0.28571	0.39683	0.15789	0.1	0	0.08475	0.44	0.33333	0.26667
s29	0.42308	0.50877	0.25424	0.28767	0.36667	0.45455	0.18033	0.29412	0.37931	0.15385	0.09091	0.08475	0	0.31034	0.23636	0.34426
s30	0.53488	0.66667	0.48	0.5	0.45098	0.54386	0.38462	0.2381	0.38776	0.44186	0.43478	0.44	0.31034	0	0.34694	0.47826
s31	0.53571	0.37705	0.36508	0.27273	0.34375	0.34286	0.29231	0.30909	0.29032	0.39286	0.38983	0.33333	0.23636	0.34694	0	0.25424
s32	0.50943	0.34483	0.4	0.27027	0.37705	0.28358	0.29032	0.38462	0.38983	0.24528	0.21429	0.26667	0.34426	0.47826	0.25424	0
s33	0.52542	0.4375	0.39394	0.25	0.34328	0.31507	0.35294	0.34483	0.23077	0.38983	0.3871	0.33333	0.30769	0.38462	0.23077	0.35484
s34	0.64	0.45455	0.40351	0.32394	0.44828	0.4375	0.32203	0.55102	0.5	0.36	0.35849	0.33333	0.39286	0.53488	0.35714	0.28302
s35	0.48148	0.52542	0.44262	0.36	0.35484	0.5	0.49206	0.4717	0.43333	0.51852	0.40351	0.40984	0.46154	0.57447	0.4	0.36842
s36	0.52	0.45455	0.47368	0.40845	0.41379	0.46875	0.49153	0.46939	0.35714	0.6	0.4717	0.47368	0.32203	0.48837	0.39286	0.54717
s37	0.47368	0.3871	0.375	0.25641	0.29231	0.29577	0.33333	0.46429	0.36508	0.40351	0.33333	0.34375	0.32143	0.48	0.30159	0.23333
s38	0.44444	0.32203	0.37705	0.25333	0.25806	0.29412	0.36508	0.50943	0.4	0.40741	0.36842	0.37705	0.55102	0.48936	0.33333	0.26316
s39	0.65957	0.42308	0.59259	0.44118	0.52727	0.44262	0.53571	0.69565	0.54717	0.53191	0.56	0.59259	0.57895	0.7	0.43396	0.36
s40	0.72222	0.65854	0.62791	0.64912	0.72727	0.68	0.6	0.71429	0.7619	0.55556	0.58974	0.62791	0.55102	0.65517	0.66667	0.64103
s41	0.57447	0.5	0.59259	0.38235	0.38182	0.47541	0.53571	0.65217	0.50943	0.53191	0.6	0.55556	0.68182	0.65	0.43396	0.4
s42	0.66667	0.57447	0.7551	0.55556	0.6	0.64286	0.72549	0.70732	0.54167	0.7619	0.68889	0.67347	0.54167	0.71429	0.54167	0.55556
s43	0.65217	0.60784	0.54717	0.49254	0.62963	0.56667	0.56364	0.64444	0.57692	0.56522	0.5102	0.54717	0.5	0.74359	0.53846	0.55102
s44	0.62963	0.45763	0.54098	0.36	0.51613	0.38235	0.36508	0.58491	0.46667	0.44444	0.4386	0.5082	0.42857	0.61702	0.36667	0.33333
s45	0.5082	0.48485	0.41176	0.26829	0.42029	0.38667	0.28571	0.53333	0.43284	0.40984	0.4375	0.41176	0.42857	0.55556	0.37313	0.375
s46	0.52	0.45455	0.47368	0.40845	0.41379	0.46875	0.49153	0.46939	0.35714	0.6	0.4717	0.47368	0.32203	0.48837	0.39286	0.54717
s47	0.4717	0.51724	0.33333	0.32432	0.44262	0.43284	0.19355	0.30769	0.42373	0.13208	0.30435	0.34426	0.37705	0.3	0.38462	0.37931
s48	0.36111	0.39683	0.37313	0.52381	0.25714	0.28358	0.46667	0.67347	0.4	0.56364	0.59322	0.40299	0.27869	0.33333	0.42373	0.21212

Table B- 14 Genetic distance among 48 acession of *Amorphophallus* spp. from all set of primers (continue).

Sample	s33	s34	s35	s36	s37	s38	s39	s40	s41	s42	s43	s44	s45	s46	s47	s48
s1	0.66667	0.94983	0.62791	0.53846	0.52174	0.48837	0.61111	0.84	0.55556	0.54839	0.71429	0.67442	0.72	0.53846	0.85714	0.39683
s2	0.57692	0.76744	0.61702	0.44186	0.48	0.44681	0.6	0.7931	0.55	0.54286	0.69231	0.65957	0.66667	0.44186	0.73913	0.29412
s3	0.32353	0.42373	0.52381	0.42373	0.24242	0.30159	0.57143	0.64444	0.46429	0.68627	0.6	0.46032	0.4	0.42373	0.35484	0.37143
s4	0.54717	0.63636	0.70833	0.59091	0.4902	0.54167	0.80488	0.86667	0.80488	0.83333	0.75	0.79167	0.74545	0.59091	0.48936	0.21429
s5	0.46667	0.45098	0.56364	0.60784	0.37931	0.41818	0.66667	0.62162	0.58333	0.81395	0.61702	0.52727	0.41935	0.60784	0.33333	0.23944
s6	0.45455	0.52174	0.52	0.47826	0.4717	0.44	0.62791	0.625	0.48837	0.68421	0.61905	0.6	0.57895	0.47826	0.5102	0.22078
s7	0.65217	0.78378	0.65854	0.56757	0.63636	0.5122	0.82353	0.82609	0.82353	0.7931	0.87879	0.85366	0.875	0.56757	0.65	0.30556
s8	0.43333	0.4902	0.52727	0.45098	0.41379	0.38182	0.66667	0.72973	0.66667	0.76744	0.65957	0.63636	0.58065	0.45098	0.2963	0.48387
s9	0.53571	0.65957	0.4902	0.44681	0.44444	0.33333	0.63636	0.69697	0.63636	0.69231	0.67442	0.72549	0.72414	0.44681	0.44	0.42029
s10	0.28947	0.43284	0.43662	0.46269	0.32432	0.29577	0.53125	0.69811	0.5	0.66102	0.5873	0.46479	0.35897	0.46269	0.34286	0.30159
s11	0.32353	0.49153	0.49206	0.45763	0.33333	0.30159	0.57143	0.68889	0.53571	0.68627	0.63636	0.52381	0.48571	0.45763	0.32258	0.30303
s12	0.29412	0.45763	0.42857	0.42373	0.30303	0.2381	0.46429	0.64444	0.39286	0.60784	0.6	0.52381	0.45714	0.42373	0.41935	0.31429
s13	0.37313	0.44828	0.45161	0.44828	0.32308	0.25806	0.52727	0.68182	0.52727	0.68	0.59259	0.48387	0.44928	0.44828	0.27869	0.35385
s14	0.25	0.42857	0.43284	0.39683	0.25714	0.28358	0.5	0.71429	0.43333	0.56364	0.59322	0.40299	0.40541	0.39683	0.42424	0.53571
s15	0.47692	0.46429	0.53333	0.53571	0.42857	0.43333	0.66038	0.71429	0.69811	0.75	0.65385	0.56667	0.52239	0.53571	0.35593	0.36232
s16	0.36111	0.39683	0.37313	0.52381	0.25714	0.28358	0.46667	0.67347	0.4	0.56364	0.59322	0.40299	0.37838	0.52381	0.30303	0.21212
s17	0.52542	0.64	0.48148	0.52	0.47368	0.44444	0.65957	0.72222	0.57447	0.66667	0.65217	0.62963	0.5082	0.52	0.4717	0.36111
s18	0.4375	0.45455	0.52542	0.45455	0.3871	0.32203	0.42308	0.65854	0.5	0.57447	0.60784	0.45763	0.48485	0.45455	0.51724	0.39683
s19	0.39394	0.40351	0.44262	0.47368	0.375	0.37705	0.59259	0.62791	0.59259	0.7551	0.54717	0.54098	0.41176	0.47368	0.33333	0.37313
s20	0.25	0.32394	0.36	0.40845	0.25641	0.25333	0.44118	0.64912	0.38235	0.55556	0.49254	0.36	0.26829	0.40845	0.32432	0.52381
s21	0.34328	0.44828	0.35484	0.41379	0.29231	0.25806	0.52727	0.72727	0.38182	0.6	0.62963	0.51613	0.42029	0.41379	0.44262	0.25714
s22	0.31507	0.4375	0.5	0.46875	0.29577	0.29412	0.44262	0.68	0.47541	0.64286	0.56667	0.38235	0.38667	0.46875	0.43284	0.28358
s23	0.35294	0.32203	0.49206	0.49153	0.33333	0.36508	0.53571	0.6	0.53571	0.72549	0.56364	0.36508	0.28571	0.49153	0.19355	0.46667
s24	0.34483	0.55102	0.4717	0.46939	0.46429	0.50943	0.69565	0.71429	0.65217	0.70732	0.64444	0.58491	0.53333	0.46939	0.30769	0.67347
s25	0.23077	0.5	0.43333	0.35714	0.36508	0.4	0.54717	0.7619	0.50943	0.54167	0.57692	0.46667	0.43284	0.35714	0.42373	0.4
s26	0.38983	0.36	0.51852	0.6	0.40351	0.40741	0.53191	0.55556	0.53191	0.7619	0.56522	0.44444	0.40984	0.6	0.13208	0.56364
s27	0.3871	0.35849	0.40351	0.4717	0.33333	0.36842	0.56	0.58974	0.6	0.68889	0.5102	0.4386	0.4375	0.4717	0.30435	0.59322
s28	0.33333	0.33333	0.40984	0.47368	0.34375	0.37705	0.59259	0.62791	0.55556	0.67347	0.54717	0.5082	0.41176	0.47368	0.34426	0.40299
s29	0.30769	0.39286	0.46154	0.32203	0.32143	0.55102	0.57895	0.55102	0.68182	0.54167	0.5	0.42857	0.42857	0.32203	0.37705	0.27869
s30	0.38462	0.53488	0.57447	0.48837	0.48	0.48936	0.7	0.65517	0.65	0.71429	0.74359	0.61702	0.55556	0.48837	0.3	0.33333
s31	0.23077	0.35714	0.4	0.39286	0.30159	0.33333	0.43396	0.66667	0.43396	0.54167	0.53846	0.36667	0.37313	0.39286	0.38462	0.42373
s32	0.35484	0.28302	0.36842	0.54717	0.23333	0.26316	0.36	0.64103	0.4	0.55556	0.55102	0.33333	0.375	0.54717	0.37931	0.21212
s33	0	0.42373	0.42857	0.35593	0.30303	0.33333	0.5	0.73333	0.46429	0.52941	0.52727	0.46032	0.37143	0.35593	0.35385	0.50943
s34	0.42373	0	0.40741	0.56	0.26316	0.37037	0.3617	0.55556	0.3617	0.52381	0.43478	0.33333	0.31148	0.56	0.42308	0.34483
s35	0.42857	0.40741	0	0.48148	0.37705	0.37931	0.45098	0.65	0.41176	0.3913	0.44	0.37931	0.38462	0.48148	0.50877	0.4
s36	0.35593	0.56	0.48148	0	0.36842	0.33333	0.57447	0.72222	0.61702	0.52381	0.6087	0.59259	0.57377	0.53846	0.25424	0.27027
s37	0.30303	0.26316	0.37705	0.36842	0	0.11475	0.33333	0.53488	0.2963	0.5102	0.43396	0.31148	0.32353	0.44186	0.28767	0.37705
s38	0.33333	0.37037	0.37931	0.33333	0.11475	0	0.29412	0.5	0.2549	0.47826	0.44	0.37931	0.38462	0.42373	0.36667	0.28358
s39	0.5	0.3617	0.45098	0.57447	0.33333	0.29412	0	0.33333	0.22727	0.33333	0.30233	0.21569	0.27586	0.59091	0.45455	0.29032
s40	0.73333	0.55556	0.65	0.72222	0.53488	0.5	0.33333	0	0.39394	0.57143	0.375	0.45	0.53191	0.60784	0.18033	0.38462
s41	0.46429	0.3617	0.41176	0.61702	0.2963	0.2549	0.22727	0.39394	0	0.33333	0.34884	0.29412	0.31034	0.47826	0.29412	0.38983
s42	0.52941	0.52381	0.3913	0.52381	0.5102	0.47826	0.33333	0.57143	0.33333	0	0.47368	0.43478	0.4717	0.56757	0.37931	0.24528
s43	0.52727	0.43478	0.44	0.6087	0.43396	0.44	0.30233	0.375	0.34884	0.47368	0	0.28	0.33333	0.45098	0.15385	0.28767
s44	0.46032	0.33333	0.37931	0.59259	0.31148	0.37931	0.21569	0.45	0.29412	0.43478	0.28	0	0.10769	0.44681	0.53846	0.22222
s45	0.37143	0.31148	0.38462	0.57377	0.32353	0.38462	0.27586	0.53191	0.31034	0.4717	0.33333	0.10769	0	0.3461	0.53846	0.24675
s46	0.35593	0.56	0.48148	0.53846	0.44186	0.42373	0.59091	0.60784	0.47826	0.56757	0.45098	0.44681	0.3461	0	0.65	0.31429
s47	0.35385	0.42308	0.50877	0.25424	0.28767	0.36667	0.45455	0.18033	0.29412	0.37931	0.15385	0.53846	0.53846	0.65	0	0.22078
s48	0.50943	0.34483	0.4	0.27027	0.37705	0.28358	0.29032	0.38462	0.38983	0.24528	0.28767	0.22222	0.24675	0.31429	0.22078	0

APPENDIX C

SCAR marker sequencing data

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Chulalongkorn University

> *A. krausei* KRU-S11
TAGCAGCGAGGCAACATGGCAGCACAGACAACTACCGATCGACTGAGGG
TAGGGCAACTAGACCTACCAGTGCAACGATCCTTGAGTCGGATGACTCGA
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TAAGCCAGAGAAAATGACATACTCCGCAAGAACATAATGCACAGGGACC
TGAGGGAACACCGAGAACCCTTAGCACACCGGACACACAGACCAAGGTA
AGACAACACCTCGAGGTATCCAGGACCAATGTATCGAACTGGATAGAGCC
ACCAAGACACACAAAGCCACCCGTCAACCCCTAGGTAACCCCCGATTGAC
CGGAGCCAGACAACACCGACACAAACAACCGACGCACAGACCCAAACAC
AGCAAGCACAAGCATCCCTCGCTGCTA

> *A. krausei* KRU-S10
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TGGCGTCCATCTCCTCGCTGCTACTGGGTCATTGGCTGTTGTTGTTGTTGTT
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> *A. krausei* KRU-S12
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ACACAGGGACCTGAGGGAACACCGAGAACCCTTAGCACACCGGACACAC
AAAGAAAGACCAAGGTAAGACAACACCTCGAGGTATCCAGGACCAATGT
ATCGAACTGGATAGAGCCACCAAGACACACAAAGCCACCCGTCAACCCCT
AGGTAACCCCCGATTGACCGGAGCCAGACAACACCGACACAAACAACCG
ACGCACAGACCCAAACACAGCAAGCACAAGCATC

>*A. kachiensis* KAC-S23
TAGCAGCGAGGCACAGCAGCACGGCCCATTGGGCGGCGGACAGACACGT
GCCTTCTCACAGCCAACGTCAGGCCCACTAGCAGTCCACCCACACGGCGC
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CCATATCTCGCAGGGAGTAGCTGCCATTGAGCATGCTTTGTCAGGAGGAG
CCACGAAGCTCGATACTAGGGACACTCACCTCAGCCATACATATTAGAGA
CGGCTCAGATGTATGATACCACAGTAGACCATTCCCTCTATATATCAGGCT
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CCTCGCTGCTA

> *A. kachiensis* KAC-S48
TAGCAGCGAGGGTCAGTGGACTCGAGGCATAGCAAAAAAAAGAGAGAGG
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AAACATCCTCGCTGCTA

>*A. konjac* KJ-S15
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> *A. konjac* KJ-S41
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> *A. muelleri* S01
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>*A. muelleri* S02
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> *A. muelleri* -S03
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> *A. muelleri* -S04
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> *A. muelleri* -S05
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> *A. muelleri* -S06
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> *A. bulbilfer* S7
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> *A. bulbilfer* S8
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>*A. bulbilfer* S43
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CGCCTCCAGACGGCCACTCCCTATAATGCTCAGATGTTTCAAAGTCTTCAA
GAAAACATCCTCGCTGCTA

>*A. xiei* S9
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> *A. xiei* S40
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GCGGCTTGCCCAGGATGTTGGCTATGGGGTCTTCAGGTGATAAATTATGA
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ATTGCTGCGCTTCCAGATGAGCACTCCCTACGTTGCTCAAATGTTTCAAAG
TCTTCAAGAAAACATCCTCGCTGCTA

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[2] Mekkerdchoo, O., Holford, P., Borompichaichartkul, C., Wattananon, S., Srzednicki, G., and Prakitchaiwattana, C. (2012). Genetic variation among Amorphophallus sp. From northern Thailand and their glucomannan content. Acta Horticulturae, 989, 323-330.

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[1] Mekkerdchoo, O., Holford, P., Srzednicki, G., Prakitchaiwattana, C., Borompichaichartkul, C., and Wattananon, S. (2010). Determination of relationships and genetic variation among Amorphophallus sp. from northern part of Thailand. Proceedings of International Conference on Agricultural and Agro-Industry, Food, Health and Trade, 19 – 20 November. Mae Fha Luang University Chiang Rai, Thailand.

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