การศึกษาความสัมพันธ์ทางพันธุกรรมและโมเลกุลบ่งชี้ของไผ่ตงเขียวจากฟาร์ม มหาวิทยาลัยเทคโนโลยีสุรนารี

นางสาวกนกอร ศรีลุนช่าง

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มหาวิทยาลัยเทคโลยีสุรนารี
ปีการศึกษา 2545

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THE STUDY OF GENETIC RELATIONSHIP AND MOLECULAR MARKERS IDENTIFICATION OF SUT *Dendrocalamus asper* (PAI TONG KEAW)

MISS KANOK-ORN SRILUNCHANG

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOTECHNOLOGY

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กนกอร ศรีลุนช่าง: การศึกษาความสัมพันธ์ทางพันธุกรรม และโมเลกุลบ่งชี้ของไผ่ตงเขียว จากฟาร์มมหาวิทยาลัยเทคโนโลยีสุรนารี (THE STUDY OF GENETIC RELATIONSHIP AND MOLECULAR MARKERS IDENTIFICATION OF SUT *Dendrocalamus asper* (PAI TONG KEAW))
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ไผ่ตงเขียว,ไผ่ตงดำ และไผ่รวก จากฟาร์มมหาวิทยาลัยเทคโนโลยีสุรนารีถูกนำมาทดสอบเพื่อหาลายพิมพ์ดีเอ็นเอ, โมเลกุลบ่งชี้ และ ความสัมพันธ์ทางพันธุกรรม ด้วยเทคนิคอาร์เอพีดี (RAPD: Random Amplication Polymorphic DNA) และเทคนิคเอเอฟแอลพี (AFLP: Amplified Fragment Length Polymorphism) จากการทดลองพบว่ามีชิ้นดีเอ็นเอที่ถูกเพิ่มจำนวน 161 ชิ้นด้วยเทคนิคอาร์เอพีดี มีจำนวน 121 ชิ้น ที่แสดงความแตกต่าง จากนั้นนำข้อมูลที่ได้ไปวิเคราะห์ด้วยโปรแกรมทางคอมพิวเตอร์ NTSYS V2.1 เพื่อจัดกลุ่มหาความสัมพันธ์โดยโปรแกรม UPGMA พบว่า ไผ่ถูกจัดเป็น 17 กลุ่มที่ต่างกัน จาก 18 ตัวอย่าง และมีสายพันธ์ที่เหมือนกันอยู่หนึ่งตัวอย่าง ด้วยเทคนิคอาร์เอพีดี จากนั้นได้ทดสอบการใช้เทคนิคดังกล่าวกับไผ่ที่มีลักษณะดีทางเศรษฐกิจ (รสหวาน, หน่อใหญ่, และหน่อดก) พบว่าการใช้เทคนิคอาร์เอพีดีสามารถใช้บอกความสัมพันธ์ของไผ่พันธ์ต่างๆได้เช่นเดียวกัน นอกจากนี้ยังมีการใช้เทคนิคเอเอฟแอลพีทดสอบกับไผ่กลุ่มเดียวกันกับเทคนิคอาร์เอพีดี และมีการเก็บตัวอย่างเพิ่มเป็น 25 กลุ่มตัวอย่าง ซึ่งมี 2 ตัวอย่างที่ไม่ใช่ไผ่ตงเขียว นั่นคือ ไผ่ตงดำ และไผ่รวก (*D.asper* Tong Daum and *Thyrosostachys siamenasis*) โดยทดสอบด้วยไพร์เมอร์ 64 คู่ พบว่า 12 คู่ ที่ให้ผลดีที่สุดและนำผลดังกล่าวไปวิเคราะห์ด้วยโปรแกรมชนิดเดียวกันกับอาร์เอพีดี พบว่าหลังจากรวมข้อมูลจากการทำพีซีอาร์แล้ว สามารถแยกความสัมพันธ์ของไผ่ได้ทุกสายพันธ์ แต่ไผ่แต่ละสายพันธ์ค่อนข้างมีความใกล้เคียงกันทางพันธุกรรม และท้ายสุดไผ่ที่ไม่รู้สายพันธ์มาก่อนถูกนำมาทดสอบด้วยเทคนิคเอเอฟแอลพีด้วยจุดประสงค์ดังกล่าวข้างต้น พบว่าเทคนิคเอเอฟแอลพีสามารถบ่งชี้ความสัมพันธ์ของไผ่ที่ไม่ทราบสายพันธ์มาก่อนเมื่อเทียบกับสายพันธ์ที่เคยอ้างอิงมาแล้ว ดังนั้นเทคนิคอาร์เอพีดีและเอเอฟแอลพีจึงสามารถประยุกต์ใช้ในการหาลายพิมพ์ดีเอ็นเอ, โมเลกุลบ่งชี้ และความสัมพันธ์ทางพันธุกรรมของสิ่งมีชีวิตอื่นๆต่อไปได้

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2545

ลายมือชื่อนักศึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

MISS KANOK-ORN SRILUNCHANG: THE STUDY OF GENETIC RELATIONSHIP AND MOLECULAR MARKERS IDENTIFICATION OF SUT *Dendrocalamus asper* (PAI TONG KEAW)
THESIS ADVISOR: ASST. PROF. MARIENA KETUDAT-CAIRNS. 52 PP
ISBN 974-533-182-1

Dendrocalamus asper were tested using Random Amplification Polymorphic DNA (RAPD) by short arbitary oligonucleotide primer and Amplification Fragment Length Polymorphism (AFLP) method with the objective of fingerprinting, identifying and grouping. Twenty-three of the fifty primers from Operon kit AE, L and S were tested in initial screening experiment with eighteen genotypes of *D. asper*. Of the 161 amplified bands, 121 were polymorphic. Cluster analysis base on NTSYS V. 2.1 program using UPGMA grouped shows 17 distinct groups and similar in 1 group. Five specimen with good character (good taste, large shoots, and high of shoot) of *D. asper* were also tested by the RAPD method separately from the 18 samples. The five lines are significantly distinct from each other. Due to the limitation of the RAPD technique the AFLP method was also use for identify and group these plants. All of the *D. asper* specimen from SUT farm were tested (A11, A13, A15, A22, A25, A27, A37, A32, A34, A41, A42, A51, SUT7, SUT23, SUT25, SUT28, SUT33, SUT35, BC, KN, SA, 5AS1, and S85 along with 2 out groups (*D. asper* Tong Daum and *Thyrosostachys siamensis*). Out of 64 primers screened and 12 primers pairs were used in the analysis. The AFLP data were also analyzed by NTSYS program similar to RAPD. The AFLP information shows that all the specimen were in 23 distinct groups and 2 out groups were also separated from the other. Therefore, RAPD and AFLP can be used together in marker identification and genetic relationship of *D. asper*.

สาขาวิชาเทคโนโลยีชีวภาพ
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ลายมือชื่อนักศึกษา.....
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ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

LIST OF ABBREVIATIONS

bp	base pair	min	minute
mm	millimeter	mL	milliliter
nmol	nanomole	mM	millimolar
rpm	round per minute	cm	centimeter
et al.,	et alia (and others)	Fig.	Figure
hr	hour	mg	milligram
ng	nanogram	pmol	picomole
µl	microliter	µg	microgram
°C	degree Celsius	e.g.	for example
g	gram		

NTSYS	Numerical Taxonomy And Multivariate Analysis System
RAPD	Random Amplification Polymorphic DNA
AFLP	Amplification Fragment Length Polymorphism
SSR	Sample Sequence Repeat
RFLP	Restriction Fragment Length Polymorphism
SDS	Sodium Dodesyl Sulphate
DNA	Deoxynucleic acid
PCR	Polymerase Chain Reaction

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CHAPTER I
INTRODUCTION

Bamboo is a tree-like woody grass, which natural occurs roughly between the 46 southern and northern latitudes, excluding Europe. It thrives at altitudes from sea level to as high as 4,000 meters. Currently more than 130 genera of woody bamboos and 25 grass-like bamboos have been defined worldwide. There are over 1,300 species distributed among tropical, subtropical and mild temperate zones covering a total area of over 25 million hectares (McClure, 1996).

Bamboo is the fastest growing, most versatile woody plant in the world, and it is annually renewable if harvested in an intelligent way. This extraordinary feature is part of everyday life and culture of most of Asia, yet strangely, the Western world remains relatively uninterested and uneducated about it.

Bamboos grow successfully in desert environments provided they get enough water. Many bamboos are reasonably salt- tolerant. A large range of species of bamboo is able to grow on the banks of low-lying rivers, located less than a kilometer form a surfing beach. Growing here they must be swept from time to time by salt-laden air, and the roots of the larger species must be into brackish water.

The development of Bamboo as a multipurpose plant on worldwide scale will depend critically on basic research. Very little is known about the basic biology and genetics of bamboo. Also, basic research is almost non-existent. Our researches concentrate on the Thai economically important Bamboo species *D. asper* (Tong Keaw). We would like to contribute to a better understanding of the genetic map of *D. asper*. While much of this basic research is not directly valuable for plant production and propagation, we consider this approach as valuable and strategic in the long term, to improve the economic added value of bamboo, as a plant.

Genetic of Bamboo

Bamboos are polyploids. Temperate bamboos have 48 chromosomes, tropical ones have 72 chromosomes, so they are tetra- and hexaploids respectively, assuming a basic chromosome number of X = 12 in grasses. The chromosomes of tropical bamboos are very small and the karyotypes of temperate bamboo are very complicated. Also given the lack of breeding system, it seems almost impossible to do any serious study on the genome of bamboo (Gielis, 1998).

Morphology of Bamboo

Bamboo is an evergreen plant, it does not lose its leaves in autumn and grow fresh ones in the spring as broad-leaved trees do. Bamboo leaves stay green throughout an average winter. In early spring the young leaves grow out and the old ones are gradually lost. This characteristic of bamboo makes it particularly desirable for the gardens.

All in all bamboo is a very hardy and vigorous plant. Even when the stems and leaves have been severely damaged the plant will usually recover, although it may take years to regain its previous height.

Bamboos have another characteristic that distinguishes them from the European native trees and shrubs. The young shoot that emerges form the ground has the diameter at which the bamboo stem will remain throughout its life, which may be ten years or more. Bamboo stems do not therefore grow in width like trees, which get visibly broader each year. The lengths that shoot reaches in the growth year is also its final length. There is no year to year increase. Young developing bamboo plants produce thicker and taller stems each year, until the clump reaches maturity. The number of branches, however, does increase each year, as does the number of leaves.

The main structural parts of a bamboo plant are the underground system of rhizomes, the aerial culms and the culm branches. All of these parts are formed according to the same principle; an alternating series of nodes and internodes.

As a bamboo grows, each new internode is wrapped in a protective sheath, attached to the preceding node at the sheath ring. Once the internode has lengthened, it does not grow any further. The nodes are massive pieces of tissue, comprising the node ring, the sheath ring and usually a dormant bud. These dormant buds are the sites of emergence of new-segmented growth. A running bamboo is botanically classed as a “monopodial” species. It’s usually a temperate zone originator characterized by the development of an open network of horizonatal underground stems called rhizomes, from which the new clums grow.

Rhizomes

Rhizomes are underground stems, which grow and branch away from the bamboo plant, thus enabling new territory to be colonized. Each year, culms arise from the rhizomes to form the aerial parts of the bamboo. These rhizomes are often so tightly packed that the soil under a bamboo plant seems to be filled with them. They form a 'turf' similar to ordinary grasses, which can vary in depth depending on the species and growing condition. Although they seldom go deeper than one meter.

The rhizomes grow on, continually branching, producing new plant tissue, which differentiates alternately as node or internodes. The growing point of a rhizome is very hard, allowing it to penetrate into the surrounding soil. The true roots of a bamboo develop from the nodes of a rhizome, to supply the bamboo with water and nutrients from the surrounding soil.

Culms

Bamboo species are most readily distinguished by their culms, whose form and appearance give the whole plant its character. In some species the culms grows to several meters,

in others just a few centimeters. Some species have thick culms, other thin, some grow erect, other bend graciously. Bamboos culms are normally green, but some are yellow, brown, black, reddish, spotted or striped.

The tropical bamboo culms are stabilized by the tough nodes, as is the case in grasses and cereals. In Asia the culm is an important part of the bamboo plant, since so many objects used in daily life derive from it. One talks of bamboo wood, even though in the strict sense of the word, bamboo is not wood. Bamboo does consist of cellulose fibers, but whereas in tree wood these fiber are usually only about a millimeter long; in bamboo they are up to a centimeter. These long fibers contain lignin and silica, whereas tree fibers only have lignin. The proportion of silica in bamboo can be measured by burning the stems and measuring the silica in the ash.

A clumping bamboo is botanically as a “sympodial” species. The new growth spring up from near the base of the mother plant to form, typically, isolated clums radiation from a center and growing outward in all directions to form a circular of vegetation.

Shoot

A shoot is a young growing branch or twig when segmented into dissimilar members it becomes a stem (McClure, 1996). One of the constant joys of growing bamboo is the multiplicity of uses this beautiful plant fulfills. Most but not all bamboo shoot species are edible, and can provide a valuable addition to the daily staple of many people. Western people are often not aware of having eaten bamboo shoots, but it is a common ingredient in Thai, Chinese and Japanese food. Unfortunately, most of shoots eaten in the Western world are tinned shoots, very inferior to fresh.

The bamboo shoot grows quickly especially in the first week. It is interesting to place a rule next to growing shoot and measure an amazing growth rate of several centimeters a day. Large species in good sites can grow as much as 40 centimeters in day.

Fresh shoots from clumping (sympodial) bamboos start appearing during the midsummer season, and subject to rainfall and harvesting procedure, can continue until midwinter. This is quite different to running (monopodial) bamboos, which produce their edible shoots during late winter and spring, all within six to eight weeks.

Bamboo shoots vary in flavour and texture depending on a number of factors. These are: the species, how soon the shoot is harvested after first appearing, whether it is harvested early or late in the season, the portion of the shoot being tasted, whether the shoot is grown covered to exclude light after appearing from the ground, and how the shoot is cooked or prepared. Their great virtues are the crunchy texture that survives to some degree even if over-cooked, and their ability to take on flavors from other ingredients. The best eating species require very little preparation, and some few (e.g. *Dendrocalamus asper*, *Nastus elatus* and some others) can often be eaten raw with no preparation if light is excluded after the shoot breaks ground level.

The edible shoot is the young newly appearing growing culm shoot, which is cut off before it has a chance to continue its charge into the sky. The rhizomes of clumping bamboos are quite close to the soil surface (about 100 mm to 200 mm on large clumping bamboos or sometime even less).

Shoot size varies with the species, with smaller bamboo shoots such as *Thyrsostachys siamensis* being up to 60 mm diameter and weighing less than 150 g, and larger species shoots such as *D. asper* being up to 300 mm diameter, 600 mm tall, and weighing up to 7.2 kg each. Most of the superior species of edible bamboo are capable of producing maximum diameter clumps for that particular environment within four to five years.

The food value and composition of bamboo shoots is complex and interesting. Bamboo shoots are very high in trace elements and vitamins, including Vitamin B complex. They represent a significant contribution towards necessary body building components; however, they are extremely low in carbohydrates, fat and protein, which makes them an excellent food for the diet-conscious. A typical shoot analysis based on 100 g weight is as follows: Carbohydrate; 4.2 to

6.1 percent, Protein; 2.6 to 4 percent, Fat; 0.3 to 0.5 percent, Fibre; 0.5 to 0.9 percent, Glucose; 1.8 to 4.1 percent, Water; 89 to 93 percent, Calories (Joules); 118-197 and containing; Thiamin and Niacin (Vitamin B1, B2 complex); 0.7 to 1.4 percent; Calcium; 81 to 86 mg; Phosphorus; 42 to 59 mg; Iron; 0.5 to 1.7 mmg, Magnesium; 32 mmg, Sodium; 91 mg, Chlorine; 76 mg, Copper; 0.19 mg, Thiamin; 80 mg. Riboflavin; 0.19 mg, Niacin; 0.2 mmg, Vitamin C5; 3.2 to 5.7 mg, Choline; 8 mg, Oxalic acid; 2 mg (Cusack, 1999).

Branches

As the bamboo culm grows, protect by its sheath, branch buds develop at the nodes. Some species, the branches start developing from the top or the upper. However, the some extent if plant has sufficient light during the period of culm growth.

The number of branches at the node is an important taxonomic characteristic in identifying bamboos, although there is some variation.

Leaves

A bamboo leaf does not simply grow out from the bud, as for example would the leaf of broad-leaved tree such as beech. The bamboo leaf develops from a sheath, which encircles the stem and is called a leaf-sheath. The leaf-sheath resembles a small culm-sheath but develops a large sheath blade which functions as a proper leaf. The leaf blade are attached to their sheaths by a short, stalk-like projection of their midrib, which, when the 'leaves' fall, breaks off from the sheaths, which remain attached for much longer.

As the point of attachment of the blade to the sheath there are different combinations of features involving auricle, bristles and ligule, which are as important as the colour of the culm sheath in the identification of individual species.

A bamboo leaf is elongated and lanceolated, rounded at the base and pointed at the tip. Length and breadth vary markedly between the different genera and species. It is a characteristic of bamboos that the 'leaf' always has a stalk, unlike the leaves of other grasses. In most grasses the base of the leaf is directly wrapped around the stem, without a stalk.

Bamboo leaves vary a great deal in colour and shape, and these can be important for identification. However some species have very similar feature that the shape and colour of leaves can not be use as identification marker. The morphology of Bamboo are shown in Figure 1.

Flowering

Bamboo flowers are small, unspectacular and straw-colored to green colour, developing usually into seed, which look like wheat or even smaller grass seed. The seed is edible and very nourishing. Bamboos do not usually flower at regular, yearly intervals, like the majority of tree and shrubs, but come into flower only very rarely. The flowers are very inconspicuous and in some instances, the plant may die after flowering.

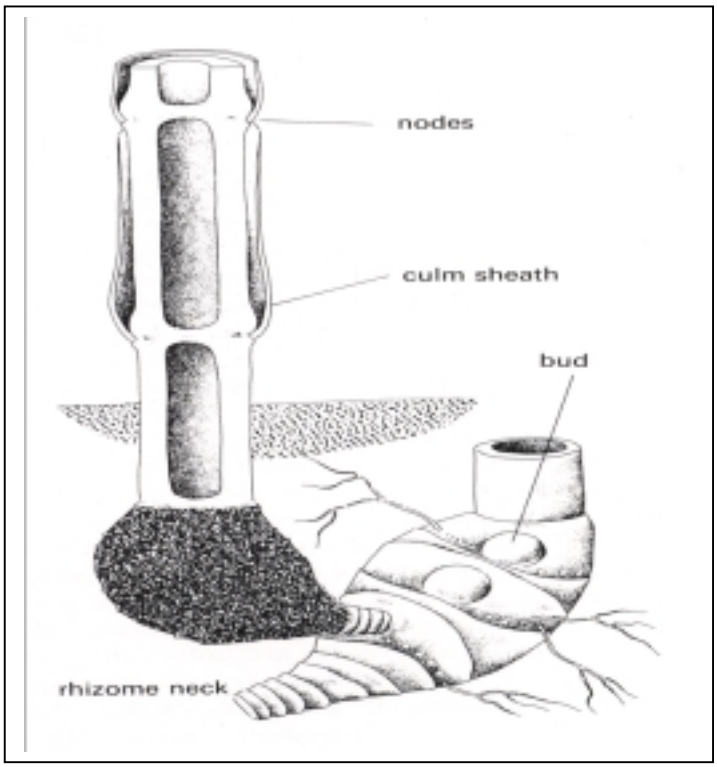
The rarity of flowering makes the identification of individual species and even their allocation to a particular genus, especially difficult. Botanical nomenclature depends upon flower structure, so one can see that studying and identifying a bamboo flower could be the work of a generation or more (Sastry, 2000).

The most extraordinary phenomenon of bamboo is its flowering and seeding cycle, which often causes chaos, death and destruction among a complete species almost simultaneously around the world. It is complex phenomenon with much variation between species, sometimes even within species.

There are three basic flowering patterns that appear dependent on genera and species. **1) Annual flowerings cycle**; bamboos following this pattern flower every year, don't die, but rarely produce a viable seed. **2) Sporadic flowerings cycle**; these bamboos flower occasionally, suffer temporarily from stress but don't die, and produce very little viable seed. **3) Gregarious flowerings cycle**; bamboos representing this pattern produce masses of viable seed, and mostly die of exhaustion in the process.

The gregarious flowering cycle phenomenon applies to most bamboo species (both sympodial and monodial), and appears to be linked to a genetic time clock within the bamboo. Most gregarious bamboos have a flowering cycle of between 30 and 120 years. For most species, their cycle variation, but for others the cycle can halve or double in time (Cusack, 1999).

Gregariously flowering bamboos produce huge volumes of viable seed, in fact they produce numerous culms dedicated to nothing but flowers and seed spikiest. The flowering process, which can take up to two years, puts great stress on the plant, and many, usually most, of the flowering plants actually die of exhaustion. Those that don’t die eventually produce small new culms, and re-build their strength over some seasons.



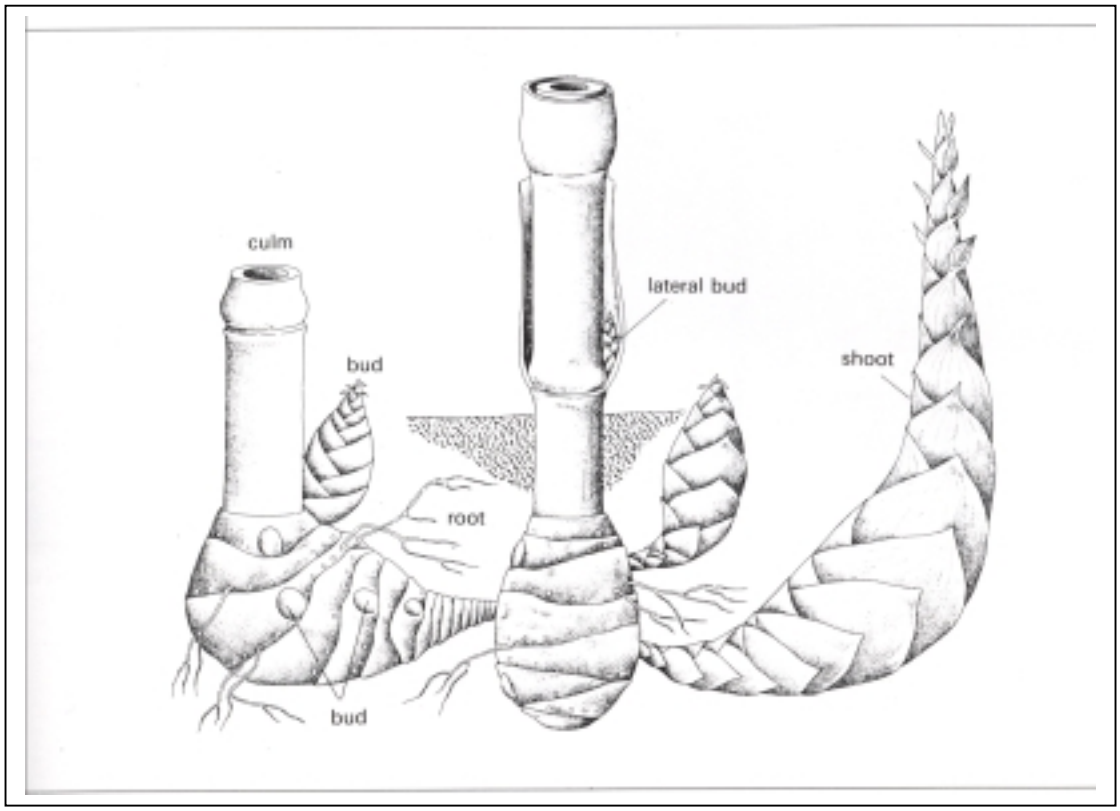


Figure 1. Morphology of *Dendrocalamus asper*
Source: Recht and Wetterwald., 1992

An example with a devastating financial impact was the flowering of approximately 40,000 hectares of *Dendrocalamus asper*, the edible shoot plantations of Thailand during the early 90's. The initial estimate of loss in the first year was US$80 million, which continue in a reduced amount until the plantations were re-established. The few plants of the same Thai cultivar (pai tong keo) planted years ago in Australia flowered the same year as their Thai brothers. In effect, seedlings from that Thai cultivar should not flower again for more than 80-100 years, but vegetative cuttings or transplants from the four other Thai cultivars (*Dendrocalamus strictus*, *Bambusa balcooa*, *D. latiflorus* and *Gigantochloa hasskarliana*), and both the Indonesian cultivours of *D. asper* are liable to flower and die at any time in the not-too-distant future (Cusack, 1999).

World consumption and Overseas Production

World consumption of bamboo shoots is obviously an estimate, but with more than 70,000 hectares planted in Japan, 90,000 hectares in Taiwan, 60,000 hectares in Thailand, and 10,000 hectares in China, together, with more than 2.8 million hectares of moso growing (mostly wild mountain stands, with some efficiently run plantation)(Seidal et al., 2000.).

Bamboo today directly contributes to the subsistence of over billion people, mostly in poor rural areas of the developing world. Woody bamboos have been used by communities throughout the developing world to satisfy basic necessities food (for both humans and their animal), fuel, shelter, agricultural tools, cooking vessels, water jugs, arts, crafts, furniture, musical instruments, weapons, hunting instruments, piping and a host of other items. In fact, wherever bamboo has been a dominant component of the vegetation, it has played an important economic and cultural role in the development of human societies.

Perhaps most basic, the seeds of bamboo and its protein-rich shoots are a popular food throughout Asia, powering a billion-dollar export industry in China, Taiwan and many Southeast Asian countries, including Japan and Thailand. The leaves and culms are frequently used in baking and cooking. Bamboo has also been used for medicine from ancient time as reported in

the Indian Vedas, Chinese Scriptures and Latin American literature. The use of bamboo as a fuel source (fuel value 4,000-6,000 cal/g) also is an age-old practice, not only for household purposes but also in small industries.

An Industrial Resource

Bamboo is one of the most versatile forest products. In countries of its natural distribution, apart from use as industrial raw material for pulp and paper (on a large scale), its most extensive utilization is for construction purposes as post, rafter, frame, partition, floor, roofing, etc. Bamboo culms are used in the round or spilt forms and as woven mats.

As an alternative to tropical timber, bamboo has the potential to contribute more widely and thus believe the pressure on tropical forests for housing, furniture, fuel and paper pulp.

Construction

The use of bamboo as an industrial raw material for the construction of human dwelling is one of the oldest uses of the bamboo culm. As noted earlier, bamboo has superior strength properties, low weight, ease of handling and processing, and low cost. A large portion of the rural housing in parts of Asia, Latin-America and Africa is constructed from bamboo. In recent years, the use of bamboo in popular housing construction has increased because of the cost and scarcity of other building materials.

Pulp and Paper

Bamboo has a higher cellulose and lower hemicellulose content when compare to wood. Because of the long bamboo fibers, the strength of bamboo paper pulps allows them to be used for a much wider variety of papers when compare to other grass pulps (bagasse, straw, etc.). Tear strength is the same as that of soft wood pulp, making it possible to produce packaging papers, and because of the slenderness of the fibers, high-quality printing papers can be made. Bamboo pulping has been well accepted by the industry, especially in Asia (including producing

dissolving pulp in India) and the full potential should be explored in countries where the resource is presently under utilized.

Today, Asia, mainly India and China, leads the way in the use of bamboo for pulp and paper. Brazil is presently the only American country that uses bamboo for making cellulose and paper. Bamboo pulp is also processed into incense paper in the Philippines for export to Taiwan.

Animal/Cattle Feed

Bamboo like wood is a composite material composed of cellulose, hemicelluloses and lignin, and that cellulose is embedded in a matrix of lignin-hemicellulose complexes. Several papers on the conversion of wood/bamboo to ruminant feed are published. It is known that for enzymatic saccharification and digestion of bamboo by ruminants, delignification or cleavage of the lignin-carbohydrate linkages is a prerequisite.

Furniture

Bamboo as a high-class furniture material has evidently generated renewed interest, especially in Asia, with the emergence of excellent bamboo handicrafts and furniture manufacturers in many places such as Indonesia, the Philippines and Thailand. A wide variety of products and designs are being exported.

Bamboo Scaffolding

Bamboo poles lashed together have been in use as scaffolding from ancient times. Bamboo is well for the purpose because of its unique property strength to weight ratio, resilience and flexibility. Bamboo scaffolding is still used extensively in Asia, Africa and Latin America to support various operations of building, painting and repair of even high-rise structures, up to 30 stories tall.

Bamboo in Thailand

In Thailand, bamboo is one of the most socio-economically-important plant species. They are used for many purposes such as food, household construction, supporting poles, basket and other handicraft making, firewood and pulping. Bamboo occurs naturally throughout the country. The species are mainly found in the mixed deciduous and tropical evergreen forests and partly found in the dry dipterocarp forest. Apart of natural forests, bamboo plantations and/or bamboo farms have been widely established for shoots and stem production throughout the country.

There are 13 genera and 60 species of bamboo recorded in Thailand (Deatwisit, 2000) and the major type of bamboos found throughout the country is the “Sympodial” (Characteristic of plant that grow horizontal) type. The important genera and species of bamboo in the Thailand can be grouped according to the utilization purpose. Ramyarongsi (1999) has grouped them in to three groups as follow:

1) **Bamboo for shoot production** (for food), (Pai Tong); *Dendrocalamus asper*, (Pai Seesuk) *Bambusa blumeana*, (Pai Ruak) *Thyrosostachys siamensis*, (Pai Ruakdam) *T. oliverii*, (Pai Bong) *D. brandisii*, (Pai Sang doi) *D. strictus* and (Pai Rai) *Gigantochloa albociliata*
2) **Bamboo stem for Construction and supporting pole**; (Pai Paa) *B. arundinaceae*, (Pai See suk) *B. blumeana*, (Pai Tong) *D. asper*, (Pai Ruakdam) *T. oliverii*, (Pai Sang doi) *D. strictus*, (Pai Sang nuan) *D. membranaceus*, (Pai Liang) *B. nana* and (Pai Phaak) *G. hasskeriana*
3) **Bamboo stem for basketing and handicraft**; (Pai Ruak) *T. siamensis*, (Pai Ruakdam) *T. oliverii*, (Pai Seesuk) *B. blumeana*, (Pai Liang) *B. nana*, (Pai Phaak) *G. hasskeriana*, (Pai Griab) *Schizostachyum humilis* and (Pai Hiae) *Cephalostachyum virgatum.*

D. asper is the most preferable for bamboo growers. They can be used for both the shoot and stem production. The shoots of *D. asper* are certainly sweet and non astringent if properly grown. The stems are very strong, good for construction purpose. The are also a relatively fast growing bamboo species.

Definition of *Dendrocalamus asper*

D. asper is a magnificent, large-leafed, vertical giant bamboo with some cultivars capable of growing to 30 m in high and 30 cm in diameter (but is not normally so large in plantation). It is the major large structural bamboo of South-East Asia, a mature clump being a truly awesome sight with its large relatively straight lower clums climbing into dense large-leafed upper growth. It is one of the strongest and most researched bamboos in the world. But very little is know in the basic science of bamboo when compare to other grasses such as rice, and maize, wheat.

In Thailand, a properly managed plantation of *D. asper* may produce 10 tones to 11 tones of young shoots/hectare/year. Thailand had 55,000 hectares of this species planted in 1994, specifically for shoot production. In 1998 the bamboo plantations reduce to 35,000 hectares due to its gregrious flowering. Most of their production is tinned or eaten locally, but some is exported as fresh, frozen, or steamed to Japan.

D. asper is a very fast-growing bamboo given reasonable conditions, good water, and some care. Within eighteen months of planting a young cutting, some reported had propagated (it had a single culm less than 3 mm diameter), it produced three large shoots in its second shooting season, the largest being more than 120 mm diameter at ground level.

It is absolutely critical that the correct cultivar of this bamboo is selected or the plantation may be destroyed by a gregarious flowering cycle in the not-too-distant future. The only cultivar that has gregariously flowered is pai tong keaw which is unlikely to flower for another 100 years or more, if the new plant has been planted from seed. The pai tong keaw also

has some advantages over the other cultivars; 1) It produces shoots about one month earlier than the others. 2) It is considered the most productive shoots production. 3) It produces a range of smaller different size shoots between 1 and 4 kilogram. This may help marketing (large ones to restaurants and smaller, less expensive ones for the caution housewife experimenting). 4) The timber is a little smaller in diameter, and it is capable of having a higher number and tonnage of timber culms than other cultivars. The very large diameter timber produced by some cultivars may not be as useful as the 50 to 120 mm diameter culms produced by pai tong keaw.

The harvesting period for *D. asper* shoots can be spread over six months or more; if the shoots are regularly harvested, and subject to enough rain or irrigation. Thailand's main shoot growing area has somewhat less than 2,000 mm/year of monsoonal rain during a five-month period, commencing at the beginning of the shoot season (Recht et al., 1992).

As mentioned earlier, bamboo is one of the most valuable forest species in Thailand. Due to pressure from a large growing population, both forest tree species (including bamboo) and the forest areas, especially the mixed deciduous forest types, have been heavily exploited for wood utilization and cleaved for land need. Without exception, bamboo resources are decreasing rapidly both quantitatively and qualitatively.

Earlier hypothesis, bamboos in Thailand are brought from cape of MALA-U. Those bamboos were grown in Prachinburi and Nakhon-Nayok long time ago. There are many varieties of Bamboos grown by farmers but the most favorite variety was *D. asper*. due to its good property such as rather broad range of seasonal budding and long harvesting period when compared to other varieties.

During 1994-1997, drought occurred widely in Thailand. *D. asper* around the country flowered and completely died. This was probably due to the flowering cycle of *D. asper* which is about 80-100 years. The death of *D. asper* brough trouble to agriculturists and farmers. Thus, Department of Agricultural Extension (DAE) and co-operative planted seedling to distribute to farmers to replace the extinct bamboo plantation. However, those seedlings were not selected for

good and high quality bamboo. After several years, the farmers realized that the newly germinated bamboos from the Department of Agricultural Extension were not of good properties. There were lots of varieties and variation and most of them with bad characteristics. Therefore, researchers from SUT have germinated more seeds and collected some good potential *D. asper* from farmer and DAE and replanted them in SUT farm for selection research (Khumleart and Sukthumrong., 1998).

Khumleart and Sukthumrong 1998, have studied *D. asper* variety selection. The characteristics of appropriate specimens were investigated such as good taste, large shoot, and high number of shoots. These specimens will be use for mass production and distribute or sell to farmers. So far five promising specimen have been selected (Khumleart and Sukthumrong., 1998). However more detail of agricultural research is needed. In the mean time molecular research of the genetic material to identify, characterize and group these specimens were performed. This study have use the DNA of these five specimens plus twenty-three other putative good specimens and two out group to study the genetic marker using RAPD and AFLP method, this study is considered to be basic research which will lead to more applied research with output for improvement of *D. asper* cultivation. This is important and will help increase bamboo resources and Bamboo production in the near future.

Genetic Identification Methods

The development of Bamboo as a multipurpose plant on world-wide scale will critically depend on basic research. Our research is concentrated on the genetic marker of *D. asper* (Tong Keaw). We would like to contribute to a better understanding of the genetic map of bamboo. While much of this basic research is not directly variable to the plant production, we consider this approach as valuable and strategic in the long term, to improve the economic added value of bamboo.

Given the long feedback times to monitor and select elite genotypes in bamboos, and given that identification of genotypes and phenotype is very difficult, the use of molecular

markers, may turn out to be very useful for different purpose. As a tool, molecular markers will save lots of time, although careful consideration must be made when and where to apply which method (Gielis, 1997).

New technological developments have expanded the range of DNA polymorphism assays for genetic mapping, marker assisted plant breeding, genome fingerprinting and for investigation of genetic relatedness. These technologies include Restriction Fragment Length Polymorphism (RFLP), Random Amplification Polymorphisms DNA marker (RAPD), Amplified Fragment Length Polymorphisms (AFLP) and Simple Sequence Repeat Polymorphisms or microsatellites (SSR) (Caetano-Anolles and Gresshoft., 1997). These methods detect polymorphism by assaying subsets of the total amount of DNA sequence variation in a genome. Polymorphisms detected with AFLP and RFLP assays reflect restriction size variation. RAPD polymorphisms result from DNA sequence variation of primer binding sites and from DNA length differences between primer binding sites. This is also true of AFLPs. SSR loci differ in the number of repetitive di-, tri- or tetranucleotide unit presents, and this length variation is detected with the polymerase chain reaction (PCR) by utilizing pairs of primers flanking each sample sequence repeat.

Since the early 1980's, RFLPs have been used successfully for a wide range of plant species. However, for some species, lack of polymorphism revealed by RFLPs has retarded progress. Furthermore, the RFLP assay is time consuming and labor intensive. The RAPD assay has alleviated some of the technical problems associated with RFLP and has been widely use to resolve problems in plant breeding and genetics. The high levels of polymorphism associated with the SSR loci have resulted in the SSR marker system being considered as the marker of choice in mammalian genetics (Welsh et al., 1991).

In order to facilitate selection of an appropriate technology for a given application, we have used two methods to study DNA marker system. A good measure of information content, which is a function of a marker system's ability to distinguish between genotypes. Both RAPDs and AFLPs have generally higher multiplex ratios than RFLPs and SSRs (Powell et al., 1996).

Main research topics in this study used the RAPD and AFLP molecular marker for precise identification, assess natural variability and for early assessment. Since RAPD approach is a powerful method for studies genetic relationship and identify molecular marker in plant (Liu et al., 1997) therefore we started with this technique. RAPD is a very general method for obtaining a molecular fingerprint of a strain or species. It is a convenient and sensitive method that is finding increasing application in such fields as epidemeology, molecular genetics, microbial ecology, molecular evolution, and taxonomy. Low-stringency PCR amplification of genomic DNA using a single short primer (10-22 bases) of arbitrary sequence is used to generate a set of fragments that is characteristic of the species or strain from the fortuitous hybridization of a pair of primers on opposite DNA strands in the appropriate orientation, and separated by such a distance allowing efficient PCR (<1,500 bp) (PCR Product Analysis, 1998). The advantages of RAPD method is that it require no prior knowledge of target template DNA sequence (Sediel et al., 2000). Only small amount of DNA is requires. And it can be detected without using any radioactively labeled probe or radiotactivity. The experiment is fast and simple to set-up. These markers would be useful for gene mapping analysis as well as for genetic resource analysis, population studies, and for relative genetic contribution analysis in genetic enhancement or conservation programs of Bamboo. The RAPD technique does not have only advantages. These techniques also have disadvantage. Due to the problem of RAPD such as; inconsistent reproducibility, limiting the comparison of data among laboratories, thus, AFLP has been used to solve some of the RAPD's problem.

AFLP is the technique represents an ingenious combination of RFLP analysis and PCR, resulting in highly informative fingerprints. AFLP is a fingerprinting technique that allows one to distinguish closely related species or subspecies that may be difficult or impossible to differentiate on the basis of morphological or biochemical characteristics. Like RAPD it requires no sequence information from the organism to be investigated. However obtaining the most useful data from AFLP analysis does involve some amount of optimization. Because stringent conditions for PCR are employed in AFLP, it is inherently more reproducible than the RAPD assay (PCR Product Analysis, 1998). In the first step, genomic DNA is digested with a restriction enzyme. In the analysis of plant genomes, where AFLP is currently finding wide use, the

genomic DNA is digested with "six cutter" *Eco*RI and the "four-cutter" *Mse*I. In a genome of 2 x 10^9 bp, such digestion would produce approximately 8 x 10^6 fragments. And then, adapters of a defined sequence are ligated to both ends of all restriction fragments. PCR is then performed using specifically designed primers that allow only a subset of restriction fragment to be amplified. To achieve this, the 5' portion of the primers is made complementary to the adapters; wherever the 3' ends extend for a few, arbitrary chosen nucleotides into the restriction fragment. Exact matching of the 3' end of a primer is necessary for amplification to occur. Therefore, only those restriction fragments are amplified, the ends of which are able to base pair with the 3' primer extension. Statistically, the occur for 1/16 of the total set of fragment in case of a one-nucleotide extension, for 1/256 in case at two, and for 1/4096 in case of three nucleotides. The amplification products are separated on highly resolving sequencing gels or silver staining gel (Weising, 1995).

In these experiment we use RAPD and AFLP technique to identify and characterize *D. asper* from SUT farm. Five out of the 25 samples are good candidate to distribution to farmers all other Thailand. We do hope that this research as a basic research will shine same light to the Bamboo research in Thailand. With our AFLP map, farmer will able to identify their good Bamboo plants.

A new technique that approaches an ideal is amplified fragment length polymorphism, a relatively cheap, easy, fast and reliable method to generate hundreds of informative genetic markers (Ulrich and Wolfen., 1999).

Objectives:

1. Use of RAPD and AFLP techniques to groups and identify *D. asper* in SUT farm.
2. To identity the genetic relationship of *D. asper* species in SUT farm.

CHAPTER II
MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 *Dendrocalamus asper* selection

Several specimen of *D. asper* (Tong Keaw) were selected from Suranaree University of Technology (SUT) farm. In this study we selected 23 clumps of *D. asper* (Tong Keaw) grown in SUT. The characteristics of each clump was investigated such as good taste, large shoot, and high number of shoots (Khumlert and Sukthumrong., 1998). *D. asper* Tong Daum (TD), *Thyrosostachys siamensis* (Pai Rauk) was also studied. Therefore, these specimens were used for the study of genetic information, identification and grouping. The samples comprise of; A11, A13, A15, A22, A25, A27, A31, A32, A34, A41, A42, A51, SUT7, SUT23, SUT25, SUT28, SUT33, SUT35, BC, KN, SA, 5AS1, and S85, and 2 out groups TD and *T. siamensis*.

2.1.2 Chemicals

All chemical used were Laboratory grade or analytical grade

1. Reagent for DNA extraction for RAPD

   Extraction buffer; 1% CTAB, 50mM Tris-HCl (pH 8.0), 10mM EDTA, 0.7 M $MgCl_2$, 0.5%PVP

   chloroform/isoamyl alcohol (24:1)

   10% CTAB ; 10% CTAB, 0.7 $MgCl_2$

   CTAB precipitation buffer; 10mM Tris-HCl (pH 8.0), 1mM EDTA, 1 M NaCl

   70% and 100% ethanol

   1/10 TE buffer; 1mM Tris-HCl (pH 8.0), 0.1mM EDTA

   phenol/chloroform/isoamyl alcohol (25:24:1)

   3M Sodium acetate

2. Reagent for DNA extraction for AFLP

   Lysis buffer; 1%CTAB, 5%PVP, 1.4M NaCl, 20mM EDTA, 10mM Tris-HCl (pH 8.0) and 350mM 2-mercaptoethanol

   phenol:chloroform (1:1)

   TE buffer; 10mM Tris-HCl (pH 8.0), 1mM EDTA

   Chloroform

   Dnase-free Rnase 10 mg/ml

   15% SDS

   95% ethanol, 70% ethanol

3. Reagent for PCR amplification

   Taq DNA polymerase in storage buffer B (Promega), size 500 u, concentration 5u/μl

   25 mM $MgCl_2$

   10X buffer; 20mM Tris-HCl (pH 8.0)m 100mM KCl, 0.1mM EDTA, 1mM DTT, 50% glycerol, 0.5% Tween® 20,and 0.5% Nonidet® -P40.

   Deoxynucleoside triphosphase, 2.5 mM

4. Reagent for agorose gel electrophoresis

   DNA marker; 1 kb Ladder marker DNA, 100 bp Ladder DNA (Promega and GibCoBRL)

   Staining Solution; 0.5 μg/ml ethidium bromide in distilled water

   Loading dye; 0.25% bromocresol purple in 50% glycerol, 0.05M Tris-acetate (pH 7.9)

5. Reagent for PAGE

   5X TBE buffer (1 Lite water); Tris-base 54 g, boric acid 27.5 g, 0.5M EDTA (pH 8.0)

   30% gel (200 ml); cross link 19:1, acrylamide 57 g, bis-acrylamide 3 g

   10% APS (1 ml water); 0.1 g Ammonium per sulfate

   TEMED

6. Reagent for Silver staining

   0.1% CTAB, 25% ammonia, 0.16% silver nitrate ($AgNO_3$), 3M NaOH (sodium hydroxyde), 2% Sodium carbonate, 40% w/v formaldehyde, 1.5-3 % glycerol

7. AFLP loading dye

   Bromophenol blue 5 mg, xylene cyanol 5 mg, formamine 10 ml, 0.5M EDTA (pH 8.0)

8. AFLP core reagent Kit

   EcoR I/*Mse* I; 1.25u/μl each 10mM Tris-HCl (pH 7.4), 50mM NaCl, 0.1mM EDTA, 1mM DTT, 0.1mg/ml BSA, 50% (v/v) glycerol, 0.1% Triton® X-100

   5X reaction buffer; 50mM Tris-HCl (pH 7.5)m 50mM Magnesium-acetate, 250mM Potassium-acetate

   AFLP-grade water

   Adapter/ligation solution; *EcoR* I/*Mse* I adapters, 0.4mM ATP, 10mM Tris-HCl (pH 7.5), 1mM DTT, 50mM KCl, 50% glycerol (v/v)

   TE buffer; 10mM Tris-HCl (pH 8.0), 0.1mM EDTA

9. AFLP Adapters and Starter Primers

   primers for AFLP were produce by GENSET Singapore Biotech. Pte Ltd.

   - Adapters primer and preparation

   *Eco*RI-adapter; Forward 5' CTCGTAGACTGCGTACC-3' ⟶

   ⟵ Reverse 3'- CATCTGACGCATGGTTAA-5'

   mixed 50 μl of forward and reverse primers to 100 μl DI water, heat to 90°C at 1 min, and allow to cool to room temperature slowly. This gives a final concentration of 25 pmoles/μl and makes enough adapter for 200 ligations reaction.

   *Mse*I adapter; forward 5' GACGATGAGTCCTGAG-3' ⟶

   ⟵ reverse 3'-TACTCAGGACTCAT-5'

   - Starter primers

   The basic *EcoR*I primer is ; 5' GACTGCGTACCAATTCx yz -3'

   where x, y, and z represent the selective bases on the 3' end of the oligo.

The basic *Mse*I primer is ; 5' GATGAGTCCTGAGTAACACx yz-3'

Like with the *Eco*RI primer x, y, and z represent the selective bases on the 3' end of the oligo.

We use 3 bp selective extensions in our *Eco*RI and *Mse*I primers.

2.2 METHODS

2.2.1 Plant DNA extraction for RAPD

DNA was extracted from 21 clumps of *D. asper* by CTAB (cetyltrimethylammonium bromide) method (Neal et al., 1993). Briefly, 500-800 mg of fresh leaf tissue were ground to fine powder with mortar and pestle in liquid nitrogen. The powder were transferred to a 15 ml microcentrifuge tubes containing 1 ml cold extraction buffer (1% CTAB, 50mM Tris-HCl, pH 8.0, 10mM EDTA, 0.7M NaCl, and 0.5% PVP). The tubes were incubated for 15-30 min in a 60°C water bath. After cooling, 1ml of chloroform/isoamyl alcohol (24:1) were added. They were mix gently, and centrifuge at 12,000 rpm for 5 min at 4°C. The supernatant were then removed to a fresh 1.5 ml centrifuge tube. One tenth volume of 10% CTAB (10% CTAB and 0.7 M NaCl) were added and then the chloroform extraction procedure were repeated as in previous step. One volume of CTAB precipitation buffer (1% CTAB, 50mM Tris-HCl, pH 8.0 and 10mM EDTA) were added to each tube then they were mixed gently and centrifuge at 12,000 rpm for 5 min at 4°C in a microcentrifuge. The supernatant was removed and the pellet was resuspended in 0.4 ml of high salt TE (10mM Tris-HCl, pH 8.0, 1mM EDTA and 1M NaCl) at 65°C for 1 hr. The DNA was then reprecipitated by adding 2 volume of ice-cold absolute ethanol. Each tube was centrifuged at 12,000 rpm for 5 min, the supernatant was removed and the precipitate was washed with 100% and 70% ethanol respectively.

The DNA was dry and dissolved overnight in 250 μl of 1/10 TE (1mM Tris-HCl, pH 8.0 and 0.1mM EDTA). One volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added and centrifuge at 12,000 rpm for 5 min. The aqueous phase was then removed to a fresh tube and extracted with chloroform/isoamyl alcohol (24:1), spin for 5 min at 12,000 rpm, and removed the aqueous phase to a fresh tube. One-tenth volume of 3 M sodium acetate was added followed by 2

volumes of absolute ethanol to precipitate the DNA. The tube was centrifuge as 12,000 rpm for 5 min before then washed with 100% and 70% ethanol, and dry respectively. The pellets were dissolved in 50 μl 1/10 TE overnight. The DNA was run in an agarose gel to determine the quality and amount.

In most studies DNA is extracted from young plant material. Both yield of DNA and quality are generally better when young leaves are used. (Gieslis et al., 1997)

2.2.2 RAPD method

A modified RAPD method was used with Perkin-Elmer thermocycler. Briefly, each 25 ul reaction consisted of buffer (50mM KCl, 10mM Tris-HCl, 1.5mM $MgCl_2$ and 0.1% TritonX-100), 2 to 10 ng template DNA, 5mM each deoxynucleotide triphosphate (dNTP), 2 pmole of 10-mer primer and 0.25 U *Taq* polymerase. The amplification condition were 35 cycles of 94°C for 30 sec, 36°C for 30 sec, and 72°C for 30 sec with a 94°C 5 min pre run and 7 min of 72°C post run (applied from Phongdara et al, 1999). Amplified DNA products were separated on 2% agarose gel in 1X TBE buffer at 80 V for 50 min, stained with ethidium bromide and visualized under UV light. Eighteen clumps of *D. asper* was initially screened for polymorhisms with 50 RAPD primers from L, AE and S Operon primer Kits. (Dha et al., 1997)

2.2.3 DNA extraction for AFLP

DNA extractions for AFLP analysis were performed following with the GIBCO BRL AFLP Analysis System I (Cat. No. 10544) protocols. Briefly, quickly freeze 0.3 to 0.5 g fresh plant tissue in liquid nitrogen and grind to fine powder using a mortar and pestle, 5 ml of lysis buffer were then added to the tissue. An equal volume of phenol:chloroform (1:1) were added to the solution and mix well by inversion. These samples were then centrifuge at 4,000 rpm for 5 min. The aqueous layer remove and place in a new tube. And then equal volumes of chloroform were added and again mix well by inversion. The sample were then centrifuged at 4,000 rpm for 5 min. The aqueous layer were removed and place in a new tube again. Two and a half volume of 95% ethanol were added and mixed well by inversion to precipitate DNA. The samples were again centrifuge at 4,000 rpm for 5 min and liquid were removed. The pellets were rinsed with 1

ml of 70% ethanol and dried. Finally, resuspend the pellet in 50 μl TE buffer [10 mM Tris-HCL (pH 8.0), 1 mM EDTA]. To determine DNA concentration, analyze a 2 μl sample on a 1% agarose gel and quantitative against a known standard.

2.2.4 AFLP assay

AFLP assays were apply performed with the GIBCO BRL AFLP Analysis System I (Cat. NO. 10544) as recommended by the manufacturer. Briefly, 150 ng of genomic DNA was digested with *Eco*R I and *Mse* I overnight and the enzymes were inactivated at 70°C for 15 min. The DNA fragments were ligated with 5 μl *Eco*R I and M*se* I adapters for 3 hr. For preselective amplification, 5 μl of 10 fold-diluted ligation mixture was amplified with 20 cycles of 94°C for 30 sec, 56°C for 60 sec and 72°C for 60. For selective amplification, 5 μl of 1/20 diluted of preselective amplified were use for the template. The mixtures were amplified for 1 cycle of 94°C for 30 sec, 65°C for 30 sec, 72°C for 60 sec, then lowering annealing temperature 0.7°C each cycle for 12 cycles; and then 30 cycles of 90°C for 30 sec, 56°C for 60 sec, 72°C for 60 sec. 9 μl of the selective amplification were added to 8 μl of AFLP loading buffer (98% formamide, 10mM EDTA, 0.1% bromphenol blue, 0.1% xylene cyanol). Then the mixture were heat at 95°C for 5 min, and 9 μl of the sample were electrophoreses in a 6% polyacrylamide sequencing gel with 8 M urea. Normally, the gel was electrophoresis at 250 constant volts for 4 hr.

2.2.5 Silver stainig

The AFLP electrophoresed gels were analyze by silver staining. Briefly, each gel was rinsed in water for 3 to 5 min. Then soak under gentle agitation for 30 min in 0.1% CTAB. The gel was then incubate in 0.3% ammonia for 15 min under gentle agitation (1.3 ml stock solution per 100 ml solution). During this step silver solution was prepared in a flask with a magnetic stirrer; dissolve 1.6 g $AgNO_3$ in 1-liter water and add 1.3 ml 3M NaOH. The solution will turn cloudy and brownish. Add 3.5 to 4.0 ml of 25% ammonia to the silver solution drop by drop till the solution has cleared and add another two drops of 25% ammonia. Then, discard of the ammonia solution from the gel, and add the freshly prepared silver solution directly to the gel.

Incubate under gentle agitation for 20 min. Prepare the developing solution (2% sodium carbonate, 0.02% formaldehyde, Sodium carbonate is first dissolved with intense stirring, than the formaldehyde is added). Rinse the gel briefly in H_2O. Then added the developer to the gel. Staining takes 5 to 25 min under gently agitation. Stop the staining process by a quick rinse with water, and fix the gel in 1.5 to 3.0% glycerol for 30 min. The gel can now be photographed or dried for further analysis (Weising et al., 1995).

2.2.6 DNA analysis

The RAPD and AFLP banding profiles of each *D. asper* was scored manually for the presence (1) or absence (0) of bands (Goto et al., 1998). These qualitative data were entered into the software package NTSYS V 2.1 program, using Qualitative data module for calculated the similarity, cluster analyses by SHAN modules and using the unweighted pair group method with arithmetic averages (UPGMA program) and a dendrogram construction was perform.

CHAPTER III
RESULTS

3.1 RAPD analysis

Fifty primers (Operon AE, L and S kits) were tested in initial screening experiments with 18 genotypes of *D. asper*. Twenty-three primers were selected on the following grounds: distinct bands, clear pattern, distinct polymorphism between species, and the presence of bands, common to all genotypes tested. Optimal PCR conditions were also investigated, as small variations in annealing temperature, DNA concentration and $MgCl_2$ concentration are known to alter even major bands in RAPD fingerprints (Ellsworth, *et al*. 1993).

Example of RAPD products, run on a 2% agarose TBE gel are shown in figure 2. The 23 primers used to create this kind of pattern for each of the 18 isolates of *D. asper* are given in table 1. Major and minor RAPD fragments ranging from 300-1500 base pairs were found to be polymorphic. A total of 160 amplified bands were produced of which 121 were polymorphic, with an average of 6.72 major bands per primers. Genetic diversity and relatedness were determined by scoring presence and absence of amplified products of certain size, after total genomic DNA had been amplified by certain primers (Smith, *et al* 1997).

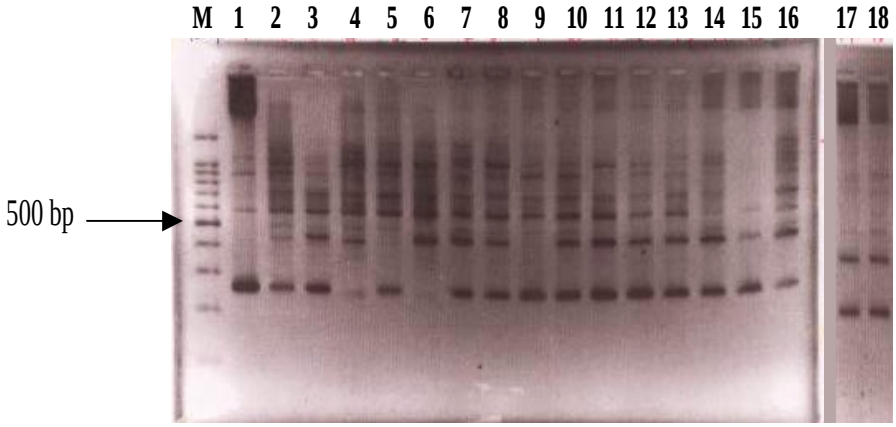


Fig. 2 RAPD-PCR fingerprint patterns of 18 clones of *D. asper*.

DNA extraction and PCR conditions were as describe in chapter II.

Lane M ; 100 bp ladder marker, Lane 1 ; A11, Lane 2 ; A13, Lane 3 ; A15, Lane 4 ; A21, Lane 5 ; A22, Lane 6 ; A25, Lane 7 ; A26, Lane 8 ; A27, Lane 9 ; A31, Lane 10 ; A32, Lane 11 ; A34, Lane 12 ; A35, Lane 13 ; A41, Lane 14 ; A42, Lane 15 ; A51, Lane 16 ; SUT 7, Lane 17 ; TD and Lane 18 ; KN

Table 1. Primer pairs used for RAPD (sequences, range of fragments, GC content and number of fragments)

number	Primer name	Sequences (5' ⟶ 3')	GC Content (%)	No. of Fragments observed	Range of fragments approximately(bp)
1	OPL 07	AGGCGGGAAC	70	7	500-1500
2	OPL 08	AGCAGGTGGA	60	7	400-1500
3	OPL 12	GGGCGGTACT	70	5	500-1200
4	OPL 17	AGCCTGAGCC	70	8	200-1400
5	OPL 18	ACCACCCACC	70	9	300-1500
6	OPL 20	TGGTGGACCA	60	7	500-1400
7	OPAE01	TGAGGGCCGT	70	7	500-1400
8	OPAE 03	CATAGAGCGG	60	6	700-1400
9	OPAE 04	CCAGCACCTTC	70	9	400-1500
10	OPAE 06	GGGGAAGACA	60	5	400-1400
11	OPAE 08	CTGGCTCAGA	60	9	800-1500
12	OPAE 11	AAGACCGGGA	60	8	400-1500
13	OPAE 14	GAGAGGCTCC	70	8	400-1500
14	OPAE 15	TGCCTGGACC	70	8	600-1500
15	OPAE 16	TCCGTGCTGA	60	10	400-1500
16	OPAE17	GGCAGGTTCA	60	5	300-1500
17	OPAE18	CTGGTGCTGA	60	7	600-1400
18	OPAE19	GACAGTCCCT	60	6	400-1500
19	OPAE20	TTGACCCCAG	60	7	600-1500
20	OPS 1	CTACTGCGCT	60	5	700-1500
21	OPS 3	CAGAGGTCCC	70	7	300-1500
22	OPS 7	TCCGATGCTG	60	3	800-1500
23	OPS 9	TCCTGGTCCC	70	7	400-1500

3.2 Analysis of RAPD data generated by a single primer

RAPD detects genetic variability between species with very high efficiency. Every primer tested yielded polymorphisms between species. To illustrate the necessity of using a wide range of primers when making an RAPD fingerprint, two dendrograms were made. One was based on the information yielded by one single PCR reaction using only one primer; OPAE 1. The other was made using twenty three of primers. In figure 3 some specimens could not be separated from each other, whereas figure 4, using more data from more primers could successfully distinguish between all test subjects.

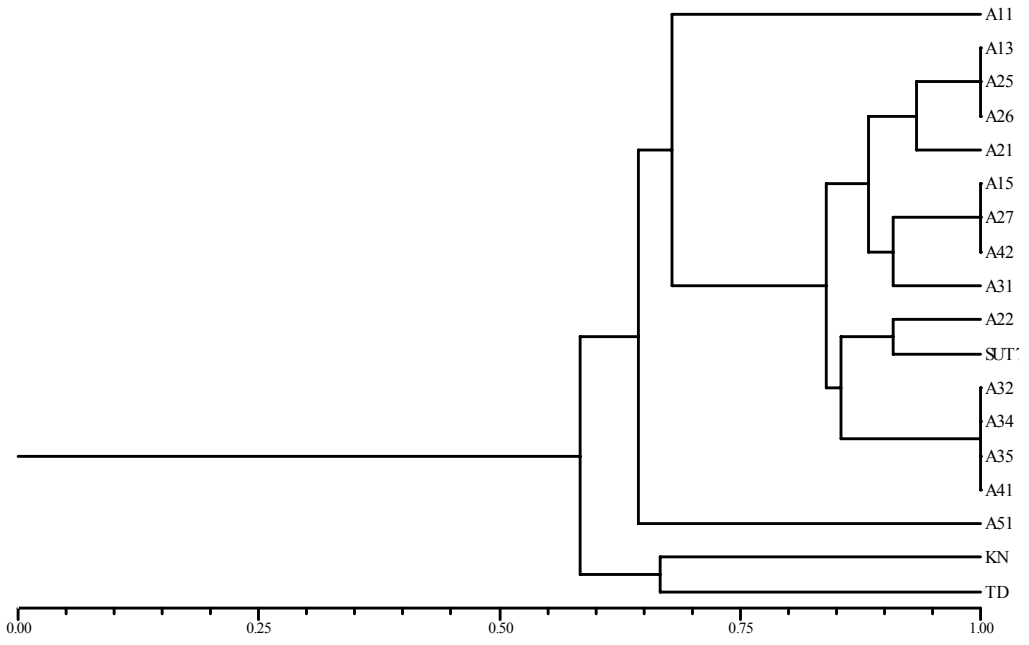


Figure 3. Dendrogram showing the relatedness of *D.asper* obtained by RAPD analysis using only one primer

When looking at the dendrogram in figure 3, the sample can be group in to five groups can be distinguished. The first consists of the specimens A13, A25, A26, A21, A15, A27, A42 and A31. The second contains A22, SUT7, A32, A34, A35 and A41. These two big groups were related with the other group A11. The fourth group is only A51. And the fifth groups are KN and TD was separated from the big groups. Here it must be noted that using this primer, the most of specimens such as A13, A25, and A26; A15, A27, A42 and A32, A34, A35, and A41 could not be separated from each other at all. Therefore, one primer it is not enough for complete separation, distinction and identification.

3.3 Analysis of RAPD data generated using various primers

A dendrogram of *D. asper* specimens collected from the SUT farm (figure 4) shows that KN and TD are unrelated to any of the other specimens. A11, A32, A34 and A35 are very closely related. A41, A51 and SUT7 are another closely related group. A third group, consisting of A13, A42, A21, A25, A15, A22, A26, A27, and A31 was found. These three groups are all fairly closely related when compare to isolates KN and TD which is a fourth group further removed from the rest.

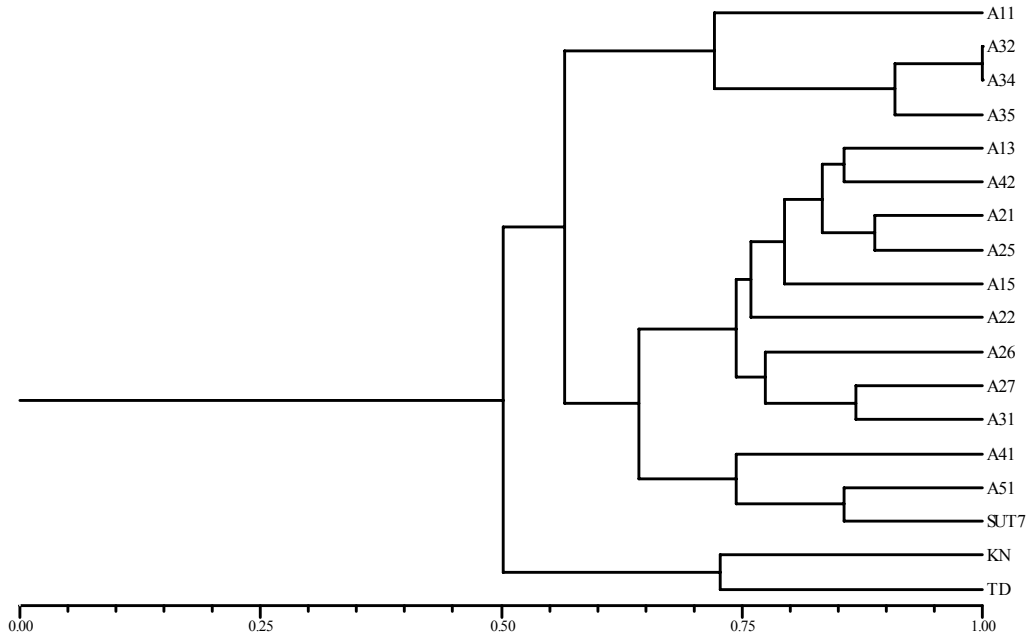


Figure 4. Dendrogram showing the relatedness of *D. asper* obtain by RAPD analysis

3.4 RAPD data of specimen which have good character

Because 5 specimens (A11, BC, KN, 5AS1 and S85) have good character such as good taste, large shoots, and high number of shoots, which are economically important. Therefore, RAPD fingerprints were made of these specimens to used as marker. These 5 specimen were selected by Khumleart, *et al*. 1998.

All steps in the procedure (DNA isolation, quantification and PCR conditions) were performed on only these 5 specimens. The optimal primers yielded 55 major and minor RAPD fragments which were scored for presence (1) or absence (0). These data was than analyzed. Example data are shown in figure 5, 6 and 7 for the RAPD polymorphism and dendrogram respectively.

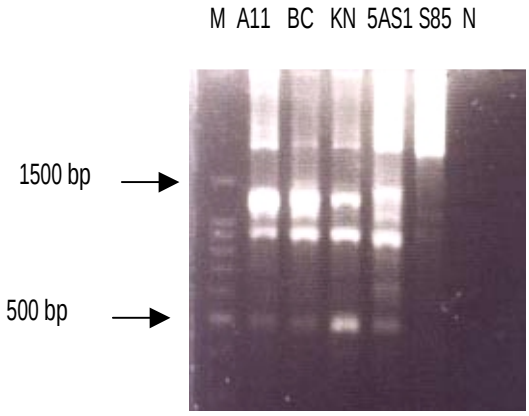


Figure 5. Agarose gel electrophoresis of amplified fragments from a RAPD reaction using primers OPAE 07 Lane M; 100 bp marker ladder, Lane1; A11, Lane2; BC, Lane3; KN, Lane4; 5AS1, Lane5; S85, and Lane N; negative control

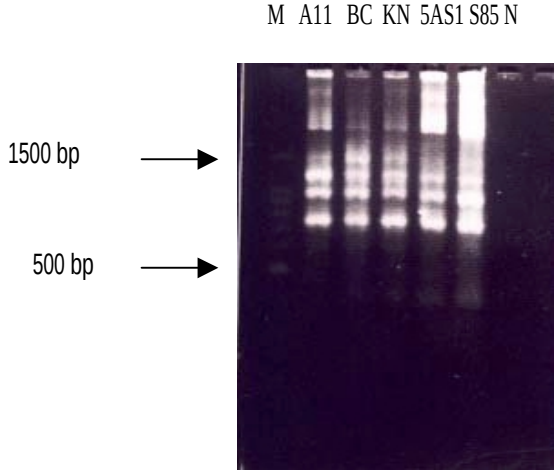


Figure 6. Agarose gel electrophoresis of amplified sequences from a RAPD reaction using primers OPL 01 Lane M; 100 bp marker ladder, Lane1; A1/1, Lane2; BC, Lane3; KN, Lane4; 5AS1, Lane5; S85, and Lane N; negative control

3.5 RAPD analyzed by computer program

When a number of primers were combined, 5AS1 and S85 were found to be similar and therefore closely related to A1/1. They are also quite closely related to KN but BC was separated from the other specimens (figure **7**). Thus, combined primers (15 primers) were tested and analyzed. The results are that every specimen can be separated. The specimens KN and S85 were close to BC. On the other hand A1/1 and S85 grouped closely together and belong in another group (figure 8)

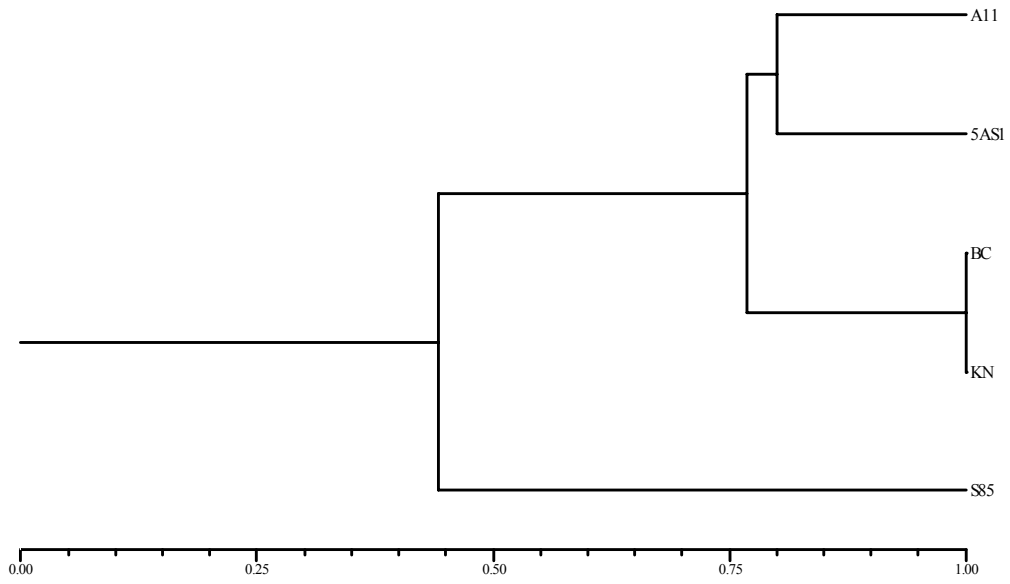


Figure 7. Dendrogram showing the relatedness of 5 good characteristic of *D.asper* obtained by RAPD analysis using primer OPL20

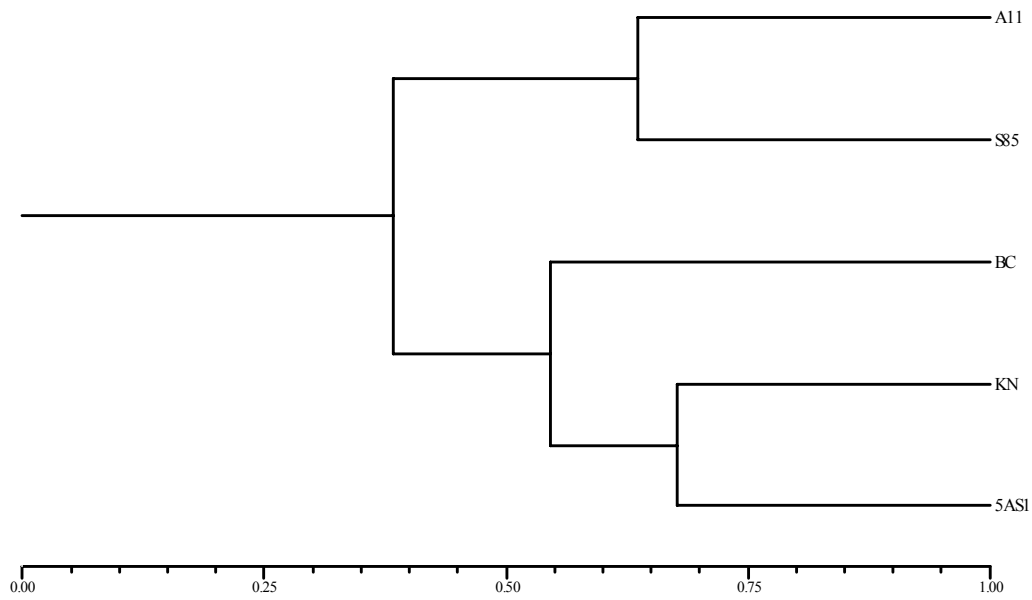


Figure 8. Dendrogram showing the relatedness of 5 good characteristic of *D.asper* obtained by RAPD analysis using primer shown in table 1

3.6 Reproducibility of RAPD

The reproducibility of our method is shown in figure 9 and figure 10. Replicate templates and PCR runs of the primers test ensured reproducibility and reliability in high molecular weight region. Primers OPAE 1, OPL15, OPL 18, OPL 20 and OPS 9 were used for the reproducibility test. The test was performed by extracting the template DNA from each sample at two different time with the same protocol (see material and method). The results show that most of the major bands are pretty much the same for each sample. With the OPAE1 and OPL 20 primers show the most highly reproducible results in figure 9 and 10 respectively.

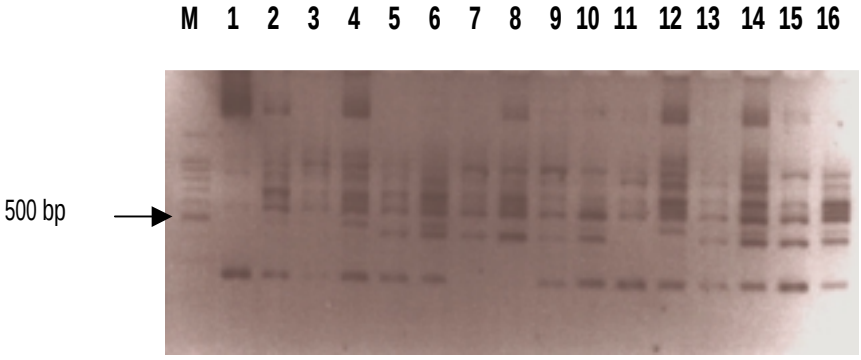


Figure 9. DNA fingerprint pattern reproducibility of RAPD-PCR by primer OPAE 01
Lane M; 100 bp marker ladder, Lane 1; A11 old and Lane 2; A11 new, Lane 3; A13 old and Lane 4; A13 new, Lane 5;A15 old and Lane6; A15 new, Lane 7; A25 old and Lane 8; A25 new, Lane 9; A27 old and Lane 10; A27 new, Lane 11; A31 old and Lane; 12 A31 new, Lane 13; A32 old and Lane 14; A32 new , Lane 15; A34 old and Lane 16; A34 new.

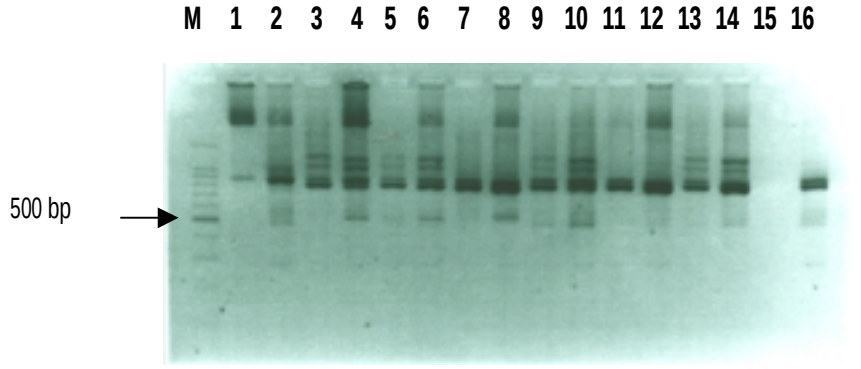


Figure 10. DNA fingerprint pattern reproducibility of RAPD-PCR by primer OPL 20
Lane M; 100 bp marker ladder, Lane 1; A11 old and Lane 2; A11 new, Lane 3; A13 old and Lane 4; A13 new, Lane 5;A15 old and Lane6; A15 new, Lane 7; A25 old and Lane 8; A25 new, Lane 9; A27 old and Lane 10; A27 new, Lane 11; A31 old and Lane; 12 A31 new, Lane 13; A32 old and Lane 14; A32 new , Lane 15; A34 old and Lane 16; A34 new.

3.7 AFLP analysis

Twenty three specimens of *D. asper* (A11, A13, A15, A22, A25, A27, A31, A32, A34, A41, A42, A51, SUT 7, SUT 23, SUT 25, SUT 28, SUT 33, SUT 35, BC, KN, SA, 5AS1 and S85) and 2 out groups (*Thyrosostachys. siamensis* and *D. asper* Tong Daum) were selected from the SUT farm for DNA fingerprints. The selected primers used in this AFLP DNA fingerprinting for analysis were shown in table 2**.**

Table 2 . Primer pairs used; sequences, range of fragment sizes, and number of fragment for AFLP analysis

Identification primer pairs	Primer sequences 5' to 3'	No. of fragment observed	Range of fragment sizes approximately (bp)
1. M-CAC E-ACA	GATGAGTCCTGAGTAACAC GACTGCGTACCAATTCACA	8	100-2000
2. M-CAA E-ACC	GATGAGTCCTGAGTAACAA GACTGCGTACCAATTCACC	14	50-2500
3. M-CAA E-AGG	GATGAGTCCTGAGTAACAA GACTGCGTACCAATTCAGG	11	100-2500
4. M-CAC E-AGC	GATGAGTCCTGAGTAACAC GACTGCGTACCAATTCAGC	8	50-2000
5. M-CTT E-AGG	GATGAGTCCTGAGTAACTT GACTGCGTACCAATTCAGG	18	25-2600
6. M-CTC E-AGG	GATGAGTCCTGAGTAACTC GACTGCGTACCAATTCAGG	10	125-2000
7. M-CAG E-AGC	GATGAGTCCTGAGTAACAG GACTGCGTACCAATTCAGC	13	50-2600
8. M-CTG E-ACC	GATGAGTCCTGAGTAACTG GACTGCGTACCAATTCACC	18	500-2600
9. M-CAG E-ACG	GATGAGTCCTGAGTAACAG GACTGCGTACCAATTCACG	15	100-1000
10. M-CTA E-ACG	GATGAGTCCTGAGTAACTA GACTGCGTACCAATTCACG	10	100-200
11. M-CTG E-ACG	GATGAGTCCTGAGTAACTG GACTGCGTACCAATTCACG	11	50-2500
12. M-CTC E-ACG	GATGAGTCCTGAGTAACTC GACTGCGTACCAATTCACG	12	100-1000

For DNA fingerprint analysis, the strategy developed by GIBCO KIT (cat. No. 10544) and modified by Kasetsart University (AFLP workshop) was used. Non-radioactive (silver stained) representative DNA fingerprinting gel is shown in Figure 11a and 11b respectively. Twelve primer pairs were selected from 64 potential primer pairs. Averages of 13 DNA fragments were analyzed per *D. asper* specimens. All fingerprint data was analyzed using the software program NTSYS V. 2.1. Bands of the top and bottom of the gel (area above and below arrow in figure 11) were ignored in the band calling process because of band compression and distortion.

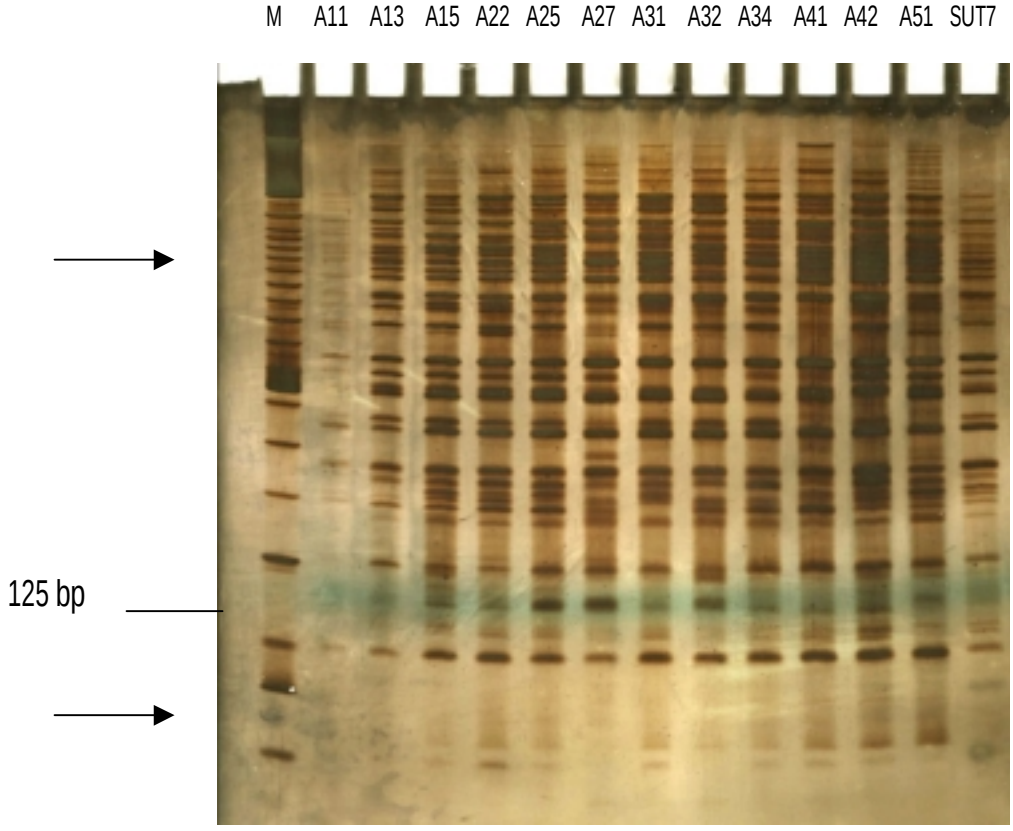


Figure 11a. AFLP fingerprint pattern of 23 specimens of *D. asper* and 2 specimens of out group using primer pair 8 from table 2 run on 6% acrylamide urea gel

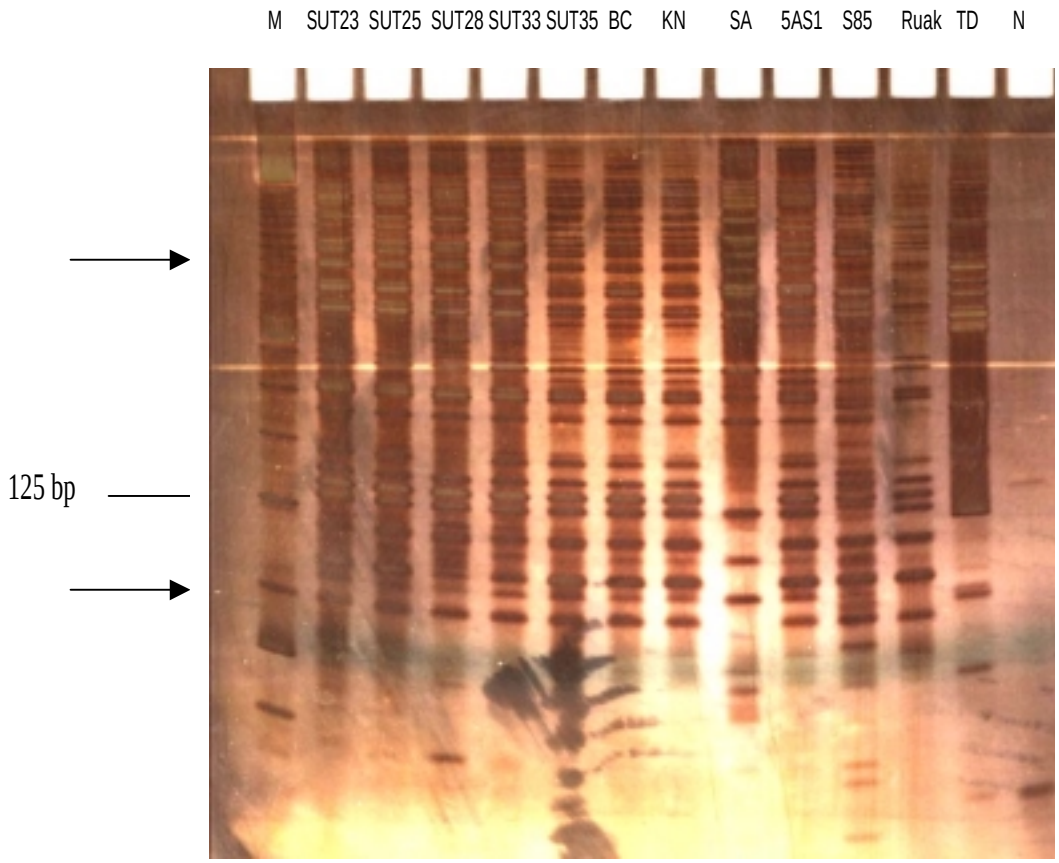


Figure 11b. AFLP fingerprint pattern of 23 specimens of *D. asper* and 2 specimens of out group using primer pair 8 from table 2 run on 6% acrylamide urea gel

When single primers do not yield distinct polymorphism a lot of primers may be used, and their information may be combined (Hu, *et al*. 1995). In our experiments good results were obtained for *D. asper* when the information from more than one primer pair was combined as was the case in RAPD analysis. When primer pair 8 were use the AFLP and dendrogram were not able to separate some of the specimen. For example A11, A22, and TD were identical; A15 and SUT7 were identical; A13, A25, A34, SUT 25 and 5AS1 were identical and SUT23, SUT35, KN and BC were identical. SA was the further remove from the group but still with only 0.65 value of coefficient. Thus, combined primers (total of 12 primers from Table 2) were tested and analyzed. The result shows we can separate each the specimen form the other. The TD was the least related to any other groups, as shown in figure 12.

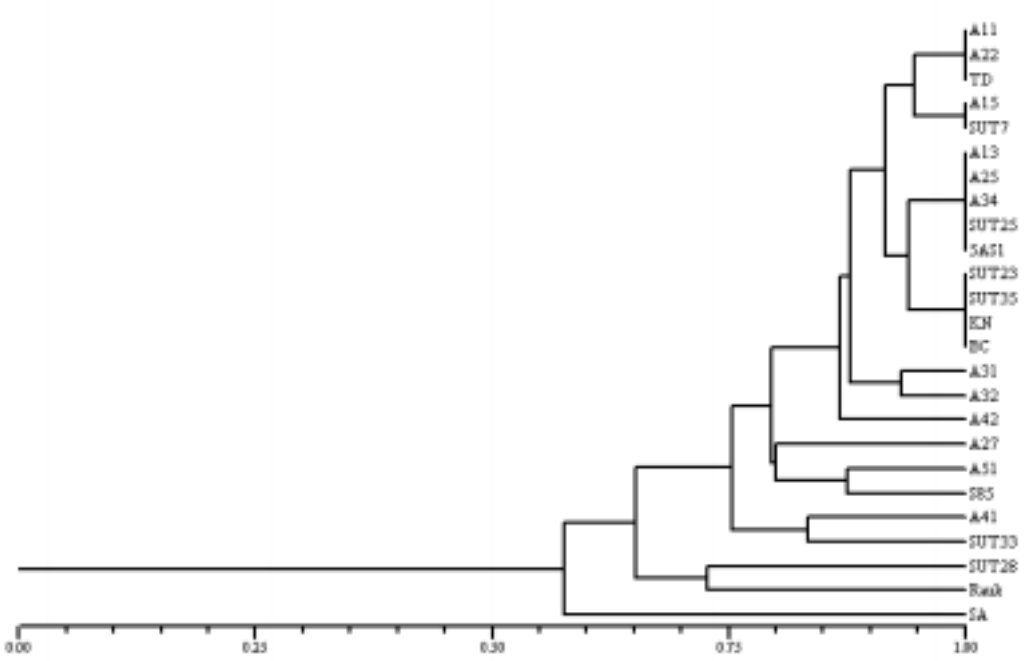


Figure 12. Show the AFLP dendrogram obtain by primer pair 8 in table 2

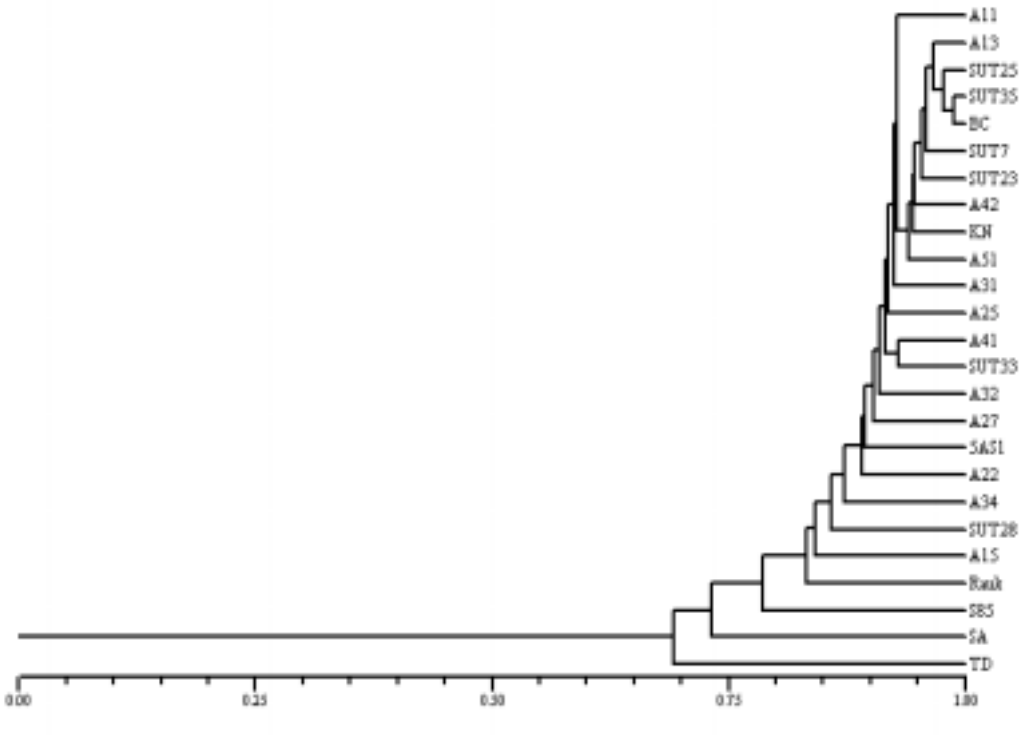


Figure 13. Show the AFLP dendrogram obtain by combined 12 primers in table 2

3.8 Identification of unknown genotypes by AFLP

Identification of unknown genotypes lists based upon the primers selected constructed with all genotype include in our study. Genotype identification of 4 *D. asper* propagate by tissue culture (by SUT researches) were needed. Since the originators of the tissue culture sample were lost, our DNA fingerprinting should be able to identify them. To tested the identification procedure the 4 unknown genotypes from *D.asper* tissues culture fingerprinted using the AFLP technique the fingerprint of A11 was also perform at the same time for comparison. Four primers were selected for testing (primer pairs 9, 10, 11, and 12 in Table 2). The four sets of primer were selected because from the previous AFLP study the resulting of these group can detected.

DNA fingerprinting gel is shown in figure 15 and the dendrogram construction is present in figure 14.

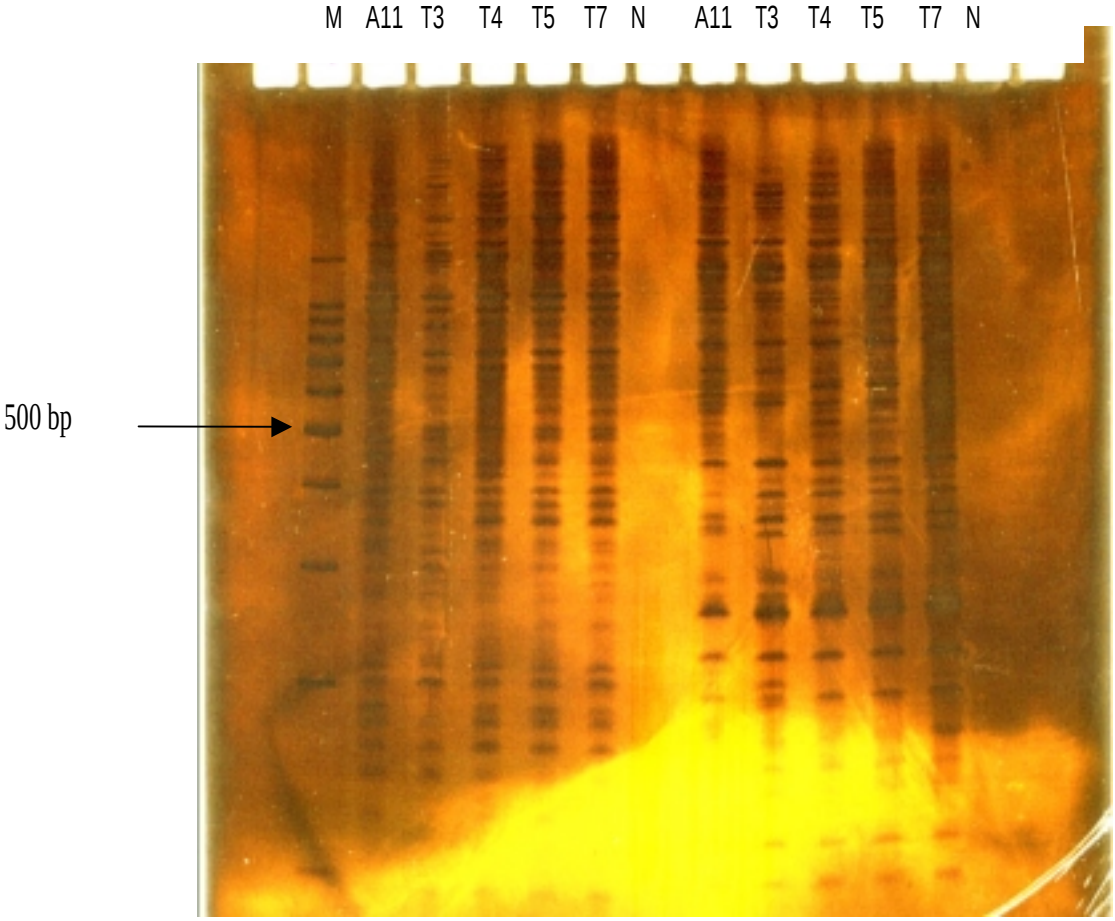


Figure 14. Fingerprint pattern of AFLP by 2 primers pair (primer11 and 12) from table 2.
M; 100 bp ladder marker and N; negative control

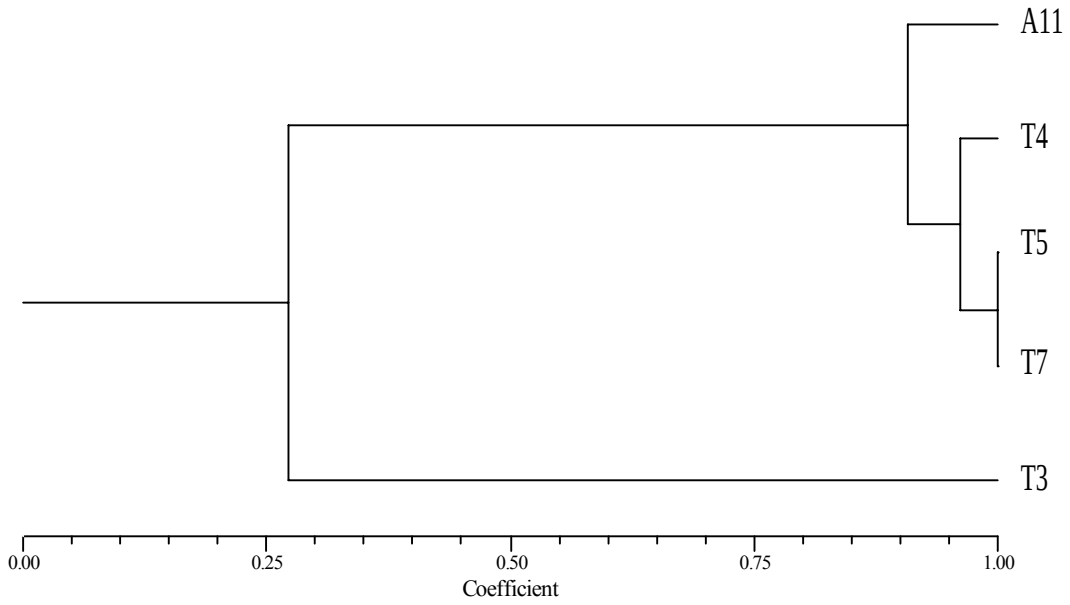


Figure 15. Dendrogram showing the relatedness of D. asper obtain by AFLP analysis

The dendrogram in figure 14 show that tissue culture 5 and 7 have the same genetic background. The tissue culture sample T4 is closely related to the T5 and T7 with only 0.97 value of coefficient. They are all related to the A11 and sample with only 0.92 value of coefficient. Therefore, we might be able to conclude that T4, T5 and T7 are tissue culture sample derived from the A11 progenitor.

3.9 Phenotypic and Genotypic analysis

The morphology of bamboo specimens used in RAPD and AFLP methods were shown in figure 16. More over the good characters such as shoot weight and sweet taste were scored in table 3. We also investigated the phenotype and genotype correlation. We were not able to find any correlation between the phenotype and genotype.

a. A11

b. KN

c. BC

Figure 16. Morphology of Bamboo specimens in SUT farm

d. SUT23 **e. KN shoot**

Figure 16. Morphology of Bamboo specimens from SUT farm

Table 3. The bamboo scored of shoot weight and sweet taste

Specimens	Shoot weight (kg/shoot)	Sweet taste (out of 10)
A11	1-1.5	8
A15	N/A	6
A21	1.5-2	4
A22	2	6
A25	N/A	7
A26	N/A	3
A27	N/A	9.5
A31	1.5-2	7
A32	N/A	4
A34	N/A	5
A35	N/A	7
A41	2-3	6
A42	2-3	7.5
A51	1.5-2	7

(N/A: data not available)

CHAPTER IV
DISCUSSION

The RAPD-PCR method can be applied to identify genetic similarity and diversity in *D. asper* using random primers. In this study, genomic DNA bands were produced by amplifying DNA, isolated form a young leaf of *D. asper*. DNA fragments sizes of 200-1500 bp were used in the scoring. Only three primers generated highly reproducibility. RAPD polymorphic fragments, producing an average of 70% polymorphic band per primer in our *D. asper* population.

The bands in the molecular weight range from 200-1500 bp generated by 23 primers were observe and shown in figure 2. Generally, the number and size of the fragment generated strictly depend on the nucleotide sequence of the primer used and upon the source of the template DNA, resulting in the genome-specific fingerprints of random DNA fragments. The polymorphism were scored by the presence or absence of DNA fragments by various primers.

The RAPD method of DNA fingerprinting has become quite popular and has been successfully to many plant and fungi population. However, several caveats of the procedure must be kept in mind. First, there is the problem of reproducibility not only among laboratories but within a laboratory over time, and this single problem, although not insurmountable, makes the development of a common database difficult. Virtually every methodological aspect of PCR can effect reproducibility. Artifactual variation can occur as a result of small differences reaction, and the concentration of magnesium in the reaction mixture. Changes in those parameters affect most notably the presence of low-intensity bands but can also affect the position and intensity of high intensity bands (Loudon et al., 1994). This may explain the general result that even in the same laboratory, using the same thermocycler and reagents, variation occurs in the low-intensity bands. Even more disturbing are reports of variation due to the *Taq* enzyme source (Meunier et al., 1993). Louden et al., 1995 who have used the RAPD methodology to fingerprint some strain of fungi isolates in a number of studies, reported that different lots of *Taq* polymerase resulted in great enough variability. Evidence was presented that one enzyme lot could not discriminate among isolated while the other could. RAPD has been used to fingerprint strains of serovars of *Bacillus thuringiensis*, the most commonly used biological insecticide. Similarly, the technique

has been used to examine clinically important strains of the other bacterial species. Plants are particularly suited to RAPD analysis. Thus the technique has been used extensively in plant breeding studies, and applications to strawberries, wheat, oat, barley, soya bean, tomato, potato and corn have all been reported (Deragon and Landry., 1992).

The results of this studies show that RAPD is a powerful technique, able to differentiate closely related species reproducibility was found with same primers. But due to the weak points of RAPD, AFLP was the other method used. AFLP is a promising modification of RAPD for fingerprinting.

The advantage of AFLP method is its efficiency and low cost when compared to RFLP technique. The selective primers used for AFLP amplification do not require information about DNA sequence same as RAPD; there fore, the cost for primer generation is low when compared to other sequence-specific such as STS and SSR marker. AFLP fingerprints will often be used as a source for DNA markers. The molecular basis for AFLP polymorphisms will most frequently be sequence polymorphisms at the nucleotide level. Single nucleotide changes will be detected by AFLP when either restriction sites themselves or nucleotides adjacent to the restriction sites are affected, causing the AFLP primers to mispair at the 3' end and preventing amplification (the selective nucleotides do not exactly match the sequence next to the restriction site). In addition, deletions, insertions and rearrangements affecting the presence or size of restriction fragments will lead to polymorphisms detected by AFLP (Vos et al., 1995). In our case, three selective nucleotide were added to each primer giving us the ability to use 64 different *EcoR* I and *Mse* I selective primers in 4096 different pair wise combination. However, with our limited time and funding 64 primer pairs were tested and 12 with good result were selected. And then analyzed the relationship by computer program (NTSYS V. 2.1), the AFLP data were classified as similar species, close related and other different strains. Huys et al., 1996 and Jiang et al., 1999 reported that AFLP is a high-resolution DNA fingerprinting technique that has been effectively used to fingerprinting of *Vibrio cholerae*. The results of these studies show that AFLP is a powerful technique able to differentiate closely related strains (Arias et at., 1997). Sixty-seven *V. cholerae* recovered form Chesapeak bay in April through July were fingerprinted (Jiang et al., 2000). Similar to the previous tested from Janssen et al., 1996 and Keim et al., 1997 which used AFLP technique for distinguish closely related bacterial strain. Due to the higher specificity of AFLP

primer when compare to the RAPD primer, the low reproducibility problem was solved. In this study, we also used the RAPD and AFLP data to calculate genetic similarities base on band sharing. Wayne et al., 1996 have shown that DNA fingerprinting using RAPD and AFLP gave a highly similar result which is correlated to our experiments.

Our results indicated that *D. asper* (Tong Keaw) grouping is closely associated to *T. siamensis* (Pai Ruak) but less related to *D. asper* (Tong daum). It would be interesting to test other species belonging to this section on a morphological basis, or which have intermediate characters.

At present it is difficult to say how many of the new species described are true species or merely forms of existing species. Our data show that variation between species is considerable with all primers tested. All the species tested could be regarded as good species, but in any case a good species definition is lacking in this genus, as it is in bamboo general. While the variability between species is large, this is not so with forms and varieties.

Nevertheless one should expect sensitive marker such as RAPD or AFLP to detect such polymorphism, since forms and varieties of a species are readily visible, and this certainly so in the groups we used.

However, if there is an unknown sample, using our methods we demonstrated that we can identify the progenitor of that sample. This is a very important aspect for the future, if SUT can develop good Bamboo strain for the Thai farmers.

For a wide range of taxa, including plants, fungi, animal and bacteria, AFLP markers have been used to uncover cryptic genetic variation of strain, or closely related species, that had been impossible to resolve with morphological or other molecular systematic characters (Russell et all., 1997).

For example, AFLP methods were shown to be superior to classic systematic methods in nematodes (Semblat et al., 1998) have allowed finer differentiation of microorganisms (Janssen et al., 1997). For close related species, AFLP markers have also been used to infer phologenetic relationships based on measures of genetic distance (Powell et al., 1996 and Tohme et al., 1996).

CHAPTER V
CONCLUSION

This study is the first report to provide essential basic genetic information of *D. asper*. In the present study, RAPD and AFLP were found to be efficient enough to reveal usable level of DNA polymorphism and were used to investigate these genetic information.

The results obtained from this research can be summarized as follow.

1. The 18 clones of *D. asper* from SUT (A11, A13, A15, A21, A22, A25, A26, A27, A31, A34, A35, A41, A42, A51, SUT7, *D. asper* Tong Daum, and KN) can identify by RAPD method. Dendorgram of RAPD patterns could divide *D. asper* species into 17 groups which were classified as similar species, closely related and other different strains.
2. Five good characteristic plants such as; good taste, large shoots and high number of shoot can be identify by RAPD. The five lines (A11, BC, KN, 5AS1 and S85) are significantly distinct from each other.
3. The 23 clones of *D. asper* from SUT [A11, A13, A15, A22, A25, A26, A27, A31, A32, A34, A41, A42, A51, SUT7, SUT 23, SUT 25, SUT 28, SUT 33, SUT 35, BC, KN, SA, 5AS1 and S85) and 2 out groups *D. asper* (Tong Daum) and *T. siamensis*] were tested by AFLP technique. AFLP information can be used to establish genetic relationships.
4. Four unknown specimen from the tissue culture samples (T3, T4, T5 and T7) were tested by AFLP and compared the fingerprinting with A11. We found that the information from AFLP can be identify the genetic relationship of these samples.

Therefore, RAPD and AFLP techniques can be used for molecular marker identification and identify the genetic relationship of *D. asper* can be identify. Moreover, RAPD and AFLP technique can be apply to the other species.

CHAPTER VI
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BIBLIOGRAPHY

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