GENETIC DIVERSITY ANALYSIS IN SUNFLOWER (*Helianthus annuus* L.) GENOTYPES USING RAPD AND SSR MARKERS

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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ปัญจมา จรรยาเลิศอดุล : การวิเคราะห์ความหลากหลายทางพันธุกรรมในทานตะวัน (*Helianthus annuus* L.) โดยเครื่องหมายอาร์เอพีดีและเครื่องหมายเอสเอสอาร์ (GENETIC DIVERSITY ANALYSIS IN SUNFLOWER (*Helianthus annuus* L.) GENOTYPES USING RAPD AND SSR MARKERS) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.หนูเดือน เมืองแสน, 106 หน้า.

เครื่องหมายดีเอ็นเอที่แตกต่างกันสองชนิด คือ เครื่องหมายอาร์เอพีดี และเครื่องหมายเอสเอสอาร์ เป็นลำดับเบสสั้นที่มีการนำมาใช้กันอย่างแพร่หลายในการจำแนกสายพันธุ์และการพัฒนาเครื่องหมายโมเลกุลสำหรับพืชหลายชนิด งานวิจัยที่นำเสนอนี้มีเป้าหมายในการศึกษาความหลากหลายทางพันธุกรรมและความสัมพันธ์ทางพันธุกรรมระหว่างจีโนไทป์ของทานตะวันที่ถูกพัฒนาขึ้นโดยมหาวิทยาลัยเทคโนโลยีสุรนารี (มทส.) โดยใช้เครื่องหมายอาร์เอพีดีและเครื่องหมายเอสเอสอาร์ ทำการประเมินสายพันธุ์แท้ 13 สายพันธุ์ พันธุ์สังเคราะห์ 8 พันธุ์ และพันธุ์ลูกผสม 3 พันธุ์ ด้วยเครื่องหมายอาร์เอพีดี 14 ไพรเมอร์และเครื่องหมายเอสเอสอาร์ 16 ไพรเมอร์ ผลการศึกษาพบว่า ทานตะวัน 24 จีโนไทป์มีค่าความแตกต่างหรือพอลิมอฟิซึม (PIC) ที่คำนวณด้วยเครื่องหมายอาร์เอพีดีมีค่าอยู่ระหว่าง 0.02-0.74 ค่าเฉลี่ย 0.40 ต่ำกว่าเครื่องหมายเอสเอสอาร์ซึ่งมีค่าอยู่ระหว่าง 0.46-0.81 ค่าเฉลี่ย 0.64 แสดงให้เห็นว่าเครื่องหมายเอสเอสอาร์มีความสามารถในการแสดงความแตกต่างหรือพอลิมอฟิซึมสูงกว่าเครื่องหมายอาร์เอพีดี เครื่องหมายเอสเอสอาร์จะมีช่วงของความสัมพันธ์ทางพันธุกรรมสูงกว่า (0.00-0.85 ค่าเฉลี่ย 0.31) เมื่อเทียบกับเครื่องหมายอาร์เอพีดี (0.00-0.49 ค่าเฉลี่ย 0.22) ซึ่งเป็นการบ่งชี้ว่าเครื่องหมายเอสเอสอาร์มีความแปรปรวนทางพันธุกรรมของทานตะวันทั้ง 24 จีโนไทป์ต่ำกว่าเครื่องหมายอาร์เอพีดี เดนโดรแกรมที่สร้างขึ้นด้วยวิธี UPGMA จากเครื่องหมายดีเอ็นเอสองชนิดแบ่งทานตะวันทั้ง 24 จีโนไทป์เป็นสองกลุ่มหลักอย่างชัดเจน เดนโดรแกรมที่จำแนกด้วยเครื่องหมายเอสเอสอาร์แสดงความสอดคล้องกับข้อมูลแหล่งที่มาของสายพันธุ์ การจัดกลุ่มจากทั้งสองระบบเครื่องหมายยังแสดงให้เห็นว่าพันธุ์ลูกผสมทางการค้าและพันธุ์จากต่างประเทศได้รับการแยกออกอย่างชัดเจนจากสายพันธุ์แท้และพันธุ์สังเคราะห์โดยมหาวิทยาลัยเทคโนโลยีสุรนารี นอกจากนี้ การวิเคราะห์พีซีโอเอซึ่งแสดงความสัมพันธ์ทางพันธุกรรมระหว่างสายพันธุ์ ให้ผลที่สอดคล้องกับการวิเคราะห์การจัดกลุ่มด้วยเดนโดรแกรม ผลที่ได้จากการศึกษาเครื่องหมายอาร์เอพีดีและเครื่องหมายเอสเอสอาร์มีความสอดคล้องกันในการศึกษาครั้งนี้ยืนยันได้จากการหาค่าสัมประสิทธิ์สหสัมพันธ์เพียร์สันระหว่างค่าความคล้ายคลึงกันทางพันธุกรรมซึ่งมีค่าไปทางบวก (0.85) ความสัมพันธ์ที่สูงนี้แสดงให้เห็นว่าการจัดกลุ่มที่เกิดจากทั้งสองระบบเครื่องหมายมีความสอดคล้องกัน ข้อมูลด้านความหลากหลายทาง

พันธุกรรมและความสัมพันธ์ระหว่างสายพันธุ์ไม่เพียงแต่มีประโยชน์มากสำหรับการอนุรักษ์เชื้อพันธุกรรมและการระบุสายพันธุ์แท้ แต่ยังมีประโยชน์ต่อการคัดเลือกสายพันธุ์พ่อแม่สำหรับการปรับปรุงพันธุ์ลูกผสมในทานตะวัน

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ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

PANJAMA JANYALERT-A-DOOL : GENETIC DIVERSITY ANALYSIS IN SUNFLOWER (*Helianthus annuus* L.) GENOTYPES USING RAPD AND SSR MARKERS. THESIS ADVISOR : ASST. PROF. NOODUAN MUANGSAN, Ph.D. 106 PP.

RANDOM AMPLIFICATION POLYMORPHIC DNA/ SIMPLE SEQUENCE REPEAT/ POLYMERASE CHAIN REACTION/ GENOTYPE/ GENETIC DIVERSITY/ GENETIC RELATIONSHIP/ SUNFLOWER

Two different DNA-based markers, random amplified polymorphic DNA (RAPD) and simple sequence repeats (SSRs), are short sequence elements that are abundant and widely used for genotype identification and marker development in many plant species. The goal of the presented research was to examine the genetic diversity and genetic relationships among sunflower genotypes developed at Suranaree University of Technology (SUT) by RAPD and SSR markers. Thirteen inbred lines, 8 synthetic and 3 hybrid varieties were assessed with 14 RAPD and 16 SSR markers. The results revealed that, among the set of 24 genotypes, the calculated PIC value for RAPD (ranging from 0.02 to 0.74 with an average 0.40) was lower than that for SSR (ranging from 0.46 to 0.81 with an average 0.64). This indicates that SSR markers have better capability to detect polymorphism than RAPD markers. SSR markers had a higher genetic similarity range (0.00-0.85 with an average 0.31) compared with RAPD markers (0.00-0.49 with an average 0.22), suggesting that SSR markers had lower genetic variation among the 24 sunflower genotypes than did RAPD markers. The dendrograms using the UPGMA algorithm based on both marker

systems divided the 24 sunflower genotypes into two main groups completely. The dendrogram based on SSR markers appears conserved with their relative history data. The clusters from both markers systems clearly showed that the commercial hybrid varieties and sunflower accessions from abroad were completely distinguished from the inbred lines and synthetic varieties developed by SUT. The results of PCoA, which was done to visualize the genetic relationships among the inbred lines, corresponded well to those obtained through UPGMA cluster analysis. The results obtained with RAPD and SSR markers were consistent in this study, as estimated by the high positive Pearson's correlation (r = 0.85) between the similarity matrices. The high correlation indicates that clusters produced based on the two marker systems were conserved. The genetic diversity and relationships data among inbred lines and varieties are not only useful for germplasm conservation and inbred line identification, but also for the selection of parental lines for hybrid breeding in sunflowers.

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LIST OF ABBREVIATIONS

AFLP	=	amplified fragment length polymorphisms
bp	=	base pair
^{o}C	=	degree Celsius
CM1	=	Chiang Mai 1
CTAB	=	Cetyltrimethyl ammonium bromide
DNA	=	deoxyribonucleic acid
EDTA	=	ethylene diamine tatra-acitic acid di-sodium salt
g	=	gram
HOC	=	high oil cross
ISSR	=	inter simple sequence repeat
kcal	=	kilocalorie
kj	=	kilojoule
LOO	=	low oil open
M	=	molar
mt	=	metric ton
µg	=	microgram
µl	=	microliter
µM	=	micromolar
mg	=	milligram
ml	=	milliliter
mM	=	millimolar

LIST OF ABBREVIATIONS (Continued)

ng	=	nanogram
min	=	minute
MOC	=	medium oil cross
MOO	=	medium oil open
nm	=	nanometer
P33	=	Pacific33
P77	=	Pacific77
%	=	percentage
PCoA	=	principle coordinate analysis
PCR	=	polymerase chain reaction
PIC	=	polymorphism information content
RAPD	=	random amplification of polymorphic DNA
RFLP	=	restriction fragment length polymorphism
rpm	=	revolutions per minute
SCAR	=	sequence characterized amplified region
sec	=	second
SSR	=	simple sequence repeats
S471	=	Suranaree471
S473	=	Suranaree473
S475	=	Suranaree475
TBE	=	tris-boric acid-disodium EDTA
UPGMA	=	unweighted pair-group method using arithmetic average

CHAPTER I

INTRODUCTION

1.1 Background/Problem

Sunflower (*Helianthus annuus* L.) is one of the most important oilseed crops grown in the world (Stefansson, 2007). During 2006, sunflower oil ranked fourth with a worldwide production after palm, soy and rapeseed oil, of about 10.6 million metric tons (FAO-STAT, 2013). In 2013, the major vegetable oils have been ranked by the USDA and sunflower oil ranked fifth after soybean, rapeseed, cottonseed and peanut (United States Department of Agriculture, 2013).

Sunflower has its origin in eastern North America, a temperate area, but the continuous breeding of sunflower makes it grow quite well in the semi-tropical or tropical areas as well. Large areas of cultivated sunflower are found in the United States, Argentina and Russia. In Asia, it is mostly grown in China and India (Abigail et al., 2004).

Sunflower seeds are very nutritious for humans and animals. The composition of sunflower seed is unsaturated fats, proteins, fiber and other important nutrients like vitamin E, vitamin K, selenium, copper, zinc, folate, iron and phytochemicals. The seed contains 30-45% oil. Sunflower oil is an excellent quality oil, because it is composed of 66-72% linoleic acid, 16-20% oleic acid, 12% saturated acids (palmitic and stearic), and less than 1% alpha-linolenic acid (Maria, 1964). One hundred g of

sunflower seed contains 20.78 g protein, 51.46 g total lipid (fat), 3.02 g ash, 20 g carbohydrate and 8.6 g fiber with total energy of 2,445 kJ. It is also an excellent source of choline (55.1 mg) and betaine (35.4 mg) (United States Department of Agriculture, 2012). In addition, sunflower oil is rich in vitamins A, D, E and K, and this is an important advantage compared to other vegetable oils (Connor and Hall, 1997).

Because of its high quality, sunflower oil is in demand, both domestically and internationally for consumption. It is also used as an ingredient in many industries such as oil, oil lacquer and cosmetics. Argentina was the second largest sunflower oil producer in the world and the largest exporter of sunflower refined oil and oil cake in 2009 (Argentinean National Food Direction, 2010). Moreover, sunflower oil cake is a manufacturing by-product, which is being almost exclusively employed for animal feeding in spite of its high content of highly digestible proteins with an important content of essential amino acids (except for lysine and sulfur amino acids) (Salgado et al., 2011). In Spain biodiesel oil of sunflower is mainly produced and the demand for sunflower oil directly competes between the use for biodiesel and the use to produce food or animal feed (Hassouneh et al., 2012). In addition, oil extracted from sunflower oil may be used as a lubricant, corrosion inhibitor, and also for manufacturing synthetic rubber, like other oilseed crop species (soybean, peanut, and castor) (Rogério et al., 2013). The residue after extraction of sunflower oil can be used as animal feed which is high in protein and easily digestible. Sunflower seeds are also used for confectionary and snack food as well as for bird and pet feed.

In Thailand, the problem of sunflower production from the past to the present is the shortage of input in terms of varieties. The varieties and seeds which Thai farmers

use to grow are hybrid varieties totally imported from abroad. In 2009, Thailand imported about 466 tons of sunflower seeds (73.46 million baht) and 8,438 tons of sunflower oil (366.8 million baht) (Office of Agricultural Economic, 2009). In 2011, Thailand imported about 11,093 tons of sunflower seeds (550.71 million baht) (Centre for Agricultural Information Office of Agricultural Economic, 2011). The seed price was 360 baht per kilogram, and 425 baht per kilogram on the market in 2011 and 2013 respectively (Pacific Seeds (Thai), 2013). High seed price increased production cost and a burden for farmers who buy seeds every year. The purchase price of sunflower seeds is 19.25 baht per kilogram (Department of Internal Trade, 2013). Farmers must buy seed varieties from abroad, which are quite expensive. To reduce production cost, creating a hybrid that yields close to the imported hybrids is an approach to solve the problem; therefore seeds are cheaper and can be stored for farmers in the coming years.

Sunflower breeding program started in Thailand in 1973 (Laosuwan, 2000). Eighteen open pollinated and hybrid varieties imported from abroad were tested at Maha Sarakham and Chiang Mai provinces and the test results were unsatisfactory. In 1986, the Vegetable Oil Development Project, Institute of Science and Technology of Thailand tested a single hybrid from abroad and found that Hysun33 gave high yield and showed best adaptation. This hybrid was then recommended for planting with a given name of Pacific 33 (Laosuwan, 1997). In 2003, the Department of Agriculture released a synthetic variety, namely Chiang Mai 1 (CM1) with yield about 203 kg/rai and 35% oil content. In addition, the Institute of Agricultural Technology, Suranaree University of Technology has set up a sunflower breeding program since 1994, aiming to create hybrid varieties for high oil and yield similar to

current hybrid varieties. Forty-eight accessions of sunflower from different sources were compiled and developed into 12 high oil inbred lines, and by crossing of these lines, 6 synthetic varieties were obtained. In 2004, two synthetic varieties, namely Suranaree 471 and Suranaree 473, were certified at the institutional level for farmers with the percentage of oil and yield similar to hybrid Pacific 33 (Srimuenwai and Laosuwan, 2008).

Genetic analysis of sunflowers is necessary because their germplasms have wide variation in characters such as yield, seed number, plant height and susceptibility to biotic and abiotic stresses. Various DNA markers have been used to assess genetic diversity in plants. Random amplification of polymorphic DNA (RAPD) markers have been used extensively for plant breeding and genetic diversity studies, because this is an uncomplicated, easy and fast method, and the cost is relatively low. RAPD markers are dominant markers and extensively used in genetic mapping (Chalmers et al., 2001) and identification of markers linked with different traits (Bai et al., 2003). Due to technical simplicity and speed, the RAPD method has been used for diversity analyses in several crops (Li and Nelson, 2001). In recent study, several hundred simple sequence repeat (SSR) markers were developed for sunflower (Paniego et al., 2002; Yu et al., 2002) and have opened the way to the analysis of molecular genetic diversity in this crop. SSR markers, due to their high polymorphism, random distribution and co-dominant, are the most reliable markers for cultivar identification and genetic diversity. Therefore, SSRs represent the current marker system of choice for characterizing sunflower germplasm (Paniego et al., 2002; Tang et al., 2002), and have been widely applied in sunflower research for identification of inbred lines, cultivars and wild species (Yu et al., 2002; Tang and Knapp, 2003).

Since SUT sunflower lines and varieties vary in characters such as yield, seed number, plant height, and head size, it is necessary to perform genetic analysis of these germplasms. Identification of sunflower lines, varieties and hybrids is mainly based on morphological traits, but a number of these traits are limited, unstable and are not always distinguishable between closely related genotypes. DNA markers seem to provide useful information about polymorphism, genetic relatedness and diversity. Several research results have shown that RAPD and SSR markers were sufficient and reliable tools for genetic diversity study and the distinguishing between closely or distantly related genotypes. Therefore, the objectives of this study were to analyze the genetic diversity in sunflower genotypes and examine genetic relationships among these genotypes by two different DNA-based marker techniques, RAPD and SSR.

1.2 Research objectives

The objectives of this thesis were:

1.2.1 To analyze the genetic diversity in sunflower genotypes by RAPD and SSR markers for genotype identification.

1.2.2 To examine genetic relationships among sunflower genotypes by RAPD and SSR markers.

1.3 Research hypothesis

The research hypotheses of this study were:

1.3.1 The genetic differences or polymorphisms of sunflower genotypes could be examined by RAPD and SSR markers.

1.3.2 RAPD and SSR markers are able to estimate the genetic relationships

among sunflower genotypes.

1.4 Scope and limitation of the study

In this study, twenty four sunflower genotypes were used. A total of 14 RAPD primers and 16 SSR primers were used to amplify DNA fragments with the PCR thermal cycle.

1.5 Expected results

The expected results from this study were:

1.5.1 DNA fingerprints of 24 sunflower genotypes were obtained, which may be useful for genotype identification.

1.5.2 The understanding of the genetic diversity and genetic relationships among sunflower genotypes developed by Suranaree University of Technology were improved which is useful for the sunflower breeding program.

1.5.3 Our understanding about the use of RAPD and SSR marker for investigation of genetic diversity and genetic relationships in sunflowers was increased.

CHAPTER II

LITERATURE REVIEW

2.1 Sunflower

2.1.1 Morphology and characteristics of sunflower

Sunflower (*Helianthus annuus* L.) is in the Asteraceae family (Figure 2.1), which contains more than 62 species worldwide (United States Department of Agriculture, 2012). The most evident characteristic of the Asteraceae family is perhaps its inflorescence: a specialized capitulum referred as the flower head. The capitulum is a contracted raceme composed of numerous individual sessile flowers, called the florets, all sharing the same receptacle (Table 2.1).

Although the source of the sunflower is in the semi-tropical or subtropical area, the improved sunflower varieties are able to adapt well. Currently, the sunflower varieties grow about 1.5 to 2.5 meters in height (while flowering), the leaves are about 20-30 leaves and the flowers are single flower heads. The leaves are large and the flowers stay upright until they bloom. After that, the flowers turn to the east.

Sunflower is a composite flower, known as a flower head actually of numerous florets crowded together. The sterile ray florets are the outer petal-bearing florets and can be yellow, red, orange, or other colors. The florets inside the circular head are called disc florets, which mature into seeds. The ray flowers are pistillate and the heads are radiate. They are usually large and yellow, orange to reddish (Figure

2.2). The fruit or achene of sunflower consists of a seed, often called the kernel, and adhering pericarp, usually called the hull (Moghaddasi, 2011).

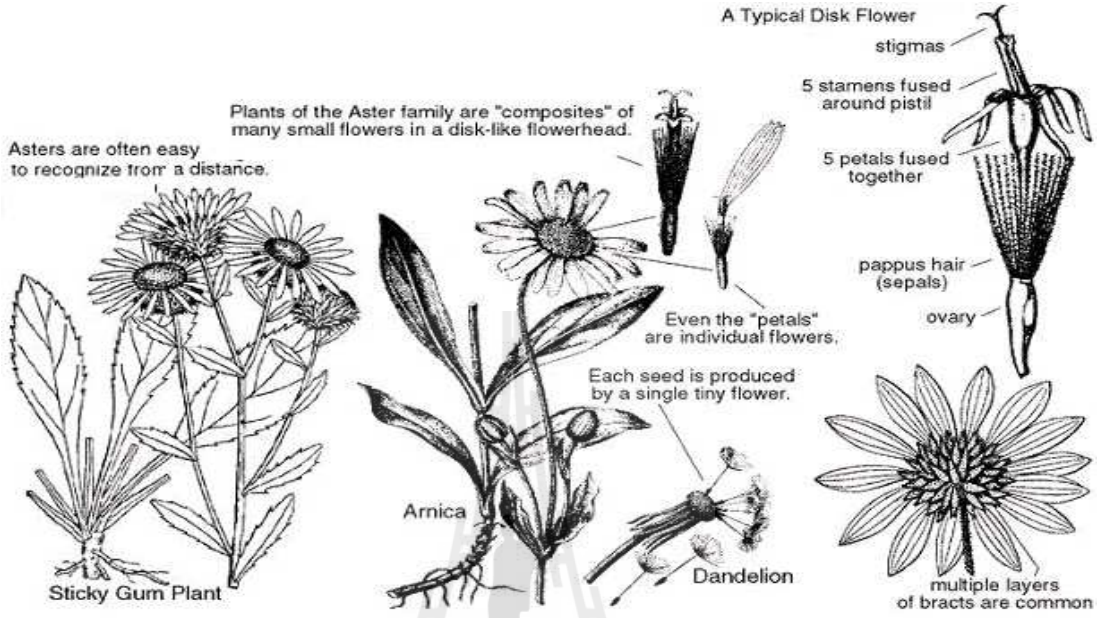

Figure 2.1 Patterns of Asteraceae (Compositae) or Sunflower family (United States Department of Agriculture, 2013).

Table 2.1 Classification of common sunflower (United States Department of Agriculture, 2011).

	Sunflower
Kingdom	Plantae - Plants
Subkingdom	Tracheobionta - Vascular plants
Superdivision	Spermatophyta - Seed plants
Division	Magnoliophyta - Flowering plants
Class	Magnoliopsida - Dicotyledons
Subclass	Asteridae
Order	Asterales
Family	Asteraceae (Compositae) - Aster family
Genus	*Helianthus* L. - sunflower
Species	*Helianthus annuus* L. - common sunflower

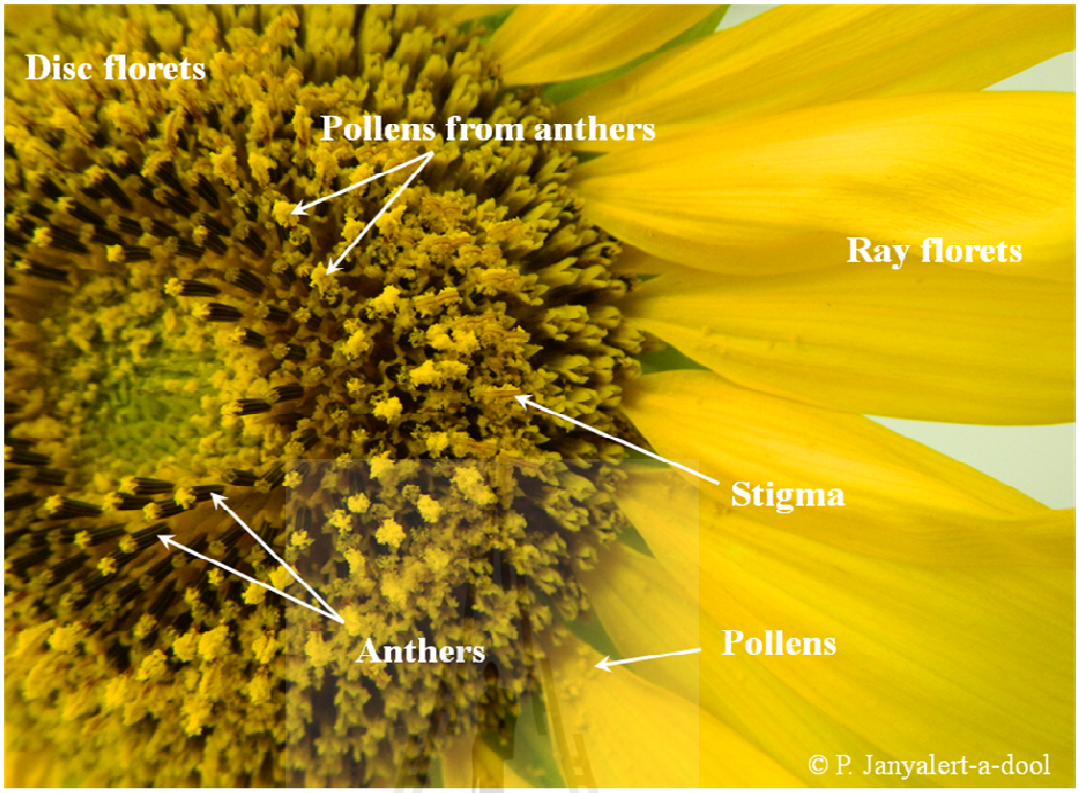

Figure 2.2 The components of sunflower.

2.1.2 Sunflower genetics

2.1.2.1 Sunflower genome

Asteraceae is the largest family within dicot plants, which contains 25,000 species (Wu et al., 2006). Among this family, *Helianthus annuus* is the cultivated sunflower for great economic importance, human nutrition, bioenergy production, and flower-farming. The Genus *Helianthus* contains 62 species and 81 accepted taxa overall (United States Department of Agriculture, 2011). *Helianthus* processes diploid (2n = 2x = 34), tetraploid (2n = 4x = 68), and hexaploid (2n = 2x = 102) species. The 14 annual species are all diploid and the 37 perennial species include 27 diploid, 4 tetraploid, 6 hexaploid and 4 mixi-ploid species. *H. ciliaris* and *H. strumosus* have both tetraploid and hexaploid forms, while *H. decapetalus* and *H.*

smithii contain diploid and tetraploid forms. The hexaploid *H. resinosus* is currently being investigated using molecular cytogenetics and markers in order to elucidate species origin through polyploidy (Carrera et al., 2004). The origins and relationships of the polyploidy hybrid species in *Helianthus* have long been of interest and remain largely unresolved, particularly for perennials (Timme et al., 2007). It is currently unknown which polyploid species are autopolyploids and which are allopolyploids (Hu et al., 2010). Recent study reported the use of the BACFISH and C-PRINS techniques for the detection of single-copy DNA sequences on sunflower chromosomes in order to evaluate the potential of both techniques for further applications in the integration of physical and genetics maps (Talia et al., 2011).

The genus *Helianthus* has a basic chromosome with n = 17, an estimated genome size is around 3.5 billion base pairs (Baack et al., 2005). Kane et al. (2011) indicated that *H. annuus* genome is dominated by repetitive elements; a recent analysis of more than 100 Gb of whole-genome shotgun sequence indicates that 78.5% of the genome consists of repeats, mainly LTR retrotransposons. The variation size of genome among *Helianthus* species is interesting, with a fourfold size difference across the diploids. Generally, the perennial species have larger genomes than most of the annuals. Among genus *Helianthus*, the genome size of the annual species is increased 50% in the three homoploid hybrid species (*H. anomalus*, *H. deserticola* and *H. paradoxus*).

2.1.2.2 Genetic variability in sunflower

The differentiation of species within *Helianthus* was explained by cross compatibility between *H. annuus* and annual *Helianthus* species and sometimes

also between *H. annuus* and perennial species (Whelan, 1978). The molecular study about the evolution of the *Helianthus* genus based on chloroplast DNA restriction analysis separated the *Helianthus* genus into four sections, first for the annual *H. agrestis*, second for the annual *H. porteri*, third (section *Helianthus*) containing all other annuals including *H. annuus*, and finally for all perennials subdivided (Schilling, 1997). Later, Schilling et al. (1997) found little differentiation among most *Helianthus* species based on ribosomal internal transcribed spacer sequences. *Helianthus* (annuals), *Atrorubentes* and *Ciliares* (both perennials) were obtained clear-cut separation of three main sections and separate positions of *H. agrestis* and *H. porteri*, using RAPD technique (Sossey-Alaoui et al., 1998). Cheres and Knapp, (1998) explained the genetic variability between cultivars and wild accessions of *H. annuus* by microsatellites. It is difficult to separate between species because of the divergence species and many species are known of hybrid origin (Rieseberg et al., 1995; Ungerer et al., 2006).

2.1.3 Nutritional value and economic importance

2.1.3.1 Oil and oil quality

The volume of vegetable oils for almost all worldwide production, they are used as both cooking oils and as straight vegetable oil or to make biodiesel. Sunflower seed oil is a major oilseed in world supply and distribution in the year 2008-2013 after soybean, rape seed, cotton seed and peanut (Table 2.2) (United States Department of Agriculture, 2013). The oil is a combination of monounsaturated and polyunsaturated fats with low saturated fat levels compared to other kinds of crop oil. It is found that sunflower seed oil is composed of 66 g polyunsaturated fatty acid

which is more than soybean oil (58 g) and peanut oil (32 g) (Table 2.3). In addition, sunflower oil also contains tocopherols, carotenoid, lecithin, and waxes as well as high vitamin E content (Table 2.4).

There are several types of sunflower oils produced, such as high oleic, standard, and mid oleic (Table 2.4). High oleic sunflower oil typically has at least 70% oleic acid, whereas mid oleic has a range between 30-70% (National Sunflower Association, 2011). In the last decade, high stearic sunflower lines have been developed in Spain to avoid the use of partially hydrogenated vegetable oils in the food industry.

Table 2.2 World supply and distribution of major oilseeds (United States Department of Agriculture, 2013)

Product	2008/09 (mt)	2009/10 (mt)	2010/11 (mt)	2011/12 (mt)	2012/13 (mt)
Copra	5.88	5.88	6.02	5.54	5.66
Cotton seed	41.08	38.91	43.56	46.62	44.97
Palm kernel	11.75	12.22	12.55	13.31	14.15
Peanut	35.07	33.74	36	35.34	37.2
Rape seed	57.81	60.96	60.55	61	60.63
Soybean	211.64	260.25	263.59	238.73	268
Sunflower seed	33.48	32.18	33.46	40.3	36.2
Total	396.7	444.13	455.72	440.83	466.8

Remark: mt = metric ton

Table 2.3 Comparative properties of common cooking fats per 100 g (National Sunflower Association, 2011).

Source	Total fat (g)	Saturated fat (g)	Monounsaturated fat (g)	Polyunsaturated fat (g)
Vegetable Shortening (hydrogenated)	71	23	8	37
Sunflower oil	100	10	20	66
Soybean oil	100	16	23	58
Peanut oil	100	17	46	32
Olive oil	100	14	73	11
Lard	100	39	45	11
Suet	94	52	32	3
Butter	81	51	21	3

Table 2.4 Sunflower oil compositions (National Sunflower Association, 2011).

Nutritional value per 100 g (3.5oz)	Sunflower oil		
	High oleic 70% and over	Standard	Mid oleic
Energy	3,699 kJ (884 kcal)	3,699 kJ (884 kcal)	3,699 kJ (884 kcal)
Carbohydrates	0 g	0 g	0 g
Fat	100 g	100 g	100 g
Saturated	9.748 g	10.3 g	9.009 g
Monounsaturated	83.594 g	19.5 g	57.344 g
Polyunsaturated	3.798 g	65.7 g	28.962 g
Vitamin E	41.08 mg (274%)	41.08 mg (274%)	41.08 mg (274%)
Vitamin K	5.4 µg (5%)	5.4 µg (5%)	5.4 µg (5%)

Remark: Percentages are relative to US recommendations for adults

2.1.3.2 Proteins

The concentration of protein from sunflower seed is of interest for human and livestock consumption, but is also usually related to the production of oil in the seed. Commercial sunflower meal has a protein concentration of approximately 440 g/kg (dehulled) and 280 g/kg (whole seed) (Dorrell and Vick, 1997). Variability among lines or genotypes for protein concentration is sufficient for selection to increase protein concentration of sunflower seeds in a breeding program. However, selection high protein usually results in lower oil concentration because of a negative correlation between the two traits. Breeding to improve protein concentration of sunflower kernels from about 240 to near 400 g/kg has been made, while maintaining acceptable oil concentration (Ivanov and Stoyanova, 1978).

2.1.4 Sunflower cultivation in Thailand

In 1988, sunflower planting was started as a commercial crop (Laosuwan, 1997). In 1988-1989, there were only 316 acres of sunflower planted area and it was increased to 58,333 acres in 1994-1995 and there were approximately 75,000 acres in 1995-1996 (Laosuwan, 1997). Total area of cultivated sunflower was raised to approximately 291,666 acres with yield about 90,000 tons in 2001 (Laosuwan, 2000).

Sunflower cultivation places in Thailand are Lop Buri province (55%), Saraburi province (17%), Nakhon Sawan province (14%), and Phetchabun province (13%) (Bamrung, 2009). Generally, farmers grow sunflowers during the period from late September to November. Then the plants are in full bloom around November and December.

However, in the early stages of the market, buying sunflower production was limited. Planting sunflowers was not receiving enough attention from farmers and research study on sunflower breeding was not continuous. Later, when farmers began to cultivate sunflower for increased trade, the government began the research and development of sunflower again.

Vegetable Oil Development Project, Institute of Science and Technology of Thailand tested a single hybrid from abroad and found that Hysun33 gave high yield and showed best adaptation in 1986. This hybrid was then recommended for planting with a given name as Pacific 33 (Laosuwan, 1997). In 2003, Department of Agriculture (DOA) released a synthetic variety, namely Chiang Mai 1 (CM1), with yielded about 203 kg/rai and 35% oil content. Institute of Agricultural Technology, Suranaree University of Technology has set up a sunflower breeding program since 1994 aiming to create hybrid varieties for high oil and yield similar to current hybrid varieties. Forty-eight accessions of sunflower from different sources were compiled and developed into 12 high oil inbred lines (1A, 2A, 3A 4A, 5A, 6A, 7A, 8A, 9A, 10A, 11A, and 12A) and by crossing of these lines, 6 synthetic varieties (HOC, HOO, MOC, MOO, LOC, and LOO) were obtained. In 2004, two synthetic varieties, Suranaree 471 and Suranaree 473, were certified at institutional level for farmers with the percentage of oil and yield similar to hybrid Pacific33 (Srimuenwai and Laosuwan, 2008).

Although efforts have been made for sunflower breeding, Thailand still has to import sunflower seeds to meet the demand in the country (Figure 2.3).

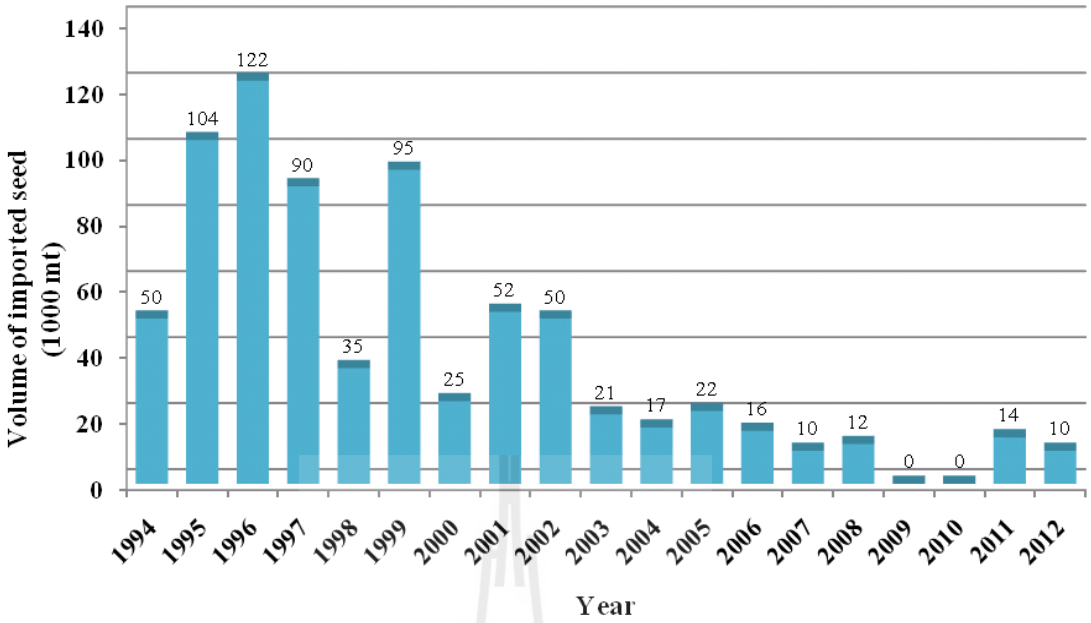

Figure 2.3 Volume of the imported sunflower seed between years 1994-2012 (United States Department of Agriculture, 2012).

2.2 DNA-based marker techniques

DNA marker refers to the sequence of DNA that is used as an indication of the identity of the organism. It may have a position on the chromosomes in the nucleus (nuclear DNA) or in organelle (mitochondria DNA or chloroplast DNA) and can be transmitted to offsprings (Huang et al., 2003). Each individual species has a unique DNA sequence. Differences or polymorphisms of a molecule of DNA sequence can be used as molecular markers (Kate-ngam, 2003).

The use of DNA as a marker to indicate the differences of living things can be done by comparing the DNA of the organisms. The technique of molecular biology commonly known as “DNA Fingerprinting” (Luong, 2009), which is the difference in the mean patterns of DNA of the organism, can be used to examine the differences, or polymorphisms of DNA of the organisms or species of plants.

DNA markers can be divided into two categories: 1) hybridization-based DNA marker and 2) polymerase chain reaction (PCR)-based marker (Kate-ngam, 2003). The former DNA marker applies a DNA probe to hybridize the restriction digested DNAs. Different hybridization patterns to DNA from different sources provide the markers. Restriction fragment length polymorphism (RFLP) is the most extensively used hybridization-based marker (Semagn et al., 2006). The RFLP markers are very reliable in linkage analysis and breeding. However, their utility has been limited due to the large amount of DNA used for restriction digestion and Southern blotting. PCR-based marker involves DNA amplification of partial DNA sequences or loci, with the help of specific primers. The PCR-based markers include random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSRs) and inter simple sequence repeat (ISSR).

2.2.1 Hybridization-based DNA marker

The differential of DNA fragment profile or polymorphism is detected by hybridizing with a chemically labelled DNA probe to a Southern blot of DNA digested by restriction enzymes. This differential profile indicated nucleotide substitutions or DNA rearrangements like insertion or deletion or single nucleotide polymorphisms.

The restriction fragments of DNA contained in agarose electrophoresis gel are placed on a filter paper wick that forms a connection between the gel and a reservoir of high-salt buffer. The membrane is placed on top of the gel and covered with a tower of paper towels that are held in place with a weight. The buffer is soaking

through the filter paper wick, gel and membrane and into the paper towels. Then, the buffer passes through the gel, the DNA fragments are carried with it into the membrane, and the DNA fragments become bound to the nitrocellulose. The fragments are transferred effectively up to 15 kb in length taking approximately 18 hours (Brown, 2001).

2.2.1.1 Restriction Fragment Length Polymorphism (RFLP)

RFLP marker is determined by the existence of alternative alleles associated with restriction fragments that differ in size from each other. The RFLP is visualized by digesting DNA with a restriction enzyme from different individuals, followed by gel electrophoresis to separate fragments according to size, then blotting and hybridization to a labeled probe that identifies the locus under investigation. RFLP is demonstrated whenever the Southern blot pattern obtained from one individual is different from the one obtained from another individual (Kate-ngam, 2003). The methodology for RFLP technique is shown in Figure 2.4.

Because of their presence throughout the plant genome, high heritability and locus specificity the RFLP markers are considered superior. Numerous samples can be simultaneously screened. DNA blots can be analyzed repeatedly by stripping and reprobing with different RFLP probes. These advantages make RFLP markers relatively highly polymorphic, codominantly inherited and highly reproducible. However, RFLP technique is not widely used because it is time consuming. It involves expensive and radioactive/toxic reagents, and requires large quantity of high quality genomic DNA. The requirement of prior sequence information for probe generation increases the complexity of the methodology. These

limitations led to the conceptualization of a new set of less technically complex methods known as PCR-based techniques (Agarwal et al., 2008).

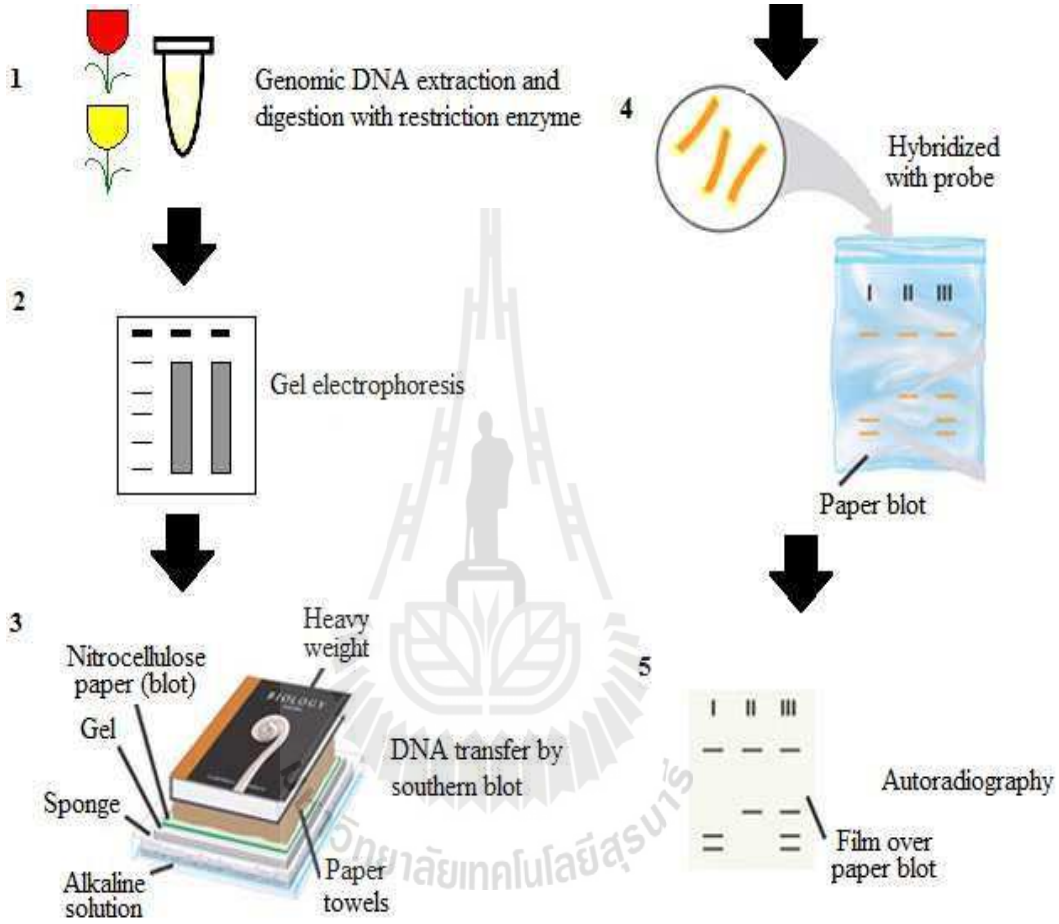

Figure 2.4 RFLP technique (modified from Kate-ngam, 2003).

The RFLP markers are useful for crop improvement (Beckmann and Soller, 1986; Tanksley, 1989), and have been used to identify crop varieties or hybrids (Beckmann and Soller, 1983; Smith and Smith, 1992) and to evaluate genetic diversity and phylogenetic relationships among cultivars and wild species (Debener et al., 1990; Kesseli et al., 1991; Livini et al., 1992; Menancio et al., 1990; Song et al., 1990).

2.2.2 Polymerase chain reaction (PCR)-based marker

The polymerase chain reaction is a simple technique for amplifying a DNA template to produce specific DNA fragments in vitro. The original process of cloning a DNA sequence into a vector and replicating it in a living cell often requires days or weeks of work, but amplification of DNA sequences by PCR requires only hours. While the other biochemical analyses, including nucleic acid detection with radioisotopes, require more amounts of input material, but the PCR process requires very little and can achieve more sensitive detection and higher levels of amplification of specific sequences in less time than previously used methods. These advantages make the PCR is extremely useful, not only in basic research, but also in commercial uses, including genetic identity testing, forensics, industrial quality control and in vitro diagnostics.

The PCR reaction includes target DNA, a thermostable DNA polymerase, oligonucleotide primer, deoxynucleotide triphosphates (dNTPs), reaction buffer and magnesium. The master mix is placed in a thermal cycler, an instrument that subjects the reaction to a series of different temperatures for set amounts of time. This series of temperature and time adjustments is referred to as one cycle of amplification. Each PCR cycle theoretically doubles the amount of targeted sequence (amplicon) in the reaction. Ten cycles theoretically multiply the amplicon by a factor of about one thousand; 20 cycles, by a factor of more than a million in a matter of hours. Each cycle includes three steps for template denaturation, primer annealing and primer extension. The initial step denatures the DNA template by heating it to 94°C or higher for 15 seconds to 2 minutes. In the denaturation process, the two intertwined strands of DNA are separated from one another, producing the necessary single-stranded

DNA template for replication by the thermostable DNA polymerase. For annealing step of a cycle, the temperature is reduced to approximately 40-60°C. The oligonucleotide primers can form stable bond with the denatured target DNA and serve as primers for the DNA polymerase. This step lasts approximately 15-60 seconds. Finally, the synthesis of new DNA begins as the reaction temperature is raised to the optimum for the DNA polymerase. For most thermostable DNA polymerases, this temperature is in the range of 70-74°C. The extension step lasts approximately 1-2 minutes. The next cycle begins with a return to 94°C for denaturation. Each step of the cycle should be optimized for each template and primer pair combination. If the temperature during the annealing and extension steps are similar, these two steps can be combined into a single step in which both primer annealing and extension take place. After 20-40 cycles, the amplified product may be analyzed for size, quantity, sequence, etc., or used in further experimental procedures.

After the invention of PCR technique, a large number of molecular markers based on PCR were generated, primarily due to its apparent simplicity and high probability of success (Agarwal et al., 2008).

2.2.2.1 Random Amplification of Polymorphic DNA (RAPD)

RAPD is a type of PCR reaction, but the segments of DNA that are amplified are random. The scientist performing RAPD creates several arbitrary, short primers (8-12 nucleotides), then proceeds with the PCR using a large template of genomic DNA, hoping that fragments will amplify. By resolving the resulting patterns, a semi-unique profile can be obtained from a RAPD reaction (Figure 2.5).

No knowledge of the DNA sequence for the targeted gene is required, due to the use of short primers that will randomly bind onto a number of complementary sites in the genomic DNA template. This makes the method popular for comparing the DNA of organisms that have not had the attention from the scientific community, or in a system in which relatively few DNA sequences have been compared. The primary drawback of RAPD markers is that they are dominant and do not estimate the heterozygosity. In recent years, RAPD has been used to characterize and trace the phylogeny of diverse plant and animal species (Bharathiraja et al., 2008). RAPD markers are able to differentiate between genetically distinct individuals, although not necessarily in a reproducible way. Rajput et al. (2006) has shown that when expressed as the percentage of RAPD bands scored in replicate data, approximately 80% reproducibility was obtained for 60 RAPD primers in wild tomato (*Lycopersicon hirsutum*). RAPD technique has been used in genetic diversity, genetic relationship and genetic characteristics in many plant species such as in *Verbesina helianthoides* (Encheva et al., 2005), bamboo (Ramanayake et al., 2007), *Phyllanthus* (Theerakulpisut et al., 2008).

Paran and Michelmore (1993) developed sequence characterized amplified region (SCAR) to overcome the reproducibility problem associated with the RAPD technique that was detected through PCR using specific oligonucleotide primers designed on the basis of the sequence data of one RAPD band and following PCR amplification under more stringent conditions.

In recent studies, the SCAR markers have been successfully used to identify several plants such as in grapevine cultivar (Vidal et al., 2000), bamboo species (Das et al., 2005), medicinal herbs species (Dnyaneshwar et al., 2006; Choi et

al., 2008), and *Lentinula edodes* strains (Li et al., 2008; Wu et al., 2010). In addition, Li et al. (2007) developed two SCAR markers for the identification of satellite chromosomes in *Cunninghamia lanceolata* seeds. Shen et al. (2011) reported that six stable informative dominant SCAR markers were developed by designing six pairs of specific SCAR primers from six sequenced polymorphic RAPD bands to identification of *C. lanceolata* clones in China.

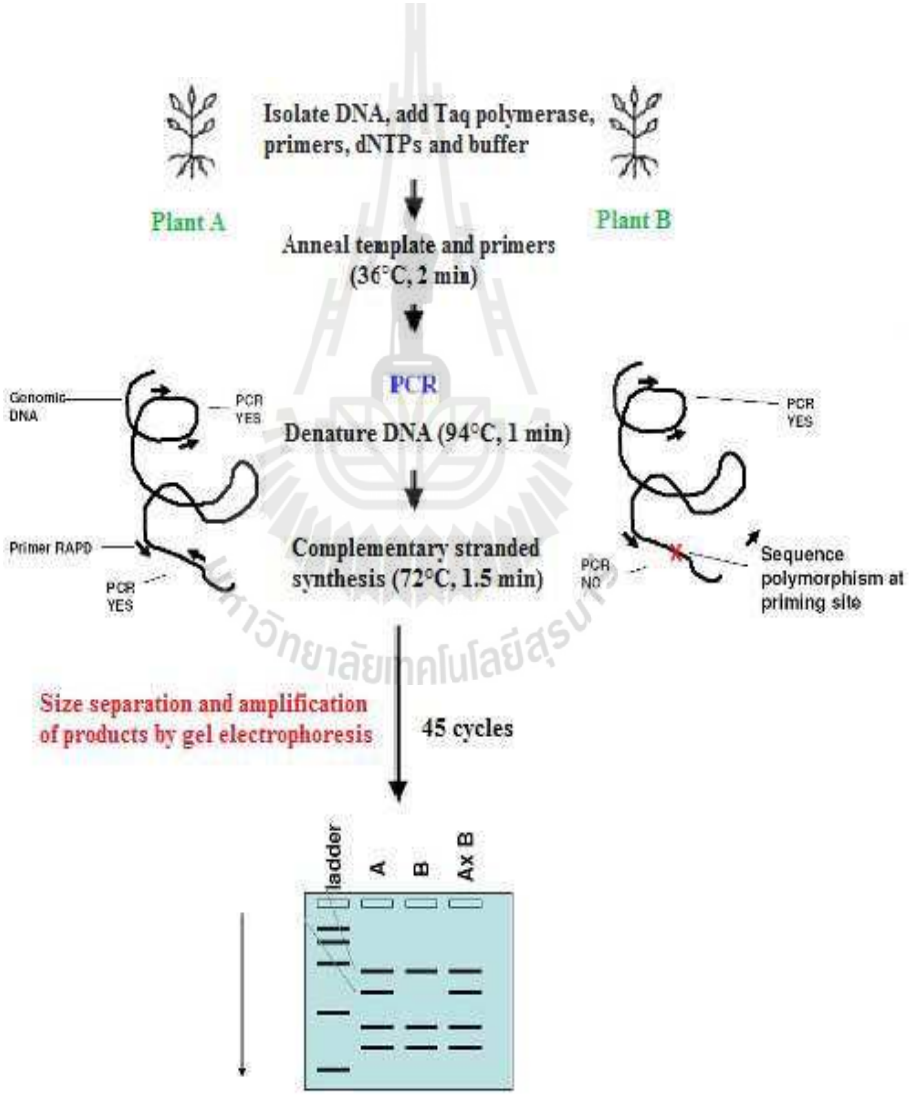

Figure 2.5 Revelation of genetic differences using RAPD (modified from Kate-ngam, 2003).

2.2.2.2 Amplified Fragment Length Polymorphisms (AFLP)

In AFLP techniques, DNA is digested with two different restriction enzymes, one of which is a frequent cutter (the four-base restriction enzyme) and the other a rare cutter (the six-base restriction enzyme). *Mse*I and *Eco*RI are best used in AT-rich genomes because they give fewer fragments in GC-rich genomes. Specific synthetic adapters for each restriction site are then ligated to the digested DNA (Figure 2.6). Both the restriction and ligation steps can be performed in a single reaction. Amplification primers length is usually 17 to 21 nucleotides, and anneal perfectly to their target sequences. A second step of amplification is then performed, using similar oligonucleotide primers but with two extra bases (e.g. AC). Thus, only a subset of the first amplification reaction will obtain subsequent amplification during the second round of PCR. The fragments are illustrated by denaturing polyacrylamide gel electrophoresis. The fingerprint and DNA bands are detected using different methods, labelled with fluorescent dyes, or stained with silver nitrate (Zhao et al., 2000).

AFLP amplifications are performed under conditions of high selectivity (at high stringency), thus eliminating the artifactual variation that is seen routinely in RAPD–PCR. Repeated AFLP amplifications show near perfect replicability and overall errors (including mispriming and scoring error) generally amount to less than 2% (Huys et al., 1996). In a careful and rigorous experiment, Jones et al. (1997) tested the replicability of AFLP markers by comparing AFLP analyses, conducted on the same samples in eight different European laboratories, and found only a single scoring difference (absence of one band among a total of 172 in the AFLP profiles). The between-laboratory error for AFLP markers, therefore, was

less than 0.6%, which was at the level of microsatellite scoring errors estimated in the same study (Mueller and Wolfenbarger, 1999).

Several studies based on AFLPs technique have been applied to DNA fingerprinting (Powell et al., 1996), genetic diversity studies (Russell et al., 1997), genome mapping (Zimnoch-Guzowska et al., 2000), and parentage analysis (Gerber et al., 2000; Lima et al., 2002).

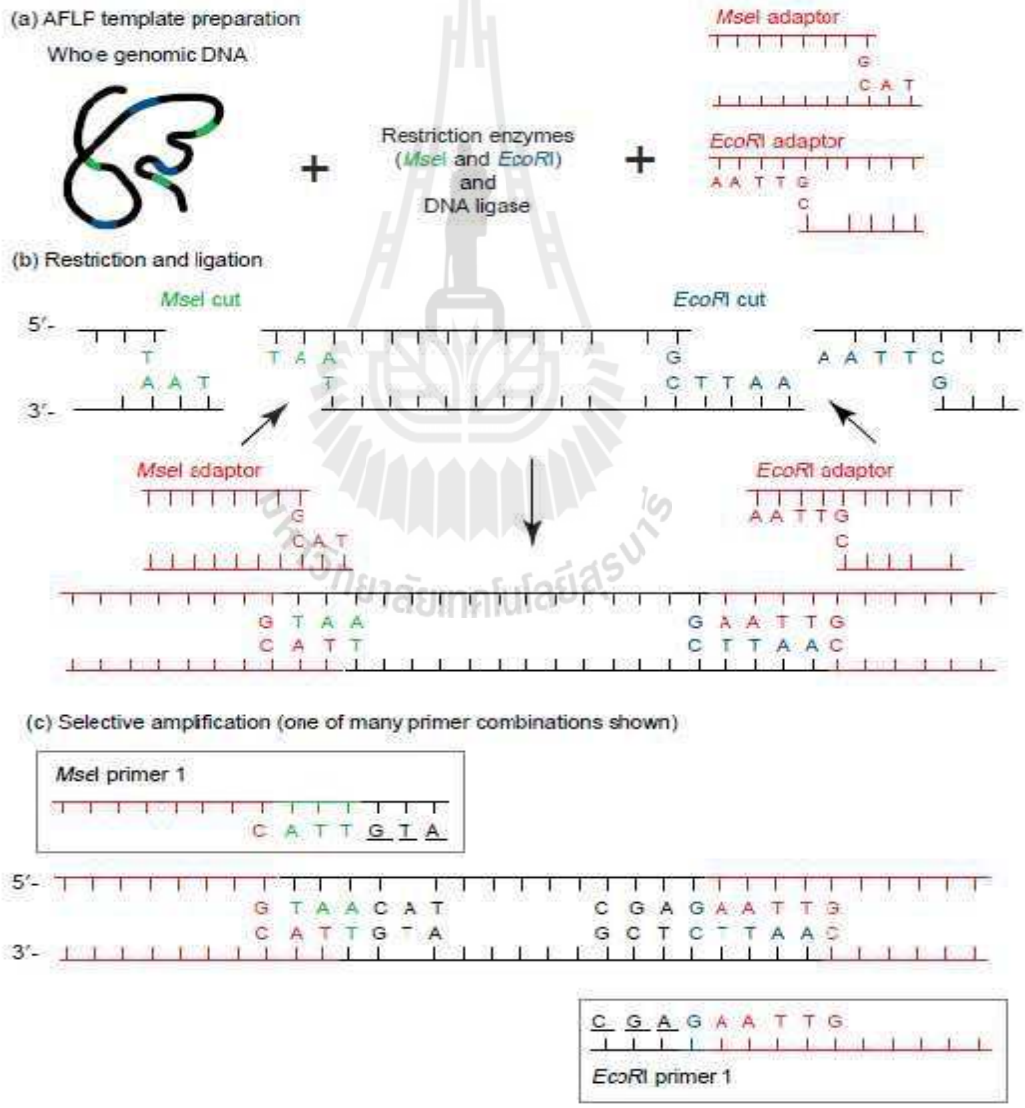

Figure 2.6 AFLP amplifications (Mueller and Wolfenbarger, 1999).

2.2.2.3 Simple Sequence Repeats (SSRs)

Simple sequence repeats (SSRs) or microsatellites are repeating sequences of 1-6 base pairs of DNA. SSRs are typically neutral and co-dominant. They are used for molecular markers in genetics, for kinship or population studies, for detection of gene duplication or deletion. One common example of a microsatellite is a $(CA)_n$ repeat (Semagn et al., 2006), where n is variable between alleles. These markers often present high levels of inter- and intra-specific polymorphism, particularly when tandem repeats number is ten or greater. The repeated sequence is often simple, consisting of two, three or four nucleotides that are repeated in tandem: di-nucleotide repeat: CACACA, tri-nucleotide repeat: ATGATGATG, tetra-nucleotide repeat: ATCTATCTATCT and can be repeated 10 to 100 times.

SSRs owe their variability to an increased rate of mutation compared to other neutral regions of DNA. Slipped strand mispairing (slippage) during DNA replication on a single DNA strand cause these high rates of mutation that can be explained most frequently. Mutation may also occur during recombination in meiosis. Some errors in slippage are rectified by proofreading mechanisms within the nucleus, but some mutations can escape repair. The size of the repeat unit, the number of repeats and the presence of variant repeats are all factors, as well as the frequency of transcription in the area of the DNA repeat. Interruption of SSRs and perhaps due to mutation can result in reduced polymorphism (Luong, 2009).

SSRs can be amplified for identification by PCR process, using the unique sequences of flanking regions as primers (Figure 2.7). With the abundance of PCR technology, primers that flank SSR loci are simple and quick to use, but the development of correctly functioning primers is often a tedious and costly process

(Tong et al., 2009). SSRs are among the most variable types of DNA sequence in the genome. In contrast to unique DNA, SSR polymorphisms derive mainly from variability in length rather than in the primary sequence. Moreover, genetic variation at many SSR loci is characterized by high heterozygosity and the presence of multiple alleles, which is in sharp contrast to unique DNA.

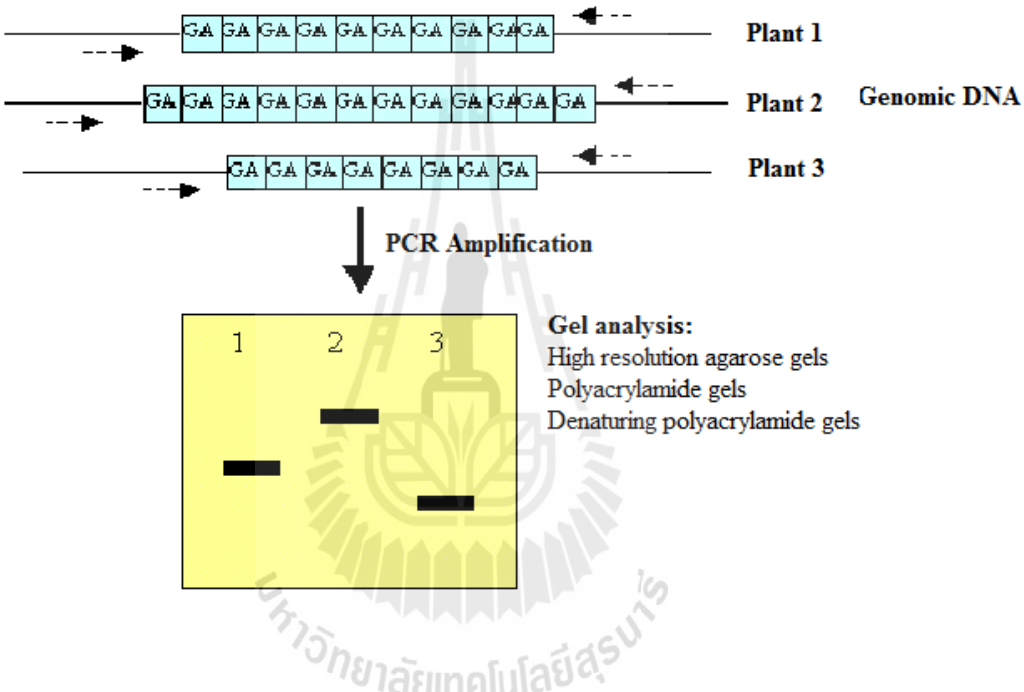

Figure 2.7 Analysis of different plant species using SSR markers (Kate-ngam, 2003).

There are several studies about DNA fingerprinting, genetic diversity, genetic relationship and genetic characteristics in many plants using SSR techniques such as in mungbean (*Vigna radiata* L.) (Gwag et al., 2006; Wang et al., 2009) and rice (*Oryza sativa* L.) (Chakravarthi and Naravaneni, 2006). Moreover, Hu et al. (2009) studied about transfer ability of SSR markers in broomcorn millet (*Panicum miliaceum* L.) and Kuleung et al. (2004) examined the transferability of SSR markers

among wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.), and triticale (X Triticosecale Wittmack).

2.2.2.4 Inter Simple Sequence Repeat (ISSR)

Inter simple sequence repeat (ISSR) technique is a PCR-based method, which involves amplification of DNA segment present at an amplifiable distance in between two identical SSR repeat regions oriented in opposite direction (Figure 2.8). The technique uses SSRs, usually 16-25 bp long, as primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly the inter-SSR sequences of different sizes. ISSRs have high reproducibility possibly due to the use of longer primers (16-25 mers) which permit the subsequent use of high annealing temperature (45-60°C) leading to higher stringency (Reddy et al., 2002). The level of polymorphism has been shown to vary with the detection method used. Polyacrylamide gel electrophoresis (PAGE) in combination with radioactivity (labelled nucleotide in PCR reaction) followed by PAGE with silver staining, are used for polymorphism detection.

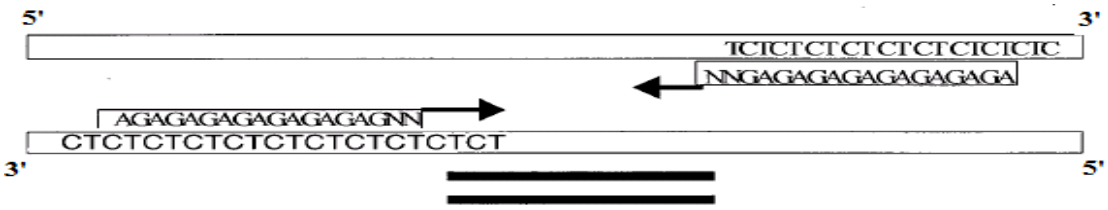

Figure 2.8 A single primer $(AG)_8$ and 3'-anchored targeting $(TC)_n$ repeat (Reddy et al., 2002).

ISSRs have been successfully used to estimate the extent of genetic diversity at inter- and intra-specific level in a wide range of crop species which include finger millet (Salimath et al., 1995), wheat (Nagaoka and Ogihara, 1997),

Plantago (Wolff and Morgan-Richards, 1998), *Vigna* (Ajibade et al., 2000), sweet potato (Huang and Sun, 2000) and rice (Joshi et al., 2000).

2.3 DNA markers in sunflower

Numerous techniques of DNA-based markers have been developed to detect polymorphisms at the DNA level in sunflower.

Several sunflower RFLP linkage maps were published recently. The first RFLP map was constructed based on segregation at 234 loci, detected by 213 probes, in an F_2 population of 289 individuals (derived from a cross between the inbred lines HA89 and ZENB8 (Berry et al., 1995). Later, Jan et al. (1998) constructed the RFLP linkage map for cultivated sunflower based on 271 loci detected by 232 cDNA probes. The gene has been located at the extreme distal end of linkage group (LG) 7 of the RFLP composite map developed by Gentzbittel et al. (1999), and the closest RFLP marker identified is 3 cM from the gene in XQR x PSC8 (Bert et al., 2002). Rojas-Barros et al. (2008) reported that RFLP markers (8A1 and 15B3) of linkage group (LG) 16 of the RHA 271 x HA 234 cultivated sunflower map anchored the b_1 LG onto the RFLP map. This result indicated that PCR-based markers tightly linked to the recessive b_1 gene. Their identification and the incorporation of the LG containing the b_1 locus onto an RFLP map will be useful for marker-assisted selection (MAS) in breeding programs and provide the bases for map-based cloning of this gene.

Rieseberg et al. (1995), using 197 RAPD markers, concluded that gene introgression in *Helianthus* involved both chromosomal and genomic barriers. Lu et al. (2000), using the RAPD bulked segregant analysis approach, reported the development of SCAR markers linked to the sunflower *Or5* locus conferring

resistance to a highly virulent Spanish population of broomrape that belongs to race E. These markers could be used either in a MAS procedure for the selection of new resistant lines or as a starting point to study the molecular genetics of broomrape resistance genes in sunflower. Later, RAPD markers were used to evaluate genetic relationships in a set of 16 inbred lines of sunflower representing the genetic stock, including restorers and maintainer lines, of the classical cytoplasmic male sterility. As the results of this study, these genotypes were grouped into eight clusters and 4 genotypes showed low similarity coefficient indicating high heterosis (Isaacs et al., 2003). Sunflower hybrids, their parental lines and open pollinated varieties were employed to identify and establish phylogenetic of relationships among genotypes and to characterize them based on RAPD markers (Nandini and Chikkadevaiah, 2005). Moreover, Iqbal et al. (2008) used eight sunflower lines through the estimation of the RAPD method for genetic diversity study. One hundred and 56 DNA fragments were generated by 20 random primers. Highly diverse lines can be used for further breeding programs to develop an ideal local hybrid of sunflower based on RAPD markers. The recent studies showed that ten mutants of two cultivars Giza1 and Giza 102 of *H. annuus* produced by sodium azide treatments and the control were evaluated by ten RAPD primers (Mostafa and Alfrmawy, 2011).

Gedil et al. (2001) published the linkage maps of AFLP. AFLP markers were used to estimate polymorphism rates, genetic similarities, and polymorphic information contents (PICs) among elite public oilseed inbred lines, and to assess the genetic diversity of inbred lines using genetic similarities estimated from AFLP fingerprints (Hongtrakul et al., 1997; Darvishzadeh, 2012). Vischi et al. (2002) studied about two above-mentioned species and populations with intermediate traits

concerning the introgressive process occurring between *H. argophyllus* and *H. debilis* ssp. comparison by morphological and AFLP analyses, between wild material and the cultivated sunflower so as to assess its potential value in breeding programs. AFLP markers were used to evaluate the genetic variability, identify and candidate genes associated with seed-quality traits under well-irrigated and water-stressed conditions in gamma induced mutants of sunflower (Haddadi et al., 2011).

SSR markers for cultivated sunflower (*H. annuus* L.) were developed by Paniego et al. (2002). A small-insert genomic library was used to perform isolation followed by hybridization screening using oligonucleotide probes containing different nucleotide arrays. The result indicated that (GA)n, (GT)n, (AT)n, followed by trinucleotides (ATT)n, (TGG)n, and (ATC)n, and the tetranucleotide (CATA)n are the most frequent motifs within polymorphic simple-sequence repeats (SSRs), and differences in the 16 reference inbred lines were obtained by 170 SSRs. In the same year, Tang et al. (2002) reported that SSR markers supplied a critical mass of DNA markers for constructing genetic maps of sunflower and created the basis for unifying and cross-referencing the multitude of genetic maps developed for wild and cultivated sunflowers. Yu et al. (2002) also developed SSR markers for cultivated sunflower from the DNA sequences of 970 clones isolated from genomic DNA libraries enriched in (CA)n, (CT)n, (CAA)n, (CATA)n, or (GATA)n and assessed the allelic diversity of SSRs among elite inbred lines. SSR sequences were used for estimation of genetic diversity and relationships of cultivated and wild sunflower, and usefulness for genotype identification. Genetic purity and “hybridity range” definition and unique alleles for some wild sunflower species were found (Solodenko and Sivolap, 2005). In addition, 18 SSR markers were used to evaluate Bulgarian sunflower

hybrids and their inbred lines (Hvarleva et al., 2007). Therefore, SSRs have been considered as the current marker system of choice for characterizing sunflower germplasm and are the most reliable markers for cultivars identification and genetic diversity (Darvishzadeh et al., 2010). Moreover, the identification of QTL associated with high oleic trait is a powerful molecular tool to facilitate sunflower breeding program progress. SSR mapping to study high oleic character in cultivated sunflower was used in some recent works (Belzile et al., 2008; Haddadi et al., 2010; Grandón et al., 2012). In the recent study, Imerovski et al. (2013) tested SSR markers from linkage group 3 (LG3) to investigate whether they could be used for identification of a particular *Or* gene (broomrape resistance genes).

Garayalde et al. (2011) attempted to evaluate the genetic diversity using ISSR marker systems to study genetic patterns and to provide data applicable to conservation and breeding uses in wild sunflower. In addition, ISSR markers have been used for genetic polymorphism study in other Asteraceae (Chapman et al., 2000; Slotta et al., 2005; Archibald et al., 2006; Mohsen and Ali, 2008).

However, the selection of one or another marker in genetic diversity studies depends on the characteristics of the material to be used and on the objectives of the given project (Cholastova et al., 2011).

CHAPTER III

MATERIALS AND METHODS

3.1 Sunflower samples and preparation

Twenty-four sunflower genotypes (Table 3.1) including 13 inbred lines (n = 3 for each line), 8 synthetic (n = 15 for each variety) and 3 hybrid varieties (n = 3 for each variety) were used in this study. A breeding program was set up to develop synthetic varieties of SUT as shown in Figure 3.1 and their parental lines as shown in Table 3.2. Sunflower seeds were germinated in germinating trays filled with pot soil (Figure 3.2), then at the age of 2 weeks, young leaves were collected and kept at -20°C for further DNA extraction.

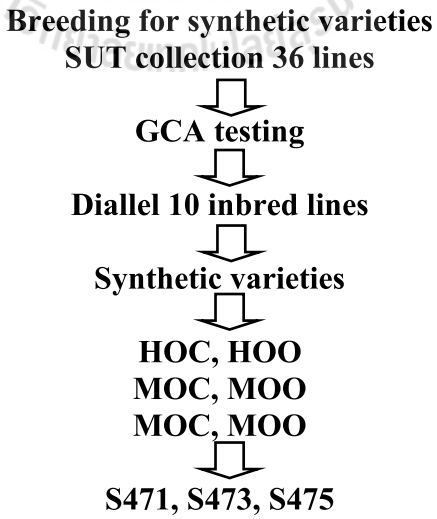

Figure 3.1 History breeding for synthetic variety.

Figure 3.2 Sunflower seedlings at the age of 1 week (a) and 2 weeks (b).

3.2 DNA extraction from sunflower

Genomic DNA was extracted from young leaf tissues of 24 sunflower genotypes grown in the trays. Sunflower leaf samples were collected at the age of 2 weeks. Then leaf samples were cleaned with 70% ethanol for surface sterilization and cut into 0.2 g placed in storage plastic bags. Sample bags were freeze dried immediately with liquid nitrogen and stored at -20°C before use. CTAB protocol using a modified version by Rogers and Bendich (1985) was used to isolate DNA from sunflower genotypes. Ground tissues were placed into 1.5 ml Eppendorf tube and added with 200 µl of hot (65°C) CTAB extraction buffer (2% CTAB (hexadecyl-trimethylammonium bromide), 100 mM Tris-HCl buffer, 20 mM EDTA, 1.4 M NaCl, pH 8.0). The extract was incubated at 65°C for 30 min with gentle inversions every 10 min, then mixed with an equal volume of chloroform : isoamyl alcohol (24:1) and the tubes were gentle mixed thoroughly to form a complete emulsion. The tubes were centrifuged at 12,000 rpm for 15 min, then the aqueous phase was transferred to new tube, and added 5 µl of 10 mg/ml RNase A, and incubated at 37°C for 30 min. The

extract was mixed with 1 volume of chloroform : isoamyl alcohol (24:1) and the tubes were gentle mixed by hand. The tubes were centrifuged at 12,000 rpm for 5 min, then the aqueous phase was transferred to new tubes. The extract was added with 1/10 volume of 3 M sodium acetate (pH 5.0) and 1 volume of cold isopropanol with gentle inversions to precipitate the DNA. The extract was placed at -20°C overnight. The tubes were centrifuged at 12,000 rpm for 20 min, then discarded the supernatant. The DNA pellet was washed with 70% ethanol. The tubes were centrifuged at 12,000 rpm for 5 min, then discarded the supernatant and dried in the air for 10-20 min. The DNA pellet was dissolved in 100 µl of TE buffer (1 M Tris-HCl pH 8.0, 0.5 M EDTA). DNA solution was kept at -20°C for long term storage (Figure 3.3).

Quantity and quality of DNA was measured at 260 and 280 nm in Nano Drop, ND-1000 Spectrophotometer (NanoDrop Technologies, USA). In addition, the quality of DNA was checked by running 5 µl of genomic DNA on 0.8% agarose gel prepared in 0.5X TBE buffer.

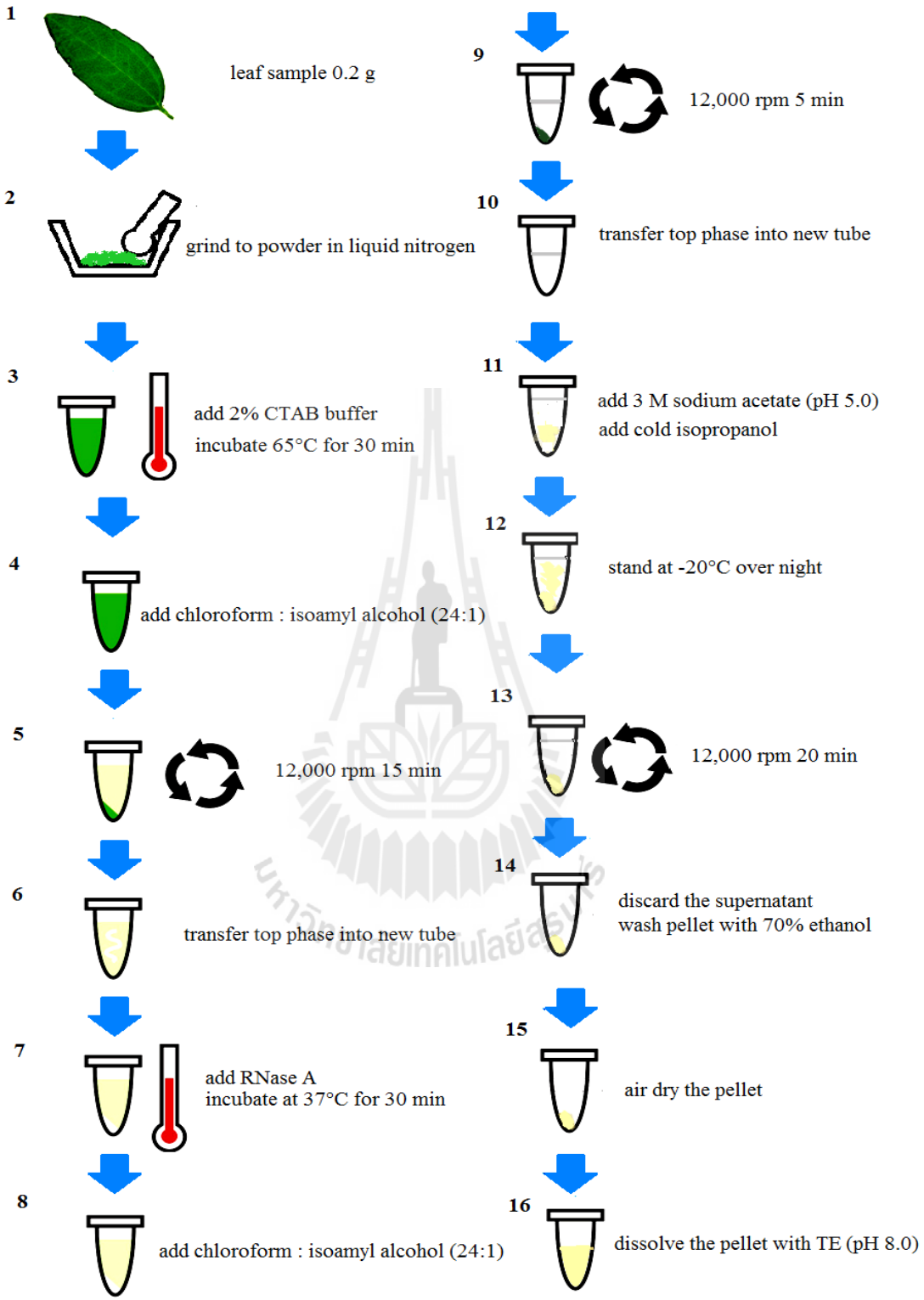

Figure 3.3 DNA extraction steps (modified from Kitijuntaropas and Wansomnuk, 2007).

Table 3.1 Sunflower lines/varieties used in this study.

Sunflower line/variety	Name	Origin	Oil content (%)
Inbred line	1A	SUT	37.25[1]
	2A	SUT	36.26[1]
	3A	SUT	35.72[1]
	4A	SUT	35.99[1]
	5A	SUT	39.02[1]
	6A	SUT	37.39[1]
	7A	SUT	39.43[1]
	8A	SUT	36.86[1]
	9A	SUT	38.34[1]
	10A	SUT	41.40[1]
	11A	SUT	40.73[1]
	12A	SUT	42.81[1]
	HA429	USDA	35.36[3]
Synthetic variety	Suranaree 471 (S471)	SUT	41.33[2]
	Suranaree 473 (S473)	SUT	39.46[2]
	Suranaree 475 (S475)	SUT	nd
	High Oil Cross (HOC)	SUT	42.80[1]
	Medium Oil Cross (MOC)	SUT	37.12[1]
	Medium Oil Open (MOO)	SUT	37.58[1]
	Low Oil Open (LOO)	SUT	37.88[1]
	Chiang Mai 1	Department of Agriculture	33.86[1]
Hybrid variety	Prado Red	USDA	nd
	Pacific 33	Pacific Seeds (Thai) Ltd.	39.00[5]
	Pacific 77	Pacific Seeds (Thai) Ltd.	44.00[4]

Note: Oil content described by [1]Laosuwan (2000); [2]Laosuwan (2005); [3]Laura (2011); [4]Pacific Seeds (Thai) (2013); [5] Department of Agriculture (2009); nd = no data

Table 3.2 The parental lines for SUT synthetic varieties and breeding method.

Line	Parental lines	Breeding method
High Oil Cross (HOC)	5A, 7A, 9A,10A, 11A, 12A	controlled pollination
High Oil Open (HOO)	5A, 7A, 9A,10A, 11A, 12A	opened pollination
Medium Oil Cross (MOC)	1A, 5A, 6A, 7A, 8A, 9A	controlled pollination
Medium Oil Open (MOO)	1A, 5A, 6A, 7A, 8A, 9A	opened pollination
Low Oil Cross (LOC)	1A, 2A, 3A, 4A, 6A	controlled pollination
Low Oil Open (LOO)	1A, 2A, 3A, 4A, 6A	opened pollination

3.3 DNA pools preparation

DNA pools were prepared for PCR reactions exclusively. The pools were prepared from a mixture of 15 accessions for each synthetic variety, 3 accessions for each inbred line and hybrid variety (Figure 3.4). The approximate concentration of these stocks is 200 ng/µl. These stocks were kept in new eppendorf tubes and stored at -20°C. The stock was diluted with de-ionized water to 10 ng/µ1 for RAPD-PCR amplification and 50 ng/µ1 for SSR-PCR amplification as DNA template.

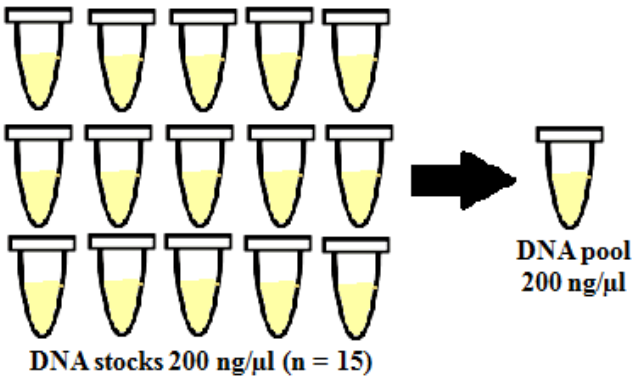

Figure 3.4 DNA pools preparation.

3.4 PCR amplification

3.4.1 RAPD-PCR amplification

Twenty-four genotypes were screened for polymorphism using 14 RAPD primers (Table 3.3). The PCR reaction volume was 25 µl containing 0.8 µl of each primer (0.2 µM), 12.5 µl of TopTaq Master Mix Kit (Qiagen, USA), 8.7 µl of de-ionized water and 10 ng template DNA (Table 3.4). Negative control with distilled water instead of DNA was included in each run in order to verify the absence of contamination using the TC-PLUS thermal cycler (Techne, UK). The touchdown PCR was used for the amplification of all investigated RAPDs as: initial denaturation 94°C for 5 min followed by 3 steps cycling of 40 cycles of 94°C for 1 min, 40°C for 1 min, 72°C for 1 min and the final extension was 72°C for 7 min.

Table 3.3 RAPD primer sequences.

Primer	Sequence 5' - 3'
S5	TGCGCCCTTC
S10	CTGCTGGGAC
S23	AGTCAGCCAC
S29	GGGTAACGCC
OPF4	GGTGATCAGG
OPF10	GGAAGCTTGG
OPF13	GGCTGCAGAA
OPF19	CCTCTAGACC
OPJ20	AAGCGGCCTC
OPX1	CTGGGCACGA
OPX2	TTCCGCCACC
OPX12	TCGCCAGCCA
OPX13	ACGGGAGCAA
OPX14	ACAGGTGCTG

Table 3.4 RAPD reaction mixture (25 µl).

Content	Stock	Final concentration	Volume for 1 tube
TopTaq master mix	2x	1x	12.5 µl
Primer	100 µM	0.2 µM	0.8 µl
DNA template	10 ng/µl	10 ng/µl	3 µl
de-ionized water			8.7 µl
Total			25 µl

3.4.2 SSR-PCR amplification

Sixteen SSR primers were selected from reports by Tang et al. (2006) and Darvishzadeh et al. (2010) and used in SSR study (Table 3.5). Three primers (ORS 1068, ORS 188 and ORS 371) associated with oil content (Tang et al. 2006) were included. PCR amplifications was performed in a volume of 25 µl containing 1 µl (2.5 mM) of forward primer, 1 µl (2.5 mM) of reverse primer, 12.5 µl of TopTaq Master Mix Kit (Qiagen, USA), 9.5 µl of de-ionized water and 50 ng template DNA (Table 3.6). A negative control with distilled water instead of DNA was included in each run in order to verify the absence of contamination using TC-PLUS thermal cyclers (Techne, UK). The touchdown PCR was used for the amplification of all investigated SSRs as: 95°C for 3 min, 1 cycle of 94°C for 30 s, 64°C for 30 s, 72°C for 45 s and followed by 10 cycles with a decrease of annealing temperature at 1°C per cycle. This was followed by 33 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 45 s. Moreover, the final extension was 20 min at 72°C (Tang et al., 2002).

Table 3.5 Primer sequences and number of alleles of the SSR loci.

Primers	Forward sequences (5'-3')	Reverse sequences (5'-3')	Number of alleles	References
ORS 160	TCCCTTCCTTTCATCGTCTGCT	TGGCAATTTGCCAAGGACC	2	Darvishzadeh et al. (2010)
ORS 16	GAGGAAATAAATCTCCGATTCA	GCAAGGACTGCAATTTAGGG	2	Darvishzadeh et al. (2010)
ORS 920	CGTTGGACGAAGAACTTGATTT	ACTTCCGTTTGTTCCGAGCTT	2	Darvishzadeh et al. (2010)
ha4149	CAAAAACCTCTCTCCGTTGGC	GACTCCAAAGTCCACCAAATC	2	Darvishzadeh et al. (2010)
ha2879	CATACCGTTCTTGTTC	CAACCTCCTAGGTCA	2	Darvishzadeh et al. (2010)
ORS 988	TTGATTTGGTGAAAGTGTGAAGC	CGAACATTATTTACATCGCTTTGTC	2	Darvishzadeh et al. (2010)
ORS 899	GCCACGTATAACTGACTATGACCA	CGAATACAGACTCGATAAACGACA	2	Darvishzadeh et al. (2010)
ORS 1088	ACTATCGAACCTCCCTCCAAAC	GGATTTCTTTCATCTTTGTGGTG	2	Darvishzadeh et al. (2010)
ORS 598	CCAAATGTGAGGTGGGAGAA	ATAGTCCCTGACGTGGATGG	3	Darvishzadeh et al. (2010)
ORS 822	CAATGCCATCTGTCATCAGCTAC	AAACAAACCTTTGGACGAAACTC	2	Darvishzadeh et al. (2010)
ORS 1068	AATTTGTCGACGGTGACGATAG	TTTTGTCATTTCATTACCCAAGG	nd	Tang et al. (2006)
ORS 188	CTTCGTAGCCAACTCCCACC	CAATGGTTGACAATGGGTTTGC	nd	Tang et al. (2006)
ORS 371	GTGTCTTCACACCACCAAACATCAACC	GGTGCCTTCTCTTCCTTGTG	nd	Tang et al. (2006)

Table 3.5 (Continued).

Primers	Forward sequences (5'-3')	Reverse sequences (5'-3')	Number of alleles	References
ORS 331	TGAAGAAGGGTTGTTGATTACAAG	GCATTGGGTTCACCATTTCT	2	Tang et al. (2006)
ORS488	CCCATTCACTCCTGTTTCCA	CTCCGGTGAGGATTTGGATT	3	Tang et al. (2006)
ORS878	TGCAAGGTATCCATATTCCACAA	TATACGCACCGGAAAGAAAGTC	2	Tang et al. (2006)

Note: nd, no data

Table 3.6 SSR reaction mixture (25 µl).

Content	Stock	Final concentration	Volume for 1 tube
TopTaq master mix	2x	1x	12.5 µl
Forward primer	2.5 µM	2.5 µM	1 µl
Reverse primer	2.5 µM	2.5 µM	1 µl
DNA template	50 ng/µl	50 ng/µl	1 µl
de-ionized water			9.5 µl
Total			25 µl

3.5 Gel electrophoresis

3.5.1 RAPD gel electrophoresis

The reaction products were resolved in 1.5% agarose gel with 0.5X TBE (40 mM Tris-borate pH 8.0, 1 mM EDTA). The running buffer was poured into the gel casting box so that the gel was submerged under approximately 0.5 inches of running buffer. The PCR products were mixed 5 µl of each sample with 1 µl MaestroSafe™ dye (Maestrogen, USA) as specified below.

MaestroSafe™ dye	1 µl
PCR reaction sample	5 µl
Total	6 µl

The mixtures were loaded into wells. In addition, DNA size standard known as 100 bp DNA Ladder (BioLabs, USA) was loaded into one lane and run at 75 volt for 35 min using iMyRun Electrophoresis (COSMO BIO, Japan). The DNA bands were visualized by placing under blue light of UltraSlim LED transilluminator (Maestrogen, USA) and photographed.

3.5.2 SSR gel electrophoresis

The reaction products were resolved in 3% agarose gel with 0.5X TBE (40 mM Tris-Borate pH 8.0, 1 mM EDTA). The running buffer was poured into the gel casting box so that the gel was submerged under approximately 0.5 inches of running buffer. The PCR products were mixed 5 µl of each sample with 1 µl MaestroSafe™ dye (Maestrogen, USA) as described above.

The mixtures were loaded into wells. In addition, DNA size standard known as 100 bp DNA Ladder (BioLabs, USA) was loaded into the lane and run at 75 volt for

35 min using iMyRun Electrophoresis (COSMO BIO, Japan). The DNA bands were visualized by placing under blue light of UltraSlim LED transilluminator (Maestrogen, USA) and photographed.

3.6 Data analysis

3.6.1 Polymorphism and scoring

DNA samples from different sunflower genotypes were amplified using a set of 14 RAPD primers and 13 SSR primer pairs for the present study. After the PCR reactions, only those primers which could amplify DNA were used for the diversity analysis.

The banding patterns were compared by visual inspection and the allele size was estimated using PhotoCapt MW, version 10.01, for Windows (Vilber Lourmat, France). Polymorphic and unambiguous bands were scored visually for their presence or absence. The amplification products were scored for the presence (1) and absence (0) of bands across the genotypes to construct a binary data matrix (Darvishzadeh et al., 2010) in Microsoft Office Excel 2007. The binary data score was used to construct a dendrogram in NTSYS-pc software. A dendrogram is hierarchical representation of the distances between each sunflower genotype used for the study.

3.7 Statistic analysis

3.7.1 Allelic polymorphism information content

Allelic polymorphism information content (PIC) was calculated as described by Anderson et al. (1992)

$$PIC = 1 - \sum_{i=1}^{n} (P_i)^2$$

Where P_i is the proportion of the population carrying the i^{th} allele, calculated for each primer.

3.7.2 Cluster analysis

The binary data score was used to construct a dendrogram. The genetic associations among accessions were evaluated by calculating the Jaccard's similarity coefficients for pair wise comparisons based on the proportions of shared bands produced by the primers. Data analyses were performed by software NTSYS-pc version 2.10p (Rohlf, 1998). The genetic similarity matrix was analyzed using Jaccard's similarity coefficient (Jaccard, 1908). Dendrogram was constructed by the unweighted pair-group method using arithmetic average (UPGMA) (Rohlf, 1998).

3.7.3 Principal coordinate analysis

Principal Coordinate Analysis or PCoA was calculated based on a total of bands or markers that were generated to produce distribution pattern of all 24 sunflower genotypes. PCoA was carried out to obtain a multidimension plot, in order to represent the diversity among the accessions. This method was used to explore and to visualize similarities or dissimilarities of data. It starts with a similarity matrix. However, all binary measures (Jaccard, Dice etc.) are distance measures and, therefore PCoA should be used (Gower, 1966). It was generated using IBM SPSS STATISTIC program, version 16 statistical software packages (IBM, USA).

3.7.4 Pearson's correlation

Pearson's correlation coefficient (r) is a measure of the strength of the association between the two variables. The similarity matrices from two markers system were used to calculate (Garcia et al., 2004). It was generated using IBM SPSS STATISTIC program, version 16 statistical software packages (IBM, USA).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Genomic DNA

Extraction of total genomic DNA from 24 sunflower genotypes was successful by using the CTAB protocol as a modified version described by Rogers and Bendich (1985). The quality of DNA from 24 sunflower genotypes was examined on 0.8% agarose gel prepared in 0.5X TBE buffer (Figure 4.1).

The quantity of DNA from all of 24 sunflower genotypes was measured at 260 and 280 nm by NanoDrop, ND-1000 Spectrophotometer as shown in Table 4.1. The results show that DNA quantity varied from 170.14 ng/µl (for LOO) to 1,454.48 ng/µl (for 11A). The ratio of absorbance at 260/230 nm ranged from 0.91 (PradoRed) to 1.95 (1A) with all samples tested being below 2.0, indicating the presence of contaminants that could be carbohydrates, proteins, chloroform and phenols. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA, in which a ratio of approximately 1.8 is generally accepted as “pure” for DNA. It was found that the 260/280 values ranged from 1.55 (5A) to 1.88 (S475) with 70% of samples tested achieving a value of 1.8 or greater. Eland et al. (2012) suggested that in reality many of DNA extractions from environmental samples do not meet the standard and further purification methods are commonly used before the DNA is used downstream.

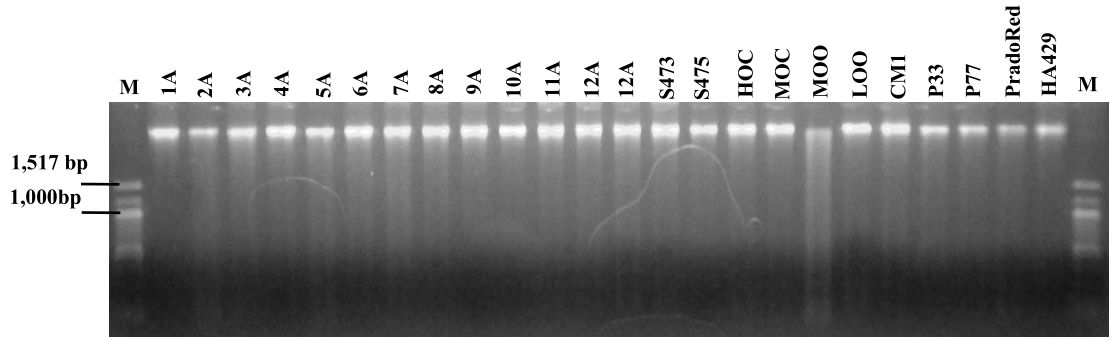

Figure 4.1 DNA quality test of 24 sunflower (M = 100 bp DNA ladder).

Assessment of genetic diversity is necessary for germplasm characterization and breeding program. Therefore genetic diversity study of *H. annuus* L. genotypes using RAPD and SSR markers were conducted. In this study, a total of 24 sunflower genotypes were used to investigate genetic diversity and their relationships with 14 RAPD primers and 16 SSR primers. The results were explained as follows.

Table 4.1 The quantity of DNA from all of 24 sunflower genotypes.

Line/Variety	260/230 nm	260/280 nm	DNA quantity (ng/µl)
1A	2.0	1.9	738.8
2A	1.2	1.6	590.5
3A	1.6	1.9	464.6
4A	1.7	1.8	532.4
5A	1.1	1.6	477.1
6A	1.5	1.8	570.5
7A	1.6	1.8	371.8
8A	1.5	1.7	387.1
9A	1.6	1.8	784.0
10A	1.4	1.7	792.7
11A	1.8	1.8	1454.5
12A	1.5	1.8	425.0
S471	1.3	1.8	205.4
S473	1.3	1.8	199.5
S475	1.6	1.9	596.8
HOC	1.3	1.8	248.2
MOC	1.6	1.8	308.2
MOO	1.0	1.6	200.8
LOO	1.1	1.8	170.1
CM1	1.4	1.8	186.4
P33	1.3	1.7	913.8
P77	1.3	1.7	594.4
PradoRed	0.9	1.8	316.0
HA429	1.4	1.8	171.7

4.2 RAPD marker analysis

Forty-eight RAPD primers were used to screen sunflower genotypes and 14 RAPD primers could amplify DNA. Therefore 14 RAPD primers were used to analyze the genetic diversity and relationship in 24 sunflower genotypes in this study.

A total of 158 bands were scored and no band was found to be monomorphic among studied genotypes as shown in Figure 4.2-4.15. Number of alleles per locus ranged from 3 for locus OPF10 to 19 for locus OPF4 with an average of 11.29 (Table 4.2). The bands size varied from minimum 196 bp for locus S5 to maximum 1,586 bp for locus OPX13. The discrimination power of each RAPD locus was estimated by polymorphic information content (PIC) values, which ranged from 0.02 for locus OPF19 to 0.74 for locus S23 and OPX14 with an average 0.40. According to a report by Yu et al. (2012), PIC from each primer was highly informative when PIC > 0.50, moderately informative when 0.25 < PIC < 0.50, and slightly informative when PIC < 0.25. The result showed that 6 from 14 RAPD markers were highly informative (PIC > 0.50), 5 RAPD markers were moderately informative (0.25 < PIC < 0.50) and 3 RAPD were slightly informative (PIC < 0.25). Markers with high PIC values are S23, OPF10, OPF13, OPX1, OPX13 and OPX14 as shown in Table 4.1. The genetic similarity coefficients, as shown in Table 4.3 among 24 sunflower genotypes varied from a maximum of 0.49 (between 7A and 8A genotypes) to a minimum of 0.00 (between 2A and Pacific77, 5A and Pacific77, 9A and PradoRed, 11A and PradoRed genotypes) with an average 0.22.

Cluster analysis was used to group the genotypes and to construct a dendogram. The similarity matrix representing the Jaccard's coefficient was used to cluster the data using the UPGMA algorithm. The UPGMA based dendogram obtained from the binary data using NTSYSpc version 2.1 program divided 24 sunflower genotypes into two main groups (Figure 4.16). The first group represents 12 inbred lines (1A, 2A, 3A, 4A, 5A, 6A, 7A, 8A, 9A, 10A, 11A and 12A) and 7 synthetic varieties (S471, S473, S475, HOC, MOC, MOO, LOO) developed by Suranaree University of

Technology. Subgroup IA contained 1A inbred line was high divergent with subgroup IIA. While the second group represents hybrid variety (CM1) developed by Department of Agriculture, commercial hybrid varieties (Pacific 33 and Pacific 77) and sunflower accessions from abroad (PradoRed and HA429). Moreover, RAPD primers S23 and OPX14 which produced the highest number of PIC showed the greatest potential to discriminate polymorphic DNA segments.

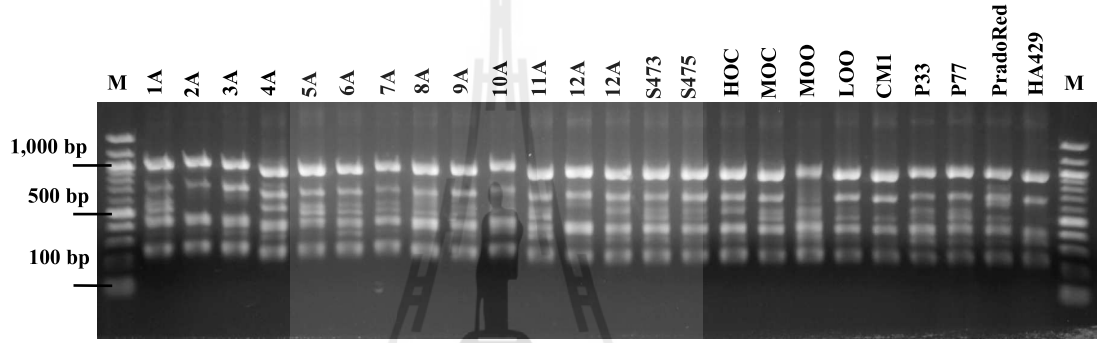

Figure 4.2 A 1.5% agarose gel showing the results of PCR amplification using primer S5 with 24 sunflower genotypes (M = 100 bp DNA ladder).

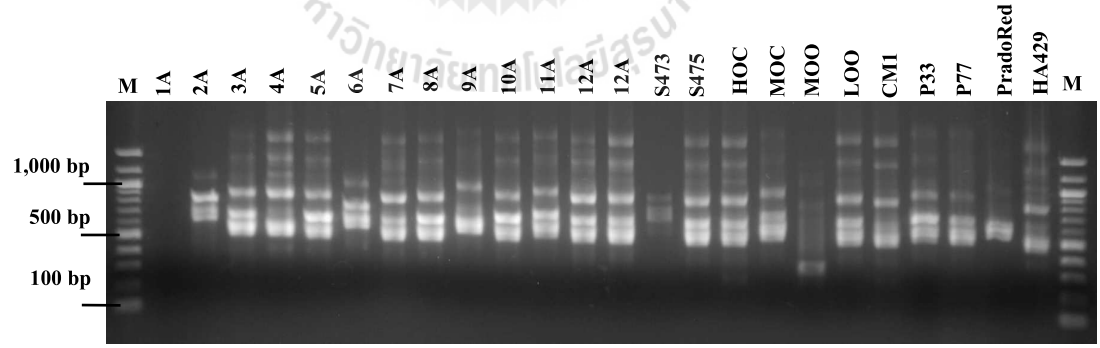

Figure 4.3 A 1.5% agarose gel showing the results of PCR amplification using primer S10 with 24 sunflower genotypes (M = 100 bp DNA ladder).

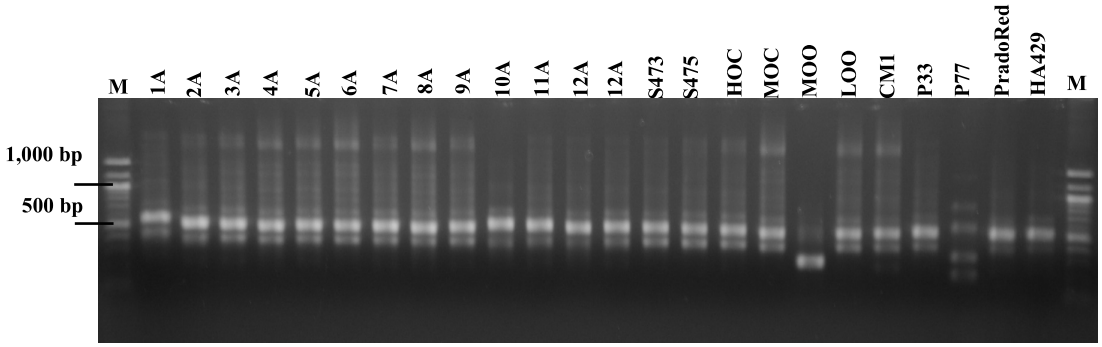

Figure 4.4 A 1.5% agarose gel showing the results of PCR amplification using primer S23 with 24 sunflower genotypes (M = 100 bp DNA ladder).

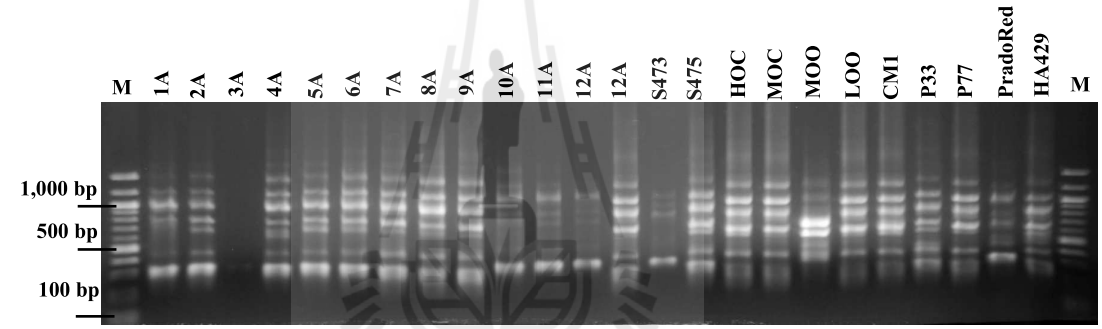

Figure 4.5 A 1.5% agarose gel showing the results of PCR amplification using primer S29 with 24 sunflower genotypes (M = 100 bp DNA ladder).

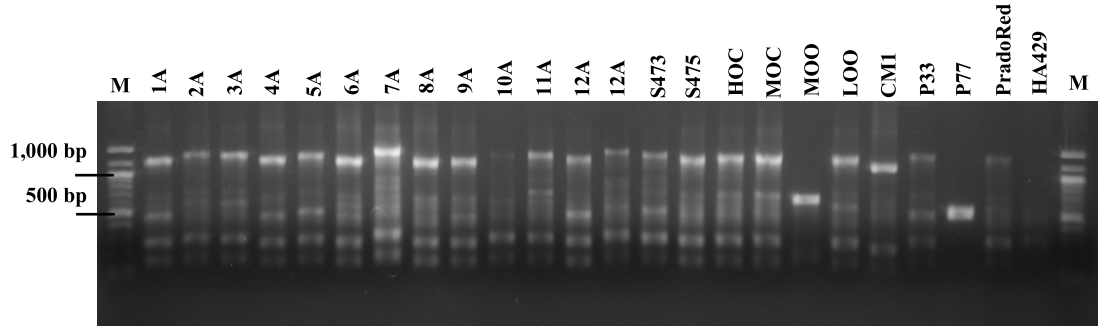

Figure 4.6 A 1.5% agarose gel showing the results of PCR amplification using primer OPF4 with 24 sunflower genotypes (M = 100 bp DNA ladder).

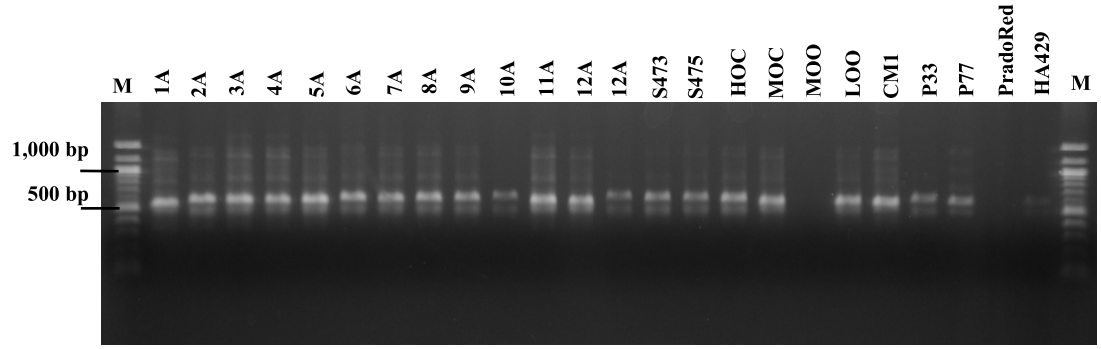

Figure 4.7 A 1.5% agarose gel showing the results of PCR amplification using primer OPF10 with 24 sunflower genotypes (M = 100 bp DNA ladder).

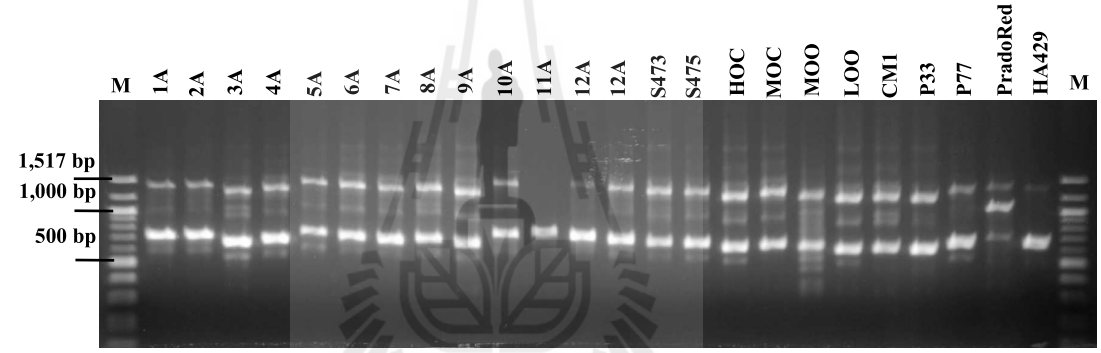

Figure 4.8 A 1.5% agarose gel showing the results of PCR amplification using primer OPF13 with 24 sunflower genotypes (M = 100 bp DNA ladder).

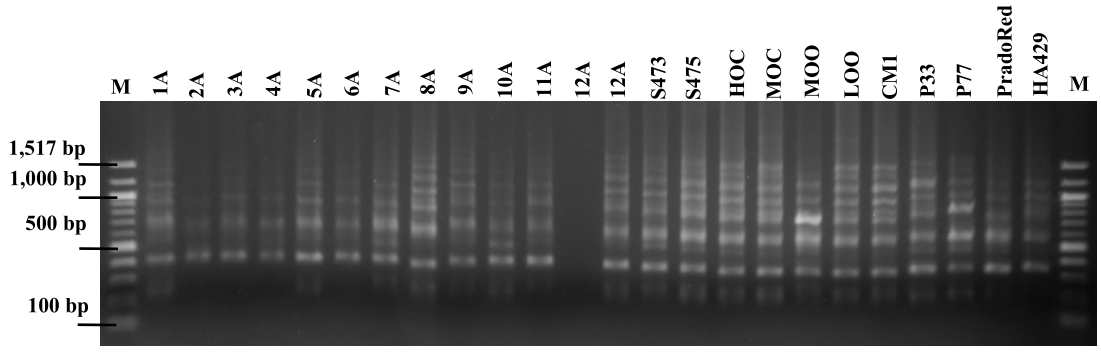

Figure 4.9 A 1.5% agarose gel showing the results of PCR amplification using primer OPF19 with 24 sunflower genotypes (M = 100 bp DNA ladder).

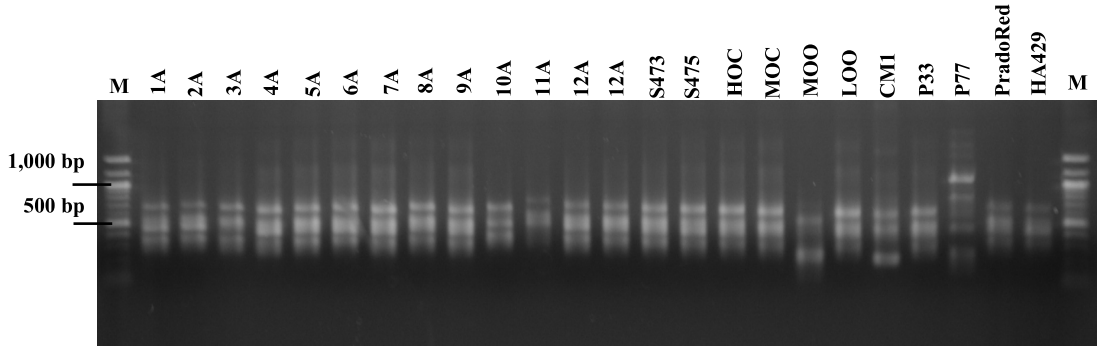

Figure 4.10 A 1.5% agarose gel showing the results of PCR amplification using primer OPJ20 with 24 sunflower genotypes (M = 100 bp DNA ladder).

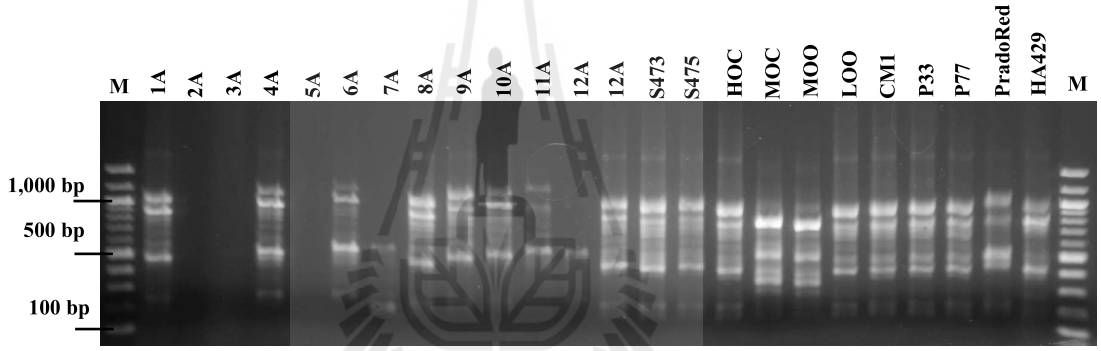

Figure 4.11 A 1.5% agarose gel showing the results of PCR amplification using primer OPX1 with 24 sunflower genotypes (M = 100 bp DNA ladder).

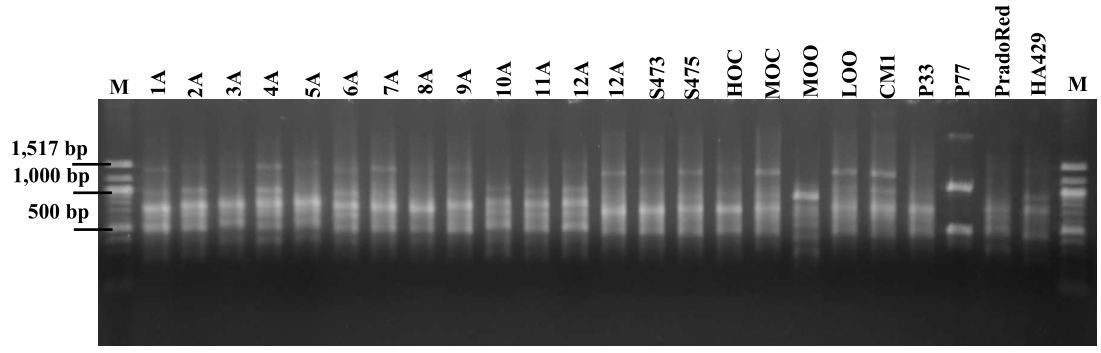

Figure 4.12 A 1.5% agarose gel showing the results of PCR amplification using primer OPX2 with 24 sunflower genotypes (M = 100 bp DNA ladder).

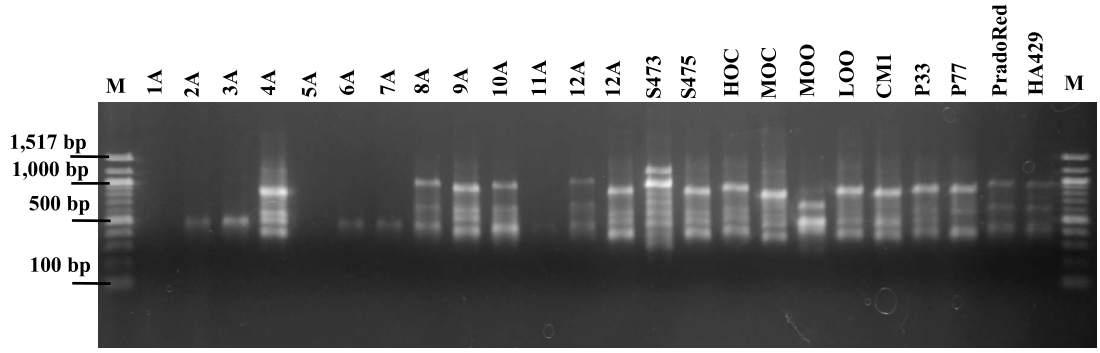

Figure 4.13 A 1.5% agarose gel showing the results of PCR amplification using primer OPX12 with 24 sunflower genotypes (M = 100 bp DNA ladder).

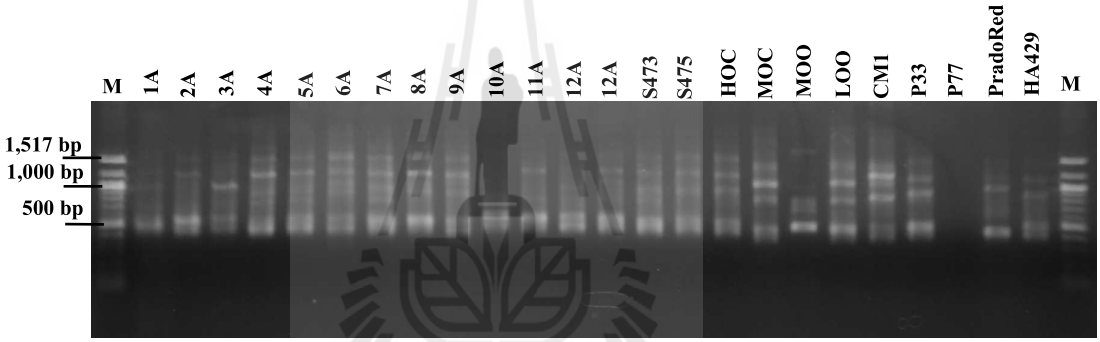

Figure 4.14 A 1.5% agarose gel showing the results of PCR amplification using primer OPX13 with 24 sunflower genotypes (M = 100 bp DNA ladder).

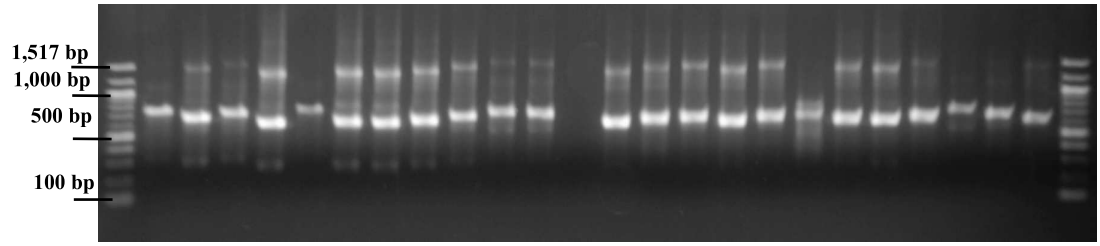

Figure 4.15 A 1.5% agarose gel showing the results of PCR amplification using primer OPX14 with 24 sunflower genotypes (M = 100 bp DNA ladder).

Table 4.2 Fourteen RAPD primers were used to amplify the genomic DNA.

Primer name	Sequence 5' - 3'	Number of alleles	Band range (bp)	Polymorphic bands	Monomorphic bands	PIC
S5	TGCGCCCTTC	18	196 - 1077	18	-	0.09
S10	CTGCTGGGAC	10	433 - 956	10	-	0.36
S23	AGTCAGCCAC	6	206 - 560	6	-	0.74
S29	GGGTAACGCC	17	300 - 1500	17	-	0.28
OPF4	GGTGATCAGG	19	300 - 1500	19	-	0.11
OPF10	GGAAGCTTGG	3	545 - 647	3	-	0.66
OPF13	GGCTGCAGAA	12	593 - 1450	12	-	0.62
OPF19	CCTCTAGACC	15	321 - 1468	15	-	0.02
OPJ20	AAGCGGCCTC	10	212 - 714	10	-	0.27
OPX1	CTGGGCACGA	13	323 - 1200	13	-	0.65
OPX2	TTCCGCCACC	7	400 - 1016	7	-	0.25
OPX12	TCGCCAGCCA	12	365 - 1174	12	-	0.29
OPX13	ACGGGAGCAA	8	400 - 1586	8	-	0.57
OPX14	ACAGGTGCTG	8	592 - 1538	8	-	0.74

Table 4.3 Jaccard's coefficient similarity matrix based on 14 RAPD primers.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	1.00																							
2	0.21	1.00																						
3	0.19	0.24	1.00																					
4	0.08	0.12	0.27	1.00																				
5	0.15	0.14	0.33	0.35	1.00																			
6	0.15	0.24	0.30	0.38	0.42	1.00																		
7	0.18	0.27	0.27	0.32	0.29	0.32	1.00																	
8	0.10	0.12	0.13	0.16	0.22	0.28	0.49	1.00																
9	0.05	0.13	0.13	0.23	0.17	0.25	0.23	0.29	1.00															
10	0.12	0.28	0.28	0.20	0.33	0.27	0.23	0.22	0.29	1.00														
11	0.10	0.18	0.16	0.13	0.22	0.23	0.13	0.17	0.20	0.27	1.00													
12	0.16	0.31	0.19	0.08	0.15	0.29	0.20	0.20	0.13	0.22	0.29	1.00												
13	0.10	0.10	0.16	0.19	0.15	0.22	0.27	0.31	0.17	0.16	0.10	0.20	1.00											
14	0.10	0.11	0.17	0.23	0.16	0.20	0.20	0.20	0.36	0.24	0.10	0.14	0.18	1.00										
15	0.02	0.09	0.13	0.25	0.15	0.25	0.17	0.20	0.38	0.24	0.09	0.15	0.38	0.33	1.00									
16	0.07	0.07	0.07	0.13	0.07	0.17	0.09	0.14	0.28	0.22	0.07	0.15	0.25	0.28	0.40	1.00								
17	0.08	0.05	0.10	0.19	0.10	0.10	0.12	0.14	0.18	0.16	0.17	0.17	0.31	0.20	0.29	0.38	1.00							
18	0.14	0.13	0.08	0.05	0.05	0.12	0.14	0.10	0.18	0.17	0.16	0.14	0.05	0.15	0.14	0.23	0.15	1.00						
19	0.12	0.12	0.15	0.16	0.12	0.20	0.22	0.17	0.23	0.21	0.21	0.21	0.26	0.15	0.29	0.45	0.39	0.26	1.00					
20	0.02	0.07	0.02	0.08	0.02	0.07	0.08	0.06	0.13	0.08	0.06	0.06	0.12	0.10	0.18	0.18	0.12	0.10	0.36	1.00				
21	0.06	0.06	0.02	0.11	0.06	0.11	0.13	0.10	0.08	0.05	0.04	0.06	0.15	0.08	0.19	0.18	0.12	0.08	0.23	0.30	1.00			
22	0.02	0.00	0.06	0.03	0.00	0.02	0.03	0.06	0.04	0.05	0.02	0.02	0.14	0.10	0.13	0.14	0.12	0.10	0.14	0.16	0.35	1.00		
23	0.05	0.05	0.08	0.04	0.02	0.06	0.06	0.06	0.00	0.06	0.00	0.05	0.11	0.06	0.07	0.05	0.13	0.06	0.07	0.13	0.13	0.13	1.00	
24	0.05	0.04	0.07	0.08	0.02	0.06	0.04	0.04	0.03	0.02	0.02	0.05	0.04	0.06	0.11	0.07	0.10	0.09	0.10	0.22	0.17	0.22	0.30	1.00

Remark; 1=1A, 2=2A, 3=3A, 4=4A, 5=5A, 6=6A, 7=7A, 8=8A, 9=9A, 10=10A, 11=11A, 12=12A, 13=S471, 14=S473, 15=S475, 16=HOC, 17=MOC, 18=MOO, 19=LOO, 20=CM1, 21=P33, 22=P77, 23=PradoRed, 24=HA429.

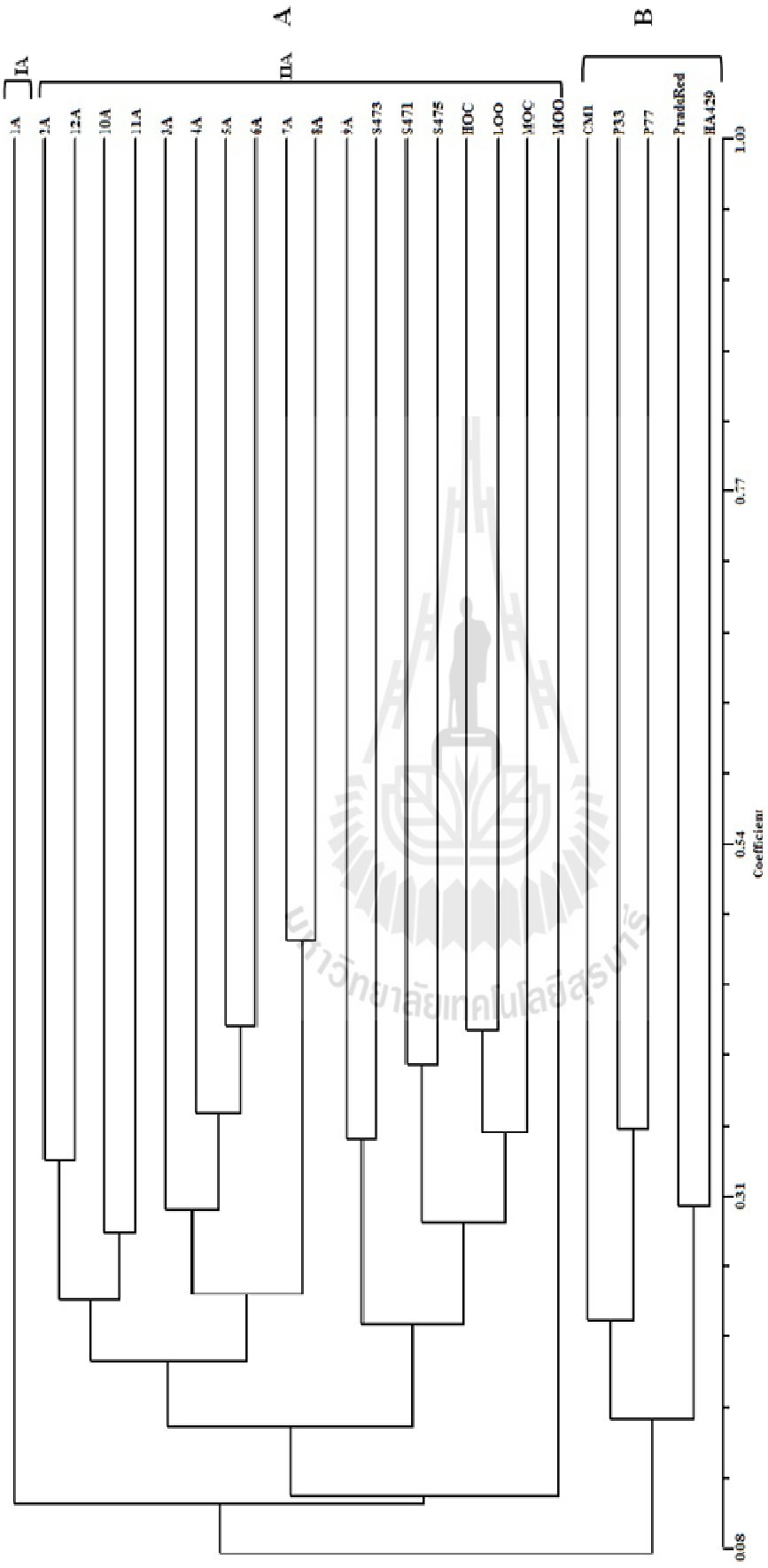

Figure 4.16 Dendrogram by UPGMA cluster generated from RAPD markers.

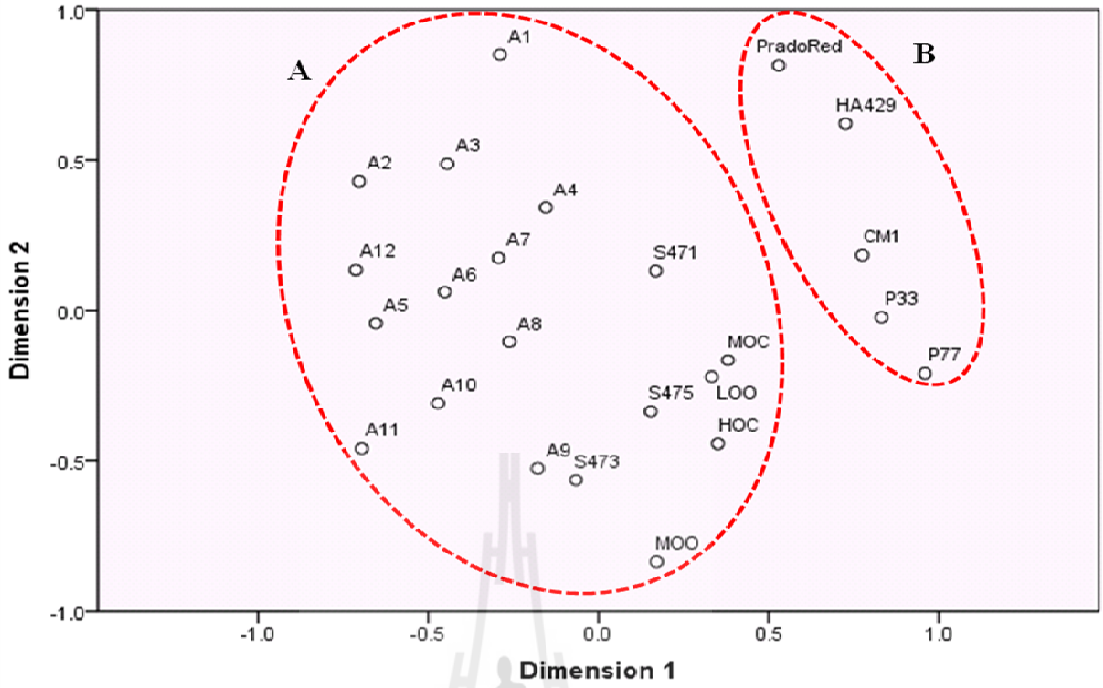

Figure 4.17 Principal Coordinates Analysis (PCoA) by RAPD markers.

The PCoA based scatter plot obtained from the similarities data using IBM SPSS statistic 19 program, 24 sunflower genotypes were separated into two main groups (Figure 4.17). PCoA was also done to visualize genetic relationships among the breeding lines. Therefore, this result supported the UPGMA result. Similar results were found in pomegranate (*Punica granatum* L.), kenaf (*Hibiscus cannabinus* L.), hop (*Humulus lupulus* L.), and the endangered Chinese endemic herb (*Saruma henryi* Oliv.) (Kim et al., 2010; Zhou et al., 2010; Ercisli et al., 2011; and Howard et al., 2011) which the PCoA data supported the UPGMA dendrogram. However, some study such as in watermelon (*Citrullus lanatus* (Thunb.) (Szamosi et al., 2009) the PCoA did not support the UPGMA dendrogram.

RAPD markers are dominant marker and have extensively used in sunflower such as for genetic diversity (Popov et al., 2002; Iqbal et al., 2008) and DNA

fingerprint study (Nandini and Chikkadevaiah, 2005), due to their technique that are simply, rapid and no prior knowledge requirement of the genome that is being analyzed. Moreover, RAPD can be employed across species using universal primers (Agarwal et al., 2008). Nevertheless, the major drawback of the method is that profiling is dependent on the reaction conditions so may vary within two different laboratories and as several discrete loci in the genome are amplified by each primer, profiles are not able to distinguish heterozygous from homozygous individuals (Bardakci, 2001).

4.3 SSR marker analysis

SSRs have established the current marker system of choice for characterizing sunflower germplasms (Tang et al., 2002; Paniego et al., 2002; Yu et al., 2002; Tang and Knapp, 2003; Zhang et al., 2005; and Hvarleva et al., 2007). Darvishzadeh et al. (2010) reported that 38 SSR markers were enough to cover sunflower genome. Based on their PIC values, 16 of 38 SSR markers were selected for polymorphism study. In this study, only 12 primers could amplify DNA and were used to analyze genetic differences and relationships among 24 sunflower genotypes. The result showed that a total of 54 alleles were detected and which no band was found to be monomorphic among studied genotypes as shown in Figure 4.18-4.29. An agarose gel electrophoresis at 3% concentration was used for visualization of the product from the amplification of SSR markers which were proofed to be an appropriate concentration as seen by the double bands for heterozygous line. Number of alleles per locus ranged from 3 for locus ORS 331 to 6 for locus ha4149 with an average of 4.50 (Table 4.4). The bands size varied from minimum 146 bp for locus ha4149 to maximum 436 bp

for locus ORS 598. The discrimination power of each SSR locus was estimated by PIC, ranged from 0.46 for locus ORS 920 to 0.81 for locus ORS 160 with an average 0.64. Moreover, 11 from 12 SSR marker set were highly informative (PIC > 0.50), while only one SSR marker was moderately informative (0.25 < PIC < 0.50). Markers with high PIC values include ORS 160, ha4149, ORS 988, ORS 899, ORS 1088, ORS 598, ORS 822, ORS 371, ORS 331, ORS 488 and ORS 878. This result is similar to a study done by Darvishzadeh et al. (2010) in which the mean for the number of allele per locus was 2.32, and PIC values ranged from 0.09 (locus ha3555) to 0.62 (locus ORS598) with an average of 0.41. They also suggested that several markers with high PIC values could be effectively used in genetic diversity studies of sunflower such as ORS160, Ha4149, ORS988, ORS899, ORS1088, ORS598 and ORS822. The genetic similarity coefficients among 24 sunflower genotypes (Table 4.5) varied from a maximum of 0.85 (between S471 and HOC genotypes) to a minimum of 0.00 (between 1A and S475, 1A and CM1, 1A and PradoRed, 1A and HA429, 2A and CM1, 3A and PradoRed, 4A and PradoRed, 5A and PradoRed, 6A and PradoRed, 7A and PradoRed, S473 and PradoRed, LOO and PradoRed genotypes) with an average 0.31.

Interestingly, ORS 1088 marker gave double bands in P33 and P77 hybrid varieties (Figure 4.29), indicating their hybrid progenies. In addition, some primers (ha4149, ORS 899 and ORS 988) evidenced the amplification of double band patterns in inbred lines (Figure 4.20, 4.26 and 4.28) which means that SUT inbred lines were not yet near isogenic lines. Furthermore, this kind of amplification profile can be explained by at least two main reasons (*i*) heterozygosity or heterogeneity of the inbred lines indicating that they are derived from heterozygous cultivar selections;

and (*ii*) presence of two (or more) duplicated SSR loci (including their bordering sequences) in the sunflower genome. This kind of amplification pattern was described before by Akagi et al. (1998) and Paniego et al. (2002).

Cluster analysis based on SSR markers divided 24 sunflower genotypes into two main groups and several sub-groups according to genetic relatedness, similar to clustering based on RAPD (Figure 4.30). Group A was comprised of all 12 inbred lines (1A, 2A, 3A, 4A, 5A, 6A, 7A, 8A, 9A, 10A, 11A and 12A) and 7 synthetic varieties (S471, S473, S475, HOC, MOC, MOO, LOO). Subgroup IA contained 2 inbred lines 1A and 2A were high divergent with subgroup IIA. In subgroup IIA, S471 is closely related to HOC with the highest similarity value (0.85), owning to HOC being the high oil cross synthetic variety which their parental inbred lines were 5A, 7A, 9A, 10A 11A and 12A. Moreover, in this subgroup all sunflower genotypes were relative. Group B represents hybrid variety (CM1) developed by Department of Agriculture, commercial hybrid varieties (Pacific 33 and Pacific 77) and sunflower accessions from abroad (PradoRed and HA429). It showed that the commercial hybrid varieties and sunflower accessions from abroad were completely distinguished from the inbred lines and synthetic varieties.

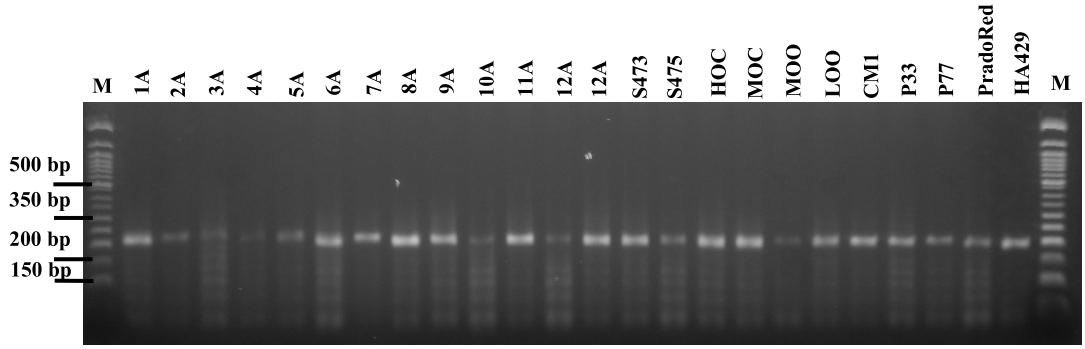

Figure 4.18 A 3% agarose gel showing the results of PCR amplification using primer ORS 160 with 24 sunflower genotypes (M = 50 bp DNA ladder).

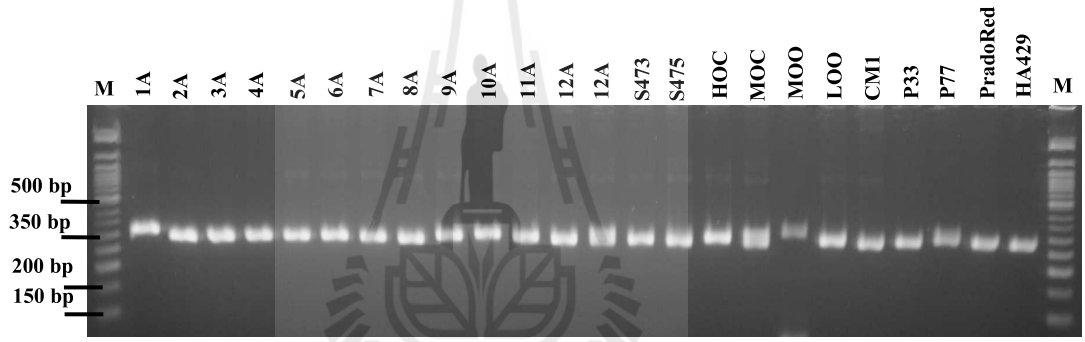

Figure 4.19 A 3% agarose gel showing the results of PCR amplification using primer ORS 371 with 24 sunflower genotypes (M = 50 bp DNA ladder).

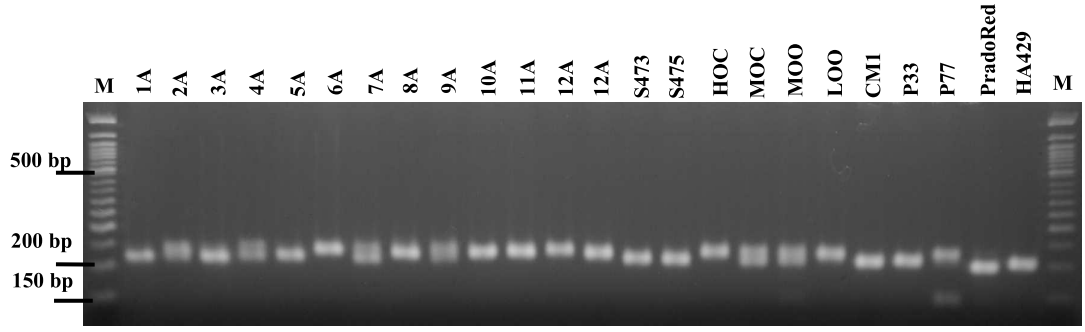

Figure 4.20 A 3% agarose gel showing the results of PCR amplification using primer ha4149 with 24 sunflower genotypes (M = 50 bp DNA ladder).

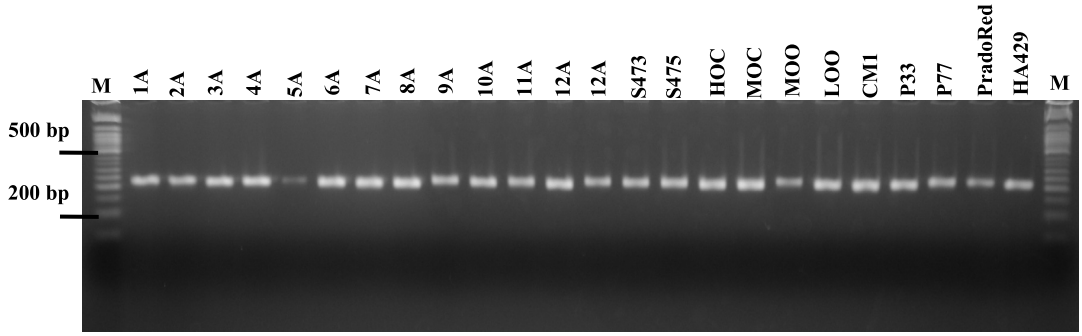

Figure 4.21 A 3% agarose gel showing the results of PCR amplification using primer ORS 331 with 24 sunflower genotypes (M = 50 bp DNA ladder).

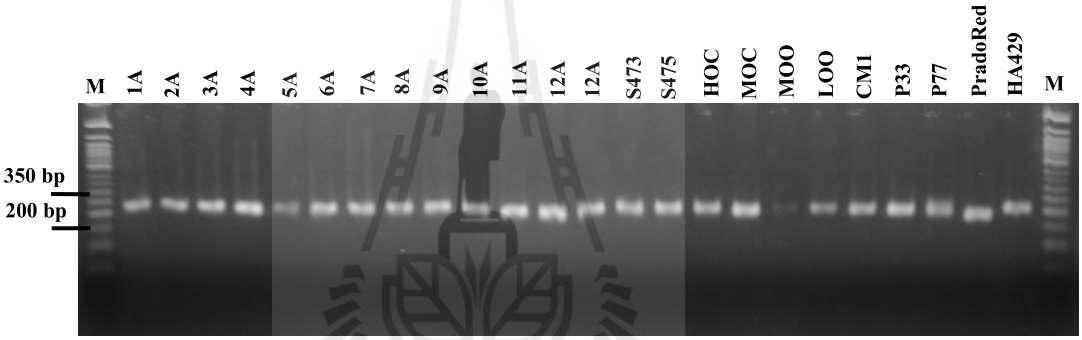

Figure 4.22 A 3% agarose gel showing the results of PCR amplification using primer ORS 488 with 24 sunflower genotypes (M = 50 bp DNA ladder).

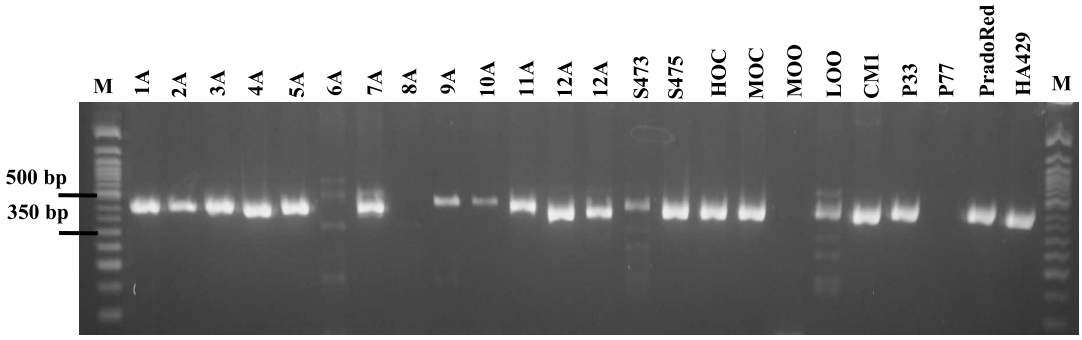

Figure 4.23 A 3% agarose gel showing the results of PCR amplification using primer ORS 598 with 24 sunflower genotypes (M = 50 bp DNA ladder).

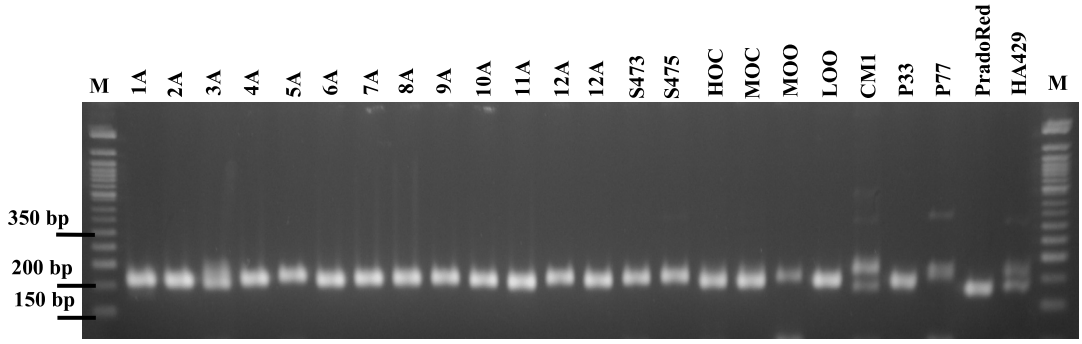

Figure 4.24 A 3% agarose gel showing the results of PCR amplification using primer ORS 822 with 24 sunflower genotypes (M = 50 bp DNA ladder).

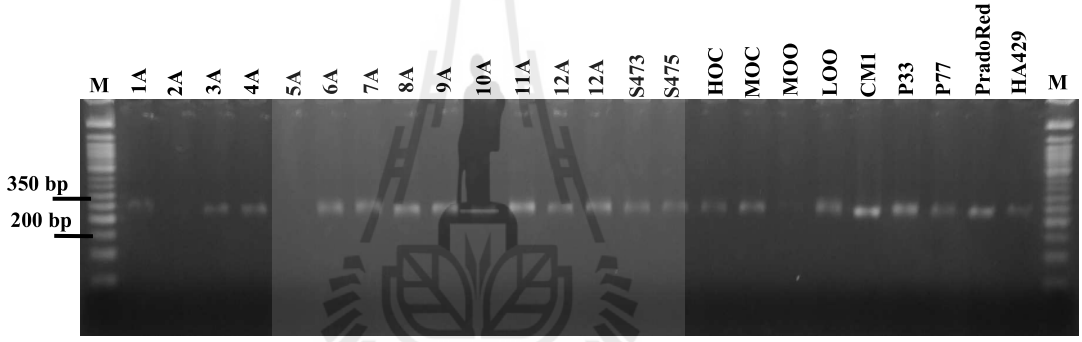

Figure 4.25 A 3% agarose gel showing the results of PCR amplification using primer ORS 878 with 24 sunflower genotypes (M = 50 bp DNA ladder).

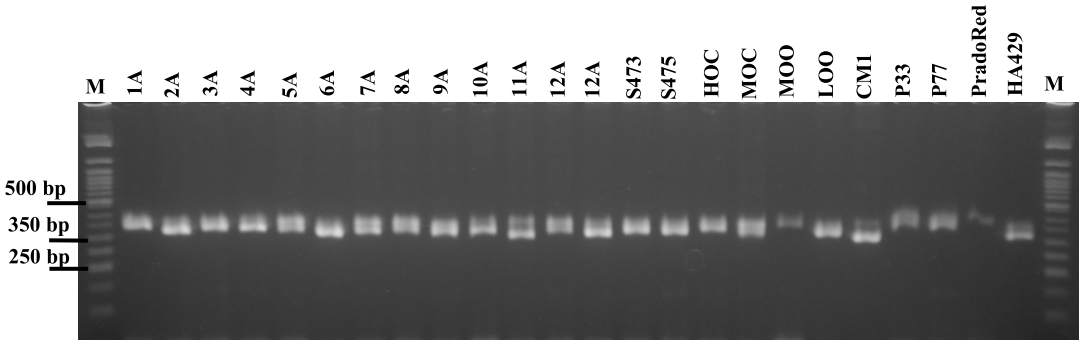

Figure 4.26 A 3% agarose gel showing the results of PCR amplification using primer ORS 899 with 24 sunflower genotypes (M = 50 bp DNA ladder).

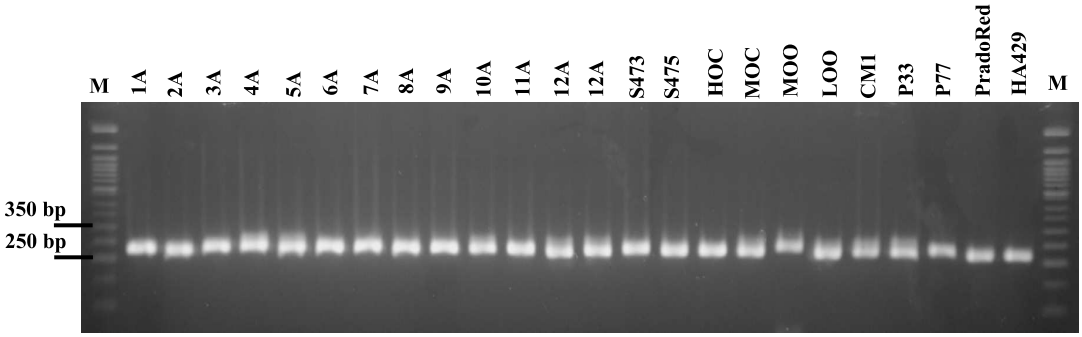

Figure 4.27 A 3% agarose gel showing the results of PCR amplification using primer ORS 920 with 24 sunflower genotypes (M = 50 bp DNA ladder).

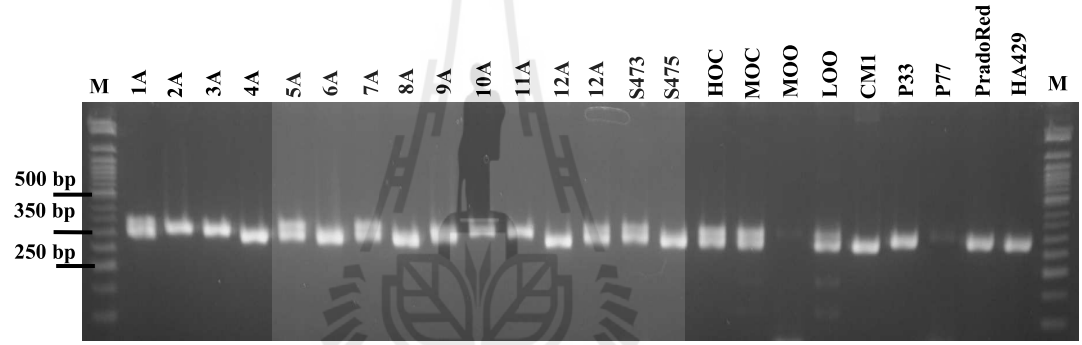

Figure 4.28 A 3% agarose gel showing the results of PCR amplification using primer ORS 988 with 24 sunflower genotypes (M = 50 bp DNA ladder).

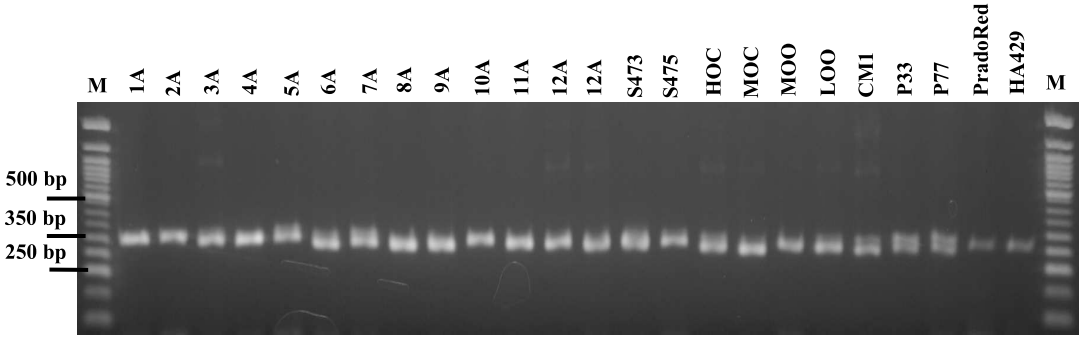

Figure 4.29 A 3% agarose gel showing the results of PCR amplification using primer ORS 1088 with 24 sunflower genotypes (M = 50 bp DNA ladder).

Table 4.4 Twelve SSR primers were used to amplify the genomic DNA.

Primer name	Forward sequence 5'-3'	Reverse sequence 5'-3'	Linkage group	Number of alleles	Band range (bp)	Polymorphic bands	Monomorphic bands	PIC
ORS 160	TCCCTTCCTTTCA TCGTCTGCT	TGGCAATTTGCCA AGGACC		4	207-230	4	-	0.81
ORS 920	CGTTGGACGAAG AACTTGATTT	ACTTCCGTTTGTT CCGAGCTT	LG16[1]	4	215-241	4	-	0.46
ha4149	CAAAAACCTCTCT CCGTTGGC	GACTCCAAAGTC CACCAAATC		6	146-200	6	-	0.68
ORS 988	TTGATTTGGTGAA AGTGTGAAGC	CGAACATTATTTA CATCGCTTTGTC	LG17[1]	5	266-336	5	-	0.57
ORS 899	GCCACGTATAACT GACTATGACCA	CGAATACAGACT CGATAAACGACA	LG16[1]	5	324-405	5	-	0.59
ORS 1088	ACTATCGAACCTC CCTCCAAAC	GGATTTCTTTCAT CTTTGTGGTG	LG10[1]	5	237-304	5	-	0.71
ORS 598	CCAAATGTGAGG TGGGAGAA	ATAGTCCCTGACG TGGATGG	LG1[1]	5	356-436	5	-	0.78
ORS 822	CAATGCCATCTGT CATCAGCTAC	AAACAAACCTTT GGACGAAACTC	LG1[1]	4	172-234	4	-	0.62

Table 4.4 (continued).

Primer name	Forward sequence 5'-3'	Reverse sequence 5'-3'	Linkage group	Number of alleles	Band range (bp)	Polymorphic bands	Monomorphic bands	PIC
ORS 371	GTGTCTTCACACC ACCAAACATCAA CC	GGTGCCTTCTCTT CCTTGTG	LG1[2]	4	291-344	4	-	0.61
ORS 331	TGAAGAAGGGTT GTTGATTACAAG	GCATTGGGTTCAC CATTTCT	LG7[1]	3	250-288	3	-	0.55
ORS488	CCCATTCACTCCT GTTTCCA	CTCCGGTGAGGA TTTGGATT	LG3[1]	5	245-290	5	-	0.74
ORS878	TGCAAGGTATCC ATATTCCACAA	TATACGCACCGG AAAGAAAGTC	LG10[1]	4	273-310	4	-	0.58

Note; [1]Tang et al. (2002); [2]Tang et al. (2006)

Table 4.5 Jaccard's coefficient similarity matrix based on 12 SSR primers.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	1.00																							
2	0.52	1.00																						
3	0.58	0.57	1.00																					
4	0.40	0.45	0.61	1.00																				
5	0.48	0.27	0.43	0.42	1.00																			
6	0.08	0.29	0.27	0.52	0.35	1.00																		
7	0.43	0.48	0.69	0.74	0.67	0.54	1.00																	
8	0.24	0.18	0.35	0.42	0.42	0.35	0.52	1.00																
9	0.31	0.35	0.17	0.40	0.48	0.58	0.43	0.48	1.00															
10	0.24	0.27	0.26	0.25	0.33	0.35	0.37	0.42	0.40	1.00														
11	0.23	0.26	0.33	0.32	0.40	0.42	0.43	0.32	0.23	0.56	1.00													
12	0.08	0.29	0.45	0.61	0.17	0.45	0.54	0.43	0.25	0.17	0.42	1.00												
13	0.15	0.26	0.17	0.32	0.40	0.50	0.36	0.32	0.54	0.56	0.62	0.25	1.00											
14	0.16	0.27	0.35	0.42	0.42	0.70	0.52	0.33	0.56	0.50	0.56	0.43	0.56	1.00										
15	0.00	0.09	0.17	0.33	0.33	0.61	0.30	0.33	0.32	0.42	0.48	0.35	0.56	0.58	1.00									
16	0.08	0.17	0.25	0.32	0.32	0.50	0.36	0.40	0.38	0.64	0.69	0.42	0.85	0.56	0.64	1.00								
17	0.20	0.22	0.36	0.41	0.28	0.36	0.44	0.21	0.27	0.41	0.53	0.50	0.47	0.48	0.48	0.60	1.00							
18	0.30	0.08	0.16	0.23	0.38	0.08	0.28	0.23	0.30	0.15	0.22	0.24	0.37	0.15	0.31	0.37	0.58	1.00						
19	0.16	0.18	0.43	0.50	0.17	0.35	0.37	0.25	0.24	0.25	0.40	0.43	0.40	0.33	0.42	0.40	0.62	0.23	1.00					
20	0.00	0.00	0.10	0.19	0.19	0.20	0.17	0.19	0.09	0.19	0.18	0.20	0.09	0.19	0.29	0.18	0.23	0.09	0.19	1.00				
21	0.15	0.08	0.24	0.31	0.15	0.24	0.28	0.23	0.07	0.46	0.37	0.24	0.30	0.23	0.38	0.37	0.39	0.14	0.38	0.61	1.00			
22	0.22	0.08	0.24	0.31	0.31	0.16	0.28	0.15	0.15	0.31	0.22	0.16	0.22	0.15	0.38	0.22	0.32	0.21	0.31	0.52	0.79	1.00		
23	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.17	0.16	0.17	0.08	0.09	0.16	0.00	0.08	0.24	0.07	0.15	0.00	0.19	0.31	0.38	1.00	
24	0.00	0.17	0.08	0.08	0.08	0.16	0.07	0.08	0.15	0.23	0.30	0.16	0.37	0.15	0.31	0.44	0.26	0.29	0.23	0.35	0.43	0.43	0.46	1.00

Remark; 1=1A, 2=2A, 3=3A, 4=4A, 5=5A, 6=6A, 7=7A, 8=8A, 9=9A, 10=10A, 11=11A, 12=12A, 13=S471, 14=S473, 15=S475, 16=HOC, 17=MOC, 18=MOO, 19=LOO, 20=CM1, 21=P33, 22=P77, 23=PradoRed, 24=HA429.

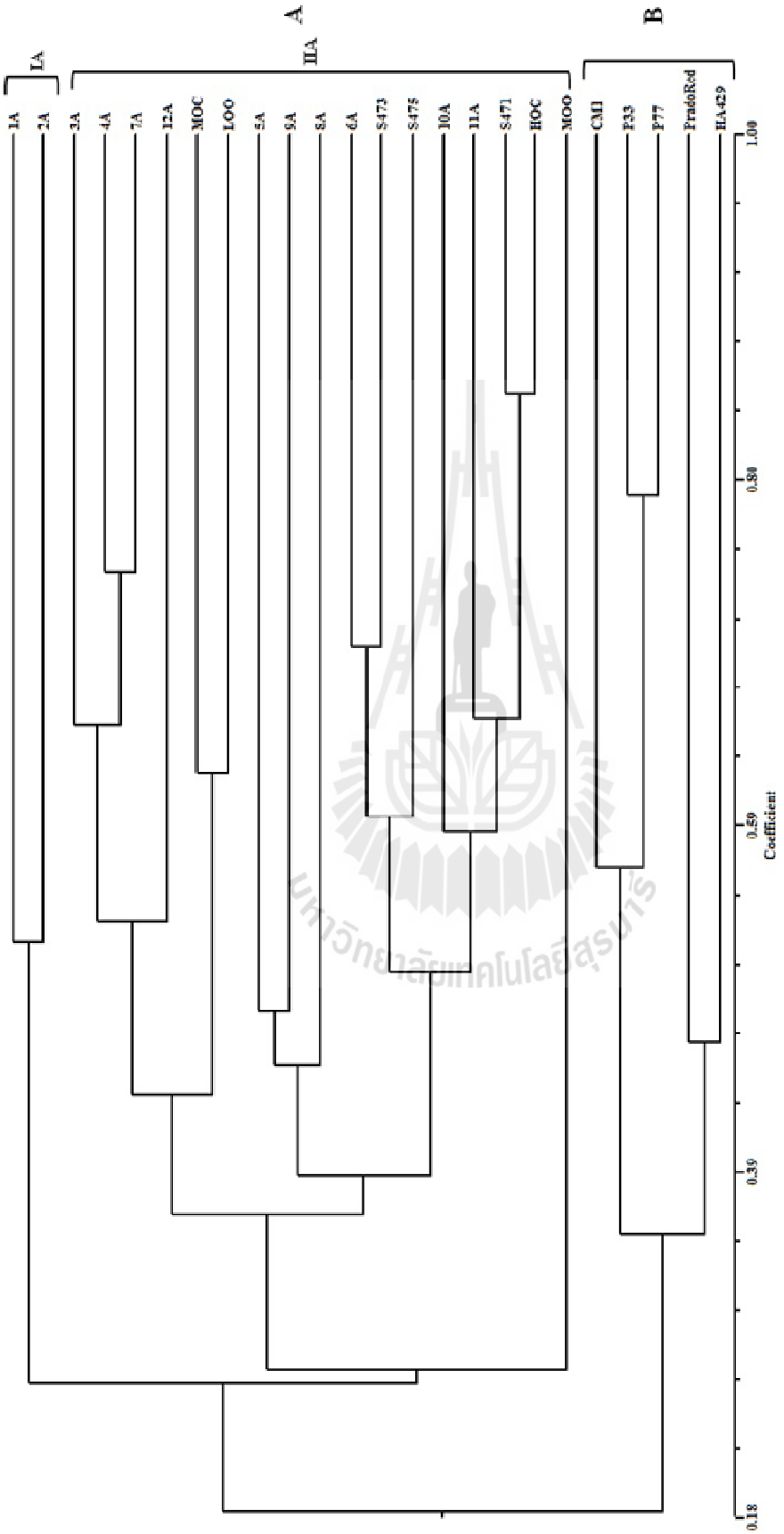

Figure 4.30 Dendrogram by UPGMA cluster generated from SSR markers.

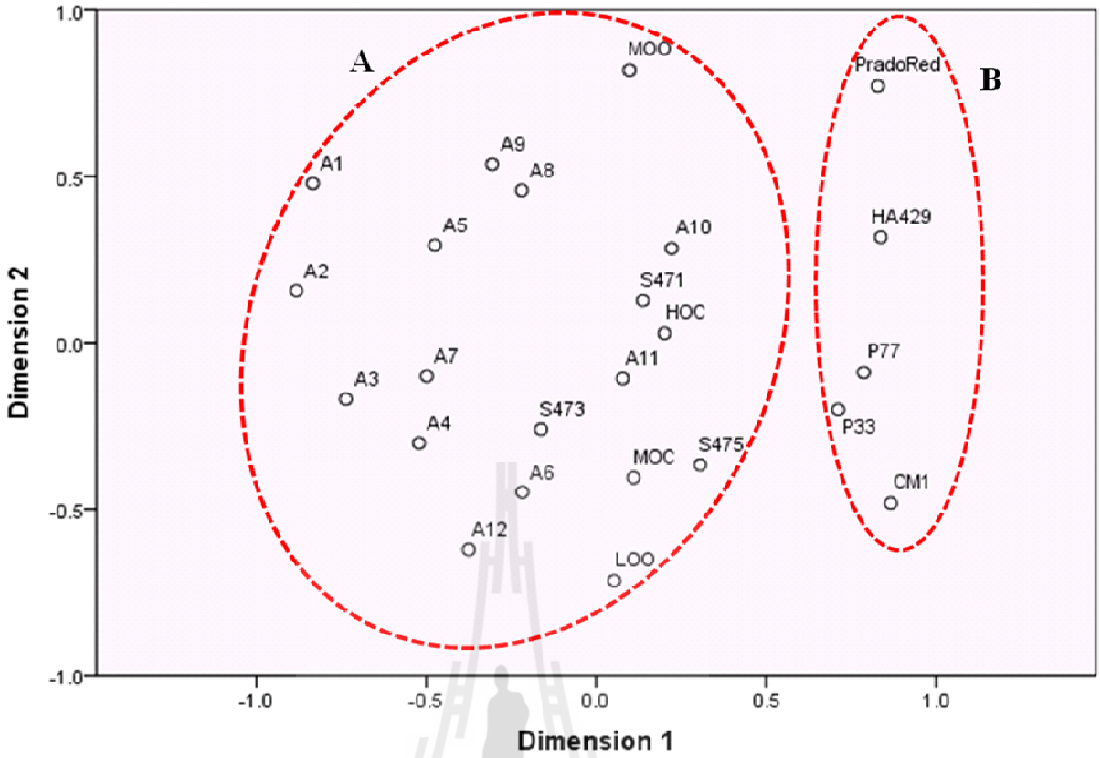

Figure 4.31 Principal Coordinates Analysis (PCoA) by SSR markers.

The PCoA based dendogram obtained from the similarity data using IBM SPSS statistic 19 program, 24 sunflower genotypes were divided in two main groups (Figure 4.31). The first group represents 12 inbred lines (1A, 2A, 3A, 4A, 5A, 6A, 7A, 8A, 9A, 10A, 11A and 12A) and 7 synthetic varieties (S471, S473, S475, HOC, MOC, MOO, LOO), while the second group represents hybrid variety (CM1), commercial hybrid varieties (Pacific 33 and Pacific 77) and sunflower accessions from abroad (PradoRed and HA429). The results of PCoA corresponded to those obtained through UPGMA cluster analysis. This result is the same as other studies using SSR and AFLP markers in common bean (*Phaseolus vulgaris*), almond (*Prunus dulcis* Miller [D.A Webb] syn *P. amygdalus* Batsch), and sunflower (*Helianthus annuus* L.) (Sorkheh et al., 2007; Kumar et al., 2009; and Darvishzadeh, 2012).

SSR markers are highly popular genetic markers because of their codominant inheritance, high abundance, enormous extent of allelic diversity (Agarwal et al., 2008). The reproducibility of SSR is such that, they can be used efficiently by different research laboratories to produce consistent data (Saghai et al., 1994).

4.4 Comparison of RAPD and SSR markers

Comparison between SSR and RAPD based analysis of genetic diversity revealed that the SSR markers detected higher polymorphism or PIC value (0.64) compared with RAPD markers (0.40) among this set of 24 sunflower genotypes and higher average genetic similarity (0.31) compared with RAPD markers (0.22) (Table 4.6). Similarly, comparison of RAPD, SSR and cytochrome P450 gene based markers, in terms of the quality of data output, indicated that SSRs and cytochrome P450 gene based markers are particularly promising for the analysis among 52 *Eleusine coracana* (finger millet) genotypes genome diversity (Panwar et al., 2010). RAPD and SSR markers were used for fingerprinting kiwifruit (*Actinidia deliciosa* A. Chev.) genotypes and for detecting undesirable genetic variation in micropropagated plants, but only SSR markers could detect genetic variation induced in micropropagated plants of cv. Tomuri (Palombi and Damiano, 2002). Cholastova et al. (2011), indicated that the effectiveness of SSR markers is greater than that of RAPD. The genetic parameters computed demonstrated the reliability of the SSR marker system. To overcome RAPD limitations in this study, some of RAPD band could be converted into sequence characterized amplified region (SCAR) marker. The results revealed that some of primers produced specific bands such as in primer S23, S29 and OPF4,

and some specific bands found in MOO genotype could be converted into SCAR marker.

The dendrograms constructed from the two markers (for SSR and RAPD markers) showed minor differences. These differences did not affect the clusters reported in the dendrograms. The dendrograms based on both marker systems divided the 24 sunflower genotypes into two main groups. In the overall, clustering pattern based on RAPD and SSR markers was not congruent in subgroup IA and IIA. The UPGMA dendrogram based on SSR markers appears conserved with their relative source data. However, the clusters from both markers system showed that the commercial hybrid varieties and sunflower accessions from abroad were completely distinguished from the inbred lines and synthetic varieties by SUT. Moreover, the results of PCoA corresponded to those obtained through UPGMA cluster analysis.

The evaluation of genetic diversity and relationship ability provides some useful information for assisting plant breeders in selecting suitable parents for the crossing program. Among factors that might have contributed to the limited correlation between results obtained by the RAPD and SSR based markers technique. RAPD has a dominant character, while SSR has a co-dominant character. There could also be differences in the effectiveness and/or number of primers used (Liu and Furnier, 1993; Pejic et al., 1998; and Sun et al., 2001).

Table 4.6 Comparison of RAPD and SSR markers.

	RAPD	**SSR**
Number of primers used	14	16
Polymorphic bands	158	54
Average number of alleles per locus	11.29	4.50
Average number of polymorphic bands	11.29	4.50
PIC range	0.02 - 0.74	0.46 - 0.81
Average PIC	0.40	0.64
Similarity coefficient range	0.00 - 0.49	0.00 - 0.85
Average of similarity coefficient	0.22	0.31

Table 4.7 RAPD and SSR correlation using Pearson correlations.

	Correlations	**RAPD**	**SSR**
RAPD	Pearson Correlation	1	0.850**
	Sig. (2-tailed)		0.000
	Sum of Squares and Cross-products	18.374	15.593
	Covariance	0.061	0.052
	N	300	300
SSR	Pearson Correlation	0.850**	1
	Sig. (2-tailed)	0.000	
	Sum of Squares and Cross-products	15.593	18.317
	Covariance	0.052	0.061
	N	300	300

** Correlation is significant at the 0.01 level.

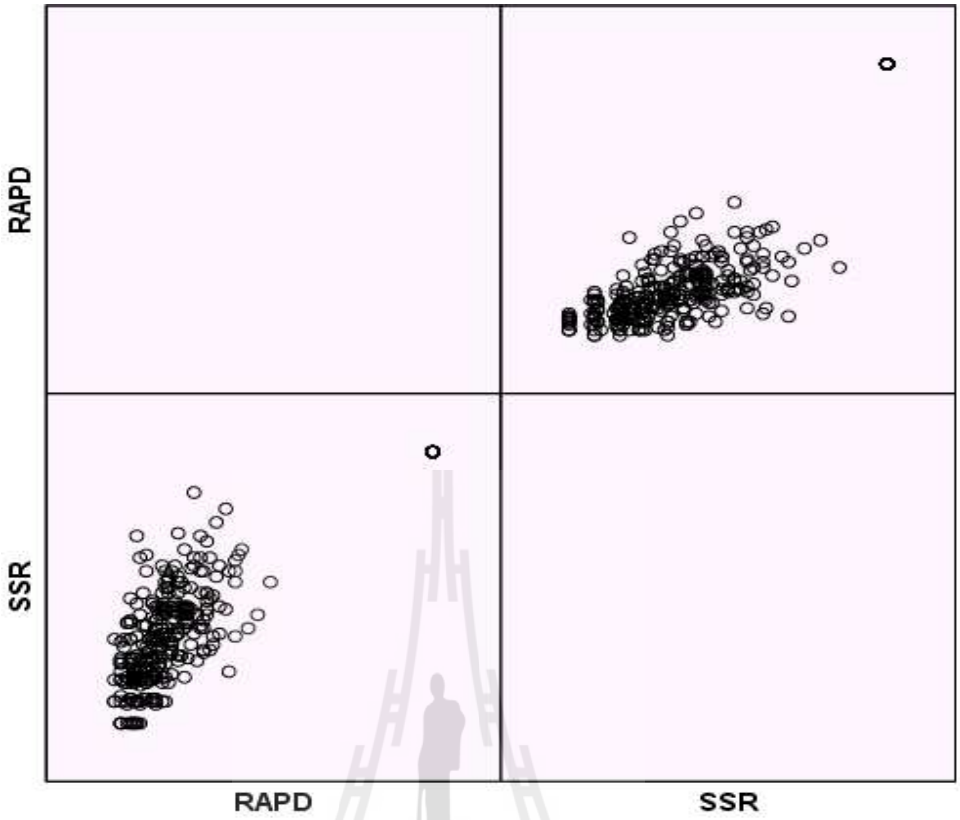

Figure 4.32 Scatter plot of correlation by SSR and RAPD markers.

The results obtained with RAPD and SSR markers were consistent in this study, estimated by the high positive Pearson's correlation (r = 0.85) between the similarity matrices (Table 4.7) and the scatter plot of correlation by SSR and RAPD markers shown in Figure 4.32. The high correlation indicated that clusters produced based on the two marker systems were conserved. This result is in accordance with other studies in maize such as Pejic et al. (1998), Sun et al. (2001), Garcia et al. (2004), and Leal et al. (2010), who found correlations of 0.57, 0.43, 0.54 and 0.55, respectively, between RAPD and SSR markers. On the other hand, there are studies that showed a low correlation between RAPD and SSR markers for rice, olive and popcorn, such as Belaj et al. (2003), Cho et al. (2004), Cholastova et al. (2011), and Kanawapee et al. (2011) who observed a correlation of 0.32, 0.24, 0.39, and 0.11, respectively.

The characteristics of the materials to be used and the objectives of the given project could be used to select of one or another marker in genetic diversity studies. The use of RAPD seems more appropriate when the objective is to cluster genotypes, because it shows similarities between the individuals. While SSR could be more appropriate for identifying genotypes. However, since they exhibited the genetic differences of each individual more extensively, these markers are important tools, in as much as they assess different DNA sequences in the genome of organisms and can indicate potential results during the selection process within breeding programs (Cholastova et al., 2011).

The genetic diversity and relationships data among breeding lines and varieties are not only usefulness for germplasm conservation and inbred line identification, but also for the selection of parental lines for hybrid breeding in crops, including sunflower (Senior et al., 1998; and Sun et al., 2001). SSRs constitute the current marker system of choice for characterizing sunflower germplasm (Paniego et al., 2002; Tang et al., 2002; Yu et al., 2002; Tang and Knapp, 2003; Zhang et al., 2005; Hvarleva et al., 2007).

CHAPTER V

CONCLUSIONS

SUT sunflower (*Helianthus annuus* L.) lines and varieties vary in characters, such as yield, seed number, plant height, and head size. It is necessary to perform genetic analysis of these germplasms. A molecular marker system for sunflower was developed to estimate the level of polymorphism, benefit for genotype identification, and predict of genetic relationships between inbred lines, synthetic varieties and hybrids. In this study, 24 sunflower genotypes, including 13 inbred lines, 8 synthetic and 3 hybrid varieties, were used to analyze the genetic diversity and estimate genetic relationship among these genotypes by two different DNA-based marker techniques, RAPD and SSR.

In this study, both RAPD and SSR makers could be used for the identification of sunflower genotypes. Both systems have some advantages. The RAPD technique has been widely used in diversity studies because, in addition to its low cost, it allows polymorphism to be detected in a simple and rapid way. SSRs are useful molecular markers because they are abundant, distributed over the genome, highly polymorphic, co-dominant, and relatively simple to interpret. The results from the study showed that there were differences between the two markers system. Regarding the 14 RAPD primers and 16 SSR primer pairs used, the calculated PIC value for RAPD was lower than SSR, indicating that SSR markers have a different polymorphic capability than RAPD markers. In addition, a higher level of polymorphism was detectable by SSR

markers than by RAPD markers. The dendrograms constructed from the two markers (for SSR and RAPD markers) showed minor differences. These differences did not affect the clusters reported in the dendrograms. The dendrograms based on both marker systems separated the 24 sunflower genotypes into two major groups. The overall clustering pattern based on RAPD and SSR markers was not in agreement in subgroups IA and IIA. The dendrograms based on SSR markers appears conserved with their origin relative data, revealed in subgroup IIA, in which S471 was closely related to HOC with the highest similarity value (0.85). The clusters showed that the commercial hybrid varieties and sunflower accessions from abroad were completely distinguished from the inbred lines and synthetic varieties developed at SUT.

Since the two markers were all based on DNA by PCR amplification, it was expected from the nature of the primers and reactions (annealing temperature) that the techniques would differ in the specific target sequences and in the number of fragments amplified. The use of RAPD seems more appropriate when the objective is to cluster genotypes, because it showed similarities between the individuals. SSR could be more appropriate for identifying genotypes, since they exhibited the genetic differences of each individual more extensively. These markers are important tools, in as much as they assess different DNA sequences in the genome of organisms and can indicate potential results during the selection process within breeding programs.

In the next step, some of RAPD bands can be converted into sequence characterized amplified region (SCAR) markers. Moreover the genetic diversity information obtained in this study will allow in the classification of germplasm into heterosis, which is especially important to hybrid breeding.

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APPENDICES

APPENDIX A

PLANT DNA EXTRACTION CHEMICAL SOLUTIONS

A.1 2% CTAB buffer

CTAB	0.2 g
1.4 M NaCl	1.6364 g
100 mM Tris-HCl (pH 8.0)	2 ml
20 mM EDTA	0.8 ml

Preparation of stock: 0.2 g NaCl was dissolved in 2 ml of 100 mM Tris-HCl (pH 8.0), added 0.8 ml of 20 mM EDTA and then added 0.2 g CTAB (Cetyltrimethyl ammonium bromide). The solution was dissolved at 65°C. Adjust volume to 20 ml with sterile distilled water. The solution was kept in 50 ml plastic tube and sterilized autoclaving at 121°C. The solution was stored at -20°C.

A.2 TE (pH 8.0)

1M Tris-HCl (pH 8.0)	1 ml
0.5 M EDTA	20 µl

Preparation of stock: 1 ml of 1M Tris-HCl (pH 8.0) added 20 µl of 0.5 M EDTA (ethylene diamine tatra-acitic acid di-sodium salt) and adjusted volume to 100 ml with sterile distilled water.

A.3 0.5 M EDTA (pH 8.0)

EDTA 93 g

Preparation of stock: 93.0 g EDTA (disodium ethylenediaminetetra acetate·$2H_2O$) in 400 ml of sterile distilled water. Adjusted pH to 8.0 with NaOH (pellet), it dissolves when pH is right and adjusted volume to 500 ml with sterile distilled water.

A.4 1 M Tris-HCl (pH 8.0)

Tris base 12.11 g

Preparation of stock: 12.11 g Tris base [tris(hydroxymethyl)aminomethane] was dissolved in sterile distilled water. Adjusted pH to 8.0 with 37% HCl .

A.5 Chloroform:Isoamyl alcohol 24:1 (v/v)

Chloroform 96 ml

Isoamyl alcohol 4 ml

Preparation of stock: the solution was prepared from a mixture of chloroform and isoamyl alcohol (v/v).

A.6 3 M Sodium acetate (pH 5.0)

Sodium acetate 12.31 g

Preparation of stock: Sodium acetate was dissolved in 30 ml water, then adjusted pH to 5.0 with glacial acetic acid. Adjusted volume to 50 ml with sterile distilled water.

A.7 RNaseA (10 mg/ml)

RNaseA	0.01 g
De-ionized water (sterile)	1 ml

Preparation of stock: RNaseA was dissolved in de-ionized water (sterile). The solution was kept in 1.5 ml Eppendorf tube and stored at -20°C.

APPENDIX B

GEL ELECTROPHORESIS CHEMICAL SOLUTIONS

B.1 5X TBE stock

Tris base	21.6 g
Boric acid	11 g
EDTA	1.66 g

Preparation of stock: each chemical component was dissolved in sterile distilled water.

B.2 0.5%, 1.5% and 3% Agarose gel

Agarose	0.5 g for 0.5%, 1.5 g for 1.5%, and 3 g for 3%
0.5X TBE	100 g

Preparation of gel: melted 0.5 g for o.5%, 1.5 g for 1.5%, and 3 g for 3% respectively agarose gel in 100 ml of 0.5X TBE buffer in the microwave for 2 min at full power, then shaked it gently to prevent undissolved agarose. The mixture was incubated at 50°C about 10 min. The cool agrose was poured into the gel casting box with 26 well comb in place and hardened for approximately 30 min. When the gel was hardened, removed the comb and placed it in the electrophoresis box

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