CHARACTERIZATION OF GENETIC IDENTITIES AND RELATIONSHIPS AMONG *BRASSICA NAPUS* USING AFLP AND SSR

Li Li

A Thesis Submitted in Partial Fulfillment of the Requirement for the

Degree of Master of Science in Biotechnology

Suranaree University of Technology

Academic Year 2010

การจำแนกความแตกต่างกันทางพันธุกรรมและการหาความสัมพันธ์ของ *BRASSICA NAPUS* ด้วยเทคนิค AFLP และ SSR

นางสาวหลี ลี่

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2553

CHARACTERIZATION OF GENETIC IDENTITIES AND RELATIONSHIPS AMONG *BRASSICA NAPUS* USING AFLP AND SSR

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

Thesis Examining Committee

_____

(Assoc. Prof. Dr. Montarop Yamabhai)

Chairperson

_____

(Asst. Prof. Dr. Chokchai Wanapu)

Member (Thesis Advisor)

_____

(Assoc. Prof. Dr. Neung Teaumroong)

Member

_____

(Asst. Prof. Dr. Mariena Ketudat-Cairns)

Member

_____ _____

(Prof. Dr. Sukit Limpijumnong) (Asst. Prof. Dr. Suwayd Ningsanond)

Vice Rector for Academic Affairs Dean of Institute of Agricultural Technology

หลี ลี่ : การจำแนกความแตกต่างกันทางพันธุ์กรรมและการหาความสัมพันธ์ของ *BRASSICA NAPUS* ด้วยเทคนิค AFLP และ SSR (CHARACTERIZATION OF GENETIC IDENTITIES AND RELATIONSHIP AMONG *BRASSICA NAPUS* USING AFLP AND SSR) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.โชคชัย วนภู, 111 หน้า.

Brassica napus เป็นพืชน้ำมันที่มีความสำคัญในมลฑลกุ้ยโจ ประเทศสาธารณรัฐประชาชนจีน ซึ่งมีพื้นที่ในการเพาะปลูกมากกว่าสายพันธุ์อื่น ได้มีการพัฒนา และคัดเลือกสายพันธุ์ของ *B. napus* เพื่อให้เหมาะสมกับสภาพภูมิอากาศ ภูมิประเทศของมลฑลกุ้ยโจ ดังนั้น ความแม่นยำในการกำหนดลักษณะของเมล็ดพันธุ์ เพื่อให้ทราบลักษณะของสายพันธุ์ตามที่ต้องการจึงมีความจำเป็นในการศึกษาครั้งนี้ โดยมุ่งเน้นในการตรวจสอบความหลากหลาย และลักษณะของสายพันธุ์ต่างๆ 25 สายพันธุ์ ด้วยเทคนิค AFLP และ SSR โดยใช้ primer 9 ชนิด พบว่าได้ชิ้นดีเอ็นเอ 193 ชิ้น จาก 73 polymorphic (38%) โดยค่าเฉลี่ยของยีนที่แสดงลักษณะเฉพาะในแต่ละชนิดมีค่าเท่ากับ 3.56 ในขณะที่ค่ามาตรฐานอยู่ที่ 1.41 และมีค่าความหลากหลายของนีส์ยีน (gene) ค่าดัชนี Shannon index ค่าความแตกต่างของพันธุกรรมอยู่ที่ 0.25, 0.62 และ 0.39 ตามลำดับ เมื่อวิเคราะห์คลัสเตอร์ด้วยเทคนิค UPGMA พบว่าค่าสัมประสิทธิ์ที่คล้ายคลึงกันอยู่ที่ 0.67 และสามารถแบ่งพืชได้เป็น 3 กลุ่ม แต่พบว่าไม่มีความชัดเจนในการจัดกลุ่ม ตามการวิเคราะห์ด้านคุณภาพของเมล็ด เมื่อใช้เทคนิค SSR พบความสัมพันธ์ทางพันธุกรรมในแต่ละสายพันธุ์ ได้ทั้งหมด 134 แถบ โดยใช้ SSR primer จำนวน 11 สาย มีลักษณะ polymorphic จำนวน 54 แถบ (40%) ยีนที่มีบทบาททางพันธุกรรมจำนวน 22 สาย มี allele เฉลี่ย 2.55 allele/ locus และ effective allele เฉลี่ย 2.01 allele/ locus ค่า fixation index ที่ 0.54 และ heterozygosity ที่ 0.45 ค่า similarity efficient จากการคำนวณในช่วง 0.43 – 0.95 โดยมีค่าเฉลี่ยอยู่ที่ 0.69

เมื่อทำการวิเคราะห์คลัสเตอร์ด้วยเทคนิค UPGMA ทั้งเทคนิค AFLP และ SSR พบว่าสามารถจำแนกพืชได้ ยกเว้นสายพันธุ์ Huayouza6 และ Huayouza9 ซึ่งไม่มีความแตกต่างกัน นอกจากนั้น ความสัมพันธ์ทางพันธุกรรมของพืชน้ำมันที่ทำการศึกษามีความแตกต่างกันเล็กน้อยกับพืชน้ำมันจากแหล่งกำเนิด ทั้งนี้เนื่องมาจาก ความคล้ายคลึงกันของพ่อแม่ โปรแกรมการปรับปรุงพันธุ์พืช และพืชที่มาจากแหล่งเดียวกันจึงทำให้มีความเหมือนกันทางพันธุกรรม นอกจากนี้ เมื่อนำตัวแปรของพันธุกรรมทั้งหมดมาคำนวณด้วยโปรแกรม POPGENE1.31 และ NTSYS-PC 2.10 พบว่า SSR เป็นเทคนิคที่เหมาะสมที่สุดในการจำแนกความหลากหลายทาง

พันธุกรรม รวมถึงความสัมพันธ์ของ *B. napus*. อย่างไรก็ตามเทคนิค AFLP ยังเป็นวิธีที่เหมาะสมในการวิเคราะห์ลายพิมพ์ดีเอ็นเอใน germplasm ของ *B. napus*

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2553 ลายมือชื่ออาจารย์ที่ปรึกษา_____

Li Li : CHARACTERIZATION OF GENETIC IDENTITIES AND RELATIONSHIPS AMONG *BRASSICA NAPUS* USING AFLP AND SSR. THESIS ADVISOR : ASST. PROF. CHOKCHAI WANAPU, Ph.D., 111 PP.

GENETIC IDENTITIES/ RELATIONSHIPS/ AFLP/ SSR/ *BRASSICA NAPUS*

Rapeseed (*Brassica napus*) is one of the most important sources of vegetable oil in Guizhou, People's Republic of China. It occupies the largest cultivated area among *Brassica* species. During long term evolution and artificial selection, a large number of *B. napus* varieties were developed and identified through regional test. Therefore, accurate characterization of seed quality traits of each cultivar and knowledge of their genetic relationships are important for germplasm conservation and utilization. This study mainly attempted to reveal the genetic diversity and relationships among 25 *B. napus* cultivars using AFLP and SSR techniques. Two parts of experiment were conducted in this study. In the first part, a powerful technique AFLP was applied to assess genetic diversity and relationships among 25 *B. napus* cultivars. Nine AFLP primer combinations generated 193 fragments, of which 73 were polymorphic with polymorphic rate of 38%. Across all the varieties, the average observed number of alleles per locus was 3.56, while the mean effective number of alleles was 1.41. Gene diversity, Shannon's information index, and genetic differentiation were 0.25, 0.62, and 0.39, respectively. At a similarity coefficient of

0.66, these 25 *B. napus* cultivars were divided into three groups using UPGMA cluster analysis. The UPGMA analysis showed no apparent clustering by seed quality characteristics and original location. In the second part, SSR technique was used for evaluating genetic diversity and relationships among *B. napus* cultivars. Total 134 bands were generated with 11 SSR primer pairs, of which 54 bands were polymorphic with polymorphism rate of 40%. Twenty two alleles were detected with an average of 2.55 alleles per locus and an average of 2.01 effective alleles per locus. High mean fixation index (0.54) and expected heterozygosity (0.45) were observed among *B. napus* cultivars. The value of similarity coefficient among all *B. napus* cultivars was calculated in a range of 0.43 to 0.95, with an average of 0.69. The UPGMA cluster analysis showed that the rapeseed cultivars could be divided into three groups at a similarity coefficient of 0.63. UPGMA analyses based on AFLP and SSR genetic similarity coefficients found that two varieties, Huayouza 6 and Huayouza 9, could not be distinguished, suggesting a very close genetic relationship between them. Likewise, the genetic relationships among *B. napus* cultivars was not closely related to their original location, may be contributed to their similar parents or breeding program, although cultivars bred by the same institute have very high level of genetic similarity. All the parameters of genetic diversity were calculated using POPGENE 1.31 software and dendrograms based on genetic similarity coefficients were constructed using NTSYS-pc 2.10 software. This study demonstrated that the SSR technique was the best choice for the evaluation of

genetic diversity and relationships among *B. napus* cultivars. However, AFLP was an optimal method for DNA fingerprinting of *B. napus* germplasm.

School of Biotechnology Student's Signature _____

Academic Year 2010 Advisor's Signature _____

ACKNOWLEDGEMENT

I would first like to express the deepest gratefulness to my thesis adviser, Asst. Prof. Dr. Chokchai Wanapu, for his valuable guidance and suggestions about my research, for his patience and encouragement to read the draft, and for the help and care when I study in Thailand.

I am also deeply indebted to Dr. Huang Xian-qun, for her constant support and guidance in this study.

Special thanks should go to my friend and colleagues, Ma Lin and Huang Tuan, for their help in providing software.

I feel grateful to my committee members, Assoc. Prof. Dr. Neung Teaumroong, Assco Prof. Dr. Montarop Yamabhai and Asst. Prof. Dr. Mariena Ketudat–Cairns for their active participation and comments on the draft.

My appreciation to the secretary of school of Biotechnology, Institute of Agricultural Science, Mrs. Prangkhaw Prukhett for her kindness and assistance.

Many thanks for all friends in F10, for their friendship and help.

Last but not the least, my gratitude also extends to my parents, for their constant love, support, encouragement without a word of complaint.

Li Li

TABLE OF CONTENTS

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF ABBREVIATIONS

AFLP	=	Amplified Fragment Length Polymorphsim
AP	=	Ammonium Persulfate
bp	=	base pair
EDTA	=	Ethylenediaminetetraacetic acid
EST	=	Expressed Sequence Tag
EtOH	=	ethanol
ddH_2O	=	double distilled water
DNA	=	Deoxynucleic acid
g	=	gram
h	=	hour
ISSR	=	Inter Simple Sequence Repeat
L	=	liter
mL	=	milliliter
mM	=	millimolar
min	=	minute
ng	=	nanogram
NIRS	=	Near Infrared Reflectance Spectroscopy
nm	=	nanometer

LIST OF ABBREVIATIONS (Continued)

NTSYS	=	Numerical Taxonomy and Multivariate Analysis System
PAGE	=	Polyacrylamide Gel Electrophoresis
PCR	=	Polymerase Chain Reaction
POPGENE	=	Population Genetic Analysis
RAPD	=	Random Amplified Polymorphic DNA
RFLP	=	Restriction Fragment Length Polymorphism
rpm	=	round per minute
s	=	second
SSR	=	Simple Sequence Repeat
TEMED	=	N, N, N', N'-tetramethylethylenediamine
UPGMA	=	Unweighted Pair Group Method with Arithmetic average
µM	=	micromolar
µL	=	microliter
u/µL	=	unit per microliter
µmol/g	=	micromoles per gram
°C	=	degree Celsius

CHAPTER I

INTRODUCTION

1.1 Significance of the study

Rapeseed also known as rape, or oilseed rape, is an important oil crop with high oil and protein contents, which provides about 12% of the world-wide edible vegetable oil supplies (Labana and Gupta, 1993). According to the Food and Agriculture Organization of the United stations (FAO), worldwide production of rapeseed (including canola) rose to 50.5 million metric tons in 2007, and is the second largest oilseed crop after soybean. China is the largest producer of oilseed rape with one hundred and ten million acres in 2000 (Cheng, 2005), planting area and production account for about one of third of world total area and production. Nowadays, the major objectives of rapeseed breeding are to develop high quality and yield variety by combining breeding with heterosis. With the application and dissemination of elite hybrid rapeseed, yield of rapeseed has been improved, and new cultivars were developed. Regional tests provide useful information for the registration and protection of rapeseed cultivars. The tested varieties represent the achievement of breeder, and reflect level of present breeding. Traditional biotechnological methods become less feasible to distinguish rapeseed varieties, due to environmental influences and low level of polymorphism. Therefore, it is necessary to use other methods for the precise cultivar identification.

Recently, several molecular approaches have already been evaluated for genetic (RFLP; Landry et al., 1991), randomly amplified polymorphic DNA (RAPD;

Kresovich et al., 1992), amplified fragment length polymorphic (AFLP; Lombard et al., 2000) and simple sequence repeat (SSR; Kresovich et al., 1995). These markers give broad and different ranges of information, which substantially differ in terms of practicability and reproducibility.

At present, there is no report that genetic diversity and relationships among rapeseed (*Brassica napus*) cultivars attending Guizhou regional tests were analyzed using AFLP and SSR markers. Therefore, this study will provide effective information for genetic analysis of rapeseed cultivars.

1.2 Research objectives

The main objective of this study is to investigate the genetic diversity and relationships among 25 *B. napus* cultivars attending Guizhou regional tests from 2007 to 2008 using AFLP and SSR markers and to provide a scientific basis for varieties assessment and utilization. The objectives are as follows:

1. To examine the usefulness of AFLPs and SSRs in differentiating 25 Guizhou cultivars of *B. napus* based on the polymorphisms.

2. To develop an effective molecular marker for *B. napus* germplasm.

1.3 Research hypothesis

The research hypothesis is if polymorphic loci were detected by AFLP and SSR markers, then genetic diversity and relationships among 25 *B. napus* cultivars could be revealed.

1.4 Scope and limitation of the study

At present, genetic analysis of *B. napus* in China using the AFLP and SSR techniques have been reported (Lei et al., 2005; Liu, 2007). Genetic analysis of *B. napus* F_1 cultivars attending Guizhou regional test still has not been available. Classification based on morphological characters and deduced genotypes based on certain agronomic characters are not reliable. In this study, AFLP and SSR markers provided important information for the characterization of AFLP and SSR in *B. napus.* By detecting the polymorphism, effective molecular marker systems were established among *B. napus* cultivars. Using these two molecular markers, useful information for further research and selection in the breeding program can be obtained. These distinct bands will result in the differentiation of cultivars and help to develop molecular markers that will facilitate breeding.

1. DNA extraction was performed by DNA quick plant system kit. Agarose gel eletrophoresis of DNA was used to show the integrity of the DNA. PCR products were detected by PAGE analysis.

2. AFLP and SSR markers were selected for characterization of *B. napus* cultivars. Combinations of *Eco*RI primers and *Mse*I primers with three nucleotides were tested for the selection amplification. SSR markers are available on the Cropnet website (http://ukcrop.net/perl/search/BrassicaDB).

3. Due to cost considerations and the relatively large amount of DNA required, the AFLP reaction was duplicated. The SSR reaction was repeated two times to test reproducibility.

1.5 Expected results

1. AFLP and SSR markers can detect substantial numbers of polymorphic loci among F_1 population of *B. napus* cultivars with a relatively small number of primer pairs.

2. DNA fingerprinting map can be constructed among these *B. napus* cultivars in Guizhou, People's Republic of China.

1.6 References

Cheng, Y. (2005). AFLP markers for the dominant genic male sterility gene and SSR fingerprint of cultivars (lines) in *Brassica napus* L. (MSc. thesis). **Oil Crop Research Institute**, Wuhan 430062 in China.

Kresovich, S., Williams, J. G. K., McFerson, J. R., Routman, E. J. and Schaal, B. A. (1992). Characterization of genetic identities and relationships of *Brassica oleracea* L. via a random amplified polymorphic DNA assay. **Theoretical and Applied Genetics**. 85: 190-196.

Kresovich, S., Szewc-McFadden, A. K., Bliek, S. M., McFerson, J. R. (1995). Abundance and characterization of simple sequence repeats (SSRs) isolated from a size-fractionated genomic library of *Brassica napus* (L.) (Rapeseed). **Theoretical and Applied Genetics**. 91:206–211.

Labana, K. S. and Gupta, M. L. (1993). Importance and origin, pp. 1–20 in Breeding Oilseed *Brassica*, edited by K. S. LABANA, S. S. BANGA and S. K. BANGA. **Springer-Verlag**, Berlin.

Landry, B. S., Hubert, N., Etoh, T., Harada, J. J. and Lincoln, S. E. (1991). A genetic map for *Brassica napus* based on restriction fragment length polymorphisms detected with expressed DNA sequences. **Genome**. 34:543—552.

Lei, T. G., Zhang, X. H., Lu, H., Li, J. N., Xu, X. F., Fan, Q. X. and Chen, Li. (2005). SSR fingerprint of yellow-seeded rapeseed varieties (*Brassica napus* L.). **Journal of Southwest Agricultural University (Natural Science)**. .27(3): 361-364.

Liu, P. W., Li, Y., He, Q. B. and Yang, G. S. (2007). Identification of genetic markers for the pol CMS restorer gene in *Brassica napus* L. **Chinese journal of oil crop sciences**. 29 (1): 14-19.

Lombard, V., Baril, C. P., Dubreuil, P., Blouet, F. and Zhang, D. (2000). Genetic relationships and fingerprinting of rapeseed cultivars by AFLP: Consequences for varietal registration. **Crop Science**. 40: 1417-1425.

CHAPTER II

LITERATURE REVIEW

2.1 Botanical description of rapeseed (*Brassica napus*)

Rapeseed (*Brassica napus*) is a bright yellow flowering member of the family *Brassicaceae* (Figure 2.1). It is an annual (spring) or biennial (winter) plant, when seed was sown late, produces fruits in early summer and the plant flowers bloom in late spring (Duke, 1983). In the old civilizations of Asia and the Mediterranean, rape oil was used for lighting purposes. Rape crop was cultivated in India about 3000 years ago and introduced to China and Japan about 500 to 200 BC (Krzymanski, 1998). Rapeseed comprises oil seeds of different species. All these species are derived from the *Brassica* genus in the Cruciferae family. *B. napus* (genome AACC, 2*n* = 38) is an amphidiploid species that was derived from spontaneous hybridizations of two diploid *Brassica* species: *B. rapa* (genome AA, 2n=20) and *B. oleracea* (genome CC, 2n=18), and contains the complete diploid chromosome sets of both parental genomes (Snowdon et al., 2002), which originated from Europe. It has dark green foliage, glaucous, smooth, or with a few scattered hairs near the margins, and partially clasping. The stems are well branched, although the degree of branching depends on variety and environmental conditions, branches originate in the axils of the highest leaves on the stem, and each terminates in an inflorescence. The inflorescence is an elongated raceme, the flowers are yellow, clustered at the top but not higher than the terminal buds, and open upwards from the base of the raceme (Musil, 1950). In China,

this crop was cultivated about 75 years ago, mainly introduced from Japan in the 1930s (Liu, 2000). Since then, the originally cultivated *B. rapa* was substantially replaced by the introduced *B. napus* because of its higher yield and better adaptation. The introduction of *B. napus* resolved the problems of low yield and severe disease on the originally cultivated *B. rapa.* The *B. napus* is self-compatible although both wind and insect pollination can occur. Large amounts of pollen are released and this is likely to contribute to both cross- and self-pollination (Harding and Harris, 1997).

Figure 2.1 Morphological characteristic of rapeseed (*Brassica napus* information from NPGS/GRIN).

2.2 Characteristics of seed quality traits and estimation using NIRS technique

2.2.1 Characteristics of seed quality traits

Erucic acid, glucosinolate, oil and protein are the main characteristics of

seed quality traits. Contributed to these characteristics, rapeseed is widely cultivated throughout the world for the production of animal feed, vegetable oil for human consumption and biodiesel.

Oil is the essential byproduct in the production of rapeseed. High oil content and production are the most important characteristics for rapeseed breeding. In traditional *Brassica* oilseeds, the occurrence of erucic acid and glucosinolate are considered as anti-nutritional factor for human consumption. Therefore, it was minimized by breeding and finally developed Canola- or '00'-quality (Lühs et al., 1999). High erucic acid rapeseed cultivars are regaining interest for industrial purposes. Erucic acid (*cis*-13-docosenoic acid, 22:1) is a very long chain fatty acid having 22 carbon atoms with one double bond at the *cis*-13 position of the carbon chain (Figure 2.2). In plants, they are mainly found as components or precursors of epicuticular waxes and in the seed oil of certain plant species (Harwood, 1980; Post-Beittenmiller, 1996). But erucic acid is found only in the seed oil and not in membrane lipid. Erucic acid accounts for 45-60% of the total fatty acid mixture in traditional *B. napus* cultivars (Frentzen, 1993). The erucic acid content depends on the embryo genotype, not the female parent, and is controlled by additive effects of two pairs of genes (Jönsson, 1977; Qi et al., 2001).

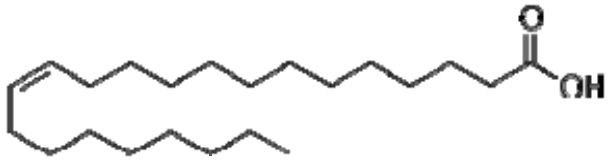


Figure 2.2 Erucic acid (Molecular formula: $C_{22}H_{42}O_2$, Molar mass: 338.57 g/mol).

Another important anti-nutritional compound is glucosinolate. Glucosinolates are a family of secondary plant metabolites particularly abundant in seeds and green tissues of the family *Brassicaceae* (Figure 2.3). Low glucosinolate meal can be used for livestock feed, and high glucosinolate meal for potential pest control (Bhardwaj et al., 1996). In the other words, either a reduction or an enhancement in glucosinolate content might be helpful in developing rapeseed as a renewable-domestic source of erucic acid (Bhardwaj and Hamama, 2000). Glucosinolate content is controlled by the maternal genotype (Kondra and Stefasom, 1970; Mou and Liu, 1990; Zhang et al., 2004).

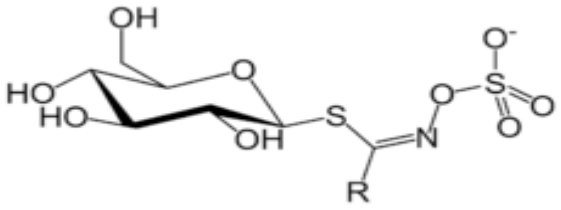


Figure 2.3 Glucosinolate structure.

After oil extraction, a residue with high protein content can be used as a valuable animal feed. The protein composition of rape meal is favorable for animal nutrition. Quality of rapeseed proteins was found to be similar to casein and superior to proteins from other vegetable sources such as soybean, pea, and wheat in some applications (Sarwar et al., 1984).

2.2.2 Estimation of seed quality traits of *B. napus* cultivars using NIRS technique

Improvement of seed quality is one of the most important objectives in *Brassica* breeding for satisfying future edible oil requirements (Becker et al., 1999).

Compositional analysis of oil seeds plays an important role for the quality control. Seed quality traits like oil, protein and glucosinolate contents as well as fatty acid composition can be modified by classical breeding and gene technological approaches. However, the conventional method for determining seed quality traits is time-consuming and labor-intensive: it includes grinding, oil extraction, sample pretreatment, and gas chromatographic (GC) analysis.

Near infrared reflectance spectroscopy (NIRS) is a spectroscopic method that uses the near-infrared region of the electromagnetic spectrum (from about 800 nm to 2500 nm), which is based on molecular overtone and combination vibrations. Such transitions are forbidden by the selection rules of quantum mechanics. As a result, the molar absorptivity in the near IR region is typically quite small. Therefore, NIRS is not a particularly sensitive technique, but it can be very useful in probing bulk material without sample preparation. Instrumentation for NIRS is partially similar to instruments for the visible and mid-IR ranges. There is a source, a detector, and a dispersive element (such as a prism, or, more common, a diffraction grating) to allow the intensity at different wavelengths to be recorded. Common incandescent or quartz halogen light bulbs are most often used as broadband sources of near-infrared radiation for analytical applications. The type of detector used depends primarily on the range of wavelengths to be measured. Usually 2D array detector with a acousto-optic tunable filter is used for chemical imaging. The ability of NIRS to determine these various compounds in plant samples is due to vibrational and rotational energies associated with H bonds (Osborne and Fearn, 1986). The quantitative and qualitative composition of seed quality traits are affected by growing conditions, which also determine the final yield (Stone and Savin, 1999). Therefore,

in this research, a rapid, simple, and non-destructive method near infrared reflectance spectroscopy (NIRS) was used to determine the seed quality traits of *B. napus* like oil, protein, erucic acid and glucosinolate contents (Orman et al., 1992).

In this study, erucic acid, glucosinolate, oil and protein contents were determined by a spectrophotometer NIRSystems FOSS 6500 (Silver Spring, USA) with a transport device for a ring sample cell which had an optical grade quartz glass cover on one side, and expressed as the percentage of erucic aicd on total oil, percentage of glucosinolate (μmol/g), oil and protein on total dry seed weight (%). The instrument was operated at constant temperature within the scanning room of 23±2ºC. Briefly, over 4 g seeds of each intact sample were packed and flattened in a close sample cell. The NIR reflectance spectra were measured directly from samples in a wavelength range from 1100-2500 nm at 2 nm intervals. The NIRS reflectance (R) readings were converted to absorbance (A) values: A = log (1/R). Each sample spectrum was obtained by averaging 32 scans. All these data of seed quality traits were provided by Guizhou Seed Management Station.

Table 2.1 The mean erucic acid, glucosinolate, oil, and protein contents of 25 *B. napus* cultivars.

Code	Variety	Erucic acid %	Glucosinolate μmol/g	Oil content %	Protein%
A1	Youyan 1517	0.5	27.27	40.36	26.52
A2	B-52	2.6	24.92	39.37	29.10
A3	Youyan 10	1.3	27.74	44.58	25.87
A4	You 05-2	0.5	38.86	41.90	26.76
A5	Jinyou 8	0.1	26.27	39.62	28.66
A6	H2139	0.2	28.83	41.98	27.81
A7	You 3115	0.4	27.64	43.72	26.22
A8	Qianza 6-18	0.6	32.58	40.54	28.13
A9	You 9559	0.4	33.28	42.42	26.43
A10	ZWH-1	1.46	45.12	40.37	25.45
A11	H0802	0	16.49	41.73	26.69
A12	Qianza 2501	2.0	32.43	41.44	27.03
B1	Qianza 222	0.1	16.59	41.32	26.36
B2	NR061	0	17.72	41.58	27.75
B4	Gui BF2-3	0.2	20.33	41.80	27.89
B5	IF5-9	0	14.56	38.79	26.71
B6	NR168	0	14.59	40.70	26.57
B7	You 06-1	0.01	40.11	41.78	27.08
B8	Mianza 04-52	0	18.08	38.13	26.93
B9	Jinuou 068	0.2	29.21	40.70	27.57
B10	Shenyou 6970	0	21.28	39.71	27.24
B11	Qianza J5005	0.6	25.85	43.44	27.45
B12	You 06-3	0	15.85	39.78	27.09
SC4	Huayouza 6	0.76	29.89	42.56	20.19
SC5	Huayouza 9	0.25	29.58	38.46	23.08

As shown in Table 2.1, significant differences existed among tested *B. napus* cultivars for contents of erucic acid, glucosinolate, oil, and protein. Among *B. napus* cultivars, B-52 had the highest erucic acid (2.6%) and protein (29.10%), ZWH-1 had the highest glucosinolate (45.12 μmol/g), and Youyan 10 had the highest oil content (44.58%). While seven cultivars (H0802, NR061, IF5-9, NR168, Mianza 04-52, Shenyou 6970, and You 06-3 showed the lowest value (zero) of erucic acid, IF

5-9 had the lowest glucosinolate content (14.56 μmol/g), Mianza 04-52 had the lowest oil content (38.13%), and Huayouza 6 had the lowest protein content (20.19%). These lines could form the basis of a breeding program to increase or decrease the amount of erucic acid, glucosinolate, oil and protein.

2.2.2.1 Erucic acid content

Kaushik (1998) reported that erucic acid and glucosinolates are the two toxic substances found in rapeseed mustard seeds. In the present study, low erucic acid was detected in all *B. napus* cultivars. The erucic acid content among the cultivars ranged from 0 to 2.6% with an average of 0.49% (Table 2.1), which showed the lowest value in H0802, NR061, IF5-9, Mianza 04-52, Shenyou 6970 and You 06-3. Therefore, 25 *B. napus* cultivars exhibited good seed quality of rapeseed. Abbas et al. (2008) reported that erucic acid content among *B. napus* and *B. campestris* genotypes ranged from 32.9% to 57.98%, while in the F_2 progenies from cross combinations *B. napus* and *B. campestris*, the erucic acid content ranged from 43.60% to 47.5% of the total fatty acids. The erucic acid content variation among cultivars is more because each cultivar has different concentrations of erucic acid. While erucic acid is a valuable resource for the non-food industry, this long-chain fatty acid is not desired in oil for human consumption (Ofori et al., 2008). With the shift to rapeseed 00 (low erucic acid and low glucosinolate) in rapeseed breeding, the low erucic acid content of the resulting rapeseed oil and its specific fatty acid composition make it a highly appreciated edible oil.

2.2.2.2 Glucosinolate

The glucosinolate content of the 25 *B. napus* genotypes ranged from 14.56 μmol/g to 45.12 μmol/g with a mean of 29.84 μmol/g (Table 2.1). Low glucosinolate content was measured in rapeseed cultivars, which is consistent with

previous reports (15.8 - 39.0 μmol/g) (Petisco et al., 2010). The present quality assessment studies in *B. napus* indicated the existence of a wide variation with respect to various parameters among the genotypes, which can serve a prominent role to the rapeseed breeders in designing future breeding program. Low glucosinolates are preferred for traditional markets to allow the use of the meal as a livestock feed.

2.2.2.3 Oil content

High oil content is one of the most important characteristics for *B. napus* breeding. Oil content naturally varies between varieties. The data of oil content of 25 *B. napus* varieties revealed that oil content values ranged from 38.13% to 44.58% with an average of 41.07% (Table 2.1). The highest oil contents (44.58%) were observed in cultivar Youyan 10, while the lowest oil contents (38.13%) were produced in Mianza 04-52. Previous researches (Velasco et al., 1999; Abbas et al., 2008) also reported that high oil content was also found using NIRS for screening of quality traits in rapeseed. These differences may be due to variation in genotypes or environmental influences. Moreover, the utilization of heterosis is an effective way to improve seed oil content in *B. napus*.

2.2.2.4 Protein content

The protein content of rapeseed cultivars was not variable greatly. Protein varied from 20.19% to 29.10% with a mean of 26.66% (Table 2.1). Data showed that the maximum protein content (29.10%) was observed in B-52. The minimum protein contents (20.19%) were shown in Huayouza 6. These results were in agreement with the findings of Abbas et al. (2008) who reported the protein value ranging from 22.3% to 27.52% and their F_2 progenies ranging from 23.50% to 29.60% in seed of *Brassica* genotypes.

The oilseed of good quality contains 40-44% of oil, low erucic acid (less than 2%), low glucosinolate (less than 30 μmol/g) and the meal contains 43-48% of protein (Ghodsvali et al., 2005). In this study, these data suggested that the seed quality of *B. napus* cultivars was high, due to low erucic acid and glucosinolate contents, but high oil and protein contents. Furthermore, modern breeding programs are aimed at revealing high-yielding rapeseed varieties with an improved ratio of fatty acid as well as large-seeded varieties with an increased oil content resistant to diseases and lodging. It was found that the oil content of F_1 hybrid seeds in rapeseed was mainly controlled by the maternal genotype (Wang et al., 2010). Therefore, selection of parent and maternal materials play an important role in the improvement for seed quality in *B. napus.* The extensive genetic differences in the ecucic acid, glucosinolate, oil, and protein contents have laid the foundation for higher genetic diversity in the tested rapeseed cultivars. Moreover, the results obtained in this work demonstrated the good performance of NIRS technology in the quantification of oil, protein, and total glucosinolates in seed samples of *B. napus* without sample preparation.

2.3 Cultivation

Rapeseed is adapted to cool, moist regions. It does not tolerate drought. Air and soil temperatures influence rapeseed plant growth and productivity. The optimum temperature for maximal growth and development is around 20ºC, and it is best grown between 12ºC and 30ºC. Seedbed preparation is very important to rapeseed because of its lack of early competitiveness (Oplinger et al., 1989). Rapeseed can be seeded in either the fall or the spring depending on the type of variety. Rapeseed grows well on a wide variety of well-drained soils, prefers a pH between 5.5 and 8.3,

and is moderately tolerant of saline soils. Seedbed preparation is usually done with a shallow (4-5 inch) tillage operation. It is usually seeded with the small seed attachment of a grain drill to a depth of 1/2 to 1 inch. Rows should be spaced 30- 40 cm apart (James, 1983). Seed of Polish types germinate readily when moisture and temperature conditions are suitable. Rapeseed responds well to nitrogen, phosphorus, potassium, and sulfur fertilizer with higher yields. After emergence, seedlings prefer relatively cool temperatures up to flowering; high temperatures at flowering will hasten the development of plant, reducing the time from flowering to maturity. Although rapeseed crops can be attacked by a number of insect pests, insect control must be carefully designed to reduce unnecessary and costly pesticide applications, chances of resistance buildup in insects, and damage to honeybees and native pollinating insect. Notably, oilseed rape should not be grown on the same field more often than once every four years, to prevent the occurence of diseases, insects, and weeds. When the color of seed changes from yellow to brown, mature seed can be harvested.

2.4 Yield and Economics

Brassica species play an important role in agriculture and horticulture, as well as contributing both to the economy and health of populations around the world. Rapeseed has worldwide importance in agriculture, providing vegetable oil in the world because of its high oil content (35%-50% w/v) in the seeds. The global production of edible oils has increased greatly. In 2005-2006, palm oil makes up the largest proportion (31% w/w) of world-wide vegetable oil production followed by soybean oil (29% w/w). Rapeseed is in third place with 14% (w/w) of total production

(http://dili.pudong-edu.sh.cn/Upload/ACW/FuJian/2008924/F116231.doc). World production of oilseed rape has increased very rapidly over the last 20 years. China with 25% of world production is the largest producer of oilseed rape with nearly 10 million ton every year (http://www.ipipotash.org/udocs/No%2016%20Oilseed%20rape.pdf). Worldwide production of rapeseed was around 57.8 million metric tons in 2008 (http://www.agmrc.org/commodities__products/grains_oilseeds/rapeseed.cfm), and ranks second in the world production of oilseed crops. Canada was the top rapeseed producing country, producing 12.6 million metric tons, and China was second, producing about 12.1 million tons. In China, rapeseed oil is an edible oil for thousands of years, forming many elite landraces with good agronomic traits for food oil production, such as high oil content, short life cycle, and natural self-incompatibility (prevent self-fertilization and encourage outcrossing). In Europe, rapeseed is primarily cultivated for animal feed (due to its very high lipid and medium protein content), and for the production of vegetable oil for bio-diesel (due to its rapid biological decomposition). Due to its economic importance, much effort has been put into devising methods for identifying varieties and improving production (Cooke, 1999).

2.5 Rapeseed in Guizhou

Guizhou province is situated at Yunnan-Guizhou Plateau, the northwest parts have higher altitude than the southeast. The culitivars of *B. napus* are broadly distributed in parts of Guizhou with average altitude 137-2200 m, the east longitude 105°12′ to 109°12′ and north latitude 25°37′ to 28°18′. In the past years, common oilseed rape breeding program was initiated with the major objectives of developing

high yielding, pest and disease resistant varieties and exploitation of hybrid vigour by induction of male sterility. During the long term evolution and artificial selection, high quality and yield *B. napus* varieties were developed. At present, cultivated area is about 7 million acre, of which *B. napus* makes up 70-75%, *B. juncea* is 10% and *B. campestris* is 15%. It demonstrated that *B. napus* is widely used in rapeseed breeding programs, due to their high yield. However, *B. napus* cultivars fingerprinting map remains unevaluated and the genotypes selected and exploited for extensive planting are very much limited. In the past years, there were various techniques for studying the genetic variability of crop germplasm, including morphological traits, total seed proteins and isozymes. Different biochemical technologies have proven potentially useful for genetic analysis of *B. napus*. These include HPLC analysis of leaf glucosinolates (Adams et al., 1988), starch-gel electrophoresis of enzyme (Mündges et al., 1990) and the comparison of seed oil fatty acid profile by Gas liquid chromatography analysis (White and Law, 1991). However, biochemical and morphological makers reveal only limited polymorphisms among closely related genotypes, and influenced by environmental conditions. Therefore, new techniques that detect more polymorphisms than morphological traits must be established for genetic characterization within species or between species. In comparison with morphological traits, molecular makers have many advantages. The molecular markers are not subject to environmental change, making them especially informative and superior to traditional methods of genotyping such as the use of morphological traits, and biochemical markers (Tanksley et al., 1989; Messmer et al., 1993; Melchinger et al., 1994).

2.6 Application of Molecular Marker in Rapeseed

2.6.1 Restriction Fragment Length Polymorphism (RFLP)

Molecular markers have been successfully used for genetic analysis，each with specific advantages and disadvantages. In the late 1980s, the first RFLP linkage maps for *B. oleracea* were constructed based on the segregation of 258 restriction fragment length polymorphism loci in a broccoli × cabbage F_2 population (Slocum et al., 1990), the result of the duplicated loci showed that a fairly high degree of genetic rearrangement occurred in the evolution of *B. oleracea.* Later genetic linkage maps of *B. rapa* (Song et al., 1991) and *B. napus* (Landry et al., 1991) were also established by RFLP for genetic map. Diers et al. (1994) and Meng et al. (1996) revealed gene diversity and relationship among *B. napus* cultivars using RFLP markers respectively. These results demonstrated that RFLP markers are well suited for the construction of linkage maps, and have also been used for the analysis of genetic diversity because of their high specificity (Chyi et al., 1992) and their co-dominant nature. Nevertheless, this technique is labor intensive, time consuming and expensive.

2.6.2 Random amplified polymorphic DNA (RAPD)

With the development of PCR, RAPD, Amplified fragment length polymorphism (AFLP), and Simple Sequence Repeat (SSR) markers have been widely used for genetic information. RAPD technique gained development due to its simple and rapid, and require no prior knowledge of sequence (Karp et al., 1997). Kresovich et al. (1992) reported that twenty-five decamer oligonucleotide primers for RAPD were screened individually with a test array composed of individuals representing a range of genetic relationships in *B. oleracea* L. Wu et al. (1997) examined the genetic diversity and the close relationship among 40 cultivars of *B.*

napus from seven provinces and other countries using RAPD technique. Therefore, RAPD and RFLP markers have been shown to be valuable tools for detecting patterns of DNA polymorphism among and within *Brasscia* species, and fingerprinting *Brassica* varieties. However, the utilization of RAPDs for genome analysis seems to be restricted by the dominant character, lack of reproducibility (Jones et al., 1997; Karp et al., 1997).

2.6.3 Inter Simple Sequence Repeat (ISSR)

A variant of microsatellites called ISSR involves the amplification of DNA region located between two microsatellites loci (Zietkiewicz et al., 1994). ISSR markers are PCR based like RAPD but are more reproducible than RAPD due to their better stringency (high annealing temperature), require no gene sequence information and targets microsatellite motif (Bornet and Branchard, 2001; Thimmappaiah et al., 2009). ISSR markers have been used for cultivar identification (Hu and Quiros, 1991; Yonemoto et al., 2006) and diversity analysis (Qiao et al., 2006; Ma et al., 2003). But it appears to be insufficiently polymorphic (McGregor et al., 2000).

2.6.4 Amplified Fragment Length Polymorphism (AFLP)

Another marker system developed recently is the AFLP. The AFLP analysis is also a DNA technique by which the total plant DNA is digested and amplified by PCR (Vos et al., 1995). Gerber et al. (2000) suggested that the high numbers of polymorphic *loci* revealed by AFLP methods counterbalance the loss of information resulting from dominance, while Garcia-Mas et al. (2000) showed that AFLP had higher efficiency in detecting polymorphism than either RAPD or RFLP markers. AFLP technique produces highly reproducible, dominant markers, but also more laborious and time consuming. It has been widely applied in mapping (Lin et al., 1996;

Mohan et al., 1997), DNA fingerprinting (Powell et al., 1996), analyses of genetic relationships (Milbourne et al., 1997), and genetic diversity (Russell et al., 1997). AFLP markers have also been employed for *Brassica* genome genetic analysis. Zhao et al. (2005), applying AFLP, reported comparable genetic diversity between and within different rape accession groups collected world-wide. A framework consensus map for *B. napus* L. was constructed from the integration of three double haploid (DH) mapping populations derived from crosses between or within spring- and winter-type parents (Lombard and Delourme, 2001). Moreover, AFLP was also a suitable approach for oilseed rape cultivar fingerprinting (Roman et al., 2004).

2.6.5 Simple Sequence Repeat (SSR)

A potentially powerful technique for DNA fingerprinting followed the successful PCR amplification of tandemly repeated sequences, which have been known to be polymorphic and widespread in plant genomes, referred to as SSR or microsatellite polymorphism (Cregan, 1992; Morgante and Olivieri, 1993). Micro-satellites occur frequently in most eukaryote genomes and can be very informative, multi-allelic and reproducible (Vos et al., 1995, Senior and Heun, 1993). The application of SSR techniques to crops depends on the availability of suitable microsatellite markers, which have been developed for species such as soybean (Rongwen et al., 1995), rice (Zhao and Kochert, 1993), and maize (Taramino and Tingey, 1996). A number of SSR markers have been developed in *Brassica* and shown to be polymorphic within and between different *Brassica* species (Kresovich et al., 1995 and Szewc- McFadden et al., 1996). Five loci were obtained for all microsatellites from *B. oleracea, B. napus* and *B. rapa*, polymorphism was detected for all microsatellites (Lagercrantz et al., 1993). Using SSR markers, Hasan et al.

(2003) found remarkable genetic variation in exotic vegetable and fodder rape genotypes compared with the gene pools of conventional spring and winter oilseed materials. The SSR may provide a useful method for the characterization, conservation, and utilization of oilseed rape cultivars diversity.

2.7 Application of AFLP and SSR in rapeseed

New *B. napus* cultivars are difficult to differentiate mostly because of very narrow genetic background of their parents (Zhou, 2001). To accelerate plant breeding programs, microsatellites of *B. napus* have been previously studied by Lagercrantz et al. (1993), Jin et al. (2006) and Tang et al. (2007). However, the limited number of primer pairs seems to be insufficient for detailed genetic diversity studies, particularly in advanced breeding material and elite cultivars, the genetic complexity of breeder's populations and high levels of heterozygosity in individual genotypes. Moreover, until now there is no report about AFLP analysis of special *B. napus* cultivar in Guizhou. Therefore, the ideal marker system should be established for genetic analysis of *Brassica* genomes. In this research, the selection of primers, identification of loci and their characterization in *B. napus* using AFLP and SSR markers have been studied.

2.7.1 Application of AFLP in rapeseed

The AFLP is a high multiplex PCR-based system having the potential to generate a large number of polymorphic loci (Powell et al., 1996). It combines the strengths and overcomes the weaknesses of the RFLP and RAPD methods. Like RFLP, the molecular basis of AFLP polymorphisms includes indels between restriction sites and base substitutions at restriction sites; like RAPD, it also includes

base substitutions at PCR primer binding sites. Therefore, it is a robust and reliable genetic molecular marker assay, and the number of polymorphisms detected per reaction is much higher than that revealed by RFLP or RAPD assay. The AFLP technique has been applied successfully to crops such as rice (Mackill et al., 1996), tea (Paul et al., 1997), and wheat (Barrett and Kidwell, 1998), oilseed rape (Èron et al., 2006) as well as ornamental plants such as caladium (Loh et al., 1999). It is also known that the AFLP technique has lower initial costs, and is more transferable across species than SSR methods. Techniques based on AFLPs have been applied to genome mapping, DNA fingerprinting and genetic diversity studies. Due to requirement of minimal amounts and high quality of DNA, AFLP can be used to detect the quality of tissue and DNA as following.

2.7.1.1 Genetic diversity of species or population

As AFLP technique provides a large amount of information in a short time, AFLP markers represent an extremely useful tool for genetic diversity and genetic relationship in *Brassica* crop species. Lombard et al. (2000) utilized amplified fragment length polymorphisms to genotype winter rapeseed cultivars and estimate genetic similarities. Srivastava et al. (2001) employed AFLP markers to assess the genetic diversity among 21 established natural and 9 synthetic varieties and lines of *B. juncea* originating from five continents. Roman et al. (2004) reported that AFLP markers detected a higher level of polymorphism among oilseed rape cultivar than RAPD and SSR markers.

2.7.1.2 DNA fingerprinting

DNA fingerprinting of 29 oilseed rape accessions was analyzed using RFLP and AFLP markers by Liu et al. (2006), showed that 1477 polymorphic RFLP

bands and 183 polymorphic AFLP bands from 166 enzyme-probe combinations. Chen et al. (2007) reported that four pairs of core AFLP primer detected 67 polymorphic bands among 89 oilseed rape cultivars and a primary DNA fingerprint database was established. Research results showed that AFLP is a very suitable approach for oilseed rape cultivar fingerprinting (Roman et al., 2004; Rajagopal et al., 1999). This is due to the high multiplex ratio, which is the number of information points analyzed per experiment (Powell et al., 1996).

2.7.1.3 Gene mapping and linkage

The development of genetic maps in *Brassica* is essential to understand the origin and relationship among the genomes of the cultivated *Brassica* species and can be utilized in applied genetics and breeding of *Brassica* crops. Most of the maps were constructed by means of AFLPs. Because AFLP is able to detect a large number of polymorphic bands in a single lane rather than high levels of polymorphism at each *locus* such as is the case for SSR methods. AFLPs are a powerful tool for generating linkage maps. Wang et al. (2004) reported an AFLP map for a double haploid (DH) population of 100 individuals from a cross between a Turnip Mosaic Virus (TuMV) resistant line and a TuMV sensitive line. A genetic linkage map of *B. rapa* ssp. *pekinensis* was constructed with 186 AFLP markers by using a population with 183 individuals (Zhang et al., 2006).

2.7.2 Application of SSR in rapeseed

The SSRs or Microsatellites are stretches of 1 to 6 nucleotide units repeated in tandem and randomly spread in Eucaryotic genomes (Turnpenny and Ellard, 2005). SSR is very polymorphic due to the high mutation rate affecting the number of repeat units. Such length-polymorphisms can be easily detected on high resolution

polyacrylamide gel by running PCR amplified fragments obtained using a unique pair of primers flanking the repeat (Weber and May, 1989).

The AFLP and SSR differ in their principles and applications and generate different amounts of data points. The SSRs are widely used for population genetics and hybridization analysis, because of their high mutation rate, are variable enough to discriminate at the individual level as following.

2.7.2.1 Population genetic analysis

For population structure study, data from many loci and individuals are required. The application of SSR techniques to plants depends on the availability of suitable microsatellite markers. The SSR markers may behave quite stochastically in terms of differentiation between sites and detect several hundreds of loci (Bensch and Åkesson, 2005; Samils et al., 2001; Yan et al., 1998). Morgante and Olivieri (1993) stated that the amount of information given by SSR *loci* in relation to a comparable number of RFLP *loci* is given in soybean by the estimated number of alleles (4.25 per locus for SSR as opposed to 2.15 per locus for RFLP). Wu and Tanskley (1993) reported that the heterozygosity of SSRs is seven to ten times higher than that of RFLPs.

2.7.2.2 Gene flow analysis

In populations continuously distributed over a larger area, where gene flow is mainly between nearby locations, the SSR DNA band patterns can be used to distinguish most of the crop varieties and determine genetic distances to help grouping of varieties on the basis of their ecological origin. Therefore, microsatellite markers are versatile tools for addressing issues of gene flow. Rongnoparut et al. (1999) reported the gene flow occurs among Anopheles maculatus population in

Thailand using microsatellite analysis. Microsatellite DNA variation reveals high gene flow and panmictic populations in the Adriatic shared stocks of the European squid and cuttlefish (Garoia et al., 2004). Consequently, microsatellite data could be applied to assessing levels of gene flow within and between populations, and individual oilseed rape plants. Bond et al. (2004) reported that some feral plants clustered tightly with the winter cultivars, which indicated that only a small proportion of the population was generated from recently spilt seed.

2.7.2.3 Hybridization and hybrid zones

Langevin et al. (1990) showed that hybrids between red rice and cultivated rice were taller than their parents; had more flag leaf area; produced viable seeds and fertile plants, exhibiting the dominant traits of the red-rice parent. Pu (2007) analyzed distant hybridization between rice and sorghum using SSR molecular marker, the result revealed that there was a certain homology between paddy and sorghum. Hopkins et al. (2006) found that 16 SSR markers primers development and characterization of 16 expressed sequence tags (EST)-SSR markers from *B. juncea.*

2.8 References

Abbas, S. J., Farhatullah, I. A. K., Khan, B. M. and Iqbal, M. (2008). Molecular and biochemical assessment of *Brassica napus* and indigenous *campestris* species. **Pakistan Journal of Botany**. 40(6): 2461-2469.

Adams, H., Vaughan, J. G. and Fenwick, G. R. (1988). The use of glucosinolates for cultivar identification in swede, *Brassica napus* L var *napobmssica* (L) peterm. **Journal of the Science of Food and Agriculture**. 46(3): 319-324.

Barrett, B. A. and Kidwell, K. K. (1998). AFLP-based genetic diversity assessment among wheat cultivars from the Pacific Northwest. **Crop Science Society of America**. 38: 1261–1271.

Becker, H. C., Löptien, H. and Röbbelen, G. (1999). **Breeding**: An overview. In: Biology of *Brassica Coeno* species. (ed.): C. Gòmez-Campo, Elsevier, Amsterdam, pp. 413-460.

Bensch, S. and Åkesson, M. (2005). Ten years of AFLP in ecology and evolution: why so few animals? **Molecular Ecology**. 14 (10): 2899-2914.

Bhardwaj, H. L., Hamama, A. A., Porter, D. M. and Reese Jr, P. F. (1996). **Rapeseed meal as a natural pesticide**. In: Janick, J. (ed.), Progress in New Crops. ASHS, Alexandria, VA, pp. 646–649.

Bhardwaj, H. L. and Hamama A. A. (2000). Oil, erucic acid, and glucosinolate contents in winter hardy rapeseed germplasms. **Industrial Crops and Products**. 12: 33-38.

Bond, J. M., Mogg, R. J., Squire, G. R. and Johnstone, C. (2004). Microsatellite amplification in *Brassica napus* cultivars: Cultivar variability and relationship to a long-term feral population. **Euphytica**. 139: 173–178.

Bornet, B. and Branchard, M. (2001). Nonanchored intersimple sequence repeat (ISSR) markers: reproducible and specific tools for genome fingerprinting. **Plant Molecular Biology**. 19: 209–215.

Chen, B. Y., Zhang, X. D., Wu, X. M., Liu, F. L., Lu, G. Y. and Gao, G. Z. (2007). AFLP analysis of 89 cultivars attending the national regional varietal trial. **Chinese journal of oil crop sciences**. 29(2): 9-14.

Chyi, S., Hoencke, M. E. and Sernyk, J. L. (1992). A genetic linkage map of restriction fragment length polymorphism loci for *Brassica rapa* (syn. *campestries*). **Genome**. 35: 746 - 757.

Cooke, R. J. (1999). Modern methods for the cultivar identification and the transgenic plant challenge. **Seed Science and Technology**. 27: 669 – 680.

Cregan, P. B. (1992). Simple sequence repeat DNA length polymorphisms. **Probe**. 2(1): 18 – 22.

Diers B. W. and Osborn T. C. (1994). Genetic diversity of oilseed *Brassica napus* germplasm based on restriction fragment length polymorphisms. **Theoretical and Applied Genetics**. 88: 662 – 668.

Duke, J. A. (1983). **Handbook of Energy Crops**. unpublished. Available http://www.hort.Purdue.edu/newcrop /duke _energy /Acacia_mangium.html (Accessed: April 3, 2003).

Èron, V., Oneesa, J., Áerota, L. and Escobar, R. (2006). Identification of oilseed rape cultivars using fluorescence based AFLP markers. **Indian J. Crop Science.** 1(1-2): 65-69.

Frentzen, M. (1993.) **Acyltransferases and triacylglycerols**. In: Moore TS Jr (ed.) Lipid Metabolism in Plants. CRC Press, Boca Raton, FL, pp, 195-220.

Garcia-Mas, J., Oliver, M., Gómez-Paniagua, H. and de Vicente, M. C. (2000). Comparing AFLP, RAPD and RFLP markers for measuring genetic diversity in melon. **Theoretical and Applied Genetics**. 101:860-864.

Garoia, F., Guarniero, I., Ramšak, A., Ungaro, N., Landi, M., Piccinetti, C., Mannini, P. and Tinti, F. (2004). Microsatellite DNA variation reveals high gene flow and panmictic populations in the Adriatic shared stocks of the European squid and cuttlefish (Cephalopoda). **Heredity**. 93:166-174.

Gerber. S., Mariette, S., Streiff, R., Bodénès, C. and Kremer, A. (2000). Comparison of microsatellites and amplified fragment length polymorphism markers for parentage analysis. **Molecular Ecology**. 9:1037-1048.

Ghodsvali, A., Haddad Khodaparast, M. H., Vosoughi, M. and Diosady, L. L. (2005). Preparation of canola protein materials using membrane technique and evaluation of meals functional properties. **Food Research International**. 38:223-231.

Harding, K. and Harris, P. S. (1997). **Risk assessment of the release of genetically modified plants**: a review Agron Food Industry Hi-Tech, pp.8-13.

Harwood, J. L. (1980). **Plant acyl lipids: structure, distribution and analysis**. In: Stumpf PK (ed.). The biochemistry of plants. Academic Press, New York, pp 2-56.

Hasan, M., Seyis, F., Lu¨hs, W., Badani, A. G., Friedt, W. and Snowdon, R. J. (2006). Analysis of genetic diversity in the *Brassica napus* gene pool using SSR markers. **Genetic Resources and Crop Evolution.** 53(4):793-802.

Hopkins, C., Mogg, R., Gororo, N., Salisbury, P. A., Burton, W. A., Love, C., Spangenberg, G.. C., Edwards, D. and Batley, J. (2006). An assessment of genetic diversity within and between *Brassica napus* and *Brassica juncea* lines from international germplasm collections. **Acta Horticulturae**. 706: 115-119.

Hu, J. And Quiros, C. F. (1991). Identification of broccoli and cauliflower cultivars with RAPD markers. **Plant Cell Reports**. 10: 505–511.

James, A. D. (1983). **Handbook of Energy Crops**. unpublished.

Jin, M. Y., Liu, L. Z., Fu, F. Y., Zhang, Z. S., Zhang, X. K. and Li, J. N. (2006). Construction of a genetic linkage map in *Brassica napus* based on SRAP, SSR, AFLP and TRAP. **Molecular Plant Breeding**. 4 (4): 520-526.

Jones, C. J., Edwards, K. J., Castaglione, S., Winfield, M. O., Sala, F., Bredemeijer, C, F. S., Vosman, G., Matthes, B., Daly, M., Brettschneider, A., Bettini, R., Buiatti, P., Maestri, M., Malcevschi, E., Marmiroli, A., Aert, N., Volckaert, R., Rueda, G., Linacero, J., Vazquez, A. R. and Karp, A. (1997). Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. **Molecular Breeding**. 3:381–390.

Jönsson, R. (1977). Erucic acid heredity in rapeseed (*Brassica napus* L. and *Brassica campestris* L.). **Hereditas**. 86:159–170.

Karp, A., Edwards, K. J., Bruford, M., Funk, S., Vosman, B., Morgante, M., Seberg, O., Kremer, A., Boursot, P., Arctander, P., Tautz, D. and Hewitt, G. M. (1997). Molecular technologies for biodiversity evaluation: opportunities and challenges. **Nature Biotech**. 15:625–628.

Kaushik, N. (1998). **Separation and quantification of quality parameters in rapeseed mustard**. Third International Symposium and short course on separation sciences, 15: 23-26.

Kresovich, S., Williams, J. G. K., McFerson, J. R., Routman, E. J. and Schaal, B. A. (1992). Characterization of genetic identities and relationships of *Brassica oleracea* L. via a random amplified polymorphic DNA assay. **Theoretical and Applied Genetics**. 85: 190-196.

Kresovich, S., Szewc-McFadden, A. K., Bliek, S. M. and McFerson, J. R. (1995). Abundance and characterization of simple sequence repeats (SSRs) isolated from a size-fractionated genomic library of *Brassica napus* (L.) (Rapeseed). **Theoretical and Applied Genetics**. 91:206–211.

Krzymanski, J. (1998). Agronomy of oilseed *Brassicas*. **Acta Horticulturae**. 459: 55–60.

Kondra, I. P. and Stefasom, B. R. (1970). Inheritance of the major glucosinolates of rapeseed (*Brassica napus* L.). **Canadian Journal of Plant Science**. 50:643–647.

Lagercrantz, U., Ellegren, H. and Andersson, L. (1993). The abundance of various polymorphic microsatellite motifs differs between plants and vertebrates. **Nucleic Acids Research**. 21: 1111-1115.

Landry, B. S., Hubert, N., Etoh, T., Harada, J. J. and Lincoln, S. E. (1991). A genetic map for *Brassica napus* based on restriction fragment length polymorphisms detected with expressed DNA sequences. **Genome**. 34:543—552.

Langevin, S. A., Clay, K. and Grace, J. B. (1990). The incidence and effects of hybridization between cultivated rice and its related weed red rice (Oryza sativa L.). **Evolution**. 44:1000-1008.

Leonardo, V. and Heiko, C. B. (2000). Variability for seed glucosinolates in a germplas, collection of the genus *Brassica*. **Genetic Resources and Crop Evolution**. 47: 231-238.

Lin, J. J., Jonathan, K., Jin, M., James, A. S., Hunter, S. B., Margaret, H. M., William, K., George, N. U. and Benjamin, F. M. (1996). Identification of molecular markers in soybean: comparing RFLP, RAPD, and AFLP DNA mapping techniques. **Plant Molecular Biology Reporter**. 14: 156–169.

Liu, H. L. (2000). **Genetics and Breeding in Rapeseed**. Beijing: China Agricultural University Press.

Liu, L. Z., Meng, J. L., Lin, N., Chen, Li., Tang, Z. L, Zhang, X. K. and Li, J. N. (2006). QTL Mapping of Seed Coat Color for Yellow Seeded *Brassica napus*. **Acta Genetica Sinica**. 33 (2):181-187.

Loh, J. P., Kiew, R., Kee, A., Gan, L. H. and Gan, Y. Y. (1999). Amplified fragment length polymorphism (AFLP) provides molecular markers for the identification of *Caladium bicolor* cultivars. **Annals of Botany**. 84: 155–161.

Lombard, V., Baril, C. P., Dubreuil, P., Blouet, F. and Zhang, D. (2000). Genetic relationships and fingerprinting of rapeseed cultivars by AFLP: Consequences for varietal registration. **Crop Science**. 40: 1417-1425.

Lombard, V. and Delourme, R. (2001). A consensus linkage map for rapeseed (*Brassica napus* L.): construction and integration of three individual maps from DH population. **Theoretical and Applied Genetics**. 103: 491–507.

Lühs, W., Voss, A., Han, J., Gräfin zu Münster, A., Weier, D., Wolter, F. P., Frentzen, M. and Friedt, W. (1999). **Genetic modification of erucic acid biosynthesis in *Brassica napus*.** In: Mugnozza GTS, Porceddu E, Pagnotta MA (ed.) Genetics and breeding for crop quality and resistance (Developments in Plant Breeding Vol. 8), Kluwer Academic Publishers, Dordrecht, Netherlands, pp 323-330.

Ma, C. Z., Fu, T. D., Tuevesson, S. and Gertsson, Bo. (2003). **Scientia Agricultura Sinica**. 36(11): 1403-1408.

Mackill, D. J., Zhang, Z., Redon˜a, E. D., and Colowit, P. M. (1996). Level of polymorphism and genetic mapping of AFLP markers in rice. **Genome**. 39: 969–977.

McGregor, C. E., Lambert, C. A., Greyling, M. M., Louw, J. H. and Warnich, L. (2000). A comparative assessment of DNA fingerprinting techniques (RAPD, ISSR, AFLP and SSR) in tetraploid potato (*Solanum tuberosum* L.) **Germplasm Euphytica**. 113: 135–144.

Melchinger, A. E., Graner, A., Singh, M. and Messmer, M. M. (1994). Relationships among European barley germplasm: I. Genetic diversity among winter and spring cultivars revealed by RFLPs. **Crop Science**. 34: 1191- 1199.

Meng, J. L., Sharpe, A., Bowman, C., Tian, Z. H., Fu, T. D., Qian, X. Z. and Lydiate, D. (1996). Genetic diversity of *Brassica napus* detected with RFLP markers. **Acta Genet Sinica**. 23(4):293–306.

Messmer, M. M. and Melchinger, A. E. (1993). Relationships among early European maize inbreeds: II. Comparison of pedigree and RFLP data. **Crop Science**. 33: 944-950.

Milbourne, D., Meyer, R., Bradshaw, J. E., Baird, E., Bonar, N., Provan, J., Powell, W. and Waugh, R. (1997). Comparison of PCR based marker systems for the analysis of genetic relationships in cultivated potato. **Molecular Breeding**. 3: 127–136.

Mohan, M., Nair, S., Bhagwat, A., Krishna, T. G., Yano, M., Bhatio, C. R. and Sasaki, T. (1997). Genome mapping, molecular markers and marker-assisted selection in crop plants. **Molecular Breeding**. 3: 87–10.

Morgante, M. and Olivieri, A. M. (1993). PCR-amplified microsatellites in plant genetics. **The Plant Journal**. 3: 175–182.

Mou, T. M. and Liu, H. L. (1990). Genetic analysis on the total content of glucosinolates in seeds of rape (*Brassica napus* L.). **Acta Agron Sin**. 16: 97–105.

Musil, A. F. (1950). **Identification of Brassicas by seedling growth or later vegetative stages**. USDA Circular pp. 857. 26 Michael Boland, 2010. Rapeseed. Kansas State University.

Mündges, H., Köhler, W. and Friedt, W. (1990). Identification of rapeseed cultivars (*Brassica napus*) by starch gel electrophoresis of enzymes. **Euphytica**. 45: 179-187.

Ofori, A. and Becker, H. C. (2008). **Breeding of *Brassica rapa* for biogas production**: Heterosis and combining ability of biomass yield. Bioenerg Res DOI 10.1007/ s12155-008-9001-2.

Oplinger, E. S., Hardman, L. L. Gritton, E. T., Doll, J. D. and Kelling, K. A. (1989). **Canola (rapeseed)**. Department of Agronomy and Soil Science, University of Wisconsin-Madison and Department of Agronomy and Plant Genetics, University of Minessota.

Orman, B. A., and Schumann, R. A. Jr. (1992). Nondestructive single-kernel oil determination of maize by near-infrared transmission spectroscopy. **Journal of the American Oil Chemists' Society**. 69: 1036-1038.

Osborne, B. G. and Fearn, T. (1986). **Near Infrared Spectroscopy in Food Analysis**. Wiley, New York.

Paul, S., Wachira, F. N., Powell, W. and Waugh, R. (1997). Diversity and genetic differentiation among populations of Indian and Kenyan tea (*Camellia sinensis* (L.) O. Kuntze) revealed by AFLP markers. **Theoretical and Applied Genetics**. 94: 255–263.

Petisco, C., García-Criado, B., Vázquez-de-Aldana, B. R., de Haro, A. and García-Ciudad, A. (2010). Measurement of quality parameters in intact seeds of *Brassica* species using visible and near-infrared spectroscopy. **Industrial Crops and Products**. 32:139-146.

Post-Beittenmiller, D. (1996). **Biochemistry and molecular biology of wax production in plants**. Annual Review of Plant Physiology and Plant Molecular Biology. 47: 405-430.

Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S. and Rafalski, A. (1996). The comparison of RFLP, RAPD, AFLP, and SSR (microsatellite) markers for germplasm analysis. **Molecular Breeding**. 2:225–238.

Pu, J. Z. (2007). The prilimilary study on analysis of distant hybridization between rice and sorghum using SSR molecular marker. **Journal of Zhangzhou Normal University**. 20(1): 99-103.

Qi, C. K., Gai, J. Y. and Zhang, Y. M. (2001). Major gene plus polygene inheritance of erucic acid content in *Brassica napus* L. **Acta Genet Sin**. 28:182–187.

Qiao, H., L, G. L., X, J. H. and L, W. C. (2006). Genetic diversity analysis of wax apple germplasm by ISSR markers. **Acta Hortic.Sin**. 33 (2): 392–394.

Rajagopal, J., Das, S., Bhatia, S., Srivastava, P. S. and Lakshmikumaran, M. (1999). Assessment of genetic variation within *Brassica campestris* cultivars using amplified fragment length polymorphism and random amplification of polymorphic DNA markers. **Journal of Biosciences**. 24: 433-440.

Roman, S., Lenka, D., Vladislav, C. and Jaroslava, O. (2004). Fluorescence-based AFLPs occure as the most suitable marker system for oilseed rape cultivar identification. **Journal of Applied Genetics**. 45(2):161-173.

Rongwen, J., Cregan, P. B., Akkaya, M. S., Bhagwat, A. A and Lavi, U. (1995). The use of microsatellite DNA markers for soybean genotype identification. **Theoretical and Applied Genetics**. 90: 43- 48.

Rongnoparut, P., Sirichotpakorn, N., Rattanarithikul, R., Yaicharoen, S. and Linthicum, K. J. (1999). Estimates of gene flow among Anopheles maculatus populations in Thailand using microsatellite analysis. **American Journal of Tropical Medicine and Hygiene**. 60(3): 508-515.

Russell, R. J., Fuller, J. D., Macaulay, M., Hats, B. G., Jahoor, A., Powell, W. and Waugh, R. (1997). Direct comparison of levels of genetic variation among barley accessions detected by RFLPs, AFLPs, SSRs and RAPDs. **Theoretical and Applied Genetics**. 95: 714–722.

Samils, B., Lagercrantz, U., Lascoux, M. and Gullberg, U. (2001). Genetic structure of *Melampsora epitea* populations in Swedish *Salix viminalis* plantations. **European Journal of Plant Pathology**. 107: 399-409.

Sarwar, G. R., Blair, R., Friedman, M., Gumbmann, M. R., Hackler, L. R., Pellet, P. L. and Smith, T. K. (1984). Inter- and intralaboratory variability in rat growth assays for estimating protein quality of foods. **Association of Official Analytical Chemists**. 67(5): 976-81.

Senior, M. L. and Heun, M. (1993). Mapping maize microssatellites and polymerase-chain-reaction confirmation of the targeted repeats using CT primer. **Genome**.36: 884-889.

Slocum, M. K., Figdore, S. S., Kennard, W. C., Suzuki, J. Y. and Osborn, T. C. (1990). Linkage arrangement of restriction fragment length polymorphisms in *Brassica oleracea*. **TAG Theoretical and Applied Genetics**. 80: 57-64.

Snowdon, R. J., Friedrich, T., Friedt, W. and Kohler, W. (2002). Identifying the chromosomes of the A- and C-genome diploid *Brassica species B. rapa (syn. campestris)* and *B. oleracea* in their amphidiploid *B. napus*. **Theoretical and Applied Genetics**. 104: 533–538.

Song, K. M., Suzuki, J.Y., Slocum, M. K., Williams, P. H. and Osborn, T. C. (1991). A linkage map of *Brassica rapa (syn. campestris)* based on restriction fragment length polymorphisms. **Theoretical and Applied Genetics**. 82: 296—304.

Srivastava, A., Gupta, V., Pental, D. and Pradhan A. K. (2001). AFLP-based genetic diversity assessment amongst agronomically important natural and some newly synthesized lines of *Brassica juncea*. **Theoretical and Applied Genetics**. 102: 193–199.

Stone, P. J. and Savin, R. (1999). **Grain quality and its physiological determinants**. In: Wheat: Ecology an Physiology of Yield Determination, Satorre, E. H. and Slafer, G. A. (eds.). FPP, New York, pp. 84-12.

Szewc- McFadden, A. K., Kresovich, S., Bliek, S. M., Mitchell, S. E. and McFerson, J. R. (1996). Identification of polymorphic, conserved simple repeats (SSR) in cultivated *Brassica* species. **Theoretical and Applied Genetics**. 93: 534-438.

Tang, R., Wu, Y. X., Tian, Z. P., Zhang, M., Wang, T. Q. (2007). Hybird purity identification of Guiyou 519 with yellow seeds by SSR. **Guizhou Agricultural sciences**. 35(3):10-11.

Tanksley, S. D., Young, N. D., Paterson, A.H. and Bonierval, M. W. (1989). RFLP mapping in plant breeding: new tools for an old science. **Biotechnology**. 7: 257-264.

Taramino, G. and Tingey, S. (1996). Simple sequence repeats for gemplasm analysis and mapping in maize. **Genome**. 39:277-287.

Thimmappaiah, S., Shobha, W. G., D. and Melwyn, G.. S. (2009). Assessment of genetic diversity in cashew germplasm using RAPD and ISSR markers. **Scientia Horticulturae**. 120: 411-417.

Turnpenny, P. and Ellard, S. (2005). **Emery's Elements of Medical Genetics.** 12th Edtion. Elsevier, London.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Van de Lee T, Hornes, M., Frijters, A., Pot, J., Kuiper, M. and Zabeau, M. (1995). AFLP: a new technique for DNA fingerprinting. **Nucleic Acids Research**. 23: 4407–4414.

Velasco, L., Mollers, C. and Becker, H. C. (1999). Estimation of seed weight, oil content and fatty acids composition in intact single seeds of rapeseed Brassica napus by near infrared reflectance spectroscopy. **Euphytica**. 106: 79-85.

Wang, M., Zhang, F. L., Meng, X. D., Liu, X. C., Zhao, X. Y. and Fan, Z. C. (2004). A linkage map construction for Chinese cabbage based on AFLP markers using DH population. **Acta Agriculturae Boreali-Sinica**.19: 28-33.

Wang, X. F., Liu, G. H., Yang, Q., W, Hua., Liu, J. and Wang, H. Z. (2010). Genetic analysis on oil content in rapeseed (*Brassica napus* L.). **Euphytica**. 173:17-24.

Weber and May. (1989). Abundant class of human DNA polymorphism which can be typed using the polymerase chain reaction. **American Journal of Human Genetics**. 44: 388-396.

White, J. and Law, J. R. (1991). Differentiation between varieties of oilseed rape *Brassica napus* (L.) on the basis of the fatty acid composition of the oil. **Plant Var Seeds**. 3:125–132.

Wu, N. F., Li, R. G., Wu, X. M., Zhu, Li., Fan, Y. L. and Qian, X. Z. (1997). RAPD markers and genetic diversity among 40 cultivars of *Brassica napus* in China. **Chinese Biodiversity**. 5(4): 246-250.

Wu, K. S. and Tanksley, S. D. (1993). Abundance, polymorphism and genetic mapping of microsatellites in rice. **Molecular and General Genetics**. 241: 225-235.

Yan, G., Romero-Severson, J., Walton, M., Chadee, D. D. and Severson, D. W. (1998). Population genetics of the Yellow Fever Mosquito in Trinidad: Comparison of AFLP and RFLP markers. **Molecular Ecology**. 8: 951-963.

Yonemoto, Y., Chowdhury, A. K., Kato, H., Macha, M. M. (2006). Cultivars identification and their genetic relationships in Dimocarpus longan subspecies based on RAPD markers. **Scientia Horticulturae**.109: 147–152.

Zhao, J. J, Wang, X. W., Deng, B., Lou, P., Wu, J., Sun, R. F., Xu, Z. Y., Vromans, J., Koornneef, M. and Bonnema, G. (2005). Genetic relationships within *Brassica rapa* as inferred from AFLP fingerprints. **Theoretical and Applied Genetics**. 110: 1301–1314.

Zhao, X. P. and Kochert, G. (1993). Phylogenetic distribution and genetic mapping of a (GGC) (n) microsatellite from rice (*Oryza sativa* L.). **Plant Molecular Biology**. 21:607-614.

Zhang, H. Z., Shi, C. H., Wu, J. G., Ren, Y. L. and Li, C. T. (2004). Genetic analysis of embryo, cytoplasm and maternal plant effects for glucosinolate content in rapeseed. **Acta Agron Sin**. 30: 31–35.

Zhang, X. W, Wu, J., Zhao, J. J, Song, X. F., Li, Y., Zhang, Y. G, Xu, D. H, Sun, R. F., Yang, Y. X., Xie, C. H and Wang, X. W. (2006). Identification of QTLs Related to Bolting in *Brassica rapa* ssp. *Pekinensis* (syn. *Brassica campestris* ssp. *pekinensis*). **Agricultural Sciences in China**. 5(4): 265-2.

Zhou, W. J. (2001). **Oilseed rape**. In: Zhang G. P. and Zhou W J. (eds.) Crop Cultivation. Zhejiang University Press, Hangzhou, China, pp. 153–178.

Zietkiewicz, E., Rafalski, A. and Labuda, D. (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. **Genomics**. 20: 176–183.

CHAPTER III

CHARACTERIZATION OF GENETIC DIVERSITY AND RELATIONSHIPS AMONG RAPESEED (*BRASSICA NAPUS*) CULTIVARS USING AFLP TECHNIQUE

3.1 Abstract

A powerful technique AFLP was applied to assess genetic diversity and relationships among 25 *B. napus* cultivars. Nine AFLP primer combinations generated 193 fragments, of which 73 were polymorphic. The polymorphic rate was 38%. The average number of observed alleles per locus was 3.56, while the mean number of effective alleles was 1.41. In all available materials, gene diversity, Shannon's information index, and genetic differentiation were 0.25, 0.62, and 0.39, respectively. UPGMA cluster analysis revealed that rapeseeds in this study can be divided into three groups at a similarity coefficient of 0.66. No apparent clustering by seed quality characteristic or original location suggested that the limited number of characters used for variety discrimination is encoded by a limited number of genes. However, a wide range of similarity coefficient value suggested that the *B. napus* germplasm collection represents a genetically diverse population, also demonstrated AFLP markers were useful for evaluating the genetic relationships among species in rapeseed and may serve as effective fingerprint technique for further characterization and management of rapeseed germplasm.

Keyword: genetic diversity; *B. napus*; AFLP

3.2 Introduction

In any crop improvement program, accurate identification of cultivars and knowledge of their genetic relationships are essential for successful breeding and management strategies. Registration and protection of oil seed rape cultivars relies on a relatively small number of morphological traits, quality traits and yield character. As the number of cultivars increases, the ability to distinguish them on a morphological basis alone becomes more difficult (Èron et al., 2006; Lombard et al., 2000). Considering the practice, time-consuming and cost, new markers are being developed to maintain the efficacy of registration and regional test which guarantees the quality of new cultivar for farmers and merchants.

Molecular markers have proven to be powerful tools to assess genetic diversity and relationships in rapeseed germplasm. Different markers systems have been constructed, such as RFLP (Song et al., 1991; McGrath et al., 1991; Chyi et al., 1992), RAPD (Chen and Ronald, 1999; Chen et al., 2000a; Hu and Quiros, 1991), SSR (Kresovich et al., 1995; Uzunova et al., 1999), ISSR (Ma et al., 2003). These techniques differ in their principles, applications, and generate different amounts of data points (Das et al., 1999). However, the utilization of RFLPs, RAPDs, SSRs and ISSRs for genome analysis seems to be restricted by their characteristics. For example, the cumbersome technique and low number of discriminating loci of RFLP (Jones et al., 1997), lack of reproducibility of RAPD (Karp et al., 1997), small number of loci and expensive cost of SSR (Gao et al., 2009), low reproducibility and informativeness of ISSR (McGregor et al., 2000).

Fifteen years ago, AFLP technique was developed, combining the RFLP and RAPD (Vos et al., 1995), having the potential to generate a large number of

polymorphic loci (Powell et al., 1996; Guo et al., 2002). Compared with the above mentioned molecular technique, AFLP is the most efficient method to estimate genetic diversity because of its high reproducibility, high polymorphism and multiplex ratio (Das et al., 1999; Steiger et al., 2002; Liu et al., 2005). Due to this characteristic of AFLP, the aim of the present study was designed to investigate genetic relationships of Guizhou *B. napus* germplasm, and evaluate genetic diversity among 25 *B. napus* genotypes. This is the first AFLP-based study on *B. napus* germplasm from Guizhou Province, People's Republic of China. Moreover, genetic analysis of rapeseed cultivars was analyzed with AFLP markers in this study. The information will be used to identify genotype having desirable characters for future breeding programs.

3.3 Materials and Methods

3.3.1 Materials

3.3.1.1 Plant materials

Twenty-five *B. napus* F_1 cultivars were selected from different breeding institutes, which were provided by Guizhou Seed Management Station, Guizhou, People's Republic of China. They represented a large range of *B. napus* germplasm and elite varieties. Seed germination was performed in Guizhou Key Laboratory of Agricultural Biotechnology. Young leaf samples were collected from these varieties. The collected leaves were used immediately for DNA extraction, while excess leaf materials were stored in -70ºC freezer for future use. The name and origin of *B. napus* cultivars used are given in Table 3.1

3.3.1.2 Chemical

All chemicals used were Laboratory grade or specified.

1) DNA extraction using DNA quick plant system kits (Tiangen Biotech Beijing Co., Ltd.):

Extraction buffer FP1

Extraction buffer FP2

RNase A 10 mg/mL

Ice cold isopropanol

70% EtOH

TE buffer (10 mM Tris-Cl (pH 7.5), 1 mM EDTA)

2) AFLP core reagent kit (New England Biolabs):

10 u/µL *Eco*RI

10 u/µL *Mse*I

10 mM Tris-HCl (pH 7.4)

50 mM KCl

0.1 mM EDTA

1mM DTT

200 µg/mL BSA

50% (v/v) glycerol

3) Adapter/ligation solution (Fermentas):

*Eco*RI/*Mse*I adapters

0.4 mM ATP

10 mM Tris-HCl (pH 7.5)

1 mM DTT

50 mM KCl

50% glycerol (v/v)

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

4) AFLP Adapters and Starter Primers (Generay Biotech Company, Shanghai, China)

5) Adapters primer and preparation

*Eco*RI-adapter: Forward 5' CTCGTAGACTGCGTACC 3'

Reverse 3' CATCTGACGCATGGTTAA 5'

*Mse*I- adapter: Forward 5' GACGATGAGTCCTGAG-3'

Reverse 3'TACTCAGGACTCAT-5'

6) Starter primers:

The basic *Eco*RI primer:

5' GACTGCGTACCAATTC xyz -3' (where x, y, and z represent the selective bases on the 3' end of the oligonucleotide).

The basic *Mse*I primer:

5' GATGAGTCCTGAGTAACACxyz-3', like with the *Eco*RI primer x, y, and z represent the selective bases on the 3' end of the oligonucleotide.

7) AFLP loading buffer:

Formamide-loading buffer: 98% formamide, 10 mM EDTA (pH 8.0), 0.25% bromophenol blue and 0.25% xylene cyanol

8) Reagent for PAGE:

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

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

1 X TBE buffer: 5X TBE buffer was diluted to 5 folds.

10% AP: 0.1 g ammonium persulfate in 1 mL ddH_2O

N, N, N', N'-tetramethylethylenediamine (TEMED)

9) Composition of denaturing 6 % PAGE storage solution (100 mL):

Urea	42 g
Acrylamide	5.7 g
Bisacrylamide	0.3 g
5 X TBE buffer	20 mL
10 % fresh AP	500 µL
TEMED	50 µL

Mix, bring deionized water volume to 100 mL. Warm the solution at 60°C until urea dissolved completely and filter through Whatman filter paper. Store at 4 °C in the dark.

10) Reagent for silver staining:

Fixer: 10% ethanol, 0.5% acetic acid

Silver nitrate: 0.2% silver nitrate, 0.2% formalin

Developer: 1.5% sodium hydroxide, 0.2% formalin

Stop staining: 10% ethanol, 0.5% acetic acid

3.3.2 Methods

3.3.2.1 Material sampling

A total of 25 *B. napus* cultivars were grown in pots in a green house of Guizhou Key Laboratory of Agricultural Biotechnology, People's Republic of China. Young leaves were harvested from 2 to 3 weeks old seedling, placed in sealable plastic bag and appropriately labeled. The collected leaves were used immediately for DNA extraction, while excess leaf materials were stored in -70ºC freezer for future use.

Table 3.1 List of name and origin of *B. napus* cultivars included in the analysis.

Code	Cultivar	Origin
A1	Youyan1517	Guizhou Rapeseed Institute
A2	B-52	Guizhou Seed Management Station
A3	Youyan 10	Guizhou Rapeseed Institute
A4	You 05-2	Guizhou Rapeseed Institute
A5	Jinyou 8	Guizhou Lantian Seed Industry Limited Liability Company
A6	H2139	Guizhou Key Laboratory of Agricultural Biotechnology
A7	You 3115	Guizhou Rapeseed Institute
A8	Qianza 6-18	Guizhou Oil Crop Institute
A9	You 9559	Zunyi Seed Management Station
A10	ZWH-1	Guizhou Oil Crop Institute
A11	H0802	Guizhou Rapeseed Institute
A12	Qianza 2501	Guizhou Oil Crop Institute
B1	Qianza 222	Guizhou Oil Crop Institute
B2	NR061	Zunyi Academy of Agricultural Science
B4	Gui BF2-3	Guizhou University
B5	IF5-9	Sichuan Shu Yu Agricultural Technology Development Company
B6	NR168	Zunyi Academy of Agricultural Science
B7	You 06-1	Guizhou Rapeseed Institute
B8	Mianza 04-52	Mianyang Academy of Agricultural Science
B9	Jinyou 068	Guizhou Oil Crop Institute
B10	Shenyou 6970	Shennong Technology Limited Liability Company of Guizhou University
B11	Qianza J5005	Guizhou Oil Crop Institute
B12	You 06-3	Guizhou Rapeseed Institute
SC4	Huayouza 6	Huazhong Agricultural University
SC5	Huayouza 9	Huazhong Agricultural University

3.3.2.2 DNA extraction

DNA was extracted from young leaves using DNA quick plant system kits (Tiangen Biotech Beijing Co., Ltd.) as the manufacturer's instructions. Briefly, 100 mg young leaf tissue were pulverized using liquid nitrogen and dissolved in 400 µL of buffer solution FP1 and 6 µL RNase A (10 mg/mL). The mixture was vortexed for 1 min and spun down for 10 min at room temperature. Then the mixture was vortexed with buffer solution FP2 for 1 min and centrifuged at 12,000 rpm for 5 min. The supernatant was transferred to clean microfuge tubes. The previous step could be repeated to improve the purity of DNA. The nucleic acids were precipitated by addition of 0.7 volume ice cold isopropanol and the tubes were inverted slowly to precipitate the DNA. The DNA pellet was washed with 700 µL 70% ethanol, vortexes for 5 seconds, and then centrifuged at 12,000 rpm for 2 min. The supernatant was discarded. The step of DNA purity was repeated. The DNA pellet was then dried at room temperature, and the purified DNA was resuspended in 50 µL TE buffer and heated in water bath at 65ºC.

3.3.2.3 Evaluation of quality and quantity of DNA

DNA concentration and purity were determined at 260 and 280 nm using DU 800 spectrophotometer (Beckman Coulter, USA). The DNA concentration was calculated using the formula, [DNA] = Optical density × dilution factor× constant (50 µg/mL). Purity was determined using the 260/280 nm ratio. The DNA samples were diluted with TE buffer and stored at 4ºC. The integrity of the DNA was confirmed by running on 1% (w/v) agarose electrophoresis with visualization under UV light after staining with ethidium bromide.

3.3.2.4 AFLP amplification

The AFLP amplification was carried out according to the lab of Biological centre, Beijing Academy of Agriculture and Forestry Sciences, People's Republic of China with minor modification. The restriction enzymes, adapters and primers used were listed in Table 3.2. There were five steps of AFLP technique:

Step 1: Restriction digests of genomic DNA. Genomic DNA was digested with restriction enzymes *Eco*RI and *Mse*I. The reaction was performed in a volume of 20 μL: 0.2 μL of 10 u/μL *Eco*RI and 0.1 μL of 20 u/μL *Mse*I (New England Biolabs), 2 μL DNA template (0.5 μg), 2 μL of 10 × NEB buffer , 0.2 μL of 100×BSA and 15.5 μL ddH_2O. It was carried out at 37ºC for 4 h. Enzymes were inactivated by incubation at 65°C for 20 min.

Step 2: Ligation. The *Eco*RI-adapter and *Mse*I-adapter were ligated to the ends of restriction fragments overnight at 15ºC to generate template DNA for PCR amplification. The restriction-ligation reactions were performed in a volume of 4 μL: 0.4 μL of 10 μM *Eco*RI adapter, 0.4 μL of 10 μM *Mse*I adapter, 0.24 μL of 3 u/μL T4-DNA ligase, 0.4 μL of 10 × T4-DNA buffer and 2.56 μL ddH_2O. The ligation reaction was inactivated by incubation at 65ºC for 20 min.

Step 3: Pre-amplification. A subset of each sample of DNA restriction fragment was amplified using universal primers, E00 and M00 (Table 3.2), each specific for one of the adaptors and adjacent restriction site. Pre-amplifications were done in a 20 μL volume containing 2 μL of 10 x PCR buffer, 0.4 μL of 10 mM dNTPs, 1 μL of 10 μM *Eco*RI and *Mse*I primers without any oligonucleotides, respectively, 0.2 μL 2.5 u/μL *Taq* polymerase and 2 μL restriction ligation mix. PCR reactions were performed in Master Cycler Gradient 22331 (Germany): an initial step

of 5 min at 94 ºC, 30 cycles of 94ºC for 30 s, 56ºC for 30 s and 72ºC for 60 s, and a final step of 10 min at 72ºC. PCR products were analyzed in a 1.0% agarose gel and the presence of low-molecular-weight smear indicated successful amplification.

Step 4: Selective amplification. The PCR products of the pre-amplification reaction were used as template, after dilution twenty fold in ddH_2O, for selective amplification using two AFLP primers, each containing three selective nucleotides. The *Eco*RI primers used were not radioactive labeled as in the original protocol. Instead, a modified silver-staining method was used. AFLP reaction was performed for 36 cycles with the following cycle profile: The selective amplifications were carried out using the following cycling parameters: 12 cycles of 30 s at 94ºC, 30 s at 65ºC to 56ºC (with a decreasing ramp of 0.7ºC each cycle), and 60 s at 72ºC, followed by 24 cycles of 30 s at 94ºC, 30 s at 56ºC, and 60 s at 72ºC.

Step 5: Gel electrophoresis and silver staining. Following the selective amplification, the reaction products were mixed with an equal volume of formamide-loading buffer (98% formamide, 10 mM EDTA pH 8.0 and 0.1% bromophenol blue). The samples were denatured for 5 minutes at 94ºC, cooled on ice and run on a 6% denaturing polyacrylamide gel in 1×TBE buffer. The gel was run at a constant 1500 V, 65 mA, for 2 h in a sequencing gel electrophoresis apparatus (EPS 3501, Amersham Pharmacia Biotech). The primers were not radioactively labeled; therefore, the AFLP fragments were visualized using silver staining (see Part 3, APPENDIX). The gel should be dry overnight before photographed.

Table 3.2 Oligonucleotide adapters and primers and their sequences for AFLP primer combinations.

Oligonucleotide	**Sequence (5′-3′)**		
Adapters			
*Eco*R Ⅰ adapter	CTCGTAGACTGCGTACC		
	AATTGGTACGCAGTCTAC		
Mse Ⅰ adapter	GACGATGAGTCCTGAG		
	TACTCAGGACTCAT		
Pre-selective primers			
E00	GACTGCGTACCAATTC		
M00	GATGAGTCCTGAGTAA		
Selective primers			
E9	E00+CCC	M31	M00+ATC
E10	E00+CCA	M32	M00+ACT
E11	E00+CCG	M33	M00+TCG
E12	E00+CCT	M34	M00+TGC
E13	E00+CGG	M35	M00+ACG
E14	E00+CGA	M36	M00+AGC
E15	E00+CGT	M37	M00+CTA
E16	E00+CGC	M38	M00+GTC

3.3.2.5 Band scoring and data analysis

Profiles for each cultivar and marker system were constructed by scoring 0 or 1 for absence or presence of bands respectively. Each AFLP band was assumed to correspond to a dominant allele at a single locus. Only clear, unambiguous and reproducible bands were scored for the data analysis. All weak or poor bands

were not recorded. The size of fragment was calculated based on DNA ladder pBR322 DNA-Msp Ⅰ Digest (New England BioLabs). These data were entered into the software package NTSYS-pc 2.10 (Rohlf, 2000) and analyzed using qualitative routine to generate genetic similarity coefficient. The genetic similarity among all cultivars was calculated using Dice similarity coefficient (Nei and Li, 1979), according to the formula:

$S_{i,j}$ = 2a/(2a+ b + c), where $S_{i,j}$ represents the similarity estimate between the genotypes *i* and *j*, based on the AFLP data, a is the number of bands presented in both *i* and *j*, b is the number of bands present in *i* and absent in *j*, and c is the number of bands present in *j* and absent in *i*.

The dendrogram was constructed with unweighted pair group method using arithmetic average (UPGMA) algorithm (Sneath and Sokal, 1973) based on similarity coefficient, the sequential hierarchical, and nested clustering routine in the NTSYS program.

POPGENE version 1.31 software (Yeh et al., 1999) was used to calculate the parameters of genetic diversity, including the observed number of alleles (NA), effective number of alleles per locus (NE; Hartl and Clark, 1989), gene diversity (H = expected heterozygosity, $H = 1-\Sigma p_i^2$, where p_i is the frequency of the presence or absence of the band, Nei, 1973) and Shannon's information index (I) for phenotypic diversity quantifying the degree of AFLP polymorphism within populations ($I = -\sum_i p_i \log_2 p_i$, where p_i is the frequency of the presence or absence of a AFLP band; Lewontin, 1972). At the species level, the coefficient of gene differentiation (G_{ST}) is defined as the proportion of the interpopulational gene

diversity, and was calculated using Nei's gene diversity method (Nei, 1973), according to the formula:

$G_{ST} = D_{ST}/ H_{T,} H_T = H_S + D_{ST}$, where, H_T is the total gene diversity, H_S is the gene diversity within the population, and D_{ST} is the gene diversity between populations.

3.4 Result and discussion

3.4.1 Quantification and visualization of the DNA

DNA concentration and purity were determined using absorption maximum wavelength at 260 nm and 260/280 ratio, respectively. The AFLP analysis relies on high yields of pure DNA samples. In this study, all samples of DNA had the value of 1.7 - 2.0 at 260/280 ratio, suggesting high purity, and met the requirement of AFLP analysis. The integrity of DNA was confirmed by 1.0% agarose electrophoresis with visualization under UV light after staining with ethidium bromide (shown in Figure 3.1). The presence of a highly resolved high molecular weight bands indicated that the quality of DNA produced by DNA quick plant system kit was sufficiently high for complete restriction digestion with *Eco*RI and *Mse*I (Figure 3.2) and successful AFLP fingerprinting with different primer pairs (Figure 3.4).

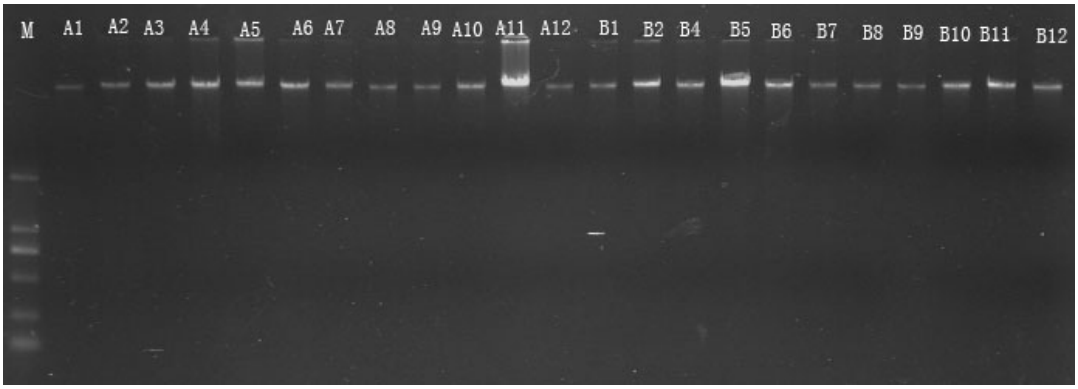


Figure 3.1 The integrity of DNA was estimated in 1.0% agarose gel. Lane M (left) showed a 2 kb DNA ladder marker. Lanes A1-A12 and B1-B12 represented the integrity of genomic DNA of *B. napus* cultivars shown in Table 3.1.

3.4.2 Restriction digestion of genomic DNA and ligation

The AFLP procedure was performed as described by Vos et al. (1995), and modified by Biological Centre of Beijing Academy of Agriculture and Forestry Sciences. 2 μL restriction-ligation products were monitored in a 1.0% agarose gel. The presence of smear in a range of 100-2000 bp indicated successful restriction digestion (Figure 3.2), and could be used as template of pre-amplification. In the study, we recommended a digestion for 4 h at 37ºC to ensure complete digestion of DNA. Usually restriction and ligation take place in a single reaction. Ligation of the adapter to the restricted DNA alters the restriction site in order to prevent a second restriction from taking place after ligation has occurred.

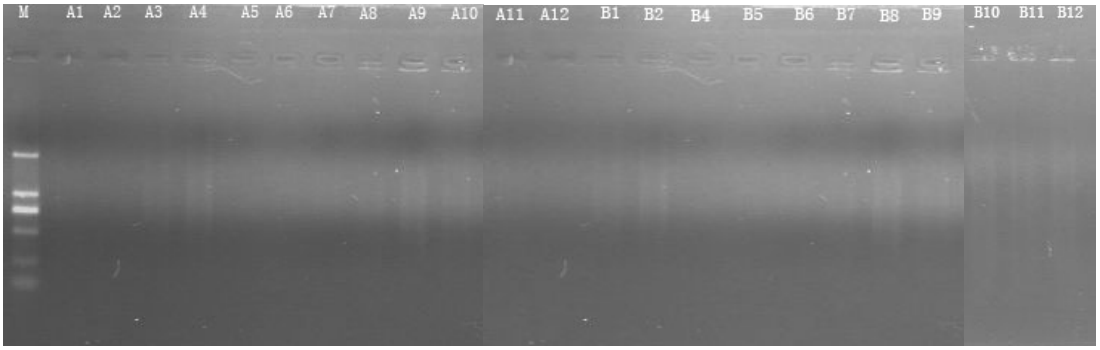


Figure 3.2 Restriction digestion and ligation products of DNA with *Eco*RI and *Mse*I enzymes, as visualized on a 1.0% agarose gel with ethidium bromide (110 V for 60 min). Lane M (left) showed a 2 kb DNA ladder marker. Lanes A1- A12 and B1-B12 showed restriction digest products of *B. napus* DNA.

3.4.3 Pre-amplification

DNA was amplified with the *Eco*RI and *Mse*I primers without any nucleotide. Pre-amplification provided a large number of templates for selective-amplification and purified the template selectively, so that clear, reproducible and stable fragments were produced. However, *Eco*RI and *Mse*I without any oligonucleotides had poor selective amplification. Thus, fragments are often compression and not separated. Therefore, it was recommended that pre-amplification steps with *Eco*RI+1/*Mse*I+1 are used to avoid mismatches (Vos et al., 1995; Qi and Lindhout, 1997). Next to that, the final selective PCR amplification with AFLP primer combination should also have moderate selectivity. The electrophoresis of pre-amplification was shown in Figure 3.3. The size of products ranged below 1 kb, which indicated the successful amplification for selective amplification.

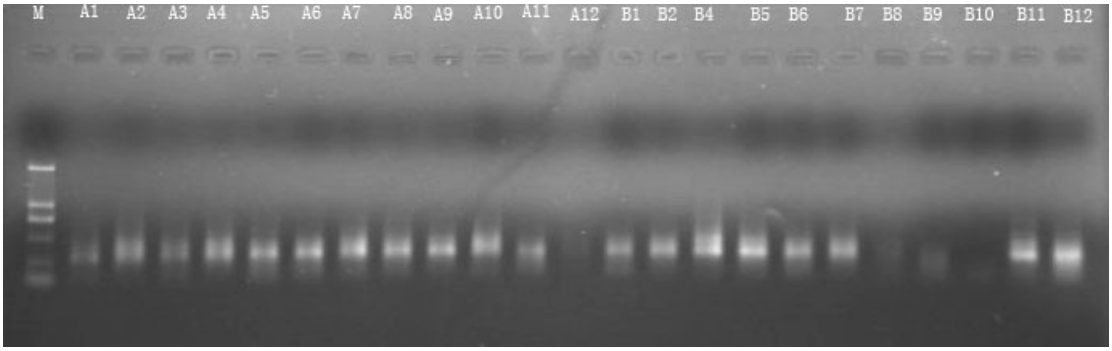


Figure 3.3 The electrophoresis of pre-amplification. Lane M (left) showed a DNA ladder marker. Lanes A1-A12 and B1-B12 showed pre-amplification of DNA of *B. napus* cultivars shown in Table 3.1.

3.4.4 AFLP selective amplification

AFLP selective amplification was carried out using AFLP primer combinations with three selective nucleotides at the *Eco*RI end (*Eco*RI +3) and *Mse*I end (*Mse*I +3), respectively. These primer combinations characterized by six selective nucleotides produced scoreable bands and were used in this study. The gels were stained with silver nitrate and represented DNA fingerprinting of rapeseed cultivars (shown in Figure 3.4). Bands labeled with arrow (Figure 3.4) were ignored in the band calling process because of band compression, weakness and distortion.

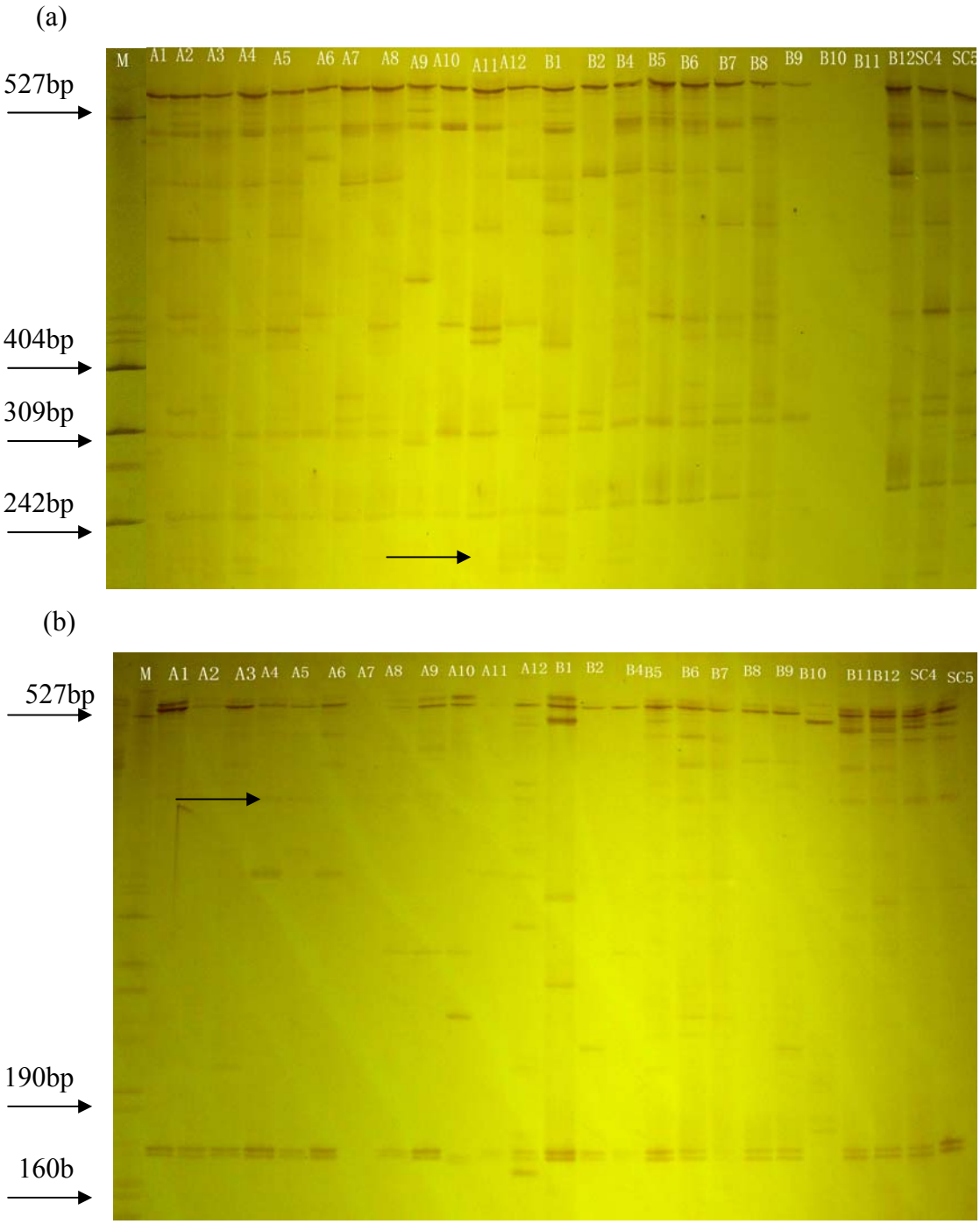


Figure 3.4 Amplification products of 25 *B. napus* cultivars using AFLP primer combinations. (a) E9/M33; (b) E16/M38. Lane M: DNA ladder pBR322 DNA-Msp Ⅰ Digest. Lanes A1-A12, B1-B12, SC4, and SC5 represented the amplification results of 25 *B. napus* cultivars shown in Table 3.1.

3.4.5 Primer selection and AFLP polymorphism analysis

Nine of the twelve primer combinations gave clear and reproducible amplification patterns. Among the three discarded primer combinations, two primer combinations amplified too many bands for accurate scoring; although it could be argued that these primer combinations are highly informative, the difference in electrophoretic mobility between bands was very small and increased the risk of misalignment. Another combination was not very informative as they produced smear bands.

The AFLP technique is effective, economical and combines the reliability of restriction fragment length polymorphism (RFLP) and the power of PCR. It generally produces polymorphism several folds higher than RFLP or any other PCR based marker system. Nine primer combinations were selected from 12 pairs of *Eco*RI / *Mse*I primers based on their robust amplification patterns and specific polymorphism. All of the primers could generate a significant number of polymorphisms within the samples surveyed. The number of polymorphic bands detected by single primer combination for each cultivar is reported in Table 3.4. A total of 193 bands were generated ranging in size from 76 to 635 bp, of which 73 unambiguous were polymorphic (Table 3.3). Each individual tested had a unique AFLP band patterns (except for Huayouza 6 and Huayouza 9 had very similar fingerprint), suggesting the high efficiency of cultivar identification using AFLP markers. Further, it also showed genetic variability existed among rapeseed cultivars. The primer combinations differed in their ability to detect polymorphism within populations. A maximum of 14 polymorphic bands was amplified with E9/M33 (E+CCC/M+TCG) primer combination, and a minimum of 5 polymorphic bands was

produced with E10/M39 (E+CCA/M+ATG) primer combination. An average of 8.1 polymorphic bands was generated for each primer combination. A typical AFLP image was illustrated in Figure 3.4 and showed that most cultivars (except for Huayouz 6 and Huayouza 9) could be distinguished by a specific set of AFLP bands. Huayouza 6 and Huayouza 9 had remarkably similar AFLP bands. The percentage of polymorphic bands were relatively lower than observed in other *Brasssica* studies (Genet et al., 2005; Zhao et al., 2005; Wang et al., 2008), but higher than some reports (Èron et al., 2006; Cheng et al., 2007; Yu et al., 2007). This discrepancy might be related to the genotypes used and the selective of AFLP primer with scorable bands. Therefore, a relative high level of polymorphism (38%) in this study indicated the high discriminatory power of AFLP markers in rapeseed population investigated. The discriminatory power of AFLP was, however, dependent on the primer combination used. Lombard et al. (2000) found that only two combinations of AFLP primers from a total number of 17 tested ones could distinguish 83 oil seed rape cultivars. Seyis et al. (2003) investigated a total of 467 polymorphic AFLP bands using only three AFLP primer combinations to estimate genetic diversity and relationships among 165 resynthesized rapeseed lines. Roman et al. (2004) reported that AFLP markers detected a very higher level of polymorphism among oilseed rape cultivar than RAPD and SSR markers. These data confirmed that AFLP is capable of detecting substantial numbers of polymorphic loci with a relatively small number of primer pairs and useful for assessment of genetic structures of rapeseed in selective breeding programs.

Table 3.3 Analysis of the level of polymorphism with nine AFLP selective primers among 25 *B. napus* cultivars.

Primer	Primer combination	Total bands	Polymorphic bands	Polymorphic rate
E9/M33	E00+CCC/M00+TCG	20	14	70%
E10/M39	E00+CCA/M00+ATG	20	5	25%
E12/M37	E00+CCT/M00+CTA	24	8	33%
E12/M39	E00+CCT/M00+ATG	15	6	40%
E13/M37	E00+CGG/M00+CTA	24	8	33%
E13/M39	E00+CGG/M00+ATG	20	6	30%
E15/M36	E00+CGT/M00+AGC	28	11	39%
E16/M37	E00+CGC/M00+CTA	20	7	35%
E16/M38	E00+CGC/M00+GTC	22	8	36%
Total		193	73	38%
Average		21.4	8.1	38%

Polymorphic rate = (number of polymorphic bands/number of total bands) × 100%

3.4.6 Genetic similarity analysis

Genetic similarity coefficient between pairs of varieties based on Dice similarity coefficients revealed a significant level of diversity within the cultivars evaluated (Table 3.4). The average genetic similarity coefficient (S_{ij}) among all 25 cultivars evaluated was 0.73 with a ranged of 0.50 to 0.96, which indicated that a considerable genetic diversity existed in the cultivars and their genetic relationships were complex. The highest S_{ij} (0.96) between Huayouza 6 and Huayouza 9 showed less genetic variation and close genetic relationship, while the lowest S_{ij} (0.50) was observed between You06-1 and Jinyou068, suggesting a remarkable genetic variation.

It is noted that Shenyou6970 shared the same S_{ij} (0.56) with NR168 and Mianza04-62, which indicated that their genetic variation was considerable. The S_{ij} (0.96) between Huayouza 6 and Huayouza 9 was the highest, and their genetic similarity was the greatest. The higher S_{ij} (0.91) between You06-3 and Huayouza 6, NR061 and GuiBF2-3 proved that their close genetic relationship. This was contradiction with their considerable seed quality variation. The cluster results indicated that the molecular classification was not completely consistent with the seed quality classification.

Table 3.4 Genetic similarity coefficient of *B. napus* cultivars from the Dice similarity coefficient.

Rows\Cols	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	B1	B2	B4	B5	B6	B7	B8	B9	B10	B11	B12	SC4	SC5
A1	1.00																								
A2	0.78	1.00																							
A3	0.74	0.84	1.00																						
A4	0.74	0.84	0.75	1.00																					
A5	0.78	0.85	0.81	0.81	1.00																				
A6	0.71	0.81	0.75	0.80	0.81	1.00																			
A7	0.78	0.80	0.79	0.81	0.85	0.86	1.00																		
A8	0.73	0.75	0.76	0.69	0.83	0.71	0.75	1.00																	
A9	0.71	0.66	0.65	0.68	0.69	0.68	0.71	0.69	1.00																
A10	0.69	0.66	0.65	0.75	0.71	0.70	0.71	0.64	0.68	1.00															
A11	0.70	0.82	0.78	0.86	0.80	0.78	0.84	0.67	0.68	0.71	1.00														
A12	0.74	0.76	0.75	0.78	0.81	0.73	0.76	0.74	0.68	0.65	0.76	1.00													
B1	0.76	0.79	0.70	0.75	0.76	0.70	0.74	0.66	0.63	0.58	0.73	0.78	1.00												
B2	0.79	0.79	0.78	0.75	0.81	0.78	0.81	0.69	0.70	0.63	0.73	0.78	0.75	1.00											
B4	0.85	0.77	0.78	0.76	0.87	0.78	0.86	0.77	0.76	0.67	0.76	0.78	0.76	0.91	1.00										
B5	0.81	0.81	0.78	0.78	0.86	0.83	0.84	0.81	0.65	0.65	0.76	0.80	0.75	0.80	0.84	1.00									
B6	0.73	0.78	0.71	0.74	0.80	0.79	0.78	0.75	0.59	0.61	0.72	0.74	0.71	0.79	0.80	0.89	1.00								
B7	0.69	0.79	0.70	0.70	0.81	0.75	0.76	0.71	0.60	0.63	0.71	0.70	0.75	0.70	0.75	0.80	0.81	1.00							
B8	0.63	0.73	0.69	0.74	0.73	0.69	0.70	0.58	0.61	0.69	0.77	0.66	0.61	0.66	0.70	0.66	0.68	0.69	1.00						
B9	0.61	0.64	0.70	0.70	0.69	0.63	0.64	0.59	0.58	0.60	0.71	0.70	0.58	0.70	0.68	0.60	0.59	0.50	0.69	1.00					
B10	0.64	0.71	0.73	0.62	0.67	0.61	0.64	0.64	0.59	0.59	0.66	0.67	0.59	0.62	0.62	0.62	0.56	0.59	0.56	0.65	1.00				
B11	0.71	0.71	0.70	0.68	0.69	0.73	0.74	0.64	0.68	0.65	0.71	0.75	0.65	0.75	0.73	0.68	0.66	0.60	0.69	0.65	0.71	1.00			
B12	0.73	0.83	0.74	0.76	0.83	0.79	0.75	0.73	0.61	0.69	0.72	0.76	0.71	0.74	0.75	0.81	0.83	0.81	0.73	0.64	0.65	0.71	1.00		
SC4	0.79	0.84	0.75	0.78	0.84	0.80	0.79	0.79	0.68	0.65	0.76	0.75	0.73	0.75	0.81	0.88	0.81	0.80	0.66	0.60	0.62	0.70	0.91	1.00	
SC5	0.75	0.80	0.74	0.76	0.80	0.76	0.78	0.75	0.66	0.61	0.75	0.74	0.71	0.71	0.77	0.84	0.80	0.79	0.68	0.61	0.61	0.69	0.88	0.96	1.00

3.4.7 Genetic diversity analysis

The observed number of alleles (NA), number of effective alleles (NE), gene diversity, Shannon's information index (I), genetic differentiation (G_{ST}), calculated for each AFLP loci were shown in Table 3.5. Across 25 *B. napus* cultivars, 9 AFLP primer combinations produced a total of 16 alleles in nine loci. The effective allele number is another index for estimating the genetic variations within a population, which reflects the interaction between the alleles per loci. Typically, the observed allele numbers were larger than the effective allele numbers. This result was consistent with the same findings. In this study, the average NA was 3.56, while the mean NE was 1.41. There was no significant difference between them. Gene diversity index (H, expected heterozygosity) varied from 0.07 to 0.50 with an average of 0.25, indicating a high genetic variability. High heterozygosity means lots of genetic variability. Low heterozygosity means little genetic variability. Shannon's information index (I) ranged from 0.38 to 0.93 with an average of 0.62 across all loci supported the existence of a considerate level of genetic variation in 25 rapeseed cultivars. Estimation of genetic variation in the rapeseed might be consistent with the fact that it is a self pollinated plant. We are aware that the relatively small number of 73 fragments may result in biases in the estimation of genetic diversity (Szmidt et al., 1996; Isabel et al., 1999). E16/M37 had the highest level of diversity with the values of NE = 1.99, H = 0.50, I = 0.82. E12/M39 exhibited the lowest diversity values of NE = 1.08, H = 0.07 and I = 0.37. Consequently, the E16/M37 combination seems to be the most informative either to discriminate among cultivars or to survey the genetic diversity. Likewise, the genetic differentiation among populations (G_{ST}) was 0.39 as estimated by partitioning of the total gene diversity, indicating that high level of

genetic variability existed among populations, although negative value of -8.22 was observed in locus E10/M39. A high level of population differentiation may be explained by several factors, including the species breeding system, genetic drift or genetic isolation of populations (Hogbin and Peakall, 1999; Cao et al., 2006). All markers showed very high G_{ST} value, indicating their suitability to differentiate among populations. The extent of variation in rapeseed might be explained by the heterozygosity existing in the natural populations and the method used in the rapeseed selection program (Seyis et al., 2003). The genetic diversity revealed at AFLPs is attributed to the differences in distribution patterns of markers in individuals. Therefore, it was believed that genetic diversity of a crop depend on the life history traits of species, such as such as breeding systems, seed dispersal mechanisms, geographic ranges and life forms, but affected by environmental factors (Loveless and Hamrick,1984; Hamrick and Godt, 1996).

Table 3.5 Characteristics of AFLP markers used in studying the genetic diversity of *B. napus* cultivars.

Locus	NA	NE	H	I	G_{ST}
E9/M33	4	1.26	0.21	0.62	1.38
E10/M39	3	1.08	0.07	0.38	-8.22
E12/M37	4	1.27	0.21	0.62	1.38
E12/M39	3	1.08	0.07	0.37	2.14
E13/M37	4	1.27	0.21	0.62	1.38
E13/M39	4	1.08	0.07	0.43	2.09
E15/M36	4	1.87	0.47	0.93	1.19
E16/M37	3	1.99	0.50	0.82	1.08
E16/M38	3	1.79	0.44	0.77	1.10
Mean	3.56	1.41	0.25	0.62	0.39

NA = Observed number of alleles

NE = Effective number of alleles (Kimura and Crow, 1964)

H = Gene diversity (Nei, 1973)

I = Shannon's Information index (Lewontin, 1972)

G_{ST} = genetic differentiation

3.4.8 Genetic relationships among *B. napus* cultivars

A UPGMA dendrogram constructed on the basis of Dice similarity coefficient was shown in Figure 3.5. No apparent clustering by seed quality characteristics (erucic acid, glucosinolate content, oil content, and protein), or original location was observed within these sub-groups. It is not surprising, considering that the limited number of characters used for variety discrimination is encoded by a limited number of genes, which can originate new phenotypes as a

consequence of simple mutation events or non-heritable changes: i.e. transposons or epigenetic effects (Portis et al., 2004). Meanwhile, it demonstrated that plant breeders often implement material from international sources in their breeding program to maximize genetic diversity (Seyis et al., 2003). All the genotypes could be distinguished except for the varieties Huayouza 6 and Huayouza 9. These two cultivars were most similar in DNA level with 71 same out of 73 bands, which indicated close genetic background and minimal differentiation with each other. The close genetic relationship of these two cultivars hinted that they might share a common ancestor, although there is no record regarding their parental trees. Therefore, close -bred varieties which share a high proportion of gene variants will be more difficult to discriminate than those based on genetically divergent parents (Forster et al., 2001). At a similarity coefficient of 0.66, all cultivars were divided into three groups. Group Ⅰ contained twenty two varieties. It was found that cultivar from same breeding institute was clustered together, suggesting closer genetic relationship. For example, Qianza 2501 and Qianza 222, You 05-2 and H0802. It may be concluded that the cultivars from the same institute shared the same or similar parentages with seeding selection or vegetative propagation. However, some cultivars from different institute were also closer, such as B-52 and Jingyou 8, IF5-9 and NR168, H2139 and You 3115. Normally, if the cultivars reflect the two germplasm introductions, these cultivars would not be expected to be so closely related. Therefore, it also may be concluded that from the above results that many of rapeseed germplasm used in the study were based on a small gene pool although randomly sampled from different institute. Therefore, it demonstrated that *B. napus* has a relatively narrow genetic background. However, we found that a high degree

of genetic variation among *B. napus* cultivars was still detected. The extent of variation in *B. napus* might be explained by the heterozygosity existing in the natural populations and the method used in the *B. napus* selection program (Hamilton and Fukunaga, 1959). Maximum variability for selection in segregating populations may be achieved by utilizing genotypes from different clusters as parents for crosses. Youyan 10, You 06-1, Qianza 6-18 and You 9559 were separated alone, suggesting distinctiveness from other cultivars. The genotypes from different breeding institutes were obviously separated, which suggested significant genetic divergence between these cultivars. It was noted that some cultivars from same institute show divergence (Qianza 6-18 and ZWH-1). All the cultivars from the same origin resource did not completely belong to one cluster, which suggested that the cultivars were not completely clustered by their origin, an extensive gene exchange may exist among all the cultivars. The possible explanation was that accessions from the same geographical origin shared the same or similar parentages with seeding selection or vegetative propagation, and more genetic exchange existed within populations of the same institute due to cross pollination. Similar result was reported early on other species (Zhou et al., 2007). Group Ⅱ consisted of Qianza J5005 and Shenyou 6970 cultivars were close to each other, indicating a highly close genetic relationship. In group Ⅲ only Jinyou 068 formed one group, which indicated this cultivar are distinct from other cultivar and had special genetic background. Divergent genotypes may have good breeding values. Therefore, Jinyou 068 could be highlighted peculiar materials in breeding programs.

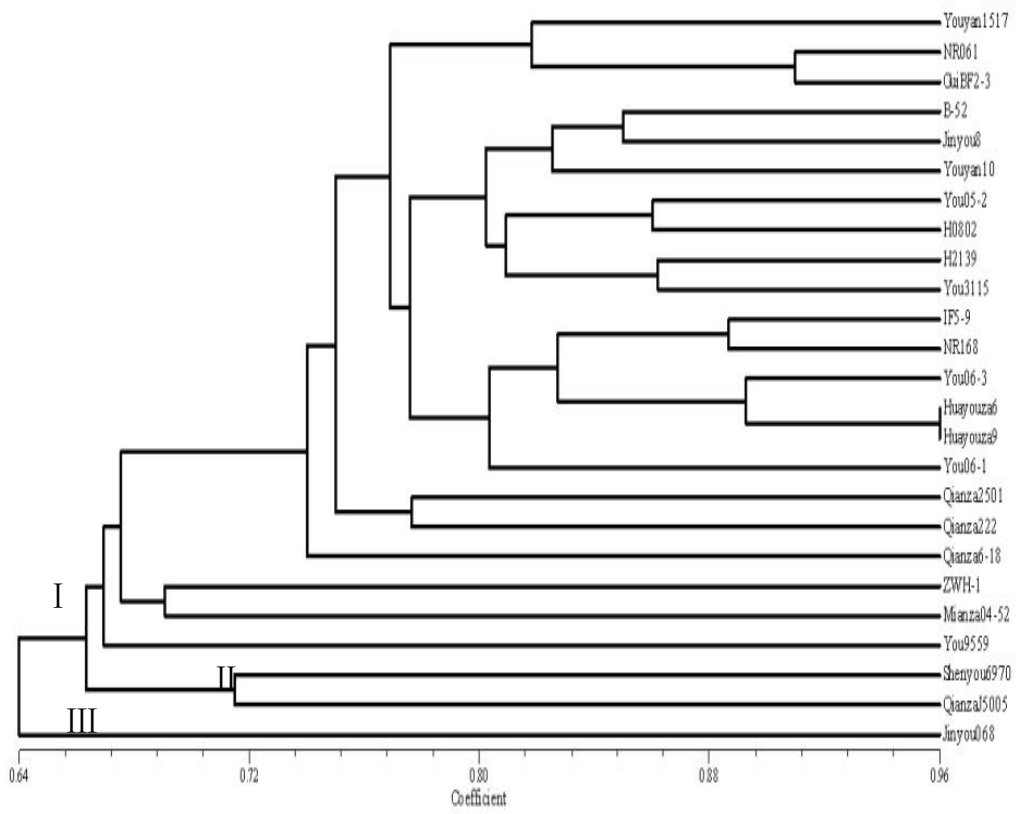


Figure 3.5 A dendrogram generated from UPGMA clustering analysis among 25 *B. napus* cultivars attending regional tests on the basis of Dice similarity coefficient.

3.5 Conclusion

The AFLP process comprises five consecutive steps (DNA extraction, restriction/ligation, pre-amplification, amplification and electrophoresis) and only a success of all these steps will lead to good electropherograms. Compared with other molecular markers, like microsatellites or RAPD that just consist in a single PCR, AFLP markers are more sophisticated. The implication of enzymes (restriction and ligation enzymes) makes the method particularly sensitive and sometimes problematic. Due to highly reproducible banding patterns and large number of polymorphic fragments revealed by AFLP markers, it is widely applied for cultivar

identification and determination of genetic relationships among cultivars (Gao et al., 2006). Fingerprints of *B. napus* are useful for seed producers, growers and users to check the identity and purity of seed rapeseed, and protect the new released from breeding programs. In the study, AFLP markers provided useful information for DNA fingerprinting of *B. napus* germplasm, which showed a relative high level of polymorphism and further revealed high level of heterozygosity among *B. napus* cultivars. However, this study also showed that the AFLP technique could not perfectly discriminate *B. napus* varieties based on their origin or seed quality traits. The genetic cluster analysis showed that cultivars bred by the same breeder have the highest level of genetic similarity, because of their similar parents. But some cultivar provided from different institute were also close together, indicating their close genetic relationship, due to their similar parents or breeding program. Moreover, these results from this study show that Guizhou has rich *B. napus* germplasm by seedling propagation and natural and human selection, indicated that *B. napus* has more extensive application prospect in rapeseed breeding. In order to understand of the presence of genetic variability in *Brassica* germplasm and utilize the existing variability for improvement of *Brassica* crop in Guizhou, more biochemical and molecular data should be required. This study of *B. napus* was analyzed with AFLP markers will aid not only the characterization and management of germplasm, but will also be useful to improve the current breeding strategies.

3.6 References

Cao, C. P., Finkeldey, R., Siregar, I., Siregar, U. and Gailing, O. (2006). Genetic diversity within and among populations of *Shorea leprosula* Miq. and *Shorea parvifolia* Dyer (Dipterocarpaceae) in Indonesia detected by AFLPs. **Tree Genet Genome**. 2: 225–239.

Chen, D. H. and Ronald, P. C. (1999). A rapid DNA minipreparation method suitable for AFLP and other PCR application. **Plant Molecular Biology Reporter**. 17: 53-57.

Chen, X. P., Yan, L., Ding, Y. and Gao, W. X. (2000a). Analysis of germplasms of Hubei barley by randomly amplified polymorphic DNA (RAPD). **Journal of Wuhan University.** 46: 249-252.

Cheng, B. Y, Zhang, D. X., Wu, X. M., Liu, F. L., Lu, G. Y. and Gao, G. Z. (2007). AFLP analysis of 89 cultivars attending the national regional varietal trial. **Chinese journal of oil crop sciences**. 29(2): 9 -14.

Chyi, S., Hoencke, M. E. and Sernyk, J. L. (1992). A genetic linkage map of restriction fragment length polymorphism loci for *Brassica rapa* (syn. *Campestries*). **Genome**. 35: 746 - 757.

Das, S., Rajagopal, J., Bhatia, S., Srivastava, P. S. and Lakshmikumaran, M. (1999). Assessment of genetic variation within *Brassica campestris* cultivars using amplified fragment length polymorphism and random amplification of polymorphic DNA markers. **Journal of Biosciences**. 24: 433 - 440.

Èron, V., Oneesa, J., Áerota, L. and Escobar, R. (2006). Identification of oilseed rape cultivars using fluorescence based AFLP markers. **Indian J. Crop Science.** 1(1-2): 65-69.

Forster, J. W., Jones, E. S., Kölliker, R., Drayton, M. C., Dupal, M. P., Guthridge, K. M. and Smith, K. F. (2001). **DNA profiling in outbreeding forage species**. In: R. Henry (ed.), Plant Genotyping – The DNA Fingerprinting of Plants. CABI Press, in press.

Gao, J. M., Zhang, S. G., Qi, L. W., Zhang, Y., Wang, C. G. and Song, W. Q. (2006). ISSR and AFLP identification and genetic relationships of Chinese elite accessions from the genus *Populus*. **Annals of Forest Science**. 63(5):48–55.

Gao, D., Du, F. and Zhu, Y. Y. (2009). Low-background and high-resolution contracted silver-stained method in polyacrylamide gels electrophoresis. **Hereditas** (Beijing). 31(6): 668-673.

Genet, T., Viljoen, C. D. and Labuschagne, M. T. (2005). Genetic analysis of Ethiopian mustard genotypes using amplified fragment length polymorphism (AFLP). **African Journal of Biotechnology**. 4 (9): 891-897.

Guo, W. W, Cheng, Y. J and Deng, X. X. (2002) Regeneration and molecular characterization of intergeneric somatic hybrids between Citrus reticulata and Poncirus trifoliata. **Plant Cell Reports**. 20: 829 – 834.

Hamilton, R. A. and Fukunaga, E. T. (1959). Growing macadamia nuts in Hawaii. **University of Hawaii, Agricultural Experiment Station Bulletin**. 121: 5–15.

Hamrick, J. L. and Godt, M. J. W. (1996). **Effects of life history traits on genetic diversity in plant species**. Philosophical Transactions: Biological Sciences. 351: 1291–1298.

Hartl, D. L. and Clark, A. G. (1989). **Principles of population genetics**, 2nd edition**.** Sunderland (MA): Sinauer Associates.

Hogbin, P. M. and Peakall, R. (1999). Evaluation of the conservation of genetic research to the management of endangered plant *Zieria prostrata*. **Conservation Biology**. 13: 514- 522.

Hu, J. and Quiros, C. F. (1991). Identification of broccoli and cauliflower cultivars with RAPD markers. **Plant Cell Reports**. 10: 505–511.

Isabel, N., Beaulieu, J., Theriault, P. and Bousquet, J. (1999). Direct evidence for biased gene diversity estimates from dominant random amplified polymorphic DNA (RAPD) fingerprints. **Molecular Ecology**. 8: 477-483.

Jones, C. J., Edwards, K. J., Castaglione, S., Winfield, M. O., Sala, F., Bredemeijer, C, F. S., Vosman, G., Matthes, B., Daly, M., Brettschneider, A., Bettini, R., Buiatti, P., Maestri, M., Malcevschi, E., Marmiroli, A., Aert, N., Volckaert, R., Rueda, G., Linacero, J., Vazquez, A. R. and Karp, A. (1997). Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. **Molecular Breeding**. 3:381–390.

Karp, A., Edwards, K. J., Bruford, M., Funk, S., Vosman, B., Morgante, M., Seberg, O., Kremer, A., Boursot, P., Arctander, P., Tautz, D. and Hewitt, G. M. (1997). Molecular technologies for biodiversity evaluation: opportunities and challenges. **Nature Biotech**. 15:625–628.

Kimura, M. and Crow, J. F. (1964). The number of alleles that can be maintained in a finite population. **Genetics**. 49:725-38.

Kresovich, S., Szewc-McFadden, A. K., Bliek, S. M. and McFerson, J. R. (1995). Abundance and characterization of simple sequence repeats (SSRs) isolated from a size-fractionated genomic library of *Brassica napus* (L.) (Rapeseed). **Theoretical and Applied Genetics**. 91:206–211.

Lewontin, R. C. (1972). **The apportionment of human diversity**. In: Dobzhansky, T.H., Hecht, M. K., Steere, W. C. (eds.) Evolutinary biology. Vol(6). Appleton-Cenrury-Crofts, New York, pp 381–398.

Liu, Y. G., Chen, S. L., Li, B. F., Wang, Z. J. and Liu, Z. J. (2005). Analysis of genetic variation in selected stocks of hatchery flounder, *Paralichthys olivaceus*, using AFLP markers. **Biochemical Systematics and Ecology**. 33: 993-1005.

Lombard, V., Baril, C. P., Dubreuil, P., Blouet, F. and Zhang, D. (2000). Genetic relationships and fingerprinting of rapeseed cultivars by AFLP: Consequences for varietal registration. **Crop Science**. 40: 1417-1425.

Loveless, M. D. and Hamrick, J. L. (1984). Ecological determinants of genetic structure in plant populations. **Annual Review of Ecology and Systematics**. 15: 65–95.

Ma, C. Z., Fu, T. D., Tuevesson, S. and Gertsson, Bo. (2003). Genetic diversity of Chinese and Swedish rapeseed (*Brassica napus* L.) analysed by Inter-simple Sequence Repeats (ISSRs). **Scientia Agricultura Sinica**. 36(11): 1403-1408.

McGrath, J. M. and Quiros, C. F. (1992). Genetic diversity at isozyme and RFLP loci in *Brassica campestris* as related to crop type and geographical origin. **Theoretical and Applied Genetics**. 83: 783–790.

McGregor, C. E., Lambert, C. A., Greyling, M. M., Louw, J. H. and Warnich, L. (2000). A comparative assessment of DNA fingerprinting techniques (RAPD, ISSR, AFLP and SSR) in tetraploid potato (*Solanum tuberosum* L.) germplasm. **Euphytica**. 113: 135–144.

Nei, M. (1973). Analysis of gene diversity in subdivided populations. **Proc. Nat. Aca. Sci**. USA. 70:3321–3323.

Nei, M. and Li, W. H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. **Proc. Natl. Acad. Sci**. USA. 76: 5269-5273.

Portis, E., Comino, C., Lenzi, A., Lombardi, P., Tesi, R. and Lanteri, S. (2004). Amplified fragment length polymorphism for variety identification and genetic diversity assessment in oleander *(Nerium oleander* L.). **Euphytica**. 136:125-137.

Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S. and Rafalski, A. (1996). The comparison of RFLP, RAPD, AFLP, and SSR (microsatellite) markers for germplasm analysis. **Molecular Breeding**. 2:225–238.

Qi, X. and Lindhout, P. (1997). Development of AFLP markers in barley. **Molecular and General Genetics**. 254 : 330-336.

Rohlf, F. J. (2000). **NTSYS-pc: numerical taxonomy and multivariate analysis system, version 2.1**. Exeter Software, New York.

Roman, S., Lenka, D., Vladislav, C. and Jaroslava, O. (2004). Fluorescence-based AFLPs occure as the most suitable marker system for oilseed rape cultivar identification. **Journal of Applied Genetics**. 45(2):161-173.

Seyis, F., Snowdon, R. J., Lu¨hs, W. and Friedt, W. (2003). Molecular characterisation of novel resynthesised rapeseed (*B. napus* L.) lines and analysis of their genetic diversity in comparison to spring rapeseed cultivars. **Plant Breeding**. 122: 473- 478.

Sneath, R. R. and Sokal, R. R. (1973). **Numerical Taxonomy**. In: Freeman, W.H. Freeman and Company, California. Francisco.

Song, K. M., Suzuki, J.Y., Slocum, M. K., Williams, P. H. and Osborn, T. C. (1991). A linkage map of *Brassica rapa (syn. campestris)* based on restriction fragment length polymorphisms. **Theoretical and Applied Genetics**. 82: 296—304.

Steiger, D. L., Steiger, C., Nagai, P. H., Moore, C. W., Morden, R. V. O. and Ming, R. (2002). AFLP analysis of genetic diversity within and among *Coffea arabica* cultivars. **Theoretical and Applied Genetics**.105: 209–215.

Szmidt, A. E. and Wang, X. R. (1993). Molecular systematics and genetic differentiation of *Pinus sylvestris* (L.) and *P. densiflora* (Sieb. et Zucc.). **Theoretical and Applied Genetics**. 86: 159–165.

Uzunova, M. I. and Ecke, W. (1999). Abundance polymorphism and genetic mapping of microsatellites in oilseed rape (*Brassica napus* L.). **Plant Breed**. 118: 323-326.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Van de Lee T, Hornes, M., Frijters, A., Pot, J., Kuiper, M. and Zabeau, M. (1995). AFLP: a new technique for DNA fingerprinting. **Nucleic Acids Research**. 23: 4407–4414.

Wang, J. L., Dan, B., Cheng, H. Y., Fang, H. L., Chang, T. J., Li, P. and Luan, Y. F. (2008). AFLP analysis on genetic diversity of wild and cultivated rapeseed (*B. campetris* and *B. juncea*) in Tibet. **Chinese journal of oil crop sciences**. 30(1): 10-16.

Yeh, F. C., Yang, R. C. and Boyle, T. (1999). **Popgene version 1.31**. Microsoft window-based freeware for population genetic analysis. Department of Renewable Resources, University of Alberta, Edmonton, Alberta, Canada. Available free at http://www.ualberta.ca/～fyeh/.

Yu, C. Y., Leišová, L., Kučera, V., VyvadiLová, M., Ovesná, J., DotLačil, L. and Hu, S. W. (2007). Assessment of genetic diversity of yellow-seeded rapeseed (*Brassica napus* L.) accessions by AFLP markers. **Czech Journal of Genetics and Plant Breeding**. 43(3): 105-112.

Zhao, J. J, Wang, X. W., Deng, B., Lou, P., Wu, J., Sun, R. F., Xu, Z. Y., Vromans, J., Koornneef, M. and Bonnema, G. (2005). Genetic relationships within *Brassica rapa* as inferred from AFLP fingerprints. **Theoretical and Applied Genetics**. 110: 1301–1314.

Zhou, M. Q., Zhao, K. G. and Chen, L. Q. (2007). Genetic diversity of Calycanthaceae accessions estimated using AFLP markers. **Scientia Horticulturae**. 112(3): 331-338.

CHAPTER IV

ANALYSIS OF GENETIC DIVERSITY AND RELATIONSHIPS AMONG 25 RAPESEED (*BRASSICA NAPUS*) CULTIVARS USING SSR MARKERS

4.1 Abstract

Eleven simple sequence repeat (SSR) markers were used for genetic relationship of 25 rapeseed (*B. napus*) cultivars. Total 134 bands were generated with 11 primers, of which 54 bands were polymorphic. The polymorphic rate is 40%. Twenty two alleles were detected with an average of 2.55 alleles per locus and an average of 2.01 effective alleles per locus. High observed heterozygosity showed outcrossing within the populations investigated. Mean fixation index (Fst) of 0.54 indicates a high level of genetic differentiation. Based on molecular data, the value of similarity coefficient among all rapeseed cultivars were calculated in a range of 0.43 to 0.95, with an average of 0.69. UPGMA cluster analysis showed that the rapeseed cultivars could be divided into three groups at a similarity coefficient of 0.63. Only two varieties Huayouza 6 and Huayouza 9 could not be distinguished. The genetic relationships among rapeseed cultivars was not closely related to their original location, which showed they possibly derived from similar parent and breeding program. Moreover, some cultivars bred by the same institute had very high level of genetic similarity. The practical implications of this study would provide a science basis for varieties assessment and utilization.

Keyword: genetic diversity; rapeseed; SSR analysis

4.2 Introduction

Rapeseed is a major crop in Guizhou, People's Republic of China. In 2005, rapeseed cultivation area in Guizhou was about 7 million acre, of which *B. napus*, *B. juncea* and *B. campestris* make up 75%, 10%, 15%, respectively. Recently, a large number of *B. napus* varieties were identified through regional test and widely grown. New *B. napus* cultivars are difficult to differentiate mostly because of very narrow genetic background of their parents (Zhou, 2001). Traditional morphological, phenological, and isozyme analysis reveal only limited polymorphisms among closely related genotypes and are influenced by environmental conditions. Therefore, an effective technique that estimate the level of genetic diversity among *B. napus* cultivars should be explored for genetic characterization. In recent years, molecular genetic techniques using DNA polymorphism have been increasingly used to characterize and identify novel germplasm for use in crop breeding (O'Neill et al., 2003). Several molecular marker systems have been considered useful tools for plant breeding and genetic analysis (Gupta and Varshney 2000; O'Neill et al., 2003). SSR is characterized as co-dominant, abundant, and highly polymorphic markers in genomes, which make it prior to most of other molecular markers (Hasan et al., 2006), and have been used in *Brassica* research for such topics as seed-coat color mapping (Padmaja et al., 2005), varietal identification (Tonguc and Griffiths, 2004), analysis of variation in plant populations and germplasm collections(Raybould et al., 1999; Westman and Kresovich,1995) and levels of heterozygosity in individual genotypes (Jones et al., 2003). In this study, we estimated the genetic diversity and relationship among 25 *B. napus* cultivars from different institute for the following objectives: (i) evaluate the

discrimination power of SSR markers to identify rapeseed cultivars; (ii) analyze genetic diversity and relationships among cultivars by calculating genetic parameters.

4.3 Materials and methods

4.3.1. Plant material

The name and origin of 25 *B. napus* cultivars were shown in CHAPTER III Table 3.1.

4.3.2 Chemical

All chemicals were Laboratory grade or specified.

(1) Reagent for PCR amplification (Tiangen, Beijing Co., Ltd.)

Taq DNA polymerase in storage buffer

10X PCR buffer (containing 25 mM $MgCl_2$)

10 mM dNTPs

10 μM Primer

(2) Reagent for agarose gel electrophoresis (Tiangen, Beijing Co., Ltd)

DNA marker: 1 kb DNA Ladder, 100 bp DNA Ladder

Staining Solution: 2.5 μg/mL ethidium bromide

6× DNA electrophoretic loading buffer: 0.25% bromophenol blue in 40% glycerol, and demonized water

(3) Reagent for Non-PAGE

5X TBE buffer (1 L)

Tris-base	54 g
Boric acid	27.5 g
0.5M EDTA (pH 8.0)	20 mL

30% acrylamide gel (100mL):

Acrylamide	29 g
Bis-acrylamide	1.0 g
Deionized water	100 mL

10% non-denaturing PAGE gel (75mL):

30% acrylamide gel	24.5 mL
5 X TBE buffer	15 mL
Deionized water	34.5 mL
10% AP	375 μL
TEMED	37.5 μL

4.3.3 Method

4.3.3.1 DNA extraction

The procedure has been described in CHAPTER Ⅲ 3.3.2.2.

4.3.3.2 SSR primer

Seventy eight SSR primer sets developed from *B. napus*, *B. oleracea, B. rapa,* and *B. nigra* were available for this study. All SSR primers were synthesized by Generay biotech, Shanghai, China. Microsatellites are selected from the information available in public domain (Piquemal et al., 2005) and the Cropnet website (http://ukcrop.net/perl /search/BrassicaDB). Repeats, primer sequences and in part the map position for the loci are available on the Cropnet website. The SSR primer pairs used in this study were shown in Table 4.1.

4.3.3.3 SSR PCR amplification

PCR amplification was carried out in a volume of 20 μL

containing 2.5 μL of 30 - 50 ng/μL DNA , 0.4 μL of 10 μM each primer, 0.4 μL of 10 mM dNTPs, 2.0 μL of 10×reaction buffer (containing 25 mM $MgCl_2$), 0.2 μL of 2.5 u/μL *Tag* polymerase and 14.1 μL distilled water. PCR reactions were performed in Mycycler^{TM} Thermal Cycler (USA)： an initial step of 5 min at 94ºC, 35 cycles of 45 s at 94ºC, 45 s at 53ºC and 1 min at 72ºC, and a final step of 10 min at 72ºC.

4.3.3.4 Gel electrophoresis

The PCR products were separated and checked on 10% non-denaturing polyacrylamide gel by silver staining. The gels were run with 1×TBE buffer and 110 V constant power was applied for 1 h using DYC-24 B gel electrophoresis apparatus (Beijing Liuyi Instrumental Factory, People's Republic of China), then the results were visualized by silver staining (see Part 3, APPENDIX).

4.3.3.5 Band scoring and Data analysis

Genetic similarity among 25 *B. napus* cultivars based on Dice similarity coefficient (Nei and Li, 1979) was used for dendrogram construction with the unweighted pair-group method with arithmetic averages (UPGMA) by employing the computer program NTSYS-pc v. 2.10 (Rohlf, 2000).

Basing on the estimated fragment sizes of each marker and each genotype, the genetic diversity parameters of mean number of alleles (NA), mean number of effective alleles (NE), Shannon's information index (I), observed heterozygosity (Ho), expected heterozygosity (He) and F-statistic (Wright, 1965) were calculated using the software POPOGENE version 1.31 (Yeh et al., 1999).

The F statistic is a measure of the difference between the mean heterozygosity among the subdivisions in a population, and the potential frequency of heterozygotes if all members of the population mixed freely and non-assortatively

(Hartl and Clark, 1997). The fixation index ranges from 0 (indicating no differentiation between the overall population and its subpopulations) to a theoretical maximum of 1. A zero value implies complete panmixis, that the two populations are interbreeding freely. A value of one would imply the two populations are completely separate. F-statistics were calculated under the infinite allele model:

$$1- F_{IT} = (1- F_{IS}) (1- F_{ST})$$

Whereas, F_{ST}: the Wright's fixation index, which often expressed as the proportion of genetic diversity due to allele frequency differences among populations.

F_{IS}: the within population inbreeding, which measures the correlation of allele frequencies among individuals within populations.

F_{IT}: the overall inbreeding that measures the correlation of allele frequencies within individuals in different populations (Holsinger et al., 2009).

Table 4.1 List of the microsatellite sequence, motifs, number of repeats and size range of the amplified bands approximated from molecular weight markers (http://ukcrop.net/perl /search/BrassicaDB).

Microsatellite	Sequence	Motif	Number of repeats	Size range
Na10-B07	F:GCCTTAGATTAGATGGTCGCC R:ACTTCAGCTCCGATTTGCC	CT	29	104-161
Na10-B11	F:TTTAACAACAACCGTCACGC R:CTCCTCCTCCATCAATCTGC	CT	28	126-181
Na10-H03	F:GAGCTGGCTCATTCAACTCC R:CACAATTTCTCAGACAAAACGG	GGC	6	48-65
Na12-A02	F:AGCCTTGTTGCTTTTCAACG R:AGTGAATCGATGATCTCGCC	CT	16	196-227
Na12-D09	F:ACTGAAACTTACTAAAAGAGAGA R:TCTAGAAACACCAGCAGTGGC	GA	24	18-65
Na12-E02	F:TTGAAGTAGTTGGAGTAATTGGA R:CAGCAGCCACAACCTTACG	TTG	13	59-97
Na14-H12	F:CACATTGGCACGTATCCATC R:GGCTGATCGAACACAAATAAG	AC	16	156-188
Ra3-D04	F:AAAAGGACCTACCAATTTCGTG R:CGACCCAAACTGAGCCATAC	AG	38	137-212
Ra3-H09	F:GTGGTAACGACGGTCCATTC R:ACCACGACGAAGACTCATCC	TGG	3	146-154
FITO-063	F:GTTCAGTTCCCAGATTCCTAA R:TTTCCTCTTCCTTCTCTCTTC	CCG	15	267-700
FITO-136	F:CCTCCTCCTCAGACTTACACT R:TCACATCCACCATAACCTTT	CTC	12	130-133

4.4 Result and discussion

4.4.1 SSR primer screening

The polymorphism survey using SSR primers for assaying *B.napus* cultivars was carefully analyzed (Table 4.2). Almost 86% of the published primers failed to generate a specific PCR product, consequently reliable allelic data could not be gathered. 78 SSR primers selected from information available in public domain, 11 SSR primers showed polymorphic banding pattern in rapeseed. The remaining 67 SSR primer pairs were either unspecific as shown by smear or superfluous bands (40 primer pairs) or did not amplify at all (27 primer pairs). The fact that these primers did not amplify the DNA of the taxa from the genus rapeseed is possibly due to the occurrence of mutations in the flanking regions where the primers hybridize or because this locus is absent in the specimens studied and possibly in the species analyzed (Patricia and Cristina, 2006). Products could be amplified using 11 SSR primers because the mutation is inside the microsatellite region and not where the primers hybridize. Therefore, although many microsatellite markers are available, a large number of markers were unsuitable for population analysis. Similar findings were reported as we have revealed here (Szewc-McFadden et al., 1996; Uzunova and Ecke, 1999; Bond et al., 2004).

4.4.2 Polymorphism of SSR markers

Eleven out of an original 78 SSR primer pairs were selected based on their amplification strength, polymorphism and resolution. Thus were used for the analysis of the 25 *B. napus* cultivars. The 11 selected primer pairs generated distinctive products in the range of 100 -1200 bp. A total of 134 bands were produced with an average of 12.2 major bands per primers, of which 54 were polymorphic.

Polymorphic bands per primer varied from 2 to 9 with a mean of 4.9 bands. The polymorphic rate was 40%. In general, polymorphism in amphidiploids is less than that observed in diploid species. Previous reports revealed that the level of polymorphism for *B. napus* is less than 45% (Cheung et al., 1997; Kresovich et al., 1995; Uzunova et al., 1995), whereas in *B. oleracea* it can be higher than 80% (Cheung et al., 1997), and in *B. juncea* a polymorphism of approximately 60% (Cheung et al., 1997). Lower levels of polymorphism in amphidiploids may be attributed to the lower level of out-crossing due to a weak and often non-existing self-incompatibility system (Rakow and Woods, 1987; Weerakoon et al., 2010). In this study, the polymorphism revealed by AFLP and SSR markers is lower than 45%, which supported the previous reports. However, SSR exhibited a higher level of polymorphism (40%) than AFLP (38%). This could be attributed to the different mechanisms of polymorphisms detection using different marker systems. SSR markers detect multiple alleles at a given locus while AFLP detect multiple loci distributed throughout the genome. On the other hand, it can be explained by this mechanism that replication slippage is thought to occur more frequently than nucleotide mutations and insertion/deletion events, which generate the polymorphisms detectable by AFLP (Powell et al., 1996). This result is in agreement with other studies comparing the level of polymorphism detected with AFLP and SSR markers (Cheng, 2005; Maughan et al., 1995; Salimath et al., 1995; Powell et al., 1996). The data of the study indicated that the SSR methodology detected polymorphism more efficiently than the AFLP approach. Representative banding patterns, generated using primer FITO-063 and Ra3-H09 were shown in Figure 4.1.

Table 4.2 Amplification results of eleven SSR primers.

Primer	Total bands	Polymorphic bands	Polymorphic rate
Na10-B07	12	3	25%
Na10-B11	12	5	42%
Na10-H03	8	6	75%
Na12-A02	22	7	32%
Na12-D09	13	4	31%
Na12-E02	13	5	38%
Na14-H12	7	2	29%
Ra3-D04	15	4	27%
Ra3-H09	14	9	64%
FITO-063	10	5	50%
FITO-136	8	4	50%
Total	134	54	40%
Average	12.2	4.9	40%

Polymorphic rate = (number of polymorphic bands/number of total bands) × 100%

4.4.3 DNA fingerprint of *B. napus* cultivars based on SSR markers

It was found that representative bands were available in fingerprint of each cultivar by electrophoresis analysis. The fingerprint map of 25 rapeseed varieties was constructed on the major fragments of FITO-063, Na10-H03, Na12-A02, Na12-E02, Na14-H12 and Ra3-H09 (Table 4.3). Amplification patterns obtained on the non-denaturing polyacrylamide gels were clear and easy to score (Figure 4.1). There was a marked difference between the size of the alleles for most of the markers. This can be explained by an insertion/deletion polymorphism rather than differences in the number of repeats of the microsatellite motif.

Table 4.3 SSR fingerprint of 25 *B. napus* cultivars using six SSR primers

Primer	Youyan1517	B-52	Youyan10	You 05-2	Jinyou 8	H2139	You 3115	Qianza6-18	You 9559	ZWH-1	H0802	Qianza2501
FITO-063-180bp	0	0	0	0	0	0	0	0	0	1	0	0
FITO-063-295bp	0	0	0	0	0	0	0	0	0	0	0	1
Na10-H03-200bp	0	1	1	1	0	0	0	0	0	0	0	0
Na10-H03-210bp	1	1	1	1	0	1	0	0	0	0	0	0
Na10-H03-240bp	1	1	1	0	1	1	0	1	0	1	1	1
Na12-A02-120bp	0	0	0	1	0	0	0	1	0	0	0	0
Na12-A02-180bp	1	1	1	1	1	1	1	1	1	1	1	1
Na12-A02-190bp	1	1	1	0	1	1	0	0	0	0	1	1
Na12-E02-100bp	1	1	1	1	0	1	1	1	1	1	1	1
Na12-E02-140bp	1	0	0	1	1	1	0	0	0	0	0	1
Na12-E02-210bp	1	1	1	1	1	1	1	1	1	1	1	1
Na14-H12-1100bp	1	1	1	1	1	1	1	1	1	0	1	0
Na14-H12-1250bp	1	0	0	1	0	0	0	0	0	1	0	1
Ra3-H09-100bp	1	0	1	1	1	0	1	1	1	1	1	1
Ra3-H09-120bp	0	1	0	0	0	0	0	0	1	0	0	0
Ra3-H09-130bp	0	1	0	0	0	0	0	0	0	0	0	0

0 represented the absence of band and 1 represented the presence of band

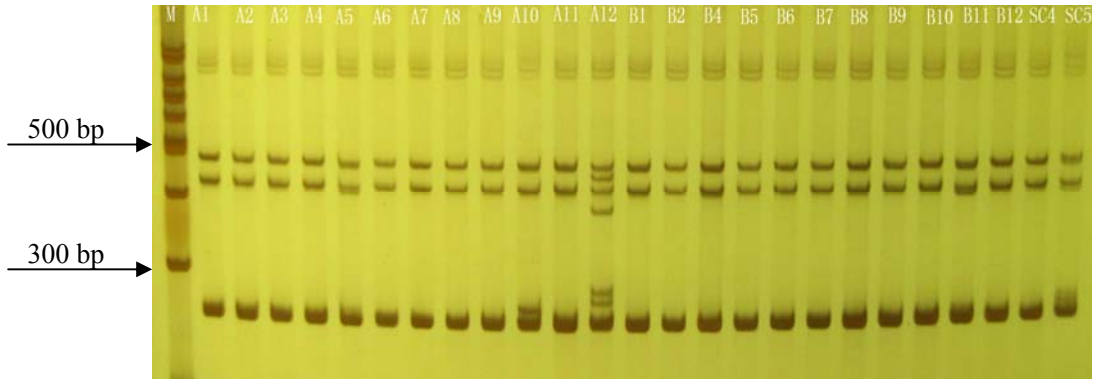
Table 4.3 (Continued).

Primer	Qianza 222	NR 061	GuiB F2-3	IF 5-9	NR 168	You 06-1	Mianza 04-52	Jinyou 068	Shenyou 6970	QianzaJ 5005	You 06-3	Huayouza6	Huayouza9
FITO-063-180bp	0	0	0	0	0	0	0	0	0	0	0	0	0
FITO-063-295bp	0	0	0	0	0	0	0	0	0	0	0	0	0
Na10-H03-200bp	1	1	0	1	0	1	0	0	0	0	0	0	0
Na10-H03-210bp	1	1	0	1	0	1	0	0	0	0	0	0	0
Na10-H03-240bp	0	1	1	1	1	1	1	1	1	0	1	0	0
Na12-A02-120bp	0	0	0	0	0	0	0	0	0	0	0	0	0
Na12-A02-180bp	1	1	0	1	1	1	1	1	1	1	1	1	1
Na12-A02-190bp	0	1	1	0	1	0	1	1	1	1	1	1	1
Na12-E02-100bp	1	1	0	1	1	1	1	1	1	1	1	1	1
Na12-E02-140bp	0	1	0	1	0	0	0	1	0	0	0	1	1
Na12-E02-210bp	1	1	0	1	1	1	1	1	1	1	1	1	1
Na14-H12-1100bp	1	1	0	1	1	1	0	1	1	1	1	1	1
Na14-H12-1250bp	0	0	1	1	0	0	1	0	0	0	0	1	1
Ra3-H09-100bp	1	0	1	1	0	0	0	1	1	1	0	1	1
Ra3-H09-120bp	0	1	0	0	0	1	1	0	0	0	0	0	0
Ra3-H09-130bp	0	0	0	0	1	0	0	0	0	0	1	0	0

0 represented the absence of band and 1 represented the presence of band

As shown in Table 4.3, six SSR primers could identify all cultivars except the cultivars Huayouza 6 and Huayouza 9. Therefore, these primers are insufficient for complete separation, distinction and identification. When a number of primers (11 primers) were combined, Huayouza 6 and Huayouza 9 still could not be separated due to their closest genetic relationship. Low number of SSR markers used in our study could be a possible reason for this result (Ren et al., 2003; Hudcovicova and Kraic, 2003; Moghaddam et al., 2009). It is likely that the primer-anchoring sequences flanking the microsatellite loci might differ sufficiently to prevent product amplification (Creste et al., 2004). In other words, this suggests that a larger number of markers probably lead to a more accurate genetic relationship among rapeseed cultivars. In fact, the majority of the tested cultivars were uniquely identified both by their AFLP fingerprints and by their multilocus SSR profiles. Huayouza 6 and Huayouza 9 sharing a highly similar fingerprint could not resolved by both techniques due to their close genetic relationship. Thus, our results showed the number of AFLP and SSR primers seemed to be insufficient for all rapeseed cultivar identification. Therefore, it is necessary to utilize a larger number of AFLP primer combinations and SSR primer pairs on a wide range of cultivars to meet the requirement of research (Reale et al., 2006). Data from more primers could successfully distinguish all the tested cultivars.

(a)



(b)

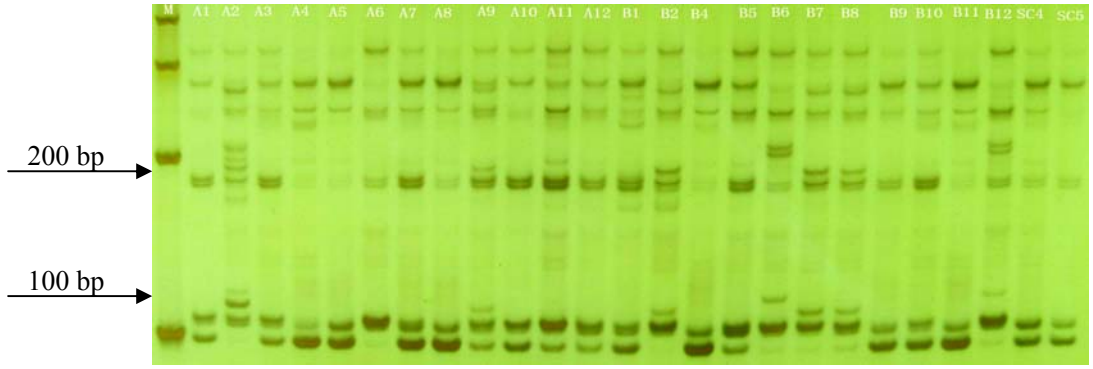


Figure 4.1 PCR amplification products of 25 *B. napus* cultivars using SSR primer. (a) FITO-063, (b) Ra3-H09 was visible in 10% non-denaturing PAGE. M: 100bp DNA ladder marker, A1,Youyan 1517; A2, B-52; A3, Youyan 10; A4, You 05-2; A5, Jinyou 8; A6, H1239; A7, You 3115; A8, Qianza 6-18; A9, You 9559; A10, ZWH-1; A11, H0802; A12, Qianza 2501; B1, Qianza 222; B2, NR061; B4, Gui BF2-3; B5, IF-9; B6, NR168; B7, You 06-1; B8, Mianza 04-52; B9, Jinyou 068; B10, Shenyou 6970; B11, Qianza J5005; B12, You 06-3; SC4, Hunyouza 6; SC5, Huayouza 9.

Table 4.4 Characteristics of SSR markers used in studying the genetic diversity of *B. napus* cultivars.

Code	Locus	NA	NE	I	Ho	He
1	FITO-063	2.00	2.00	0.69	0.33	0.50
2	FITO-136	2.00	1.99	0.69	0.49	0.50
3	Na10-B07	2.00	1.44	0.48	0.69	0.30
4	Na10-B11	2.00	1.54	0.54	0.64	0.35
5	Na10-H03	4.00	3.00	1.21	0.32	0.67
6	Na12-A02	3.00	2.64	1.02	0.37	0.62
7	Na12-D09	3.00	2.02	0.81	0.48	0.51
8	Na12-E02	2.00	1.49	0.51	0.66	0.33
9	Na14-H12	2.00	1.57	0.55	0.63	0.36
10	Ra3-D04	2.00	1.13	0.23	0.88	0.12
11	Ra3-H09	4.00	3.27	1.26	0.29	0.69
	Mean	2.55	2.01	0.73	0.53	0.45

NA = Observed number of alleles

NE = Effective number of alleles (Kimura and Crow, 1964)

I = Shannon's Information index (Lewontin, 1972)

Ho= observed heterozygosity

He= expected heterozygosity (Levene, 1949)

Table 4.5 F-statistics for the 11 microsatellite loci.

Code	Locus	F_{IS}	F_{IT}	F_{ST}
1	FITO-063	****	1.00	1.00
2	FITO-136	-1.00	-0.69	0.16
3	Na10-B07	-1.00	0.66	0.83
4	Na10-B11	-1.00	0.36	0.68
5	Na10-H03	-1.00	-0.02	0.49
6	Na12-A02	-1.00	-0.35	0.32
7	Na12-D09	-1.00	0.03	0.51
8	Na12-E02	-1.00	-0.05	0.48
9	Na14-H02	-1.00	0.56	0.78
10	Ra3-D04	-1.00	0..87	0.94
11	Ra3-H09	-1.00	-0.27	0.37
	Mean	-1.00	0.08	0.54

F_{ST} = Wright's fixation index as a measure of heterozygote deficiency or excess

4.4.4 Genetic diversity analysis

The mean NA, NE, I, Ho, and He calculated for each microsatellite were shown in Table 4.4. Across 25 rapeseed cultivars, 11 SSR primers produced a total of 22 alleles in 11 loci (average of 2.5 alleles per locus). Other researchers have reported the average number of alleles per locus as 2 (Uzunova and Ecke, 1999), 3.9 (Rudolph et al., 2000) and 4.44 (Tonguc and Griffiths, 2004). The number of alleles per locus observed (NA) in our study ranged from 2 to 4 with an average of 2.55 alleles per locus, while the average number of effective alleles was 2.01. Ra3-D04 showed lower discrimination ability, showing fewer NE and I than other loci. This result could be

explained by the ‘short allele dominance’ reported by Wattier et al. (1998) where, in heterozygotes including a short and a long allele, only the short allele is sufficiently amplified in the PCR reaction to be detected. Ho ranged from 0.29 to 0.88 (mean of 0.53), while He ranged from 0.12 to 0.69 (mean of 0.45). If the Ho is lower than He, it is attributed the discrepancy to forces such as inbreeding. If Ho is higher than He, it suspects an isolate-breaking effect (the mixing of two previously isolated populations). In this study, the He (0.45) was lower than Ho (0.53) indicating a tendency towards out-breeding within the investigated population. Actually, ***B. napus*** is a facultative outcrossing species (Hasan et al., 2006). This result agreed with this view. Additionally, Shannon’s information index (I) ranged from 0.23 to 1.26 with an average of 0.73. The locus of Ra3-D04 showed the lowest 0.23, while Ra3-H09 had the highest value (1.26). These results demonstrated that a relatively high level of genetic diversity present among the rapeseed cultivars under investigation. Moreover, the fixation index (F_{ST}) ranged from 0.16 to 1.0 with a mean of 0.54, indicating a very great genetic differentiation within the population analyzed (Table 4.5). Even though negative value of F_{IT} was noticed for five of loci (FITO-136, Na10-H03, Na12-A02, Na12-E02, and Ra3-H09), F_{IT} for most of the remaining loci was high. Negative F_{IS} values were observed in all loci. These results indicated heterozygote excess.

The SSR markers detected more alleles (22 alleles) than AFLP markers (16 alleles). Dominant markers can only identify two alleles per locus, with a detectable maximum level of heterozygosity of 0.5 (Maguire et al., 2002). Thus the mean expected heterozygosity (He) level based on AFLP (0.24) was, as expected, lower than the heterozygosity for SSRs (0.45). Shannon’ information index (I) detected by SSR (0.73) is higher than in AFLP (0.62), indicating a higher level of genetic diversity

than AFLP. It contributed to the lower genetic similarity estimates based on SSR markers (mean 0.69) as compared to AFLP markers (mean 0.73). Therefore, these results indicate the immense potential of the 11 SSR markers from the *B. napus* microsatellite kit (for genetic diversity studies *B. napus* from different geographical regions). Furthermore, using microsatellites allowed estimation based on level of heterozygosity which was not possible using dominant markers such as AFLP or RAPD.

4.4.5 Genetic similarity analysis

Genetic similarity was calculated using cluster analysis (UPGMA method). The UPGMA similarity matrixes were calculated from 54 polymorphic bands. High level of genetic diversity was observed, the similarity coefficient value ranged from 0.43 to 0.95 with a mean of 0.69 (Table 4.6). Cultivars You 05-2 and ZWH-1 showed the lowest similarity (0.43) while Huayouza 6 and Huayouza 9 were the most similar (0.95) for the 115 amplification products compared. In addition, NR168 and You 06-3 showed a higher value of genetic similarity (0.93), indicating a very close genetic relationship. Likewise, H0802 and You 06-3; NR168 and Shenyou 6970; Jinyou 8 and Jinyou 068 also shared the same similarity coefficient (0.89), suggesting closely related with each other, may due to their similar parent materials in breeding program.

Table 4.6 Genetic similarity coefficient among *B. napus* cultivars based on Dice similarity coefficient

Rows\Cols	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	B1	B2	B4	B5	B6	B7	B8	B9	B10	B11	B12	SC4	SC5
A1	1.00																								
A2	0.64	1.00																							
A3	0.80	0.70	1.00																						
A4	0.70	0.57	0.68	1.00																					
A5	0.750	0.70	0.73	0.64	1.00																				
A6	0.84	0.75	0.73	0.59	0.82	1.00																			
A7	0.55	0.59	0.61	0.66	0.66	0.57	1.00																		
A8	0.68	0.59	0.70	0.70	0.75	0.66	0.73	1.00																	
A9	0.73	0.64	0.66	0.70	0.70	0.75	0.77	0.77	1.00																
A10	0.68	0.64	0.66	0.43	0.66	0.70	0.64	0.59	0.64	1.00															
A11	0.77	0.73	0.84	0.66	0.80	0.80	0.59	0.73	0.68	0.64	1.00														
A12	0.77	0.50	0.66	0.70	0.66	0.66	0.59	0.77	0.68	0.55	0.73	1.00													
B1	0.80	0.66	0.86	0.64	0.68	0.77	0.61	0.61	0.75	0.70	0.75	0.57	1.00												
B2	0.77	0.77	0.75	0.66	0.80	0.80	0.55	0.59	0.64	0.55	0.73	0.64	0.70	1.00											
B4	0.55	0.45	0.61	0.48	0.70	0.52	0.55	0.73	0.55	0.64	0.59	0.64	0.57	0.50	1.00										
B5	0.75	0.52	0.64	0.77	0.59	0.68	0.57	0.61	0.66	0.52	0.61	0.70	0.59	0.61	0.48	1.00									
B6	0.68	0.77	0.80	0.57	0.84	0.80	0.64	0.68	0.68	0.68	0.82	0.59	0.70	0.77	0.64	0.52	1.00								
B7	0.59	0.73	0.70	0.70	0.66	0.70	0.59	0.68	0.73	0.45	0.73	0.64	0.57	0.73	0.55	0.70	0.73	1.00							
B8	0.70	0.66	0.59	0.50	0.68	0.82	0.52	0.66	0.75	0.75	0.70	0.66	0.64	0.66	0.66	0.64	0.70	0.70	1.00						
B9	0.77	0.64	0.75	0.66	0.89	0.80	0.64	0.68	0.64	0.64	0.82	0.73	0.70	0.82	0.64	0.66	0.82	0.64	0.66	1.00					
B10	0.70	0.75	0.77	0.64	0.86	0.77	0.66	0.70	0.70	0.70	0.89	0.61	0.68	0.75	0.61	0.55	0.89	0.75	0.68	0.80	1.00				
B11	0.77	0.68	0.70	0.66	0.84	0.84	0.73	0.73	0.86	0.73	0.77	0.59	0.84	0.68	0.59	0.61	0.77	0.59	0.75	0.77	0.80	1.00			
B12	0.70	0.80	0.82	0.59	0.77	0.77	0.61	0.70	0.66	0.66	0.89	0.61	0.73	0.70	0.61	0.50	0.93	0.70	0.68	0.75	0.86	0.75	1.00		
SC4	0.64	0.55	0.66	0.70	0.70	0.61	0.59	0.64	0.59	0.55	0.77	0.64	0.66	0.59	0.64	0.57	0.68	0.59	0.57	0.77	0.70	0.68	0.70	1.00	
SC5	0.59	0.50	0.61	0.66	0.66	0.57	0.55	0.59	0.55	0.50	0.73	0.59	0.61	0.55	0.59	0.52	0.64	0.55	0.52	0.73	0.66	0.64	0.66	0.95	1.00

4.4.6 Genetic relationships among 25 *B. napus* cultivars

The dendrogram from the UPGMA clustering methods of 25 rapeseed genotypes based on S_{ij} matrix is shown in Figure 4.2, which illustrated the unambiguous identification of different varieties and their grouping. No apparent clustering by original location was observed. The results that genetic similarity between random two cultivars shared was lower than 1, which showed rapeseed cultivars could be discriminated using SSR markers. Further, SSR marker is a reliable and accurate method for varieties identification. At a similarity coefficient of 0.63, all cultivars were divided into three groups. Group Ⅰ consisted of 22 cultivars. Unexpectedly, it was found that cultivars bred from different breeding institute clustered closer, due to the similar parent or breeding program. At a similarity coefficient of 0.64, Group Ⅰ was divided into two sub-clusters (A and B). Sub-cluster A consisted of 18 cultivars, the higher similarity coefficient (0.93) between NR168 and You 06-3 suggested the closer genetic relationship and background. Sub-cluster B contained four cultivars You 05-2, IF5-9, Qianza 6-18 and Qianza 2501. Qianza 6-18 and Qianza 2501 provided by the same institute (Guizhou oil crop institute) were closer related. The varieties from different or same breeding institute were grouped together, which showed that there was no clear relevance between regional location and genetic differentiating. Group Ⅱ only included two cultivars Huayouza 6 and Huayouza 9 with highest similarity coefficient (0.95) showed identical SSR fingerprint for all but 2 of 54 loci, indicating a very close genetic relationship. It was explained that they originated from the same institute using same parents in breeding program. Group Ⅲ GuiBF2-3 alone formed a separated cluster, which was highly genetically distinct from other rapeseed cultivars. The presence of unique fingerprint

in GuiBF2-3 highlighted the peculiarity of this genetic material in comparison to the most representative rapeseed cultivars. In addition, the narrow clustering of special genotype pairs with very similar SSR fingerprint (e.g. You 9559 and Qianza J5005, H0802 and Shenyou6970, Jinyou 8 and Jinyou 068) reflects the close genetic relationship among these varieties.

The clustering obtained with AFLP and SSR data were not identical. However, accessions having common parents were grouped together in both the techniques. This might be due to the fact that although equal numbers of AFLP and SSR primers were used, the number of polymorphic bands generated by AFLP primers was much more than alleles obtained with SSR primers. These results are comparable with earlier reports by Bowers and Meredith (1997) and Merdinoglu et al. (2005), who obtained topologically different dendrograms while analyzing grape varieties with different marker types. Mahmound et al. (2005) found that dendrograms of Egyptian rice genotypes derived from different techniques (RAPD, SSR and AFLP) gave minor differences in clustering patterns.

These results demonstrated that SSR is a reliable tool for evaluating genetic diversity and relationship between rapeseed cultivars, which was in agreement with previous studies. Xu et al. (2008) reported genetic diversity and relationship among 102 winter rapeseed cultivars were evaluated using 18 SSR primers. Lei et al. (2005) and Liu et al. (2007) analyzed the genetic diversity and relationship of yellow-seeded rapeseed varieties and double-lower respectively. Zhou et al. (2006) revealed the genetic relationship between Chinese and Swedish *B. napus* by SSRs.

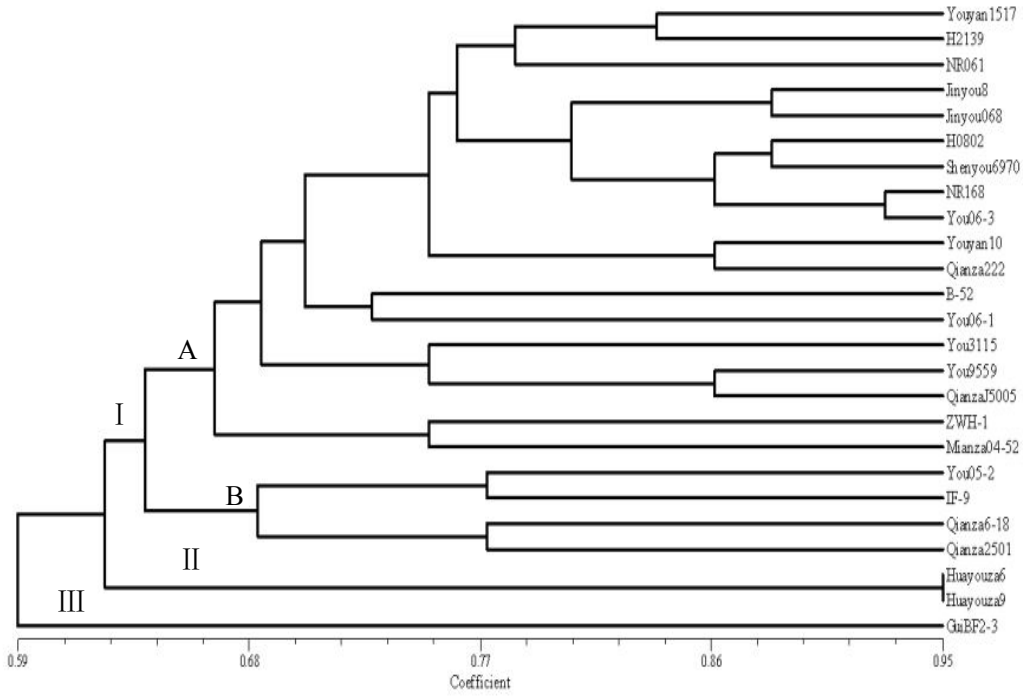


Figure 4.2 A dendrogram generated from UPGMA clustering analysis among 25 *B. napus* cultivars attending regional tests on the basis of Dice similarity coefficient.

4.5 Conclusion

In conclusion, an efficient system for the genetic diversity and relationships of rapeseed varieties cultivated in Guizhou was developed using SSR markers. The high level of genetic diversity among *B. napus* cultivars using 11 SSR markers would provide new genes for yield, adaptation, high value uses and characters in rapeseed production. Therefore, these markers can be proposed to distinguish *B. napus* germplasm. Meanwhile, this approach is very useful to choose parental genotypes for crosses, and to optimize germplasm conservation and management of diversity.

In the past, many authors have published about the genetic diversity and relationship of rapeseed cultivars using AFLP and SSR markers (Powell et al., 1996;

Snowdon and Friedt., 2004; Cheng, 2005). These results of these works clearly demonstrate that AFLP and SSR markers provided useful information on the level of polymorphism and diversity in rapeseed, showing their utility in the characterization of germplasm.

Comparison with these two techniques, these results of this study demonstrated that the SSR technique was the best choice for the evaluation of diversity and assessing the genetic relationships among rapeseed cultivars, because SSR is a co-dominant marker, and is usually highly polymorphic in the number of repeat units and shows a single locus pattern which allows the comparison of populations based on their allele frequencies (Bruford et al., 1996). However, it is predictable that significantly high polymorphism would be found for AFLPs if it were possible to score the large number of restriction fragments. Therefore, our results also showed that AFLP is an optimal method for DNA fingerprinting of rapeseed germplasm, due to multiple loci distributed throughout the genome. However, there are some disadvantages of AFLP markers due to its dominant character. Homozygotes are not directly distinguishable from heterozygotes using dominant markers and thus heterozygosity has to be calculated indirectly by assuming that gene frequencies in the studied populations (Lynch and Milligan, 1994; Travis et al., 1996). Therefore, AFLPs and SSRs could be used in a complementary way to unambiguously distinguish varieties. For the first stage, SSR is applied to separate most varieties; then, at the second stage, AFLP is further applied to characterize the most similar ones due to a higher discrimination power.

4.6 References

Bond, J. M., Mogg, R. J., Squire, G. R. and Johnstone, C. (2004). Microsatellite amplification in *Brassica napus* cultivars: Cultivar variability and relationship to a long-term feral population. **Euphytica**. 139: 173–178.

Bowers, E. B. B and Meredith, C. P. (1997). **Comparison of RFLP, AFLP and SSR markers for analyzing phonetic relationships among cultivars of *Vitis vinifera***, Presented at International Plant and Animal Genome V conference San Diego, CA, January 12–16.

Bruford, M. W., Cheesman, D. J., Coote, T., Green, H. A. A., Haines, S. A., O'Ryan, C. and Williams, T. R. (1996). **Microsatellites and their application to conservation genetics**. In: Smith TB and Wayne RK (eds.) Molecular Genetic Approaches in Conservation. Oxford University Press, New York, pp 278-277.

Cheng, Y. (2005). AFLP markers for the dominant genic male sterility gene and SSR fingerprint of cultivars (lines) in *Brassica napus* L., (MSc. thesis). **Oil Crop Research Institute**, Wuhan 430062 in China.

Cheung, W. Y., Champagne, G.., Hubert, N. and Landry, B. S. (1997). Comparison of the genetic maps of *Brassica napus* and *Brassica oleracea*. **Theoretical and Applied Genetics**. 94: 569–582.

Cregan, P. B. (1992). Simple sequence repeat DNA length polymorphisms. **Probe**. 2(1): 18 – 22.

Creste, A. Tulmann Neto, R. Vencovsky, S. de Oliveira Silva. and Figueira, A. (2004). Genetic diversity of *Musa* diploid and triploid accessions from the Brazilian banana breeding program estimated by microsatellite markers. **Genetic Resources and Crop Evolution** 51: 723–733.

Gupta, P. K. and Varshney, R. K. (2000). The development and use of microsatellite markers for genetic analysis and plant breeding with emphasis on bread wheat. **Euphytica**.113:163–185.

Hartl, D. L. and Clark, A. G. (1997). **Principles of population genetics**, 3rd edition. Sunderland (MA): Sinauer Associates.

Hasan, M., Seyis, F., Lu¨hs, W., Badani, A. G., Friedt, W. and Snowdon, R. J. (2006). Analysis of genetic diversity in the *Brassica napus* gene pool using SSR markers. **Genetic Resources and Crop Evolution.** 53(4):793-802.

Holsinger, K. E. and Bruce, S. W. (2009). Genetics in geographically structured populations: defining, estimating and interpreting FST. **Nature Reveiw Genetics**. 10 (9): 639–650.

Hudcovicova, M. and Kraic, J. (2003). Utilization of SSRs for characterization of the soybean (Glycine max L. Merr.) genetic resources. **Czech Journal of Genetics Plant Breeding**. 39: 120-126.

Jones, C., Merrett, S., Thomas, V. A., Barker, T. H., Lakhani, S. R. (2003). Comparative genomic hybridization analysis of bilateral hyperplasia of usual type of the breast. **Journal of Pathology**. 199: 152–156.

Kresovich, S., Szewc-McFadden, A. K., Bliek, S. M., McFerson, J. R. (1995). Abundance and characterization of simple sequence repeats (SSRs) isolated from a size-fractionated genomic library of *Brassica napus* (L.) (Rapeseed). **Theoretical and Applied Genetics**. 91:206–211.

Lei, T. G., Zhang, X. H., Lu, H., Li, J. N., Xu, X. F., Fan, Q. X. and Chen, Li. (2005). SSR fingerprint of yellow-seeded rapeseed varieties (*Brassica napus* L.). **Journal of Southwest Agricultural University (Natural Science)**. .27(3): 361-364.

Liu, P. W., Li, Y., He, Q. B. and Yang, G. S. (2007). Identification of genetic markers for the pol CMS restorer gene in *Brassica napus* L. **Chinese journal of oil crop sciences**. 29 (1): 14-19.

Lynch, M. and Milligan, B. G. (1994). Analysis of population genetic structure with RAPD markers. **Molecular Ecology**. 3: 91–99.

Maguire, T. L., Peakall, R. and Saenger, P. (2002). Comparative analysis of genetic diversity in the mangrove species Avicennia marina (Forsk.) Vierh. (Avicenniaceae) detected by AFLPs and SSRs. **Theoretical and Applied Genetics**. 104: 388–398.

Mahmoud, M. S., Sawsan, S. Y., Naglaa, A. A., Hany, S. B. and Ahmed, M. E. S. (2005). Genetic analysis of some Egyptian rice genotypes using RAPD, SSR and AFLP. **African Journal of Biotechnology**. 4(9): 882-890.

Maughan, P. J., Maroof, M. A. S. and Buss, G.. R. (1995). Microsatellite and amplified sequence length polymorphisms in cultivated and wild soybean. **Genome**. 38: 715-723.

Merdinoglu, D., Butterlin, G., Bevilacqua, L., Chiquet, V., Adam-Blondon, A. F. and Decroocq, S. (2005). Development and characterization of a large set of microsatellite markers in grapevine (*Vitis vinifera* L.) suitable for multiplex PCR. **Molecular Breeding**. 15:349–366.

Moghaddam, M., Mohammmadi, S. A., Mohebalipour, N., Toorchi, M., Aharizad, S. and Javidfar, F. (2009). Assessment of genetic diversity in rapeseed cultivars as revealed by RAPD and microsatellite markers. **African Journal of Biotechnology**. 8(14):3160-3167.

Nei, M. and Li, W. H. (1979). Mathematical model for studying genetic variation in

terms of restriction endonucleases. **Proc. Natl. Acad. Sci**. USA. 76: 5269-5273.

O'Neill, N. R., van Berkum, P., Lin, J. J., Kuo, J., Ude, G.. N., Kenworthy, W. and Saunders, J. A. (1997). Application of amplified restriction fragment length polymorphism for genetic characterization of Colletotrichum pathogens of Alfalfa. **Phytopathology**. 87: 745–750.

Padmaja, K. L, Arumugam, N., Gupta, V., Mukhopadhyay, A., Sodhi, Y. S., Pental D, Pradhan, A. K. (2005). Mapping and tagging of seed coat colour and the identification of microsatellite markers for marker assisted manipulation of the trait in *Brassica juncea* .**Theoretical and Applied Genetics**. 111: 8–14.

Patricia, J. F. and Cristina, Y. M. (2006). Molecular markers for population genetic analysed in the family Psittacidae (Psittaciformes, Aves). **Genetic and Molecular Biology**. 29(2): 231-240.

Piquemal, J., Cinquin, E., Couton, F., Rondeau, C., Seigoret, E., doucet, I., Perret, D., Villeger, M.J., Vincourt, P. and Blanchard, P. (2005). Construction of an oilseed rape (*Brassica napus* L.) genetic map with SSR markers. **Theoretical and Applied Genetics**. 111: 1514-1523.

Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S. and Rafalski, A. (1996). The comparison of RFLP, RAPD, AFLP, and SSR (microsatellite) markers for germplasm analysis. **Molecular Breeding**. 2:225–238.

Rakow, G., and Woods, D. L. (1987). Outcrossing in rape and mustard under Saskatchewan prairie conditions. **Canadian Journal of Plant Science**. 67: 147-151.

Raybould, A. F., Mogg, R. J., Clarke, R. T., Gliddon, C. J. and Gray, A. (1999).

Variation and population structure at microsatellite and isozyme loci in wild cabbage (*Brassica oleracea* L.) in Dorset (UK). **Genetic resources and crop evolution**. 46: 351–360.

Reale, S., Pilla, F. and Angiolillo, A. (2006). Genetic analysis of the Italian Vitis vinifera cultivar 'Tintilia' and related cultivars using SSR markers. **Journal of Horticultural Science and Biotechnology**. 81: 989–994.

Ren, F. G., Lu, B. R., Li, S. Q., Huang, J. Y. and Zhu, Y. G. (2003). A comparative study of genetic relationships among the AA-genome Oryza species using RAPD and SSR markers. **Theoretical and Applied Genetics**. 108: 113-120.

Rohlf, F. J. (2000). **NTSYS-pc: numerical taxonomy and multivariate analysis system, version 2.1**. Exeter Software, New York.

Rudolph, B., Uzunova, M. I., Ecke, W. (2000). **Development of microsatellite markers for the analysis of genetic diversity in rapeseed (*Brassica napus* L.)**. 3rd ISHS International Symposium on *Brassica*, 12th Crucifer Genetics Workshop. 5-9 Sep. Horticulture Research International Wellesbourne, CV35 9EF, UK.

Salimath, S. S., Olivieri, A. C., Godwin, I. D. and Bennetzen, J. L. (1995). Assessment of genome origins and genetic diversity in the genus Eleusine with DNA markers. **Genome**. 38: 757-763.

Snowdon, R. J., Friedrich, T., Friedt, W. and Kohler, W. (2002). Identifying the chromosomes of the A- and C-genome diploid *Brassica species B. rapa (syn. campestris)* and *B. oleracea* in their amphidiploid *B. napus*. **Theoretical and Applied Genetics**. 104: 533–538.

Szewc- McFadden, A. K., Kresovich, S., Bliek, S. M., Mitchell, S. E. and McFerson, J. R.

(1996). Identification of polymorphic, conserved simple repeats (SSR) in cultivated *Brassica* species. **Theoretical and Applied Genetics**. 93: 534-438.

Tonguc, M. and Griffiths, P. D. (2004). Genetic relationships of *Brassica* vegetables determined using database derived simple sequence repeats. **Euphytica**. 137: 193-201.

Travis, S. E., Maschinski, J. and Keim, P. (1996). An analysis of genetic variation in *Astragalus cremnophylax* var. *cremnophylax*, a critically endangered plant, using AFLP markers. **Molecular Ecology**. 5: 735-745.

Uzunova, M., Ecke, W., Weissleder, K. and Robbelen, G. (1995). Mapping the genome of rapeseed (*Brassica napus* L.) I. Construction of an RFLP linkage map and localization of QTLs for seed glucosinate content. **Theoretical and Applied Genetics**. 90: 194–204.

Uzunova, M. I. and Ecke, W. (1999). Abundance polymorphism and genetic mapping of microsatellites in oilseed rape (*Brassica napus* L.). **Plant Breed**. 118: 323-326.

Wattier, R., Engel, C. R., Saumitou-Laprade, P., Valero, M. (1998). Short allele dominance as a source of heterozygote deficiency at microsatellite loci: experimental evidence at the dinucleotide locus Gv1CT in Gracilaria gracilis (Rhodophyta). **Molecular Ecology**. 7: 1569–1573.

Weerakoon, S. R., Jagathpriya Weerasena, O. V. D. S, Peiris, P. K. D. and Somaratne, S. (2010). Assessment of genetic variation within Mustard (Brassica juncea (L.) Czern & Coss) germplasm in Sri Lanka using Fluorescent-based Amplified Fragment Length Polymorphic DNA markers. **International Journal of Biotechnology and Biochemistry**. 6(5): 757-768.

Westman, A. and Kresovich, S. (1999). Simple sequence repeat (SSR)-based marker

variation in *Brassica nigra* genebank accessions and weed populations. **Euphytica**. 109: 85–92.

Wright, S. (1965). The interpretation of population structure by F-statistic with special regard to systems of mating. **Evolution**. 19: 395–420.

Xu, K.，Zhang, D. X.，Wu, X. M.，Lu, G. Y.，Liu, F. L.，Chen, B. Y. and Gao, G. Z. (2008). SSR analysis of new winter rapeseed cultivars in the national field tests．**Chinese Journal of Oil Crop Sciences**. 30（1）：29－34.

Yeh, F. C., Yang, R. C. and Boyle, T. (1999). **Popgene version 1.31**. Microsoft window-based freeware for population genetic analysis. Department of Renewable Resources, University of Alberta, Edmonton, Alberta, Canada. Available free at http://www.ualberta.ca/～fyeh/.

Zhou, W. J. (2001). **Oilseed rape**. In: Zhang G. P. and Zhou W J. (eds.) Crop Cultivation. Zhejiang University Press, Hangzhou, China, pp. 153–178.

Zhou, W. J., Zhang, G. Q., Tuvesson, S., Dayteg, C. and Gertsson, B. (2006). Genetic survey of Chinese and Swedish oilseed rape (*Brassica napus* L.) by simple sequence repeats (SSRs). **Genetic Resource and Crop Evolution**. 53: 443-447.

CHAPTER V

CONCLUSION

In this study, two techniques (AFLP and SSR) were successfully used to reveal characterization of the genetic identities and relationships among 25 *Brassica napus* cultivars. Therefore, they provided effective genetic information of *B. napus* germplasm for future breeding program.

The results obtained from this research can be concluded as follow:

1. The polymorphism among 25 *B. napus* cultivars revealed by AFLP and SSR markers is lower than 45%. Moreover, SSR exhibited a higher level of polymorphism (40%) than AFLP (38%). The data of the study indicated that the SSR methodology detected polymorphism more efficiently than the AFLP approach.

2. The clustering of 25 *B. napus* cultivars obtained with AFLP and SSR data were not identical. The UPGMA analysis showed that no apparent clustering by their original location. However, it was found that varieties bred from same institute were often grouped together in both the techniques. This might be due to common parent or similar breeding program for utilization of *B. napus* germplasm. Huayouza 6 and Huayouza 9 sharing a highly similar fingerprint could not resolved by both techniques due to their close genetic relationship. Therefore, it is necessary to utilize a larger number of AFLP primer combinations and SSR primer pairs on a wide range of cultivars to meet the requirement of research.

3. Comparison with these two techniques, these results of this study demonstrated that the SSR technique was the best choice for the evaluation of diversity and assessing the genetic relationships among *B. napus* cultivars. However, AFLP was an optimal method for DNA fingerprinting of *B. napus* germplasm.

APPENDIX

1. Preparation of the glass plates

Long plate for Bind-silane

a) Clean the plate with laboratory detergent. Rinse well with distilled water.

b) Clean the upper surface with 100% ethanol. Using blue roll, polish until it squeaks.

c) Apply 20 μl Bind-silane to the upper surface of the plate and spread evenly using blue roll.

d) Wipe with warm water and then 100% ethanol, and let air dry.

Short plate for treatment with Repel-silane:

a) Wear gloves. Clean the plate with laboratory detergent (type used for cleaning radioactivity – e.g. 25% Lipsol) and warm water. Rinse.

b) Clean the upper surface with 100% ethanol. Using blue rolls of tissues, polish until it ‘squeaks’.

c) Apply a few drops of Repel-silane to the upper surface of the plate and spread evenly using blue roll.

d) Wipe with warm water and then 100% ethanol, and let air dry.

Chang the groove between long plate and short plate

2. Setting up and casting a polyacrylamide gel using sequencing apparatus involves the following steps:

a) Swirl around. Working quickly (maximum 5 min), pour the gel mix on long plate c carefully to avoid bubbles, while sliding in the short plate (or use a 50ml syringe)

b) Insert comb into to of plate: if using sharkstooth/sawtooth comb, place flat side against the top of the gel, displacing some liquid.

c) Leave to polymerise for about 1 hour.

d) Remove top spacer, and wash well with distilled water from a wash bottle to remove both unpolymerized acrylamide and crystallized urea. This is very important. Wash bottom of gel to removed unpolymerized acrylamide.

3. Silver staining:

Briefly, soak the gel for15 min in fixing solution. Then transfer it into 0.2% $AgNO_3$ and leave shaking for 8 min. The gel will turn cloudy and brownish. Rinse in 3-4 sec in distilled water. Soak gel in fresh developing solution under gentle shaking until band intensities are adequate. The staining will be stopped in fixing solution again for 2-3 min. Wash gel for1 min in distilled water. The gel is photographed after drying.

BIOGRAPHY

Miss Li Li was born on March 17th, 1982 in Guizhou, China. In 2000, she studied in Department of Biotechnology, School of Life Science, Guizhou University. She graduated with a Bachelor's of Science in Biotechnology in 2004. After that, she works as a research assistant in the institute of Biotechnology, Guizhou Academic of Agricultural Science, and made four publications in the core of Chinese journal. In 2007, she was Master's student in School of Biotechnology at Suranaree University of Technology. During Master's student, she had an experience in oral presentation in title "SSR analysis of genetic diversity and relationship among 25 rapeseed cultivars" at the TSB 2010 International Conference on Biotechnology for Healthy Living (the 22nd Annual Meeting of the Thai Society for Biotechnology) October 20-22, 2010, Prince of Songkla University, Trang Campus. She also made a poster presentation in title "genetic variation of *Brassica napus* cultivars using SSR markers" at the 3rd SUT Grade Conference 2010, November 21-23, 2010, Suranaree University of Technology, Nakhon Ratchasima, Thailand.