Comparison of AFLP and SSR for Genetic Diversity Analysis of *Brassica napus* Hybrids

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Abstract

The AFLP and SSR markers were used to estimate the genetic diversity of 25 *Brassica napus* hybrids attending Guizhou regional test. The fingerprints obtained with both the AFLP and SSR markers revealed high levels of heterozygosity. Nine AFLP primer combinations produced 16 loci, while 11 SSR primer pairs generated 22 loci. The mean of expected heterozygosity, Shannon's information index, and genetic differentiation obtained by SSR were higher than those by AFLP, indicating that the SSR methodology evaluated genetic diversity among *B. napus* more efficiently than the AFLP approach. The higher level of genetic diversity detected by SSR markers was contributed to the lower genetic similarity estimates based on SSR markers (mean 0.69) as compared to AFLP markers (mean 0.73). While the AFLP technique was suitable for identification and DNA fingerprinting of *B. napus* germplasm. Based on AFLP and SSR analysis, it was concluded that *B. napus* hybrids had high level genetic diversity.

Keyword: AFLP, *Brassica napus*, Genetic diversity, Hybrid, SSR

1. Introduction

1.1 Brassica napus of Guizhou

Brassica napus is one of the most important sources of vegetable oil in Guizhou, China, and is the second most

important rapeseed crop in the world after soybean. In 2006, rapeseed cultivation area in Guizhou was about 1.27 million acre, of which *B. napus*, *B. juncea* and *B. campestris* make up 70% - 75%, 10%, and 15%, respectively (Rao et al., 2005). During long term natural and artificial selection, a large number of *B. napus* hybrids were developed. With the application and dissemination of elite hybrid rapeseed, yield of *B. napus* has been improved and new cultivars are introduced to update the assortment. Regional tests provide useful information for the registration and protection of *B. napus* hybrids. The candidate varieties represent the achievement of breeder and reflect level of present breeding. However, the genetic diversity of *B. napus* hybrids attending Guizhou regional test remains unevaluated, and the genotypes selected and exploited for extensive planting are very much limited.

1.2 Significance of research

In the past, there were various techniques for studying the genetic variability of crop germplasm, including morphological traits, total seed proteins and isozymes, potentially useful for genetic analysis of *B. napus*. As the number of various hybrids increases, these traditional methods seem to be limited to distinguish them, due to environmental influences and low level of polymorphism (Èron et al., 2002; Lombard et al., 2000). Therefore, it is necessary to use other methods for a precise hybrid description.

1.3 Superiority of molecular markers

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 (Tanksley et al., 1989; Messmer et al., 1993; Melchinger et al., 1994), including restriction fragment length polymorphism (RFLP) markers (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; Uzunova and Ecke, 1999). These markers give broad and different ranges of information and substantially differ in terms of practicability and reproducibility.

1.4 Aim of research

The AFLP and SSR are two powerful DNA fingerprinting techniques. A number of polymorphic fragments can be detected in an experiment and there is a higher reproducibility of banding patterns by AFLP. SSR markers have several advantages over other molecular markers for their co-dominant inheritance, large number of alleles per locus, and abundance in genomes. However, there are few reports concerning of AFLP and SSR techniques for genetic diversity and relationship among *B. napus* hybrids. Therefore, we have compared the level of information provided by AFLP and SSR markers for estimating genetic relationships among *B. napus* hybrids. This study will provide useful information for *Brassica* breeding program.

2. Research methods

2.1 Plant materials

Twenty-five *B. napus* hybrids were selected from different breeding institutes and provided by Guizhou Seed Management Station, China, which represented a large range of *B. napus* germplasm and elite varieties. Young leaf samples were collected from these varieties. The leaf samples were frozen in liquid nitrogen and stored at -70°C until needed for DNA extraction. The name and origin of *B. napus* hybrids used were given in Table 1.

2.2 DNA extraction

Genomic DNA was extracted from young leaflets of 2-week-old seedlings using DNA quick plant system kits (Tiangen Biotech Beijing Co., Ltd.). The quality and quantity of the DNA were determined at 260 and 280 nm using DU800 spectrophotometer (Beckman Coulter, USA), and visualized by agarose gel eletrophoresis.

2.3 AFLP analysis

The AFLP assay was carried out according to Vos et al. (1995) with minor modifications. Briefly, 100-300 ng of DNA was double-restricted at 37°C 4 h with *Eco*RI and *Mse*I restriction enzymes in a total volume of 20 μL and then ligated with *Eco*RI and *Mse*I adapters. This was followed by a pre-amplification step using universe primer *Eco*RI (5′ GACTGCGTACCAATTC3′) and *Mse*I (5′GATGAGTCCTGAGTAA3′). It was performed in a Master Cycler Gradient 22331 (Germany): an initial step of 5 min at 94°C, 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 60 s, and a final step of 10 min at 72°C. For selective amplification, 5 μL of a 20-fold diluted preamplification mixture was amplified in the same thermocycler as preamplification consisting of 12 cycles of 30 s at 94°C, 30 s at 65°C to 56°C (with a decreasing ramp of 0.7°C each cycle), and 60 s at 72°C, then by 24 cycles of 30 s at 94°C, 30 s at 56°C, and 60 s at 72°C using *Eco*RI and *Mse*I with three selective nucleotides. Amplification products were visualized on a 6% denaturing polyacrylamide gel at a constant 1500 V, 65 mA for

2 h in a sequencing gel electrophoresis apparatus (EPS 3501, Amersham Pharmacia Biotech, USA) using sliver staining.

2.4 SSR analysis

11 SSR primer sets developed from *B. napus*, *and B. rapa* were available for this study (shown in Table 2). All SSR primers were synthesized by Generay Biotech Co. Ltd. (Shanghai, China). Repeats, primer sequences and in part the map position for the loci are available on the Cropnet website (http: // ukcrop. net/ perl/ ace/ search /BrassicaDB). PCR amplification was carried out in a volume of 20 μL containing 2.5 μL of 30 ng/μL DNA, 0.4 μL of 10 μM of each primer, 0.4 μL of 10 mM dNTPs, 2.0 μL of 10×reaction buffer (containing 25mM Mg^{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: an initial step of 5 min at 94°C, 35 cycles of 45 s at 94°C, 45 s at 53°C and 1 min at 72°C, and a final step of 10 min at 72°C. The PCR products were separated and visualized on 10% non-denaturing polyacrylamide gel by silver staining.

2.5 Band scoring and data analysis

The AFLP and SSR reproducible fragments were scored as 0 or 1 for absence or presence of fragments, respectively. The dendrograms were constructed using unweighted pair group method with arithmetic average (UPGMA) based on Dice similarity coefficient (Nei and Li, 1979), the sequential hierarchical, and nested clustering routine in the NTSYS – pc 2.01 program (Rohlf, 2000).POPGENE version 1.31 software (Yeh et al., 1999) was used to calculate the parameters of genetic diversity, including the percentage of polymorphic bands, effective number of alleles per locus (NA; Hartl and Clark, 1989), expected heterozygosity (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, if a locus consists of two alleles as applicable in dominant marker analyses (e.g., RAPD and AFLP), the 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. For co-dominant marker (SSR), F_{ST} is analogous to the G_{ST}, and 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 and Bruce, 2009).

3. Analysis results

3.1 Polymorphism detected by AFLP and SSR markers

A total of 193 bands were generated with 9 AFLP markers ranging in size from 76 to 635 bp, of which 73 unambiguous were polymorphic with a mean polymorphic rate of 38% (Table 3). An average of 8.1 polymorphic bands was generated for each AFLP assay unit. A maximum of 14 polymorphic bands was amplified with E9/M33 (E+CCC/M+TCG) primer combination (shown in Figure 1. A), and a minimum of 5 polymorphic bands was produced with E10/M39 (E+CCA/M+ATG) primer combination. The 134 bands were produced using 11 SSR markers, of which 54 is polymorphic with an average polymorphic rate of 40%. Polymorphic bands per SSR assay unit were 4.9. The fragment size ranged from 100 to 1200 bp. The maximum number of polymorphic bands was obtained using Na12-A02, while the minimum number was observed using FITO-063 primer (shown in Figure 1. B).

3.2 Genetic diversity revealed by AFLP and SSR markers

Each of technique revealed a large number of loci, relying on their characteristic to identify each cultivar. Across 25 *B. napus* hybrids, 9 AFLP primer combinations generated a total of 16 alleles in 9 loci with the mean effective number of 1.41 alleles per locus. The H, I and G_{ST} were 0.24, 0.62, and 0.39, respectively. 11 SSR primers produced a total of 22 alleles in 11 loci with the average effective number of 2.01 alleles per locus. The H, I and F_{ST} were 0.45, 0.73, and 0.54, respectively.

3.3 Dendrograms based on AFLP and SSR analyses

The AFLP analysis presented an average genetic similarity coefficient of 0.73 while the SSR analysis showed a mean similarity coefficient of 0.69. The genetic similarity data obtained from AFLP and SSR data were used to investigate the difference among *B. napus* hybrids at the DNA level. The dendrograms depicting relationship

among the tested hybrids based on AFLP and SSR data were constructed in Figure 2.C. and D., respectively. Both dendrogram were divided into three groups. Both group Ⅰ of AFLP and SSR contained 22 hybrids. Group Ⅱ of AFLP included two hybrids Shenyou 6970 and Qianza J5005, whereas group Ⅱ of SSR consisted of Huayouza 6 and Huayouza 9. The last group of the AFLP and SSR analyses had only hybrids Jinyou068 and Gui BF2-3, respectively. Cluster analysis showed that there is difference in clustering based on the AFLP and SSR techniques. For example, in AFLP cluster analysis, Qianza 2501 and Qianza 222 were grouped closely, while Qianza 2501 and Qianza 6-18 were close together in SSR cluster, although these three hybrids were bred by the same institute. It was also noted that hybrids bred from different institutes clustered together using AFLP and SSR markers (e.g. NR061 and Gui BF2-3 in AFLP, NR168 and You 06-3 in SSR). Moreover, in both genetic analyses, two hybrids of Huayouza 6 and Huayouza 9 from same institute could not be separated with each other, sharing the highest similar fingerprint, suggesting their very close genetic relationships.

4. Discussion

4.1 Comparison of polymorhism

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 non-existing self-incompatibility system (Rakow and Woods, 1987; Weerakoon et al., 2010). In our study, the polymorphism revealed by the AFLP and SSR markers is lower than 45%, which supported the previous reports. However, the SSR methodology exhibited a higher level of polymorphism (40%) than AFLP approach (38%). Although the percentage of polymorphic bands of the AFLP was lower than that of the SSR, but the polymorphic bands detected by each AFLP primer (7.78) were much higher than SSR (4.9). 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 (Maughan et al., 1995, Salimath et al., 1995, Powell et al., 1996). Mean number of effective alleles per locus detected with AFLP was 3.56 compared with by SSR was 1.41. In fact, the majority of the tested hybrids were uniquely identified both by their AFLP fingerprints and by their multilocus SSR profiles (Fossati et al., 2005). The two hybrids of Huayouza 6 and Huayouza 9 sharing a highly similar fingerprint could not resolved by both techniques due to their close genetic relationship. Therefore, it seems that more primers could lead to accurate identification, and exploitation of primers should be met to the requirement of research.

4.2 Genetic diversity analysis

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 level based on AFLP (0.24) was, as expected, lower than the heterozygosity for SSRs (0.45). The mean of expected heterozygosity, Shannon's information index, and genetic differentiation obtained by the SSR were higher than those by the AFLP, indicating that the SSR methodology evaluate genetic diversity among *B. napus* more efficiently than the AFLP approach. The higher level of genetic diversity detected by SSR markers was contributed to the lower genetic similarity estimates based on SSR markers (mean 0.69) as compared to AFLP markers (mean 0.73). The great genetic differentiation among *B. napus* populations demonstrated that a high level of genetic variability existed among them. 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). Moreover, based on the AFLP and SSR analyses, it was concluded that *B. napus* had high level of genetic diversity.

4.3 Dendrogram analysis based on AFLP and SSR markers

The clustering obtained with the AFLP and SSR data were not identical. However, there is a common phenomenon that no apparent clustering by 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 (Portis et al., 2004). Hybrids 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. The difference of clustering using different marker system was also investigated in other studies. Mahmound et al. (2005) found that dendrograms of Egyptian rice genotypes derived from different techniques (RPAD, SSR, and AFLP) gave minor differences in clustering patterns. Merdinoglu et al. (2005) who obtained topologically different dendrograms while analyzing grape varieties with different marker types.

5. Conclusion

Many authors have published in the past about the genetic diversity of rapeseed cultivars using AFLP and SSR markers (Powell et al., 1996; Jin et al., 2006). The results of this work clearly demonstrate that both AFLP and SSR markers can be successfully used for genetic diversity and relationship among *B. napus*, although only limited numbers of hybrids and primer combinations were analyzed. It is necessary to utilize a larger number of AFLP primer combinations and SSR primer pairs on a wide range of cultivars to distinguish all the hybrids.The SSR technique was the best choice for the evaluation of diversity and assessing the genetic relationships among *B. napus* hybrids, AFLP technique was an optimal method for DNA fingerprinting of *B. napus* germplasm. Moreover, the AFLP and SSR techniques could be used in a complementary way to unambiguously distinguish hybrids. For the first stage, the SSR is used to distinguish most hybrids; then, at the second stage, the AFLP is further employed to characterize the most similar ones due to the high polymorphism.

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Table 1. List of name and origin of *B. napus* hybrids

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

Table 2. AFLP and SSR primers used in this study

Code	AFLP primer	Primer combinations	Primer sequence (5′- 3′)
1	E9/M33	E+CCC/M+TCG	E+CCC: GACTGCGTACCAATTCCCC M+TCG:GATGAGTCCTGAGTAATCG
2	E10.M39	E+CCA/M+ATG	E+CCA: GACTGCGTACCAATTCCCA M+ATG:GATGAGTCCTGAGTAAATG
3	E12/M37	E+CCT/M+CTA	E+CCT:GACTGCGTACCAATTCCCCT M+CTA:GATGAGTCCTGAGTAACTA
4	E12/M39	E+CCT/M+ATG	E+CCT: GACTGCGTACCAATTCCCT M+ATG:GATGAGTCCTGAGTAAATG
5	E13/M37	E+CGG/M+CTA	E+CGG: GACTGCGTACCAATTCCGG M+CTA:GATGAGTCCTGAGTAACTA
6	E13/M39	E+CGG/M+ATG	E+CGG:GACTGCGTACCAATTCCGG M+ATG:GATGAGTCCTGAGTAAATG
7	E15/M36	E+CGT/M+AGC	E+CGT: GACTGCGTACCAATTCCGT M+AGC:GATGAGTCCTGAGTAAAGC
8	E16/M37	E+CGC/M+CTA	E+CGC: GACTGCGTACCAATTCCGC M+CTA: GATGAGTCCTGAGTAACTA
9	E16/M38	E+CGC/M+GTC	E+CGC: GACTGCGTACCAATTCCGC M+GTC:GATGAGTCCTGAGTAAGTC
		SSR primers	
1		FITO-063	F: GTTCAGTTCCCAGATTCCTAA R: TTTCCTCTTCCTTCTCTCTTC
2		FITO-136	F: CCTCCTCCTCAGACTTACACT R: TCACATCCACCATAACCTTT
3		Na10-B07	F: GCCTTAGATTAGATGGTCGCC R: ACTTCAGCTCCGATTTGCC
4		Na10-B11	F: TTTAACAACAACCGTCACGC R: CTCCTCCTCCATCAATCTGC
5		Na10-H03	F: GAGCTGGCTCATTCAACTCC R: CACAATTTCTCAGACAAAACGG
6		Na12-A02	F: AGCCTTGTTGCTTTTCAACG R: AGTGAATCGATGATCTCGCC
7		Na12-D09	F:ACTGAAACTTACTAAAAGAGAGA R: TCTAGAAACACCAGCAGTGGC
8		Na12-E02	F:TTGAAGTAGTTGGAGTAATTGGA R: CAGCAGCCACAACCTTACG
9		Na14-H12	F:CACATTGGCACGTATCCATC R: GGCTGATCGAACACAAATAAG
10		Ra3-D04	F: AAAAGGACCTACCAATTTCGTG R: CGACCCAAACTGAGCCATAC
11		Ra3-H09	F:GTGGTAACGACGGTCCATTC R:ACCACGACGAAGACTCATCC

E: *Eco*RI (5′GACTGCGTACCAATTC3′), M: *Mse*I (5′GATGAGTCCTGAGTAA3′),
F: Forward primer, R: Reverse primer

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Table 3. Genetic diversity of 25 *B. napus* hybrids based on 9 AFLP markers and 11 SSR markers

Parameters	AFLP marker	SSR marker
Number of primers	9 AFLP primer combinations	11 SSR primer pairs
Total number of bands	193	134
Number of polymorphic bands	73	54
Polymorphism rate	38%	40%
Number of bands per assay unit	21.4	12.2
Number of polymorphic bands per assay unit	8.1	4.9
Number of loci	16	22
Mean observed number of alleles	3.56	2.55
Mean effective number of alleles	1.41	2.01
Expected heterozygosity	0.24	0.45
Shannon's information index	0.62	0.73
Genetic differentiation	0.39	0.54

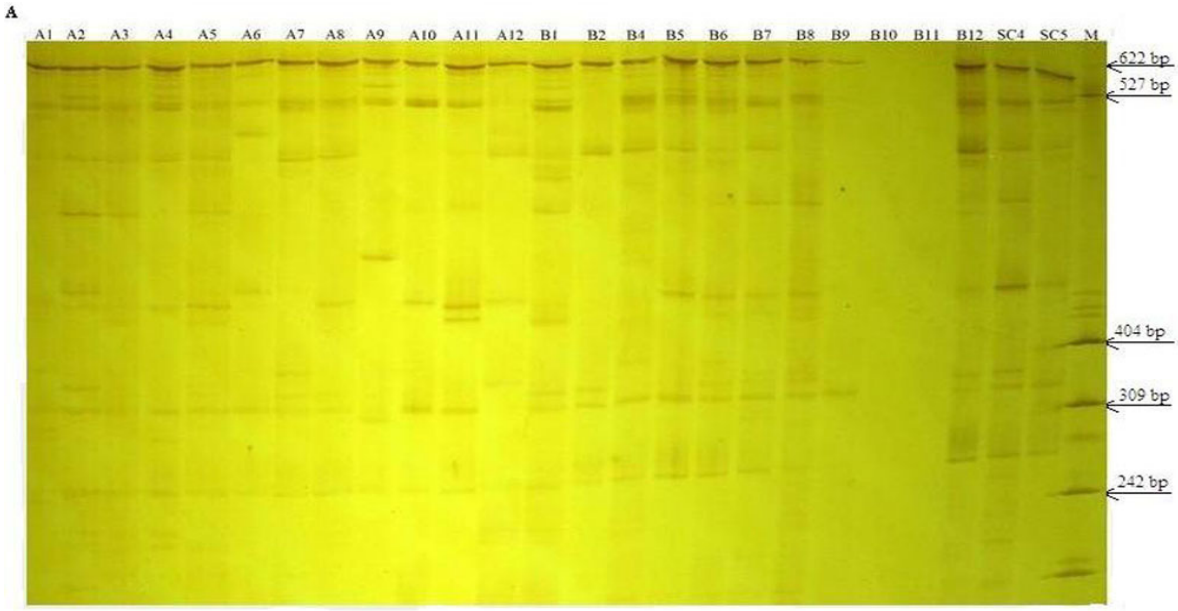

Figure 1A. PCR amplification products of 25 *B. napus* hybrids using AFLP primer E9/M33 were visible in 6% denaturing PAGE gel. M: DNA ladder pBR322 DNA-MspIDigest. Lanes A1-A12, B1-B12, SC4, and SC5 represented the amplification results of 25 *B. napus* hybrids

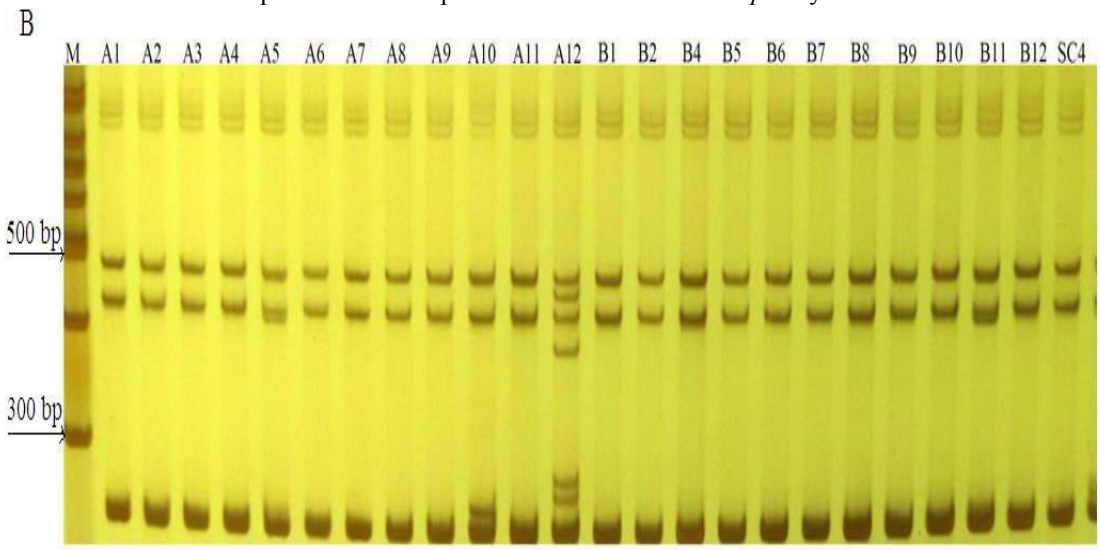

Figure 1B. PCR amplification products of 25 *B. napus* hybrids using SSR primer FITO-063 were visible in 10% non-denaturing PAGE gel. M: 100bp DNA ladder marker. Lanes A1-A12, B1-B12, SC4, and SC5 represented the amplification results of 25 *B. napus* hybrids

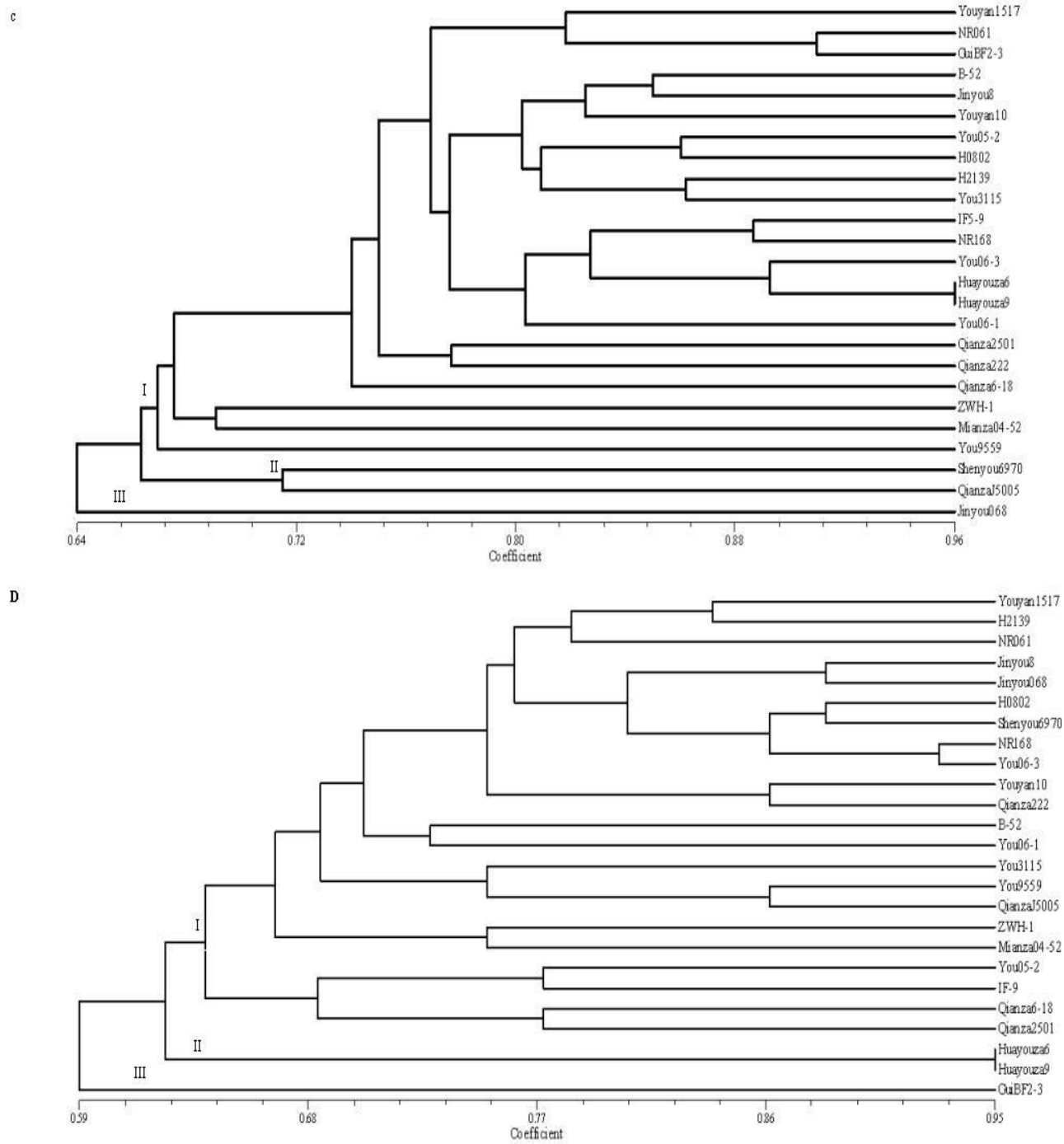

Figure 2. Dendrograms of 25 *B. napus* hybrids based on 73 AFLP markers and 54 SSR markers. (C) Dendrogram was constructed by 73 AFLP markers; (D) Dendrogram was constructed by 54 SSR markers

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