Assessment of Genetic Diversity of Yellow-seeded Rapeseed (*Brassica napus* L.) Accessions by AFLP Markers

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Abstract: The genetic diversity of 35 yellow-seeded *Brassica napus* L. accessions originating from China, Czech Republic and Poland was assessed by means of Amplified Fragment Length Polymorphism (AFLP) markers based on multiplex PCR using multi-colour fluorescent-labelled primers. Five brown-seeded accessions originating from China and France were selected as outliers. In total, 632 peaks were generated by AFLP reaction using 18 primer combinations. Only distinctly polymorphic markers among them were scored. In total, 242 polymorphic markers were detected with an average of 13.4 markers per primer combination. The AFLP analysis separated forty studied accessions into Chinese and European groups by UPGMA clustering and Principal Coordinates Analysis (PCA). The grouping of accessions based on the cluster analysis and PCA was generally consistent with known pedigree information and geographic origin. Notable geographical divergence was found between Chinese and European yellow-seeded accessions. This information is useful for yellow-seeded hybrid breeding and encouraging breeders to exchange their germplasm as to enlarge the genetic diversity of breeding accessions.

Keywords: AFLP; *Brassica napus* L.; genetic diversity; yellow seed trait

Improvements in the yield as well as in the quality of oil and meal of rapeseed (*Brassica napus* L.) are two important objectives for rapeseed breeders. It is widely proved that yellow-seeded types have higher oil and protein, lower fibre and dull pigment contents than brown-seeded ones (Stringam *et al*. 1974; Shirzadegan 1986; Rashid *et al.* 1994). Thus, the yellow seed colour is a desirable trait for rapeseed because it can enhance the dietary feed value and decrease the processing cost. However, the yellow-seeded character seldom occurs in *B. napus* naturally. Attempts have been made to develop yellow-seeded materials of *B. napus* (AACC) by transferring the genes conferring the yellow-seed trait from *B. rapa* (AA), *B. oleracea* (CC), *B. juncea* (AABB), or *B. carinata* (BBCC) (Chen *et al.* 1988; Liu 1992; Meng *et al.* 1998; Seyis *et al.* 2003). Some spontaneous yellow-seeded mutants were also found later in *B. napus* from cultivars Midas, Start, Topas (Liu 1992), and Polo, when they were introduced into China. Unfortunately, the yellow-seed character in *B. napus* was frequently found difficult to stabilize (Shirzadegan 1986; Liu 1992). Therefore, doubled haploid (DH) technology was used to stabilize yellow-seed colour and some DH lines proved to have distinctly better seed colour in comparison with the initial hybrid material (Vyvadilová *et al*. 1999).

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Like most agricultural crops, rapeseed has been improved over a long period and the genetic basis of advanced cultivars has been narrowed nowadays. The enhancement of genetically diverse gene pools is an essential requirement in plant breeding. Yellow-seeded rapeseed strains from interspecific crosses represent a potentially important resource to expand genetic diversity in the gene pool of *B. napus*. The seed colour of these materials seems to be inherited in a different genetic pattern and these materials have formed a distinct type from the brown seeded type. Up to now, the diversity information of yellow-seeded accessions has not been studied well. Zhang *et al.* (2003) revealed some genetic variation in biochemical traits and RAPDs (Random Amplified Polymorphic DNA) among 14 yellow-seeded accessions and Seyis *et al.* (2003) detected wide genetic diversity in 143 resynthesized rapeseed lines originating from the cross between Indian Yellow Sarson and cauliflower using Amplified Fragment Length Polymorphism (AFLP). However, the genetic diversity of yellow-seeded materials across different countries has not been analyzed due to the difficulty in germplasm collecting.

For hybrid breeding, the importance of the genetic structure of the parents for the expected heterosis of their hybrids is well known. Heterosis in a hybrid is based on genetic completion between divergent parents, so the information on genetic diversity could help breeders better understand the genetic structure of germplasm and to predict which cross combinations would produce good F_1 hybrids (Yu *et al.* 2005). For the breeding of yellow-seeded hybrid *B. napus*, the genetic diversity information is more important because of limitation on available yellow-seeded parents by now. Although the yellow-seeded hybrid could also be produced by crossing a brown-seeded parent with a dominant yellow-seeded type (Li *et al.* 1998; Li & Tian 1999), its seed colour was not as good as that from the yellow-seeded × yellow-seeded type. It was also found that hybrids from the crosses among yellow-seeded parents possessing the same or different dominant genes conferring yellow seeds have heterosis over the better parent on the penetration (ratio of yellow seed) and the expressivity (grade of seed colour) of yellow-seed gene (Li *et al.* 1998). This indicated that the accumulation of more yellow-seed genes in one hybrid would be the more robust to control the seed colour phenotype. However, all of it requires a very different genetic background essential for heterosis among the parents. For this intention, the rapeseed research groups in Northwest A&F University, Yangling, and Crop Research Institute, Prague-Ruzyně, have collected many *B. napus* accessions with different genes conferring yellow seed originating from China, Czech Republic and Poland. These accessions were used as the source to develop yellow-seeded restorer or maintainer lines for cytoplasmic male sterility (CMS). Revealing the genetic diversity among them is a necessary step for the good utilization of these yellow-seeded materials in the breeding program.

DNA markers such as AFLP play an important role in genetic diversity studies due to abundant production and elimination of environmental effects. The high genetic diversity obtained demonstrates the high efficiency of AFLP markers for genome analysis in *Brassica* (Lombard *et al.* 2000). Especially, AFLP with multiplex PCR using mixed multi-colour fluorescent-labelled primers saves the expensive reagents and allows a high throughput assay of the products (Sobotka *et al.* 2004). The objective of this study was to assess the measure of the genetic diversity of yellow-seeded *B. napus* accessions collected from China, Czech Republic and Poland by AFLPs with multi-colour fluorescent-labelled primers.

MATERIAL AND METHODS

Materials

Thirty-one yellow-seeded rapeseed accessions were used, including 16 Chinese original accessions, 14 doubled haploid lines from Slapy Breeding Station and Crop Research Institute, Czech Republic, and a spontaneous variant POLO-Y from the brown-seeded Polish cultivar Polo, which was kindly provided by Dr. Genlou Sun from Saint Mary's University, Halifax, Canada. The Chinese lines came from cultivars Yuhuang 1 and Yuhuang 2 (Li *et al.* 1998), Huangza 1 and Huangza 2 (Li & Tian 1999), Ningyou 10, Youyan9 and Youyan10 and from some breeding materials of Northwest A&F University, respectively. The pure yellow-seeded Czech DH lines were derived from partially yellow-seeded lines of *B. napus* resynthesized from yellowish-seeded *B. oleracea*, var. Furchenkohl and *B. rapa* var. Yellow Sarson (Vyvadilová *et al.* 1999). The inheritance of the yellow-seed trait of these DH lines was thought to be recessive,

similarly like in Yellow Sarson. Nevertheless, some yellow-seeded plants were found in two hybrids SLZ55C and C753 from the crosses between Chinese and Czech accessions. These two hybrids and another two N310-CZ and SLZS originating in the crosses of Chinese and Czech yellow-seeded materials were included as well. Five brown-seeded cultivars including two Chinese RY10, ZA2015 and three French ones, namely Caracas, Catonic and Californium, were chosen as outliers to indicate genetic distance between yellow-seeded and brown-seeded groups (Table 1).

DNA extraction and AFLP assays

The plants of all accessions were cultivated for one month in the greenhouse. Leaves from 20 seedlings for each accession were pooled together for DNA isolation. Genomic DNA was extracted according to the protocol of Doyle and Dickson (1987). AFLP was performed using the protocol described by Sobotka *et al.* (2004) and the Applied Biosystems AFLP Protocol (Applied Biosystems, Foster City, California, USA). Eighteen primer combinations involving six single MseI+3 and three fluorescently labelled EcoRI+3 primers (AAC, AAG, and ACA) were used (Table 2). After selective amplification, PCR products were analyzed using capillary electrophoresis on an ABI PRISM 310 sequencer. Electrophoretograms were analyzed by Gene Scan and Genotyper software (Applied Biosystems). The number of polymorphic peaks and their percentage from all detected peaks for each primer combination were recorded.

Statistical analyses

The total number of peaks with strong intensity (robust and high peaks) was counted and only clear-cut polymorphic (absent or present) peaks (markers) were scored and a binary matrix for AFLP data was established. Illegible markers with a molecular weight lower than 80 bp were excluded from the data matrix.

Nei and Li's dissimilarity coefficients (Nei & Li 1979) were computed from the binary matrix. Dissimilarity coefficient = $1 - 2c/(a+b)$, in which a is the number of bands of accession a, b is the number of bands of accession b and c means the total number of shared bands between two accessions a and b.

Dendrograms were constructed based on the dissimilarity matrices and UPGMA (Unweighted Pair Group Method with Arithmetic Mean) to show the structure of the diversity. Principal Coordinates Analysis (PCA) further analyzed the structure of the genetic diversity. All statistical analyses were performed with the software of NTSYSpc© computer program (Rohlf 2001).

RESULTS AND DISCUSSION

AFLP polymorphism and power of discrimination

The genetic diversity of yellow-seeded *B. napus* was evaluated in resynthesized lines using AFLP (Seyis *et al.* 2003) and in accessions derived from interspecific hybridization using biochemical and RAPD markers (Zhang *et al.* 2003). However, due to the difficulty in collecting the yellow-seeded germplasm, no further study was done to assess the genetic diversity of yellow-seeded *B. napus* accessions that were improved in quality and originated from various countries. This paper is the first study, which involved some advanced breeding materials of yellow-seeded *B. napus* collected from different breeding programs and countries.

Only 242 peaks were polymorphic out of the detected 723 AFLP markers (Table 2). The number of polymorphic markers per primer combination ranged from four to 22, with an average of 13.4 markers. The number of polymorphic markers per primer combination in this experiment was markedly lower compared to other research (Lombard *et al.* 2000; Seyis *et al.* 2003) because we discarded all other markers of minor peaks and other 35 markers that had an ambiguous peak in some samples, which could result in dilemmatic score. Another reason may be that the accessions in our experiment have a lower genetic diversity.

Dissimilarity values among accessions ranged from 0.028 to 0.516 for AFLP, with an average of 0.327. Cluster analysis allowed differentiation among the various lines. The dendrogram obtained by cluster analysis on the Nei and Li's distance matrix separated the Chinese from non-Chinese accessions (Figure 1). The first group included 21 accessions that were partitioned into three small clusters. The first cluster contained only the mutant POLO-Y, the second cluster included 14 Czech accessions, two hybrids SLZ55C and C753 from the crosses of Chinese and Czech accessions,

Table 1. The list of *Brassica napus* L. accessions used in the experiment, their origin and yellow-seedness inheritance

Code of accessions	Pedigree	Inheritance of yellow-seedness	Breeder or origin
YH03	Yuhuang 1	dominant	SWAU, China
YH04	Yuhuang 1	dominant	SWAU, China
YH1	Yuhuang 1	dominant	SWAU, China
YH2	Yuhuang 2	dominant	SWAU, China
HZ1	Huangza 1	partially dominant	HRRC, China
HZ2	Huangza 2	partially dominant	HRRC, China
Y0308	You0308	recessive	Guizhou, China
YY9	Youyan9	recessive	Guizhou, China
YY10	Youyan10	recessive	Guizhou, China
B23	Youyan10	recessive	NWAFU, China
B25	Youyan10	recessive	NWAFU, China
HUANG-C	Qinyou7 × Ningyou 10	partially dominant	NWAFU, China
C18	Qinyou7 × Ningyou 10	partially dominant	NWAFU, China
C19	Qinyou7 × Ningyou 10	partially dominant	NWAFU, China
B795	Qinyou 8 × Huangza2000-4	partially dominant	NWAFU, China
N310B	Huang 310	partially dominant	NWAFU, China
N310-CZ	N310B × Cz-var66		NWAFU, China
SLZ-S	1079 cms × Cz-var55		NWAFU, China
SLZ55C	Cz-var55 × D156		NWAFU, China
C753	D169 × SLZ02		NWAFU, China
SLZ55	Bn 153 × SL 511/1 DH line	recessive	Slapy, CRI, Czech Republic
SLZ66	Bn 153 × SL 511/1 DH line	recessive	Slapy, CRI, Czech Republic
SLZ02	Bn 153 × SL 511/1 DH line	recessive	Slapy, CRI, Czech Republic
SLZ1	Bn 153 × SL 511/1 DH line	recessive	Slapy, CRI, Czech Republic
SLZ2	Bn 153 × SL 511/1 DH line	recessive	Slapy, CRI, Czech Republic
SLZ3	Bn 153 × SL 511/1 DH line	recessive	Slapy, CRI, Czech Republic
SLZ4	Bn 153 × SL 511/1 DH line	recessive	Slapy, CRI, Czech Republic
SLZ6	Bn 153 × SL 511/1 DH line	recessive	Slapy, CRI, Czech Republic
SLZ8	Bn 153 × SL 511/1 DH line	recessive	Slapy, CRI, Czech Republic
SLZ14	Bn 153 × SL 511/1 DH line	recessive	Slapy, CRI, Czech Republic
SLZ23	Bn 153 × SL 511/1 DH line	recessive	Slapy, CRI, Czech Republic
SLZ28	Bn 153 × SL 511/1 DH line	recessive	Slapy, CRI, Czech Republic
SLZ29	Bn 153 × SL 511/1 DH line	recessive	Slapy, CRI, Czech Republic
SL28	Bn 153 × SL 511/1 DH line	recessive	Slapy, CRI, Czech Republic
POLO-Y	Polo	unknown	Poland
RY10	Rongyou10 Brown-seeded		SIAS, China
ZA2015	Rongyou11 Brown-seeded		SIAS, China
CARAC	Caracas Brown-seeded		Monsanto SAS, France
CATON	Catonic Brown-seeded		Monsanto SAS, France
CALIF	Californium Brown-seeded		Monsanto SAS, France

SWAU – Southwest Agriculture University, Chongqing, China; NWAFU – Northwest A&F University, Shaanxi, China; HRRC – Hybrid Rapeseed Research Centre, Shaanxi, China; SIAS – Second Institute of Agriculture Sciences, Sichuan, China; CRI – Crop Research Institute, Prague, Czech Republic; Slapy – Breeding Station of SEMPRA PRAHA Comp., Czech Republic

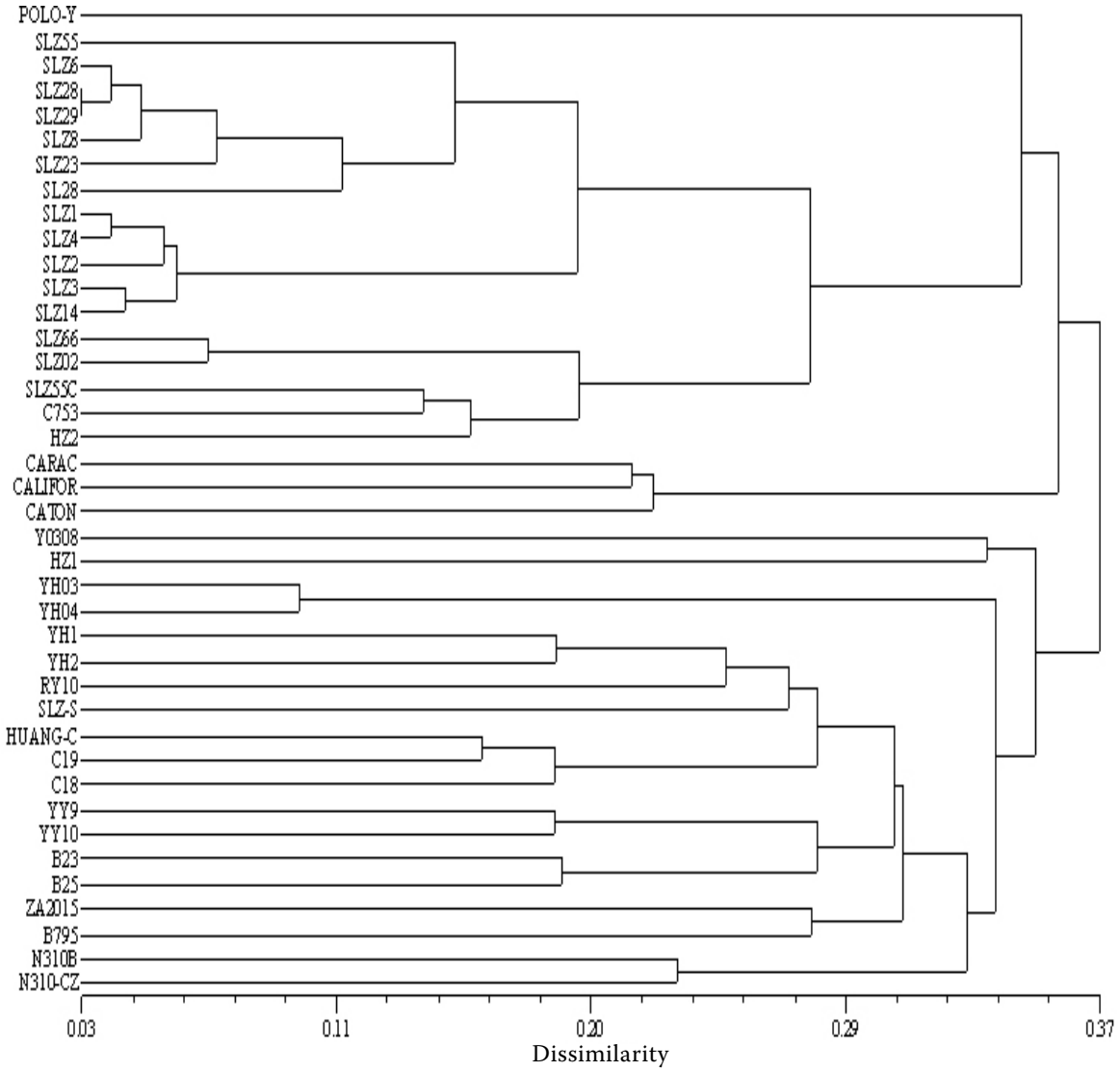

Figure 1. Clustering dendrogram of 40 rapeseed accessions based on 242 AFLP markers

and Chinese accession HZ2, and the third cluster included three brown-seeded French varieties from Monsanto, i.e. Caracas, Catonic, and Californium. The average genetic dissimilarity among Czech DH lines is very low due to the same origin. The second group included all 18 Chinese accessions and two hybrids N310-CZ, SLZS from the crosses of Chinese and Czech accessions. It was notable that the three brown-seeded French varieties Caracas, Catonic, and Californium were clustered together with Czech yellow-seeded DH lines while two Chinese brown-seeded hybrids RY10 and ZA2015 with the same female parent were isolated by different Chinese yellow-seeded accessions. This indicated that the genetic differences were influenced more by geographical origin than by seed-coat colour. A possible reason is that advanced breeding accessions originating from interspecific hybridization were introduced into common breeding materials by repeated backcrosses with advanced European cultivars. As it was expected, the sublines or accessions with similar pedigree or developed by the same breeders had a similar genetic basis as the AFLPs revealed. Therefore, for the production of yellow-seeded high yielding hybrids it is necessary to use materials of different origin. For instance, the Czech DH materials and Chinese accessions Y0308, YY9, and YY10 from Guizhou province could be used as maintainers of *Polima* CMS, and dominant genes conditioning the yellow-seed colour from Yuhuang1 and Ningyou 10 may be introduced into different restorers. A high yield

Table 2. The number and proportions of polymorphic fragments obtained with eighteen AFLP primer combinations in *Brassica napus* L.

Primer combinations	Total number of peaks	Number of polymorphic peaks	Percentage of polymorphic peaks
CTC/ACA	43	13	30.2
CTC/AAG	37	15	40.5
CTC/AAC	37	19	51.4
CTT/ACA	50	22	44.0
CTT/AAG	41	16	39.0
CTT/AAC	33	8	24.2
CAC/ACA	37	14	37.8
CAC/AAG	34	19	55.9
CAC/AAC	38	8	21.1
CTA/ACA	25	4	16.0
CTA/AAG	37	17	45.9
CTA/AAC	42	15	35.7
CAG/ACA	28	10	35.7
CAG/AAG	40	9	22.5
CAG/AAC	35	22	62.9
CTG/ACA	15	8	53.3
CTG/AAG	34	12	35.3
CTG/AAC	26	11	42.3
Total	632	242	38.3

potential was found in some cross combinations, for example in N310-CZ, SLZS, SLZ55C, and C753. Li *et al.* (1998) proved that most hybrids between the parents possessing different yellow-seed genes gave heterosis over the better parent for yellow seed colour. We also found that such yellow-seed combination as N310-CZ had the better seed-coat colour than its parents. Therefore the pyramiding of dominant yellow-seed genes will be a promising approach for both conventional and hybrid breeding. Nevertheless, some putative inhibitor genes for yellow seed in a specific genetic background (Li *et al.* 1998) could be involved in this approach. A further investigation on the heterosis of biological and agronomic traits among these yellow-seeded materials continues.

Principal Coordinates Analysis (PCA) is one of the most important methods of ordination analysis. It constructs a new set of orthogonal coordinate axes so that the projection of points (accessions) onto them has maximum variance. Associations among the 40 accessions revealed by PCA are presented in Figure 2. Principal Coordinates Analysis revealed the global structure similar to the dendrogram analysis but the distribution of these accessions was shown more clearly on three dimensions. The three previous small clusters included in the first group in cluster analysis were clearly isolated. Three brown-seeded French cultivars were placed at the negative end of the third dimension, 14 Czech accessions in the second cluster were partitioned into three small blocks along the second dimension, but the previous first cluster POLO-Y was put together with SLZ55C, C753, and HZ2 from the second cluster. It is worth mentioning that the 15 yellow-seeded and 2 brown-seeded Chinese accession, and two hybrids N310-CZ, SLZS were located far from the Czech group along the first principal coordinate showing their genetic distinctness in comparison with the Czech genetic pool. Therefore, the geographical origin was more spread along the first principal coordinate, i.e. genetic diversity is higher along this coordinate. It can be concluded from the above analyses that the grouping of accessions based on the cluster analysis and PCA was gener-

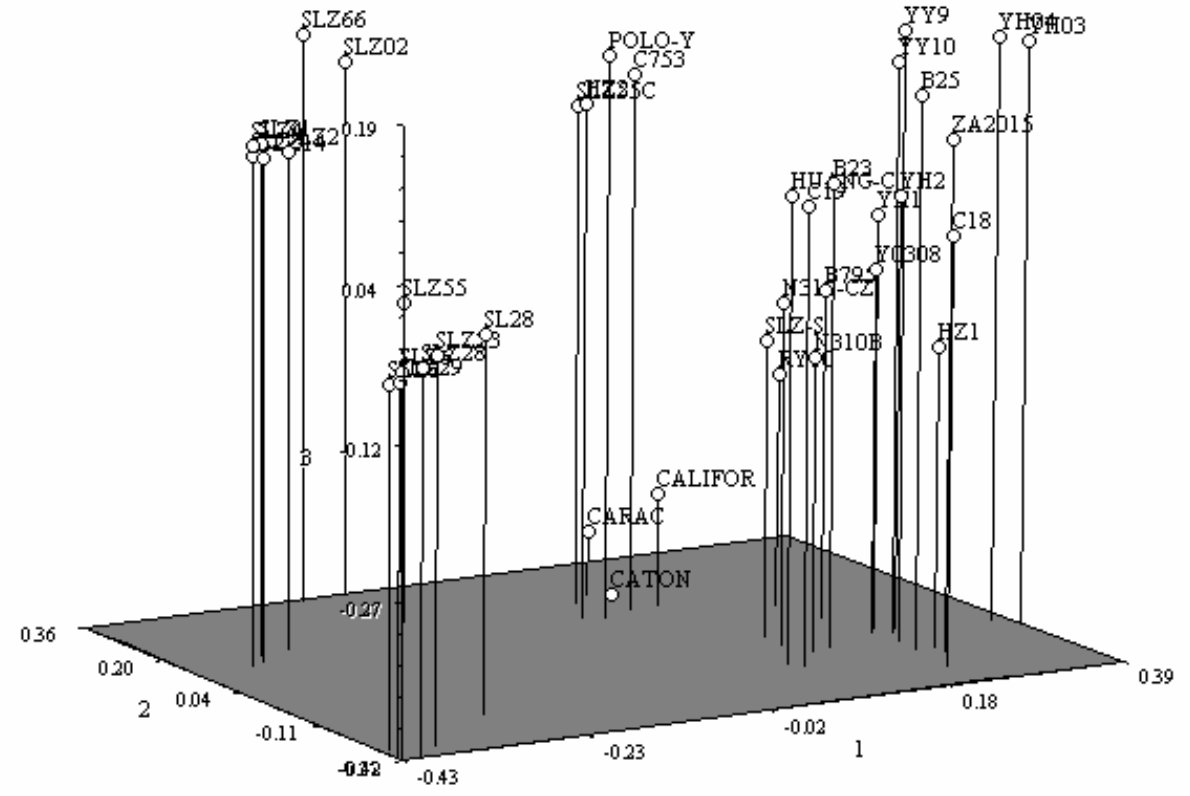

Figure 2. Principal coordinates analysis of 40 accessions based on the dissimilarity of AFLP data (the figure was rotated to its optimal visual angle)

ally consistent with known pedigree information and geographic origin.

In conclusion, a wide genetic diversity was observed between Chinese and European original brown-seeded accessions using RAPD markers (Hu *et al.* 2003), and a significant genetic diversity was found between Chinese and European yellow-seeded accessions as well. This information is helpful for rapeseed breeders of both sides to exchange the germplasm to enlarge the genetic diversity of breeding accessions.

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