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ORIGINAL PAPER

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Genetic distances revealed by morphological characters, isozymes, proteins and RAPD markers and their relationships with hybrid performance in oilseed rape (*Brassica napus* L.)

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Abstract Genetic distances (GDs) based on morphological characters, isozymes and storage proteins, and random amplified polymorphic DNAs (RAPD) were used to predict the performance and heterosis of crosses in oilseed rape (*Brassica napus* L.). Six male-sterile lines carrying the widely used Shaan2A cytoplasm were crossed with five restorer lines to produce 30 F_1 hybrids. These 30 hybrids and their parents were evaluated for seven agronomically important traits and their mid-parent heterosis (MPH) at Yangling, Shaanxi province in Northwest China for 2 years. Genetic similarity among the parents based on 34 isozyme and seven protein markers was higher than that based on 136 RAPDs and/or 48 morphological markers. No significant correlation was detected among these three sets of data. Associations between the different estimates of GDs and F_1 performance for some agronomic traits were significant, but not for seed yield. In order to enhance the predicting efficiency, we selected 114 significant markers and 43 favoring markers following statistical comparison of the mean values of the yield components between the heterozygous group (where the marker is present only in one parent of each hybrid) and the homozygous group (where the marker is either present or absent in both parents of each hybrid) of the 30 hybrids. Parental GD based on total polymorphic markers (GD_{total}, indicating general heterozygosity), significant markers (GD_{sign}, indicating specific heterozygosity) and favoring markers (GD_{favor}, indicating favoring-marker heterozygosity) were calculated. The correlation between GD_{favor} or GD_{sign} and hybrid performance was higher than the correlation between GD_{total} and hybrid performance. GD_{sign} and GD_{favor} significantly correlated with plant height, seeds per silique and seed yield, but not with the MPH of the other six agronomic traits with the exception of plant height. The information obtained in this study on the genetic diversity of the parental lines does not appear to be reliable for predicting F_1 yield and heterosis.

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Introduction

Early research on heterosis prediction focused primarily on analyses of the genetic effects by comparing the quantitative traits of parents and progenies, calculating the combining ability or using geographical diversity as the criterion to select parents for producing high-yield hybrids (Lefort-Buson et al. 1989; Ali et al. 1995). However, these approaches were unable to accurately reveal differences among parents, especially in *Brassica napus*, which originated from Europe and comprises many varieties having complex pedigrees. Since the heterosis in a hybrid is contributed by genetic complementation between divergent parents, it was assumed that the genetic distance (GD) between the parents affects the amount of heterosis expressed in an F_1 hybrid (Griffing and Linstrom 1954). Schwartz (1960) and Frei et al. (1986) proposed that heterozygosity in isozymes and allozymes could be used to predict heterosis in crops. More recently, DNA molecular technologies have become the favorite tools for predicting heterosis in maize (Goldshalk et al. 1990; Smith et al. 1990; Duley and Saghai 1991; Stuber et al. 1992; Shieh and Thseng 2002; Betran et al. 2003),

rice (Xiao et al. 1995; Zhang et al. 1996), wheat (Martin et al. 1995) and oilseed rape (Diers et al. 1996; Riaz et al. 2001; Liu et al. 2002; Sheng et al. 2002). However, some of the results obtained from the different types of research are contradictory with respect to the relationship between GD and heterosis. It has been found that not all polymorphic DNA fragments contribute to heterosis due to the considerable number of fragments that are either located in non-encoded regions or have no association with agronomically important traits. Consequently, it has been proposed that the discrimination between general heterozygosity and special heterozygosity might be useful for heterosis prediction (Zhang et al. 1996).

Significant heterosis for seed yield and other agronomic traits is well documented in oilseed rape (Morice 1979; Sernyk and Stefansson 1983). Many approaches have been exploited to utilize this heterosis, among which the cytoplasmic male sterility (CMS) system has been the most successful. In China, Shaan 2A and Polima are the two most useful cytoplasmic male sterility systems for hybrid breeding in *Brassica napus*(L.). Since the successful breeding and subsequent cultivation success (widely grown in China for nearly 20 years) of the first three-line commercial hybrid, Qinyou No.2, based on the Shaan 2A CMS system (Li 1986), more than ten commercial hybrids derived from Shaan 2A CMS have been registered. However, to date, no study on the relationships between GD and the performance and heterosis of the crosses involving Shaan 2A CMS has been conducted.

The present study reports an analysis of the relationships between the GDs among several oilseed rape lines with the Shaan 2A CMS system and their hybrids' performances using morphological characters, proteins (isozymes), and random amplified polymorphic DNA (RAPD). The objectives were: (1) to estimate heterosis for agronomic traits of winter rapeseed (*B. napus* L.) lines adapted to the region near the Huanghe River and Huaihe River in North China; (2) to assess the possibility of predicting hybrid performance by GDs based on the combination of morphological characters, isozymes, proteins and RAPDs. The information obtained in this study may be useful for the improvement of the hybrid performance in breeding programs.

Materials and methods

Plant materials and field experiments

Six *Brassica napus*(L.) Shaan 2A-type CMS lines derived from six elite maintainers of diverse geographic origins were crossed with five restorer lines (Table 1) to produce 30 hybrids. These 30 F_1 hybrids, the five restorers and the six corresponding maintainer lines (substituted for the six sterile lines to evaluate the agronomic traits) were planted in a randomized complete block design with three replications during the two crop seasons of 2001–2002 and 2002–2003 at Yangling, Shaanxi Province in Northwest China. Each plot consisted of five 2-m-long rows with 0.45 m between rows and 0.15 m between plants within rows. Hybrid performance, including the seed yield of each plot, was evaluated. Five plants in the middle row of each plot were used to measure six agronomic traits: plant height, number of primary branches per plant, number of siliques per plant, number of seeds per silique (the average of ten siliques in the middle section of the main inflorescence), one-thousand-seed weight and days to flowering. The mid-parent heterosis (MPH) was computed using the formula $MPH = 100 \times (F_1 - MP)/MP$, where F_1 is the hybrid mean and MP is the mid-parent mean. Seventeen morphological characters were measured during the growing season (Table 2). Using a well-known numerical classification method described by Sneath and Sokal (1973), we classified each character into two to five rankings as genetic markers, and a total of 48 morphological markers were used to group the parents. A ranking present in a parental line was scored as 1, while absent was scored as 0.

Analyses of isozymes, proteins and RAPDs

We examined six isozyme systems: (1) acid phosphatase (ACP, E.C.3.1.3.2), peroxidase (POD, E.C.1.11.1.7), catalase (CAT, E.C.1.11.1.6), esterase (EST, E.C.3.1.1.2) in young seedlings cultivated in water for 7 days; (2) glutamate oxalacetate transaminase (GOT, E.C.2.6.1.1) and α-amylase (α-AMY, E.C.3.2.1.1) in seeds imbibed for 1 day. These enzymes were extracted following

Table 1 The winter-type *Brassica napus* parental lines used in this study

Lines	Pedigree	Origin	Lines	Pedigree	Origin
A1	Shaan 2A × B_1^n	Shaanxi, China	B4	D89	Jiangsu, China
A2	Shaan 2A × B_2^n	Shaanxi, China	B5	Zhongshuang No.2	Hubei, China
A3	Shaan 2A × B_3^n	Henan, China	B6	1500100285	Czech Republic
A4	Shaan 2A × B_4^n	Jiangsu, China	C1	L161	Shaanxi, China
A5	Shaan 2A × B_5^n	Hubei, China	C2	Qinyou No.3	Shaanxi, China
A6	Shaan 2A × B_6^n	Czech Republic	C3	227	Henan, China
B1	L451	Shaanxi, China	C4	Qinyou No.1	Shaanxi, China
B2	L89	Shaanxi, China	C5	Yuyou No.1	Henan, China
B3	L656	Henan, China			

Table 2 Morphological characters and their ranks

Characters	Rank 1	Rank 2	Rank 3	Rank 4
Cotyledon shape	Cordate	Reniform	-	-
Cotyledon conformation	Flat	Curling	-	-
Cotyledon size	< 0.8 cm	0.8–1.2	> 1.2 cm	-
Leaf color	Olivine	Green	Dark green	Glaucous
Number of seta	None	Few	More	-
Amount of wax	None	Little	More	Most
Color of central leaf	Green	Light purple	Purple	-
Conformation of seedling leaf	Concave	Flat	Convex	-
Hypocotyl color	Green	Light purple	Purple	-
Petiole bent	Straight	Bent	-	-
Petiole color	White	Purple	-	-
Number of side leaflets	1–2 pair	Three to four pairs	More than five pairs	-
Margin of leaf	Entire	Serrulate	Crenate	Dentate
Growing habit of seedling	Half erect	Prostrate	-	-
Period of flowering	< 32 days	≥32 days	-	-
Seed size (1,000 seeds)	< 2.5 g	2.5–3.5	> 3.5 g	-
Density of silique in main inflorescence	< 1.1/cm	1.1–1.6	> 1.6/cm	-

standard procedures. Polyacrylamide gel electrophoresis (PAGE) was used to produce the zymogams. The soluble storage proteins of degreased seeds were run on sodium dodecyl sulphate (SDS)-PAGE as described by Gan and Wang (1999). Electrophoresis and staining of the isozymes and proteins were as described by Vallejos (1988). The protocols for DNA isolation and RAPD were as reported by Hu et al. (2003). The decamer primers used in the RAPD-PCR analyses were purchased from the Bioasia Company (Shanghai, China).

The isozyme, protein and RAPD bands, which was polymorphic in the 11 parental lines, were scored as 0 (absent) or 1 (present) for each parent. A binary matrix was constructed to estimate the GD between a pair of parental lines using the formula of Nei and Li (1979), $GD = 1-2c/(a+b)$, in which c is the number of shared bands between two parents, and a and b are the total number of each parental plant, respectively.

Simple factor *t*-test to select significant and favoring markers

The concepts of general marker, significant marker and favoring (or increasing-effect) marker proposed by Zhang et al. (1996), Liu et al. (2002) and He et al. (2002) were employed to estimate general heterozygosity, specific heterozygosity (Zhang et al. 1996) and favoring-marker heterozygosity, respectively. For each genetic marker, we assigned the 30 F_1 hybrids to two groups: (1) a homozygous group, in which the marker was present or absent in both parents; (2) a heterozygous group, in which the marker was present only in one parent. The simple factor *t*-test was used to test the difference in three yield components (siliques per plant, seeds per silique, and 1,000-seed-weight) between the two groups. If a significant difference was detected between the two groups, the marker was considered to be a significant marker for the yield component; if the yield component of the heterozygous group was higher than that of the homozygous group, the marker was denoted a favoring marker. The statistics test was carried out using SPPS 10.0 for Windows (SPSS 1999).

Results

Genetic polymorphisms among parental lines

On polyacrylamide gel, the zymograms of each isozyme system and soluble storage proteins were revealed by different staining compounds. At the cotyledon stage of all the parental lines, the ACP isozyme showed eight polymorphic bands and one monomorphic band, both CAT and POD had four polymorphic bands and two monomorphic bands, and EST had 14 polymorphic bands and three monomorphic bands. Two of the three GOT and two of the three α-AMY bands of the imbibed rape seeds were polymorphic. In total, 18 soluble storage protein bands were detected in the degreased seed by SDS-PAGE, but only seven were polymorphic (Table 3). The percentage of biochemical (isozyme and storage protein) polymorphic bands was 66.13%.

The parents were screened with 100 RAPD primers. Of these, 34 reproducible primers were chosen and generated 241 distinct bands, of which 136 bands were polymorphic (Table 3) with an average of four polymorphic bands per primer. The polymorphic ratio was 52.18%.

Genetic distances

Genetic distances based on 48 morphological markers (GD_{mor}), on 34 isozymes and seven soluble protein bands (GD_{iso}) and on 136 RAPD bands (GD_{rapd}) were computed for each class of polymorphic markers. GD_{mor} ranged from 0.455 to 0.833, with an average of 0.648; GD_{iso} varied from 0.143 to 0.535, with an average of 0.297; GD_{rapd} was from 0.309 to 0.553, with an

Table 3 The number of isozyme, protein and RAPD polymorphic bands

Isozymes, proteins or primers (with sequence)	Polymorphic bands	Isozymes, proteins or primers (with sequence)	Polymorphic bands
ACP	8	BA353 (CCACACTACC)	4
POD	4	BA361 (CATTCGAGCC)	2
CAT	4	BA370 (GTGCAACGTG)	4
α-AMY	2	BA377 (CCCAGCTGTG)	5
GOT	2	BA379 (CACAGGCGGA)	5
EST	14	BA380 (GTGTCGCGAG)	2
Soluble storage proteins	7	BA381 (GGCATGACCT)	4
BA41 (ACCGCGAAGG)	8	BA383 (CCAGCAGCTT)	2
BA42 (GGACCCAACC)	1	BA384 (GACTGCACAC)	8
BA48 (GTGTGCCCCA)	6	BA387 (AGGCGGGAAC)	6
BA50 (GGTCTACACC)	4	BA392 (GGGCGGTACT)	5
BA60 (ACCCGGTCAC)	7	BA393 (ACCGCCTGCT)	9
BA89 (CTGACGTCAC)	3	BA395 (AAGAGAGGGG)	2
BA152 (TTATCGCCCC)	1	BA403 (GGGGGATGAG)	4
BA304 (CCGCTACCGA)	3	BA406 (CTGGGCAACT)	2
BA306 (ACGCCAGAGG)	2	BA425 (ACTGAACGCC)	2
BA307 (GAGCGAGGCT)	2	BA1098 (GGAGTGGACT)	2
BA312 (TCGCCAGCCA)	6	BA1102 (ACTTGACGGG)	8
BA345 (CTCCATGGGG)	3	BA1111 (AGATGCGCGG)	8
BA346 (TCGTTCCGCA)	2	BA1171 (CTGGCTTCTG)	2
BA352 (GTCCCGTGGT)	2	Total	177

average of 0.434. No significant correlation among these three GDs was observed. Genetic similarity among all parents based on isozymes and soluble proteins was higher than that based on morphology and RAPD data. A noteworthy observation was that the average GD_{iso} and GD_{rapd} of each restorer versus maintainers was larger than that of each maintainer versus the restorers (Fig. 1).

Relationships between GD and hybrid performance

There were significant phenotypic variations among the 30 hybrids and 11 parental lines and significant heterotic effects for seed yield and the other six traits (data not shown). GD_{mor} showed no significant correlation with any of the all seven agronomic traits. However, significant associations between GD_{iso} and number of primary branches (−0.551), GD_{iso} and 1,000-seed-weight (−0.436), GD_{iso} and days to flowering (0.428), GD_{rapd} and number of siliques per plant (−0.480), GD_{rapd} and number of seeds per silique (0.389) were detected (Table 4). GD_{total} (based on all markers) was calculated in order to estimate the general heterozygosity of each hybrid. We found that it was significantly correlated with plant height and number of siliques per plant (0.461 and −0.432, respectively).

Based on the *t*-test for agronomic traits between marker-heterozygosity group and marker-homozygosity group of the 30 hybrids, we selected 114 significant markers, including 20 morphological markers, 22 isozyme (protein) markers and 72 RAPD markers, to estimate the specific heterozygosity (GD_{sign}) for each cross. The association between GD_{sign} and hybrid performance such as plant height ($r = 0.433$, $P < 0.05$), number of seeds per silique ($r = 0.560$, $P < 0.01$) and seed

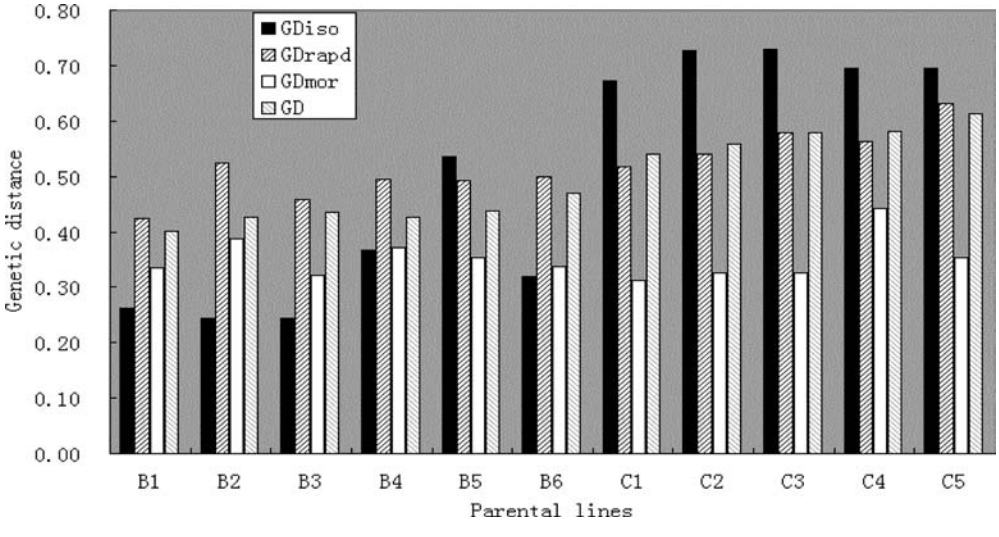

Fig. 1 Average genetic distance (*GD*) of each maintainer versus restorers or each restorer versus maintainers

Table 4 Correlation coefficients between genetic distance (GD) and hybrid performance

Traits	Plant height	Primary branches	Siliques per plant	Seeds per silique	1,000-seed weight	Days to flowering	Seed yield
GD_{mor}	0.050	0.057	−0.126	0.167	0.080	−0.041	0.126
GD_{iso}	0.130	−0.551**	−0.102	−0.037	−0.436*	0.428*	−0.144
GD_{rapd}	0.351	−0.333	−0.480*	0.389*	−0.034	0.163	0.269
GD_{total}	0.461*	−0.191	−0.432*	0.330	−0.026	0.329	0.320
GD_{sign}	0.433*	0.074	−0.228	0.560**	0.110	−0.222	0.514**
GD_{favor}	0.422*	0.225	−0.069	0.448*	0.232	−0.307	0.580**

*, ** Significant at the 0.05 and 0.01 probability levels, respectively

yield ($r = 0.514$, $P < 0.01$) were higher than the GD_{total} associated with these traits (Table 4).

Subsequently, 29 RAPD markers, seven isozyme markers and seven morphological markers, whose heterozygosity in the hybrid were favoring factors for seed yield components, were selected from the 114 significant markers in order to calculate the favoring-marker heterozygosity of each cross. The heterozygous state of four ACP, three leaf-color and two silique-density markers was positively and significantly correlated with the agronomic traits, whereas heterozygous state of the CAT and POD markers was negatively and significantly correlated with hybrid performance. No qualitative morphological markers had a significant association with the seven agronomic traits. The correlations between genetic distance based on favoring markers (GD_{favor}) and plant height ($r = 0.422$, $P < 0.05$), seeds per silique ($r = 0.448$, $P < 0.05$) and seed yield ($r = 0.580$, $P < 0.01$) were also significant as GD_{sign}. The degree of GD variation among the 30 hybrids was $GD_{favor} > GD_{sign} > GD_{total}$ (Fig. 2). Figure 3 shows the relationship of GD_{sign} and GD_{favor} with seed yield. The

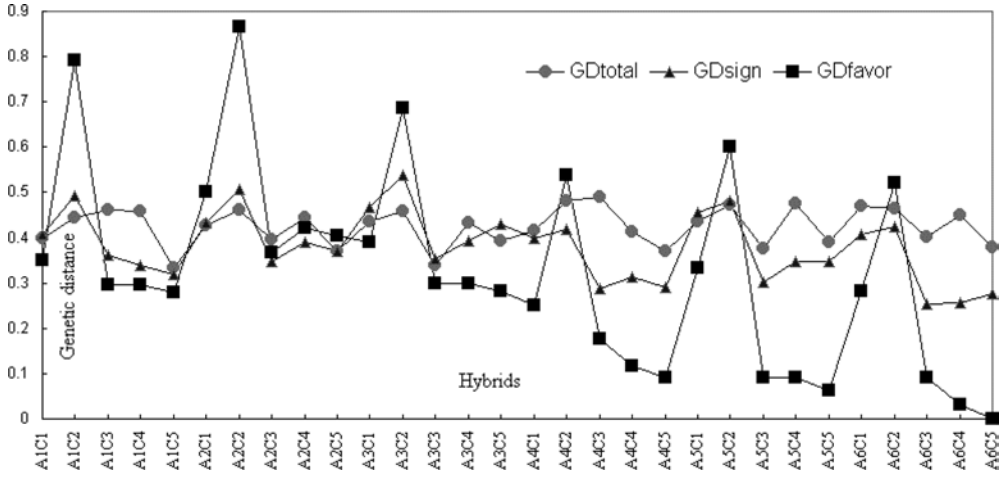

Fig. 2 GD_{total}, GD_{sign} and GD_{favor} between the two parents of each hybrid

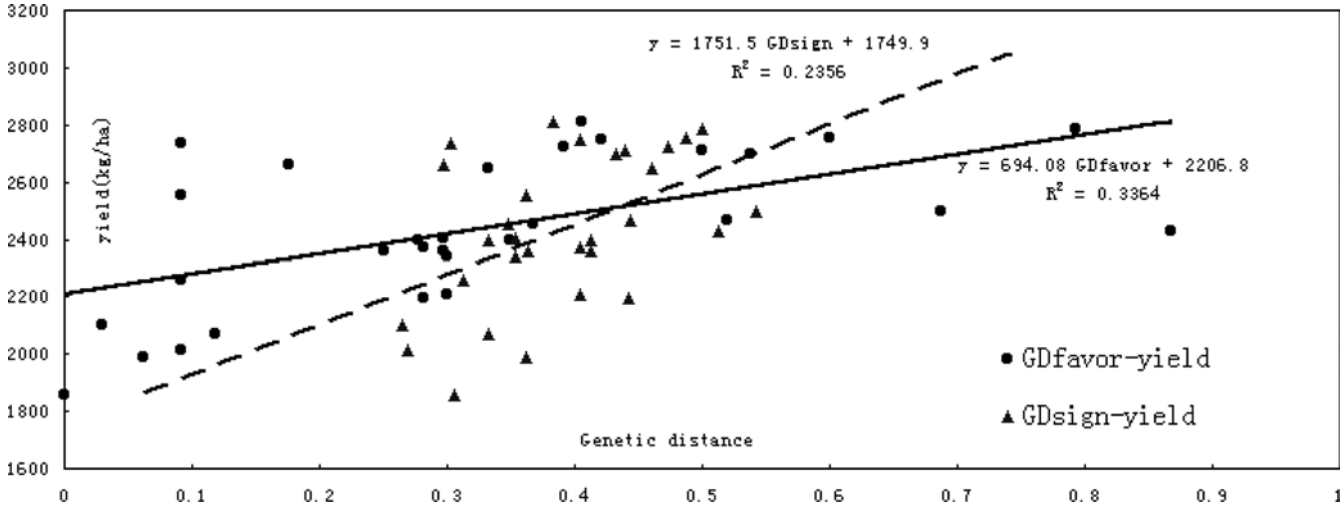

Fig. 3 The relationship of GD_{sign} and GD_{favor} with seed yield

line of increase in the hybrids' seed yield (black circle) was not consistent with the increase in the GD, suggesting that the genetic diversity approach is still not practical means for breeders to select the best crosses.

Relationships between GD and MPH

GD_{iso} was significantly correlated with the MPH of 1,000-seed-weight ($r = -0.475$, $P < 0.01$) and days to flowering ($r = 0.524$, $P < 0.01$); GD_{total} was significantly correlated with the MPH of siliques per plant ($r = -0.365$, $P < 0.05$), However, the correlation between GD_{total} and the MPH values of seed yield was not significant ($r = -0.334$). No significant relationship was observed between GD_{sign} or GD_{favor} and MPH of the other six agronomic traits except for the MPH of plant height (Table 5).

Discussion

While the collection of phenotypic information is practicable in field, the prediction of hybrid performance based on the phenotypes of quantitative traits is difficult due to the unsteadiness of the latter as the environment changes. Moreover, most quantitative traits are agronomically important traits per se, hence their heterozygous state in the parents may be adverse to the production of a superior hybrid. Most of the morphological characters used as genetic markers in this study were steady-state qualitative traits found at the seedling stage, but the heterozygosity of these qualitative characters did not correlate with hybrid performance as significantly as some quantitative traits did.

Cerna et al. (1997) reported that there was no association between GD estimated by RFLP analysis and seed yield heterosis in soybean (*Glycine max*), whereas an association was observed between isozyme loci and heterosis for seed yield. Qian et al. (1999) found that some allozyme loci associated with quantitative trait loci (QTL) directly or indirectly in maize. In our study, we also found that some isozyme loci, such as acid phosphatase, did correlate significantly with the quantitative traits of the hybrid. Despite this correlation however, the practical use of isozyme markers alone to select parents may be limited because of a lack of assayable isozyme loci.

Heterosis in a hybrid is achieved through the genetic complementation between divergent parents. As reported in the literature, the relationship between genetic heterozygosity and hybrid performance has not been consistent among the many different studies using different species, different materials or different environments. In maize, Smith et al. (1990), Stuber et al. (1992) and Betran et al. (2003) reported that yield heterosis was significantly correlated with parental molecular diversity, but Goldshalk et al. (1990), Duley and Saghai (1991) and Shieh and Thseng (2002) obtained the opposite results. Such contradictions have also been reported in rice (Xiao et al. 1995; Zhang et al. 1996; Hua et al. 2002), wheat (Martin et al. 1995; Corbellini et al. 2002), grain sorghum (Jordan et al. 2003) and sunflower (Cheres et al. 2000). The major reason for this contradiction may be the particularities of different agronomic traits and genotype-environment interaction in different crops.

There have been fewer investigations on the mechanism of heterosis, heterotic grouping and the criterion for selecting parents using molecular markers in oilseed rape than in other crops. Diers et al. (1996) analyzed the relationships between heterosis of Canadian *B. napus* accessions and RFLP distance and found that neither GD nor general combining ability was consistently correlated with heterosis for inbred dialleles and for cultivar dialleles. Sheng et al. (2002) reported that the correlation between GD and seed yield of a hybrid derived from self-incompatible lines was significant but that the determinative coefficient was very low (0.1024). However, Riaz et al. (2001) found that the GD of sequence-related amplified polymorphism (SRAP) in American *B. napus* inbred lines was significantly correlated with hybrid yield and heterosis, and Liu et al. (2002) suggested that selected favoring loci could be used to predict biomass heterosis in tri-genomic hybrids of *B.napus* × *B. campestris*. In the present study, the general heterozygosity, an integrated genetic distance of three types of genetic markers, did not provide a satisfactory prediction of hybrid performance, whereas the significant marker heterozygosity and favoring-marker heterozygosity led to a moderate improvement in prediction. When data published in previous studies are also taken into consideration, we may conclude that although some heterotic groups can be identified on the basis of marker heterozygosity, the associations between

Table 5 Correlation coefficients between GD and hybrid mid-parent heterosis

MPH	Plant height	Primary branches	Siliques per plant	Seeds per silique	1,000 seed weight	Days to flowering	Seed yield
GD_{mor}	0.035	0.404	0.197	0.012	0.074	−0.154	0.164
GD_{iso}	−0.325	−0.293	−0.066	0.196	−0.475**	0.524**	−0.114
GD_{rapd}	−0.273	−0.128	−0.328	0.305	−0.139	0.007	−0.341
GD_{total}	−0.230	−0.129	−0.345	0.034	−0.078	0.250	−0.308
GD_{sign}	0.259	0.127	−0.021	0.171	−0.129	−0.131	−0.249
GD_{favor}	0.450*	0.295	0.071	−0.005	0.082	−0.256	−0.036

*, ** Significant at the 0.05 and 0.01 probability levels, respectively

GD and hybrid performance and heterosis are inconstant. Consequently, our information on the genetic diversity of the parental lines does not appear to be a reliable basis for predicting F_1 yield and heterosis, thereby indicating the need to develop specific strategies for identifying parental lines with a high level of combining ability. An alternative approach is to search for the QTLs or differential expression genes involved in heterosis expression. Xiao et al. (1995) found that dominance was the major genetic basis of heterosis in rice, as determined by QTL analysis using molecular markers. Sun et al. (2004) found four different expression patterns of fragments to be correlated with heterosis. The results of Graham et al. (1997) indicated that some genetic factors are in repulsion phase linkage and that their effects support the dominance theory of heterosis. These results indicate that there is a very intricate association between polymorphic markers and the QTLs involved in heterosis.

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