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Morphological and genetic characteristics of hybrid combinations of *Dactylis glomerata*

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ABSTRACT. Six F_1 populations derived from crosses among 4 orchardgrass (*Dactylis glomerata* L.) cultivars were studied by morphological and simple sequence repeat molecular markers to test for hybrid vigor and a correlation between genetic distance and heterosis. Heterosis was observed for days to length of culm, leaf traits, tiller numbers, etc. Significant differences between obverse and inverse crosses were found for eleven traits. A cytoplasmic effect existed for the agronomic traits considered in this study. The correlations between genetic distance and heterosis were investigated by analyzing the performance of 3 crosses. The results showed that genetic distance was significantly correlated with tiller number (r = 0.834) and negatively correlated with length of culm (r = -0.889). However, there was no significant correlation with heterosis for the other traits, including yield; the correlation coefficient were too small to allow prediction of orchardgrass heterosis from the parental genetics.

Key words: *Dactylis glomerata*; Hybrids; Morphological characters; SSR

INTRODUCTION

Orchardgrass (*Dactylis glomerata* L.) is one of the most commonly used forage species and has become naturalized in nearly every continent. It has great vigor in root growth and regrowth characteristics, and has good forage quality (Rawnsley et al., 2002; Sanada et al., 2010). Orchardgrass breeding has been conducted by the public sector mainly in the USA, Canada, and other countries. Ochardgrass has also been widely cultivated in southern China, providing important social and ecological benefits (Xie et al., 2009).

Hybridization is frequently practiced in agriculture to make stronger, healthier plant with desirable characteristics. Some cultivars produced from hybridization have improved agronomic traits, such as high hay yield, excellent palatability, and strong stress tolerance. Moreover, in countries like in Italy, Canada, and Australia hybridization has been used in orchardgrass breeding to improve seed retention, over-summering, rust diseases resistance, and other desirable traits (Knight, 1968; Falcinelli, 1991; Casler et al., 2000). Chinese orchardgrass genetic resources are diverse and most abundant in the southwest and northwest, which, over the past 20 years, have played an important role in forage-breeding programs utilizing wild germplasms for domestication. Three cultivated varieties have recently been developed from wild germplasms, including Gulin, Baoxing, and Chuandong, whereas Kaimo was an introduced variety. They have been widely used in cultivated pasture, with high yield and good adaptability to local environments (Peng et al., 2008). Therefore, it is important to exploit available *D. glomerata* germplasms, including cultivated varieties and wild materials, for further breeding purposes. So far, there are few reports on hybridization breeding of orchardgrass and no orchardgrass hybrid variety has been released in China. Hybridization can pyramid beneficial genes, and the hybrids have higher adaptation and improved resistance traits than their parental lines (Posselt, 2010). Barclay developed the hybrid variety "Grassland Kara" derived from the cross between "Grassland Apanui" orchardgrass and 2 Portuguese populations. "Grassland Wana" was developed from the accession Bc5659, and after observing and evaluating agronomic traits, 30 promising plants were chosen and interpollinated in a field isolation (Rumball, 1982a,b). Zhong and colleagues (Zhong, 2006, 2007) obtained the orchardgrass hybrids from crosses between 2 tetraploid individuals, which represented valuable breeding materials with desirable and improved traits. Evaluation of morphological characteristics is most commonly used to test the performance of newly generated hybrid plants. Although this approach has been widely used in breeding programs, it is easily affected by the environment. By contrast, molecular markers are much more stable and accurate. Several studies have successfully used combinations of these 2 approaches to identify hybrid plants with improved traits (Astarini et al., 2008; Lakušić et al., 2009; Joung et al., 2011).

The aims of breeding new orchardgrass varieties through hybridization are improved forage yield, quality, tolerance to abiotic stress, and seasonal distribution of forage. In order to achieve these goals, 3 hybrid combinations were generated by hybridizing 4 cultivated *D. glomerata* varieties with each other to produce intraspecific hybrid orchardgrass plants. The current study was carried out to (a) evaluate the agronomic performance of the hybrid vigor derived from the crosses between orchardgrass cultivars, (b) detect the genetic relationship between parents and progenies using SSR markers, and (c) test for possible correlations between genetic distance and heterosis.

MATERIAL AND METHODS

Site studied and materials

The field test was carried out at the forage germplasm repository in Sichuan Agricultural University, in Ya'an, Sichuan Province, China (29°58'N, 102°59'E). The soil type at this site was purplish soil (pH = 5.6), containing available nitrogen at 100.63 mg/kg, phosphorus at 4.73 mg/kg, and potassium at 338.24 mg/kg. Plants were spaced at 0.5 x 1 m, and plot sizes ranged from 4 to 10 m^2 depending on the number of plants. The plants were grown under normal field conditions without additional fertilization.

Three hybrid combinations H1, H2, and H3 were obtained by crossing the following 4 cultivars of tetraploid orchardgrass (2n = 4x = 28): "Chuandong", "Gulin", "Baoxing", and "Kaimo". Three hybrid combination comprised 6 reciprocal crosses, resulting 58 individual plants in total (Table 1).

Table 1. Parents and hybrids used in this study.

Code	Female	Male	Code of the generations								
H1a	CHD	GL	H1a-1	H1a-2	H1a-3	H1a-4	H1a-5	H1a-6	H1a-7	H1a-8	
H1b	GL	CHD	H1b-1	H1b-2	H1b-4	H1b-5	H1b-6	H1b-7			
H2a	CHD	BX	H2a-1	H2a-2	H2a-3	H2a-4	H2a-5	H2a-6	H2a-7		
H2b	BX	CHD	H2b-1	H2b-2	H2b-3	H2b-4	H2b-5	H2b-6	H2b-7	H2b-8	H2b-9
			H2b-10	H2b-11	H2b-12	H2b-13	H2b-14	H2b-15	H2b-16	H2b-17	H2b-18
H3a	BX	KM	H3a-1	H3a-2	H3a-3	H3a-4	H3a-5	H3a-6			
H3b	KM	BX	H3b-2	H3b-3	H3b-4	H3b-5	H3b-6	H3b-7	H3b-8	H3b-10	H3b-11
			H3b-12	H3b-13	H3b-14	H3b-15					

CHD = Chuandong; GL = Gulin; BX = Baoxing; KM = Kaimo.

Hybridization

Four cultivars were chosen on the basis of their different morphological traits. Orchardgrass has self-incompatibility and inbreeding depression systems. Individual parental plants were grown as clones for one year and tillers separated into 12 individuals. One individual plant from each variety was used to generate the F_1 population with isolated pollination. Six reciprocal crosses were made. To compare the level of heterozygosity between parents and progenies, 3 hybrid combinations were made by randomly crossing varieties. Seeds were collected from female plants separately during June to July 2010, and were germinated in culture dishes in late August. After 3 weeks of germination and growth, the seedlings were transplanted into experiment plots.

DNA extraction

Genomic DNA was extracted from individual hybrid plant tissues (0.1-0.2 g) using the CTAB method described by Saghai-Maroof et al. (1984). The concentration of the DNA was adjusted with ddH_2O to 10 ng/μL and stored at 4°C.

SSR assays

The SSR-PCR protocol for *D. glomerata* was followed as described by Xie et al.

(2008), and performed in a 15-μL reaction volume containing 50 ng DNA template, 2.5 mM Mg^{2+}, 1U Taq DNA polymerase, 240 μM dNTP, and 0.4 μM of each primer. All reactions were carried out in a Thermo Hybaid PCR thermocycler. PCR amplification was performed as follows: 1 hold at 94°C for 4 min, followed by 35 cycles of DNA denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 1 min; the amplification was completed after final extension for 10 min at 72°C, and stored at 4°C. The PCR amplification products were separated on a 6% (w/v) denaturing polyacrylamide gel and visualized by silver staining.

Primer selection

Twenty-seven primers selected from 44 are listed in Table 2; they were synthesized by Shanghai Sangon using published sequences (Xie et al., 2008, 2009).

Table 2. Twenty-seven primers selected for SSR of *Dactylis glomerata*.

Primer No.	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
A01E02	AGCTGTGGAGAAAAAAATGA	GATGCCATTAAGTTCAAAATG
B01A05	GAGAGCGGCAGAGTTATTC	AAAGGTCGATATCTCTATTCCA
A01K14	AAGGATGGCCTGATCTTC	GCAGAGGTCTTTCTCTTGG
B01C15	GTCGATTGATGGTGTACGTA	TCTAGTGCTACTTGTATGCACC
A01B10	TCTTCCTTGGAAAACATCAA	ACTTGCTTACACGGTATCATG
A01E14	ACCCGTTTTCTATCTCCAG	GTTCTAGCGTCGTGAGGG
A02B24	GACGAGGCATGTTTGTTG	CTCTATAAAACCCATGAGCG
A03M21	TTCTACAGCTTGCACTGATG	AAGTGGACAGTTGACACTCC
A02G09	TACACGAGAGGGCAGATACT	CGTAACTTGAATCTTCCAGG
A04C24	AGCAACATATCTTACTGCAATG	ATCAAACTCGAAAAGTTGTCA
A02I05	GCAAATGTCCACACCATT	CTACCACAGCGACATCAAG
A02N22	AAACATGTCGTGGTCGTC	ATCATTTGTTATGCCGGTAG
A03K22	AGACTCTAGGGTGGCACAC	GTAGCACGCTAACGAGAGAT
A03B16	TCTGGAATCTCTCTGAAATCA	ATCTTGACCCTGATGTTCTG
A04O08	AGAGGTTAGATGGATGTAGGC	ATAGACCCATAGCATGTTGG
A03C05	TAAGAATCGATCCTCCCG	ACCTTCTTCCACTCCGTC
B01B19	AGAAGTTGGCCTGTCTCC	CTCTTCCTTCCTCCTTGG
A01F24	AAAATGTTTTATTCTCAGCCC	TGCAAGATGGAATGCTCT
B01E09	ACAACTCACAAACTCAAGAACA	GTGGACTCGGAGGAGAAG
A02A10	AGGTTACCGATAGTAAGTGGG	AGGGGATGGTTGGTTAGTAT
A01I11	CATCGTAATGACTGCTAGTCC	ACAGATCCATCGGTGGTT
B01C11	GCCATGTAACCAGAATCCTA	TGTTTGTGCATAGATCAAGC
B01D10	GGGAGATCTCAGTGGAGG	CCGTGATAACTCATAAACAGC
A02J20	TCCAATGTTACACACATAGCA	TGTGTGCGATTTTCTGTG
B01F08	ATTAGTCCGTGTCTCCCAC	TTATCGAGACCTCCAGGAG
A01L12	GGCTCAATCCTTAGACACTG	ACGAGAAATCGTCGTATTGT
A01L14	GCACAATGACACCAAATATG	ATCAGCATTGTGACCACC

Morphological analyses

Length of the culm (LC), length of the flag leaf (LF), length of the second leaf (LL), width of the flag leaf (WF), width of the second leaf (WL), culm diameter (CD), length of the internode (LI), number of the internode (NI), tiller number (T), individual yield (Y), and growing period (GP) were measured in parental and F_1 hybrid plants. The data used in this paper were collected in the blooming period with 5 replications used to calculate average values for each trait.

Data analysis

Morphological characters

Values for the eleven traits represent the means determined from the replicates. The heterosis of F_1 hybrids was estimated as mid-parent and higher-parent values by using the following formulas: mid-parent heterosis (%) = (F_1 - MP) / MP x 100% and higher-parent heterosis (%) = (F_1 – HP) / HP x 100%, where F_1 is the mean of the hybrid, MP is the mean value of parents and HP indicates the value of higher parent. The significance testing on 3 reciprocal combinations was carried out by using SPSS 17.0. The Euclidean distance cluster analysis based on morphological traits was conducted using NTSYS pc 2.10 (Rohlf, 2000).

SSR markers

Each polymorphic band detected was considered an allele. The SSR bands were scored as present "1", or absent "0". Genetic diversity analyses were carried out on the basis of these scores. Genetic similarity (GS) was calculated as described by Nei and Li (1979). Genetic distance (GD) was calculated as GD = 1 - GS. The Popgene v.1.31 software was used to analyze the data by using Nei's genetic diversity index (H), Shannon diversity index (I), and numbers of alleles (N_A) (Yeh et al., 1999). Clustering analysis of the hybrid and parental plants was conducted with NTSYS pc 2.10 using the unweighted pair group method with arithmetic average (UPGMA) method based on GDs.

Correlation analysis

The relationship between genetic distance and heterosis was analyzed using SPSS 17.0.

RESULTS

Heterozygosity comparison between parents and progenies

Heterozygosity should be compared among progeny and parents before evaluating heterosis in self-incompatible forage grasses. Therefore, 9 inter-variety crosses (shown in Table 3) were made and analyzed. The GDs among the inter-variety crossings were calculated and are listed in Table 4. The GDs among progeny from inter-variety crossing were all smaller than the GDs among the intra-variety progeny. According to these results indicating that the individual progeny plants were more heterozygous than their parent plants, heterosis could be evaluated in this study.

Hybrid performance and heterosis

Table 5 shows mid-parent heterosis (MPH), higher-parent heterosis (HPH), and coefficient of variation (CV) of eleven agronomic traits for 3 cultivar hybridizations represented by 6 reciprocal crosses of 4 *D. glomerata* cultivars. The hybrids exhibited positive

HPH for the length and width of flag leaf, length and width of second leaf, culm diameter, and individual yield. According to Table 5, greater variations were detected for MPH in width of flag leaf giving a range of 143.5-278.6%; the corresponding range for HPH was 91.8-275.6%. Most agronomic traits of the hybrid plant showed evidence for significant heterosis. Whereas the heterosis of length of culm, internode number, tiller number, and growing period was lower than for other agronomic traits, some of them showed negative heterosis for F_1 hybrids.

Table 3. Intervariety crossing.

Variety	Chuandong	Baoxing	Gulin	Kaimo
Intervariety crossing	HC-CHD1	HC-BX1	HC-GL1	HC-KM1
	HC-CHD2	HC-BX2	HC-GL2	HC-KM2
	HC-CHD3	HC-BX3	HC-GL3	HC-KM3

HC-CHD1 = Chuandong2 (CHD2) x CHD3; HC-CHD2 = CHD7 x CHD11; HC-CHD3 = CHD8 x CHD9; HC-BX1 = Baoxing3 (BX3) x BX5; HC-BX2 = BX8 x BX10; HC-BX3 = BX11 x BX12; HC-GL1 = Gulin2(GL2) x GL5; HC-GL2 = GL6 x GL7; HC-GL3 = GL9 x GL12; HC-KM1 = Kaimo3(KM3) x KM4; HC-KM2 = KM5 x KM6; HC-KM3 = KM9 x KM12.

Table 4. Average genetic distance between the intervariety crossing and significant testing.

Intervariety crossing	GD	Intervariety crossing	GD	Intervariety crossing	GD	Intervariety crossing	GD
HC-CHD1	0.369*	HC-BX1	0.318*	HC-GL1	0.632	HC-KM1	0.496
HC-CHD2	0.302*	HC-BX2	0.403*	HC-GL2	0.466*	HC-KM2	0.337*
HC-CHD3	0.297**	HC-BX3	0.375*	HC-GL3	0.381*	HC-KM3	0.421

HC-CHD1 = Chuandong2 (CHD2) x CHD3; HC-CHD2 = CHD7 x CHD11; HC-CHD3 = CHD8 x CHD9; HC-BX1 = Baoxing3 (BX3) x BX5; HC-BX2 = BX8 x BX10; HC-BX3 = BX11 x BX12; HC-GL1 = Gulin2(GL2) x GL5; HC-GL2 = GL6 x GL7; HC-GL3 = GL9 x GL12; HC-KM1 = Kaimo3(KM3) x KM4; HC-KM2 = KM5 x KM6; HC-KM3 = KM9 x KM12. * and **Significance at 0.05 and 0.01 levels, respectively.

The highest mean value of heterosis was observed in width of second leaf, followed by WF > Y > LF > CD > LL > LC > GP > T > LI > NI. Interestingly, the heterosis of length of the flag leaf and second leaf in the inverse crosses H1b, H2b, and H3b were higher than those in the obverse crosses H1a, H2a, and H3a. In contrast, the heterosis of width of the flag leaf and second leaf in obverse crosses H1a, H2a, and H3a were higher than the corresponding inverse crosses. The obverse crosses H1a and H2a had superior heterosis in the length of culm than their inverse crosses, while heterosis in cross H3b was higher than that in H3a.

To test for statistical significance, the mean value for each eleven traits was analyzed by one-way ANOVA at the 0.01 and 0.05 threshold levels. The *P* values of this analysis are shown in Table 6. Most of the analyzed traits showed significant differences between the parental and F_1 hybrid plants. Except for the length of internode and tiller number, traits of the F_1 hybrids significantly differed from their parents, which did not show any significant differences in traits among each other. The differences between obverse crosses and inverse crosses in the eleven traits were significant among 3 reciprocal crosses, suggesting significant cytoplasmic effects on these agronomic traits, especially on yield.

Table 5. Mean values of indicated parents and for the shown coefficient of variation (CV) along with mid-parent (MPH) and higher-parent heterosis (HPH) for each cross.

Characters	Average of parents	H1a			H1b		
		MPH	HPH	CV	MPH	HPH	CV
		%	%	%	%	%	%
LF (cm)	23.87	41.7	-1.3	21.2	67.1	16.4	16.2
WF (cm)	0.50	190.3	130.7	13.9	161.1	107.5	11.6
LL (cm)	37.02	13.2	-10.8	13.4	20.0	-5.4	21.7
WL (cm)	0.49	187.4	124.4	12.0	163.7	105.9	11.6
LC (cm)	103.25	-3.8	-21.6	11.9	-16.6	-32.0	8.8
CD (cm)	0.35	42.6	6.2	15.6	48.0	10.2	19.4
NI (No.)	3.83	-34.8	-37.5	20.4	-20.3	-23.6	20.9
LI (cm)	19.38	4.1	-0.1	11.0	-26.6	-35.8	18.7
T (No.)	42.5	18.5	9.5	36.6	14.5	5.8	36.0
Y (kg)	0.73	75.2	62.8	15.6	28.3	19.2	14.9
GP (days)	239.25	-1.4	-2.9	11.3	-0.9	-2.5	10.8
Characters	Average of parents	H2a			H2b		
		MPH	HPH	CV	MPH	HPH	CV
		%	%	%	%	%	%
LF (cm)	16.40	84.1	56.2	11.7	124.1	90.1	17.8
WF (cm)	0.38	278.6	275.6	11.2	278.6	275.6	10.8
LL (cm)	24.60	60.0	45.4	10.9	71.9	56.3	20.5
WL (cm)	0.37	279.9	258.1	8.9	271.0	249.8	11.1
LC (cm)	92.28	7.0	-5.8	10.8	4.8	-7.7	14.8
CD (cm)	0.25	95.9	81.8	21.3	97.0	82.8	18.9
NI (No.)	3.67	-28.6	-34.5	19.0	-34.8	-40.3	19.8
LI (cm)	20.75	-4.8	-10.9	17.5	-8.0	-13.8	19.0
T (No.)	43.5	-23.2	-30.4	18.5	-7.8	-16.4	42.2
Y (kg)	0.69	72.5	67.6	17.2	76.8	71.8	17.3
GP (days)	230.8	1.0	-1.1	13.8	-3.8	-5.7	14.1
Characters	Average of parents	H3a			H3b		
		MPH	HPH	CV	MPH	HPH	CV
		%	%	%	%	%	%
LF (cm)	24.52	58.8	31.1	16.4	63.3	34.8	17.4
WF (cm)	0.52	175.2	116.8	13.3	143.5	91.8	14.9
LL (cm)	27.47	44.9	21.3	17.4	64.0	37.7	16.7
WL (cm)	0.53	179.4	122.9	12.1	170.2	115.6	10.2
LC (cm)	103.72	-1.4	-2.4	21.9	6.8	5.7	9.8
CD (cm)	0.33	81.2	53.7	17.7	64.0	39.1	16.5
NI (No.)	3.50	-22.2	-25.8	16.9	-19.4	-23.1	13.8
LI (cm)	19.57	-11.4	-12.5	30.5	-15.1	-16.1	31.4
T (No.)	50.0	-9.3	-12.8	10.7	-28.9	-31.7	28.0
Y (kg)	0.77	115.7	101.2	16.6	106.5	92.7	15.1
GP (days)	230.0	-0.4	-2.1	8.5	-0.2	-1.9	9.1

LF = length of the flag leaf; WF = width of the flag leaf; LL = length of the second leaf; WL = width of the second leaf; LC = length of the culm; CD = culm diameter; NI = number of the internode; LI = length of the internode; T = tiller number; Y = individual yield; GP = growing period.

Analysis of SSR marker information

Forty-four pairs of SSR primers were screened, of which 27 primers producing clear and reproducible amplification bands were selected. In total, 127, 109, and 112 identifiable bands were amplified within the crosses H1, H2, and H3, respectively. The SSR amplicons ranged in size from 100-200 bp. Each pair of primers detected 3-7 different alleles in cross H1,

2-6 in H2, and 3-6 in H3, with an average allele number for H1, H2, and H3 of 5.1, 4.5, and 4.5, respectively. In total, 102 polymorphic bands were amplified from H1, 109 from H2, and 82 from H3. The polymorphic bands accounted for 80.3, 100, and 73.2% of the total alleles in H1, H2, and H3, respectively.

Table 6. Significant testing for 3 reciprocal combination.

Materials	LF (cm)	WF (cm)	LL (cm)	WL (cm)	LC (cm)	CD (cm)	NI (No.)	LI (cm)	T (No.)	Y (kg)	GP (day)
CHD	13.47$^{c, C\dagger}$	0.37$^{c, B}$	27.07$^{b, B}$	0.35$^{d, B}$	79.77$^{c, C}$	0.23$^{b, B}$	4.0$^{a, A}$	22.17$^{a, A}$	39.0$^{a, A}$	0.67$^{d, D}$	235$^{b, B}$
GL	34.27$^{ab, AB}$	0.63$^{c, B}$	46.97$^{a, A}$	0.62$^{c, B}$	126.73$^{a, A}$	0.47$^{a, A}$	3.7$^{ab, A}$	16.60$^{b, BC}$	46.0$^{a, A}$	0.78$^{c, C}$	243$^{a, A}$
H1a	33.81$^{b, B}$	1.46$^{a, A}$	41.91$^{a, A}$	1.40$^{a, A}$	99.37$^{b, B}$	0.50$^{a, A}$	2.5$^{c, B}$	20.17$^{a, AB}$	50.4$^{a, A}$	1.27$^{a, A}$	236$^{b, B}$
H1b	39.87$^{a, A}$	1.31$^{b, A}$	44.43$^{a, A}$	1.28$^{b, A}$	86.12$^{c, C}$	0.52$^{a, A}$	3.1$^{b, A}$	14.22$^{b, C}$	48.7$^{a, A}$	0.93$^{b, B}$	237$^{b, B}$
CHD	13.47$^{c, C}$	0.37$^{b, B}$	27.07$^{b, B}$	0.35$^{b, B}$	79.77$^{b, A}$	0.23$^{b, B}$	4.0$^{a, A}$	22.17$^{a, A}$	39.0$^{a, A}$	0.67$^{d, D}$	235.5$^{a, A}$
BX	19.33$^{c, C}$	0.38$^{b, B}$	22.13$^{b, B}$	0.40$^{b, B}$	104.80$^{a, A}$	0.27$^{b, B}$	3.3$^{a, A}$	19.33$^{a, A}$	48.0$^{a, A}$	0.71$^{c, C}$	226$^{c, C}$
H2a	30.20$^{b, B}$	1.43$^{a, A}$	39.35$^{a, A}$	1.42$^{a, A}$	98.76$^{a, A}$	0.49$^{a, A}$	2.6$^{b, B}$	19.75$^{a, A}$	33.4$^{a, A}$	1.19$^{b, B}$	233$^{b, B}$
H2b	36.74$^{a, A}$	1.43$^{a, A}$	42.29$^{a, A}$	1.38$^{a, A}$	96.72$^{a, A}$	0.49$^{a, A}$	2.5$^{b, B}$	19.10$^{a, A}$	40.1$^{a, A}$	1.22$^{a, A}$	222$^{d, D}$
BX	19.33$^{b, B}$	0.38$^{c, C}$	22.13$^{c, B}$	0.40$^{c, B}$	104.80$^{a, A}$	0.27$^{c, B}$	3.3$^{a, A}$	19.33$^{a, A}$	48.0$^{a, AB}$	0.71$^{d, D}$	226$^{c, C}$
KM	29.70$^{b, B}$	0.66$^{c, C}$	32.80$^{bc, B}$	0.66$^{b, B}$	102.63$^{a, A}$	0.39$^{c, B}$	3.7$^{a, A}$	19.80$^{a, A}$	52.0$^{a, A}$	0.82$^{c, C}$	234$^{a, A}$
H3a	38.94$^{a, A}$	1.43$^{a, A}$	39.80$^{b, A}$	1.48$^{a, A}$	102.24$^{a, A}$	0.59$^{a, A}$	2.7$^{b, B}$	17.33$^{a, A}$	45.3$^{a, AB}$	1.65$^{a, A}$	229$^{b, B}$
H3b	40.03$^{a, A}$	1.26$^{b, B}$	44.50$^{a, A}$	1.43$^{a, A}$	110.82$^{a, A}$	0.54$^{b, A}$	2.8$^{b, B}$	16.60$^{a, A}$	35.5$^{b, B}$	1.58$^{b, B}$	229.5$^{b, B}$

CHD = Chuandong; GL = Gulin; BX = Baoxing; KM = Kaimo. For other abbreviations, see legend to Table 5. †Means with the same capital letter are not significantly at 1% level; means with the same little letter are not significantly at 5%.

Nei's genetic diversity index and the Shannon diversity index can reveal the genetic diversity in parents and progenies. The H, I, and GD values from 3 reciprocal crosses are shown in Table 7. Progenies of H2 had a larger variance than the other F_1 hybrids because their H and I values were higher than those in H1 and H3.

Table 7 Genetic diversity indexes for F_1 hybrids and SSR-based genetic distances between hybrid parents.

Crosses	N_A	H	I	P_L	P_P	GD_p
H1	102	0.316	0.464	102	80.31	1.016
H2	89	0.418	0.604	109	100	0.907
H3	82	0.295	0.429	82	73.21	0.847

N_A = observed number of alleles; H = Nei's genetic diversity index; I = Shannon diversity index; P_L = polymorphic loci; P_p = percentage of polymorphic loci. GD_p = SSR-based genetic distances between hybrid parents.

Cluster analysis based on SSR markers

The UPGMA clustering reproducibly showed that SSR could classify H2 into 2 groups. Group I comprised the parent cultivar "Chuandong" and all the H2 hybrids, whereas "Baoxing" clustered into group II with a similarity coefficient of 0.63 (Figure 1). Moreover, UPGMA clustering divided Group I into 5 sub-groups: sub-group I_a included 8 inverse cross hybrids and 3 obverse cross hybrids, sub-group I_b contained 2 inverse cross hybrids and 2 obverse cross hybrids, and sub-group I_c comprised H2a-5 and 6 inverse cross hybrids. The other 3 hybrids clustered into sub-group I_d and sub-group I_e. At the sub-group level, the hybrids of the obverse and inverse crosses could be separated, indicating that there was significant variation between obverse and inverse crosses. Both H1 and H3 grouped into 2 main clusters; the

obverse cross in H1 tended to display paternal inheritance, whereas the H3 crosses were prone to maternal inheritance.

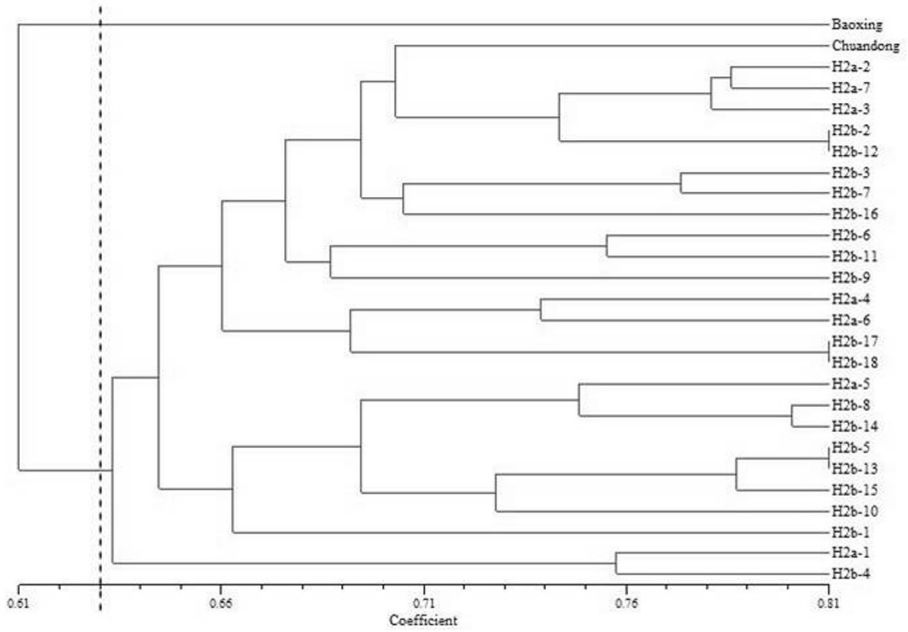

Figure 1. UPGMA dendrogram based on genetic similarity coefficients among parents, inverse and obverse F_1 hybrids of H2.

Euclidean distance cluster analysis based on morphologic characteristics

The reciprocal crosses were clustered separately based on the basis of morphological traits. At a Euclidean distance of 7.1, the H2 dendrogram indicated that the reciprocal crosses could be divided into 4 groups (Figure 2). Group I was formed by "Chuandong" and all the progenies generated by obverse cross and most inverse crosses, which indicated that the F_1 hybrids from obverse crosses inherited most traits from their female parent "Chuandong", a result similar to the UPGMA clustering. Group II contained H2b-15 and H2b-16, and H2b-1 and parent "Baoxing" clustered into groups III and IV, respectively. Likewise, the reciprocal crosses in H1 and H3 could not be distinguished clearly; this observation may be related to gene flow as seen in most cross-pollinating species as well as orchardgrass.

Correlation analysis between genetic distance and heterosis

Genetic distance based on SSR markers was computed for all crosses and parents of the 3 cultivars. The distance among parent plants indicated by the SSR data ranged from 0.847 ("Gulin" versus "Chuandong") to 1.016 ("Baoxing" versus "Kaimo") with an overall mean distance of 0.923. The results of correlation analyses are shown in Table 8, indicating that the parental GD was significant positively correlated with tiller number (r = 0.834*, * indicated

significance at 0.05 threshold level). Negative correlations were identified between genetic distance and heterosis in length of the flag leaf, width of flag leaf, length of second leaf, width of second leaf, length of the culm, culm diameter, growing period, and the number and length of internode. Moreover, none of these correlations were statistically significant, except the heterosis for length of the culm, which was negatively correlated with the parental genetic distance (r = -0.889*, * indicated significance at 0.05 threshold level). Only the individual yield was positively correlated with genetic distance but this correlation was not significant (r = 0.427). These results suggest that the parental genetic distance could only predict heterosis for length of culm and tiller numbers, but not for the other traits in this experiment.

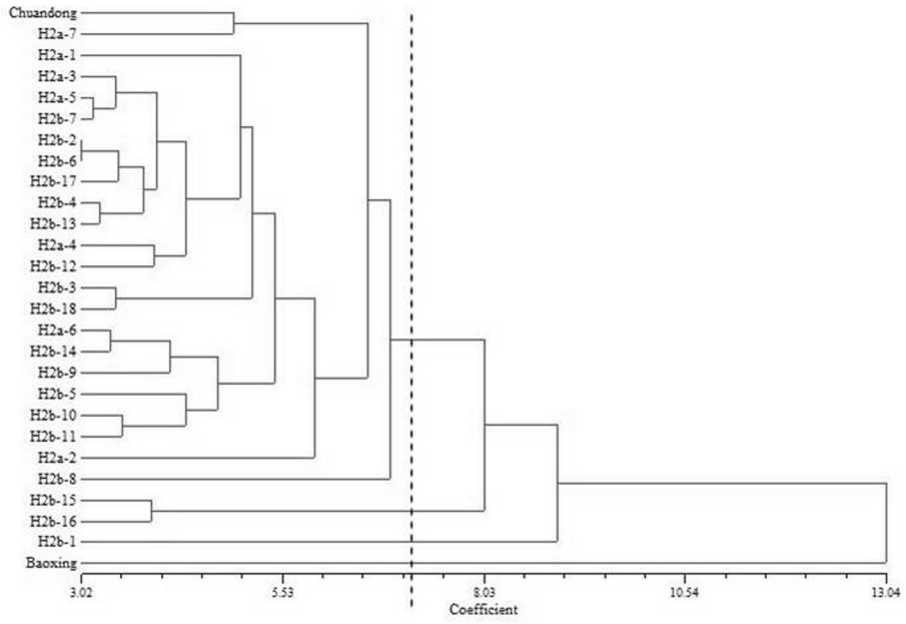

Figure 2. Dendrogram based on Euclidean distance among parents, inverse and obverse F_1 hybrids of H2.

Table 8. Correlation coefficients between genetic distance and higher-parent heterosis.

	LF	WF	LL	WL	LC	CD	NI	LI	T	Y	GP
GD_p	-0.491	-0.086	-0.728	-0.188	-0.889*†	-0.642	-0.247	-0.165	0.834*	0.427	-0.782

GD_p = SSR-based genetic distances between hybrid parents. *† and ** indicated significance at 0.05 and 0.01 level, respectively. For other abbreviations, see legend to Table 5.

DISCUSSION

Hybrid vigor existing in *Dactylis glomerata*

The description of phenotypic characteristics is indispensable in studies with hybrid

plants. The results in the present study showed that the F_1 hybrids of *D. glomerata* displayed significant heterosis in length of flag leaf, second leaf, width of flag leaf, second leaf, culms diameter, and individual yield. The characteristics of second leaf, flag leaf, and culm may influence forage yields and palatability (Monyo and Whittington, 1973). F_1 hybrid plants having tetraploid and diploid-tetraploid levels in the studies by Zhong (2006 and 2007) exhibited higher disease resistance and reproductive performance, and gave higher yields than their parent plants. Similarly, our results confirmed that the morphological traits of orchardgrass could be improved by means of hybridization of *D. glomerata* cultivars.

Correlation analysis between genetic distance and heterosis

The morphological variation analysis indicated a significant difference between the parents of the H1 cross. However, although the genetic distance between the H1 parents was the largest, the H1 hybrids did not achieve the highest heterosis among the reciprocal crosses. Genetic distance was positively correlated with tiller number, negatively correlated with length of clum, and no correlation was detected for the other traits, including yield. This may suggest that although SSR analysis maybe useful for predicting phenotypic variation, SSR-based genetic distance may not accurately predict hybrid performance. Accordingly, reports of the use of molecular markers to determine genetic differences between parents for predicting heterosis have yielded inconsistent results. Such analyses were reported by Godshalk et al. (1990) and Dudley et al. (1991). Zhao and colleagues, conducting a study on rice, found that a correlation index of SSR-based genetic distance and heterosis was too low to predict heterosis (Zhao et al., 2009). Boppenmaier et al. (1992) suggested that this lack of correlation may be due to specific marker genotypes having no significant effect on the expression of the traits or affected by epistatic effects. The marker-assisted selection in orchardgrass should be further studied to identify hybrid progenies with superior economic traits. For example, Xie et al. (2011) constructed a diploid orchardgrass linkage map of genetic markers, which may be used to identify the loci involved in agronomical traits (Xie et al., 2011), and permit selection of desirable progenies at the early growth stages. Furthermore, because orchardgrass is a cross-pollinating species, the trait segregation in its F_1 hybrids could not be accurately analyzed. Therefore, we suggest that future studies investigating hybridization in orchardgrass could be improved by using homozygous parents derived from chromosome doubling, or by eliminating self-incompatibility in *D. glomerata*, thus permitting development of inbred lines.

Clustering analysis

The figures show that the SSR-banding patterns and Euclidian clustering of the single-cross progeny individuals did not distribute evenly but instead shifted to one of the parental plants. This might be caused by linkage effects among the SSR markers. Moreover, many phenotypic traits might interact with each other, preventing an even marker distribution.

Relationship between GD and cytoplasmic effect

The genetic distance between the parents of H1 (GD = 1.016) was larger than those for the parent of H2 and H3. The test for statistical significance showed that variation between

obverse and inverse crosses in H1 was statistically significant for most morphological traits. Furthermore, greater numbers of beneficial traits in inverse than in obverse crosses were common enough to be observed, warranting further research into this interesting observation. A cytoplasmic effect exists in orchardgrass hybrids, especially in yield. Therefore, one can infer that conducting reciprocal crosses may be essential if the GD of the parent reaches some threshold in hybridization experiments. However, concise statistics of this GD in this study have not yet been calculated, and it should be detected in further studies aimed at more efficient orchardgrass breeding in the future.

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