Evaluation of genetic diversity in wild orchardgrass (*Dactylis glomerata* L.) based on AFLP markers

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Orchardgrass (*Dactylis glomerata* L.) is a highly variable perennial forage grass, widely cultivated in all temperate and subtropical growing regions of the world. Despite its economic importance, there is a lack of information on the genetic diversity within and among populations. In order to assess DNA molecular genetic variations and formulate appropriate strategies for conservation and utilization of wild orchardgrass genetic resources available in China, mainly amplified fragment length polymorphism (AFLP) DNA profiling method was used to detect the genetic diversity and relatedness among 32 wild orchardgrass accessions collected in China and two in the USA. Using nine primer combinations, 400 DNA fragments were amplified, among which 322 (80.50%) were polymorphic. Genetic similarity coefficients (GSC) for 34 accessions ranged from 0.69 to 0.93, with an average of 0.81. Further examination of the different components of genetic variation by analysis of molecular variance (AMOVA) indicated that larger proportions of variability existed within ploidy levels (87.98%) and geographical regions (85.65%). Cluster analysis using the UPGMA approach seperated the studied accessions into six major clusters. On the basis of principal coordinate analyses (PCoA) on the genetic characteristics, the studied accessions could be grouped into three main clusters. All tetraploid ($2n = 4x = 28$) accessions originating from different regions were grouped into the same cluster whereas the diploid ($2n = 2x = 14$) accessions were grouped into other two clusters associated with their geographical distributions. The results revealed by AFLP markers were concordant with the morphological variability, agronomic traits and karyotype. The results also showed that comprehensive germplasm collection in major geographic regions and exploitation of the existing variation are required to widen the genetic base and sample the full extent of the available variation in breeding strategies for orchardgrass.

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Orchardgrass (*Dactylis glomerata* L.) is a highly variable perennial forage grass, widely cultivated in all temperate and subtropical growing regions of the world. It is native to northern Africa, Europe, temperate and tropic Asia (Hultén 1968; Tolmachev et al. 1995), and is widely grown for pasture and hay. It is also an important intercrop in fruit orchards and under shading. Because of its dense network of roots, orchardgrass is recommended as a part of a seed mix for erosion control (Anderson and Brooks 1975; Mclean and Clark 1980). Orchardgrass is indigenous to China where it is primarily distributed in the southwest and northwest areas, mainly growing on forest edges, in shrubs and in sub alpine meadows at elevations ranging from 1000 to 3600 m, where diploid ($2n = 2x = 14$) and tetrapoid ($2n = 4x = 28$) cytotypes grow in sympatry (Zhang et al. 1994; Zhou et al. 2000). The interest in studying wild orchardgrass germplasm has dramatically increased over the past 20 years since the cultivated varieties selected from the local wild sources offered more advantages including higher yield and adaptability to the environment than those introduced from other countries. Thus, three cultivated varieties from wild material has been developed recently, named cv. Gulin, cv. Baoxing and cv. Chuandong, reflecting their origin regions. The genetic resources of the wild germplasm present indigenously are to be exploited and conserved for breeding purpose. In order to achieve this, it is imperative to assess the genetic variability present among wild accessions.

Variations in orchardgrass morphological features, distributional patterns, adaptive and agronomic characters, and allozymes are well documented (Lumaret et al. 1987; Lumaret and Borrientos 1990; Sahuquillo and Lumaret 1995; Volaire and Thomas 1995; Volaire 1995; Bretagnolle and Thompson 1996; Gauthier et al. 1998; Gautier and Lumaret 1999; Lindner et al. 1999; Sugiyama and Nakashima 1999; Tosun et al. 2002). DNA profiling techniques that have been successfully used in assessing genetic diversity and relatedness of orchardgrass germplasm include randomly amplified polymorphic DNA (RAPD) markers (Kolliker et al. 1999; Tuna et al. 2004; Zeng et al. 2006a) and

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inter-simple sequence repeat (ISSR) (ZENG et al. 2006b). REEVES et al. (1998) used amplified fragment length polymorphism (AFLP) DNA profiles to determine relationship between genome size and altitude of origin in natural populations of orchardgrass. Even though these studies demonstrated the usefullness of DNA profiling in assessing genetic differences in orchardgrass, none has focused on assessing genetic diversity and relationship within the wild Chinese orchardgrass.

So far, information on the genetic diversity of wild Chinese orchardgrass at the DNA molecular level is still scarce. AFLP is an efficient, reproducible technique which combines the reliability of RFLP and the power of the PCR technique (VOS et al. 1995). Many forage grasses have been analyzed by this technique, including *Lolium perenne* (GUTHRIDGE et al. 2001), *Phalaris aquatica* (ROUF et al. 2005) and *Eragrostis* species (AYELE et al. 1999). Accordingly, we selected AFLP marker: (1) to estimate the levels of genetic diversity among 32 wild orchardgrass originated from various geographic locations in China, together with two wild accessions from USA, (2) to evaluate the genetic relationships between orchardgrass accessions, and (3) to characterize germplasms to select desirable genotypes for breeding and other utilization purposes.

MATERIAL AND METHODS

Plant material and DNA extraction

A total of 34 wild orchardgrass accessions were included in the study (Table 1). Thirty-two Chinese accessions were collected from southwestern and northwestern China, among which ten were from the Sichuan province, six from the Yunnan province, five from the Guizhou province, ten from the Xinjiang municipality, one from the Jiangxi province and two American accessions from New York. Specifically, there are nine tetraploid orchardgrass and 25 diploid

Table 1. *Wild orchardgrass accessions analyzed by AFLP.*

Accession code	Origin	Chromosome no.(2n)	Geographical group*
Ya79-9	Lushan, Jiangxi Provience, China	28	SWC
Ya90-70	Kangding,Sichuan Provience, China	28	SWC
Ya91-1	Hanyuan, Sichuan Provience, China	14	SWC
Ya91-2	Baoxing, Sichuan Provience, China	28	SWC
Ya91-7	Hanyuan, Sichuan Provience, China	28	SWC
Ya91-103	Yexi, Sichuan Provience, China	14	SWC
Ya01-101	Bijie, Guizhou Provience, China	28	SWC
Ya01-103	New York, USA	28	USA
Ya01-104	New York, USA	28	USA
Ya02-101	Bijie,Guizhou Provience, China	14	SWC
Ya02-102	Zhijin, Guizhou Provience, China	14	SWC
Ya02-103	Nayong,Guizhou Provience, China	14	SWC
Ya02-104	Shuicheng,Guizhou Provience, China	14	SWC
Ya02-105	Dazhou, Sichuan Provience, China	28	SWC
Ya02-106	Baoxing, Sichuan Provience, China	14	SWC
Ya02-107	Baoxing, Sichuan Provience, China	14	SWC
Ya02-108	Baoxing, Sichuan Provience, China	14	SWC
Ya02-109	Baoxing, Sichuan Provience, China	14	SWC
Ya02-111	Zhongdian,unnan Provience, China	14	SWC
Ya02-112	Zhongdian, Yunnan Provience, China	14	SWC
Ya02-113	Deqin, Yunnan Provience, China	14	SWC
Ya02-114	Qujing, Yunnan Provience, China	14	SWC
Ya02-115	Deqin, Yunnan Provience, China	14	SWC
Ya02-116	Kunming, Yunnan Provience, China	28	SWC
Ya00849	Tianshan,Xinjiang Provience, China	14	NWC
Ya00850	Tianshan,Xinjiang Provience, China	14	NWC
Ya01032	Tianshan,Xinjiang Provience, China	14	NWC
Ya02104	Tianshan,Xinjiang Provience, China	14	NWC
Ya02106	Tianshan,Xinjiang Provience, China	14	NWC
Ya02138	Tianshan,Xinjiang Provience, China	14	NWC
Ya02207	Tianshan,Xinjiang Provience, China	14	NWC
Ya02240	Tianshan,Xinjiang Provience, China	14	NWC
Ya02271	Tianshan,Xinjiang Provience, China	14	NWC
Ya02411	Tianshan,Xinjiang Provience, China	14	NWC

*SWC – southwest of China; NWC – northwest of China.

orchardgrass. In addition, the accessions were aslo grouped in three geographical regions: SWC, NWC and USA according to their origin (Table 1).

Plants of each accession were grown in the Campus Experimental Garden in Sichuan Agricultural Univ., Yaan, Sichuan, in a 4.0×2.5 m plot containing six plants of each accession. Total genomic DNA samples was extracted from fresh leaf tissue of each accession by the CTAB cetyl-trimethyl-ammonium-bromide protocol (Doyle and Doyle 1987). The quality and quantity of the DNA were estimated by Beckman counter DU800 nucleic acid/protein analyzer.

AFLP analysis

AFLP analysis was performed as described by Vos et al. (1995). Briefly, approximately 300 ng of genomic DNA of each accession was double digested with 3.0 U *Eco*RI and 3.0 U *Mse*I restriction enzymes. Adapters for both enzymes were then ligated to the ends of restriction fragments using T4 DNA ligase (Promega). The sequence of *Eco*RI and *Mse*I primers are 5′-GACTGCGTACCAATTC and 5′-GATGAGTCCTGAGTAA, respectively. Pre-amplification started at 94°C for 2 min, 26 cycles of 1 min at 94°C followed by 1 min at 56°C and 1 min at 72°C. Pre-amplification product DNA was diluted 20-fold with sterile nanopure water, and was then used as template for the selective amplification. Nine *Eco*RI: *Mse*I AFLP selective primer combinations were selected for selective amplification (Table 2). The selective amplification PCR reaction was performed in a final volume of 20 µl containing 1 × PCR buffer, 2 mM $MgCl_2$, 2 mM dNTP, 40 ng of each of *Eco*RI primer and *Mse*I primer, 1 U Taq polymerase, and 5.0 µl preamplified template DNA. All selective amplifications were conducted using the following touchdown thermal profile: one cycle of 2 min at 94°C; 13 touchdown cycles of 30 s at 94°C, 30 s at 65°C (−0.7°C per cycle), 60 s at 72°C; 23 cycles of 30 s at 94°C, 30 s at 56°C, 60 s at 72°C. All PCR reactions were conducted on the MJ PTC-100 thermocycler. Five µl selectively amplified PCR products were mixed with 5 µl loading buffer (98% formamide, 10 mM EDTA, 0.25% xylene cyanol and 0.25% bromophenol blue), and denatured for 5 min at 95°C. AFLP fragments were separated on 5% polyacrylamide gel with 7.5 M urea, and 1× TBE buffer. Gels were run at 70 W for 2 h by using Life Technologies Model S200 electrophoresis equipment with Bio RAD Model 3000X I power supply. At the end of the eletrophoresis period, gel was stained with 0.1% silver nitrate (Bassam et al. 1991). The resulting banding was manually analyzed.

Data analysis

Polymorphic DNA bands were scored as present (1) or absent (0) across 34 accessions for each primer combination. Only clear bands were scored. Data were compiled in a binary data matrix. The level of polymorphism was described for each primer combination as a percentage of polymorphic variable loci among all analyzed loci. Relative genetic similarity coefficients GS were calculated as described by Nei and Li (1979): $GS_{ij} = 2n_{ij}/(n_i + n_j)$, where n_i and n_j were the numbers of fragments in individuals I and J, respectively, and n_{ij} was the number of the fragments shared between individuals (Nei and Li 1979). Genetic dissimilarity, GD, was calculated by GD = 1-GS using Microsoft Excel. POPGENE ver. 1.31 (Yeh et al. 1997) was used to calculate Shannon's information index (I). Analysis of molecular variance (AMOVA) was performed using ARLEQUIN ver. 3.11 (Excoffier et al. 2006) to partition the variation between diploid and tetraploid accessions, and among geographical regions

Table 2. *Numbers of total bands, polymorphic bands and percentage polymorphic bands for each of nine AFLP selective primer pairs.*

Seletive amplification primer pairs*	Total no. of bands	Polymorphic bands	% polymorphic bands
e-CAT-m-CTA	36	33	91.67
e-ACG-m-CTA	45	35	77.78
e-CAT-m-CAA	34	22	64.71
e-CAT-m-CAT	45	35	77.78
e-CAT-m-GCC	49	30	61.22
e-CCA-m-ACG	42	38	90.48
e-AGT-m-ACG	50	41	82.00
e-ACT-m-ACG	45	40	88.89
e-AAG-m-ACG	54	48	88.89
Total	400	322	
Average	44.44 ± 6.42	35.78 ± 7.34	80.50

*e is the preamplification primer sequence for *Eco*RI site (5′-GACTGCGTACCAATTC) without any selective nucleotides and m is the preamplification primer sequence for *Mse*I site (5′-GATGAGTCCTGAGTAA).

Table 3. *Genetic variability within ploidy levels and geographical regions of orchardgrass detected by AFLP analysis. PB, polymorphic bands; PPB, percentage of polymorphic bands; GD, average genetic distance between pairs of accessions within groups; H_0, Shannon's information index.*

Groups	No. of PB	PPB (%)	GD	I
Diploid	297	74.25	0.1680	0.3897 ± 0.2728
Tetraploid	263	65.75	0.1956	0.3619 ± 02892
SWC	316	79.00	0.1942	0.4255 ± 0.2582
NWC	196	49.00	0.1326	0.2671 ± 0.2943
USA	105	26.25	0.1927	0.1587 ± 0.2664

of orchardgrass. Cluster analysis was performed with the NTSYS-pc ver. 2.1 program using the unweighted pair-group mean algorithm (UPGMA) within the SAHN module. A goodness-of-fit test of the cophenetic matrix of the cluster to the similarity matrix was performed using the MXCOMP module. A principal coordinate analysis was performed using the DCENTER module of the NTSYS-pc program.

RESULTS

AFLP polymorphism

Nine AFLP primer combinations produced a total of 400 AFLP bands for 34 wild orchardgrass accessions, averaging 44.44 ± 6.42 bands per primer combination. Of the 400 bands scored, 322 were polymorphic, ranging from 22 to 41, with an average of 35.78 ± 7.34 polymorphic bands per primer combination. The percentage of polymorphism ranged from 61.22% to 91.67%, and averaged 80.50% (Table 2). The fragment size ranged from 200–2000 bp. The percentage of polymorphic bands (PPB) for diploid and tetraploid accessions was 74.25% and 65.75%, respectively. Among three geographical regions, the PPB for group SWC was 79.00%, for NWC, 49.00% and for USA, 26.25% (Table 3).

Genetic variation

The genetic similarity (GS) among 34 accessions was relatively low, ranging from 0.69 to 0.93, with an average of 0.81. The lowest GS value (0.69) obtained was between accession Ya91-1 and Ya02-116, which were collected from the Sichuan and Yunnan provinces in southwestern China. The highest GS (0.93) was between accession Ya02-113 and Ya02-114, collected from close sites only in the Yunnan province.

To obtain a more detailed view of the distribution of genetic variation within different groups, the Shannon's index (I) were estimated. Of the total gene diversity (0.4172 ± 0.2509), the Shannon's index (I) within diploid and tetraploid accessions were respectively 0.3897 and 0.3619. Among three geographical origions, the Shannon's index (I) for group SWC, group NWC and group USA were 0.4255, 0.2671 and 0.1587, repectively (Table 3).

Genetic distances (GD) for accessions belonging to the same group were calculated based on 400 markers scored (Table 3). Values for diploid and tetraploid accessions were 0.1680 and 0.1956, respectively, whereas group SWC, 0.1942, group NWC, 0.1326, group USA, 0.1927.

On the basis of the AMOVA analysis, the difference between diploid and tetraploid groups was significant, but greater variation was found among accessions within ploidy level. The variance within ploidy level accounted for 87.98% of total variance, while among ploidy level variance contributed only 12.02%. In addition, significant variation existed among accessions of different geographical regions, and it accounted for 14.35% of total AFLP variation detected, whereas 85.65% of total genetic variation was attributable to differences among accessions within regions (Table 4).

Cluster analysis

Taking the average genetic similarity of 0.81 as the cut point, the UPGMA dendrogram separated the 34 natural orchardgrass accessions into six major groups, designated as A, B, C, D, E and F (Fig. 1). The cophenetic correlation coefficient was 0.80, indicating that the dendrogram represents well the similarity matrix.

Cluster A contains six tetraploid ($2n = 4x = 28$) accessions collected from different geographical locations, which of three (Ya91-2, Ya91-7 and Ya02-105) were from Sichuan, one, Ya79-9, from Jiangxi, China, and two (Ya01-103 and Ya01-104) from New York,

Table 4. *Analysis of molecular variance (AMOVA) for 34 accessions of orchardgrass based on 400 AFLP markers.*

Source of variation	DF	Sum of squares	Variance components	Percentage of variation	P-values
Among ploidy levels	1	139.9	6.81	12.02	<0.01
Within ploidy level	32	1594.1	49.81	87.98	<0.01
Among regions	2	232.5	8.12	14.35	<0.001
Within regions	31	1501.6	48.44	85.65	<0.001

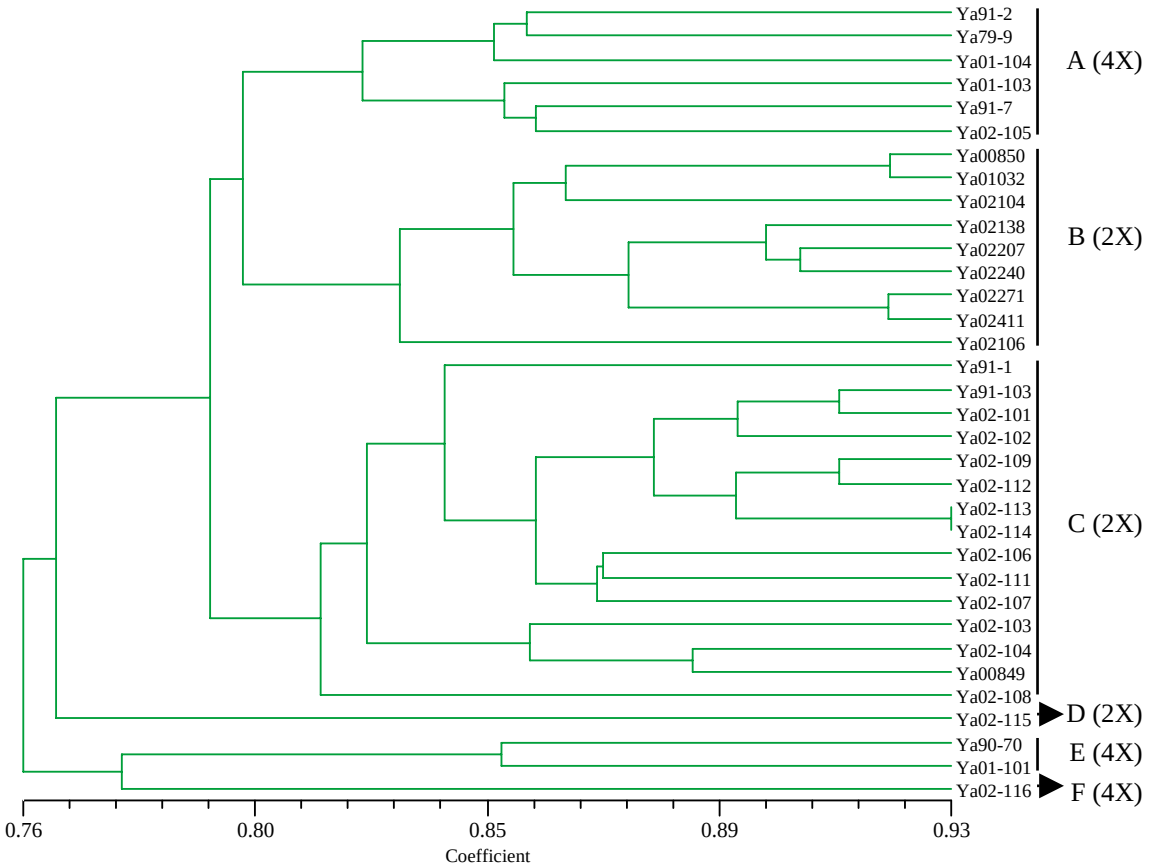

Fig. 1. UPGMA dendrogram of 34 wild orchardgrass accessions based on the genetic similarity matrix derived from 400 AFLP markers.

USA (Fig. 1). The GSC among six accessions ranged from 0.80 to 0.86. Based on the field survey, all accessions showed the similar morpho-agronomic characters as erect panicle, higher yield, earlier heading, and a higher 1000-seed weight compared with diploid accessions (Peng et al. 2007).

Cluster B consists of nine diploid ($2n = 2x = 14$) accessions from Xinjiang (Ya00850, Ya01032, Ya02104, Ya02138, Ya02207, Ya02240, Ya02271, Ya02411, Ya02106), with GSC ranging from 0.78-0.92. The field-test indicated that eight of the accessions grew slowly and could not have developed periodically with the exception of Ya02411 in the test area.

Cluster C contains a total of 15 diploid accessions mainly from southwestern China, among which six accessions originated from the Sichuan province (Ya91-1, Ya91-103, Ya02-109, Ya02-106, Ya02-107 and Ya02-108), four accessions from the Guizhou province (Ya02-101, Ya02-102, Ya02-103 and Ya02-104), and four accessions (Ya02-111, Ya02-112, Ya02-113 and Ya02-114) from the Yunnan province, respectively, except for accession Ya00849 collected from Xinjiang of northwestern China. The GSC in the cluster ranged from 0.76 to 0.93. The field-test indicated that accessions in the cluster had distinct morpho-agronomic characters compared with those in cluster A, such as pendulous panicle, lower yield, later heading, and a lower 1000-seed weight (Peng et al. 2006), except for accession Ya00849 that had traits similar with accessions in cluster B.

Cluster D contains only diploid accession Ya02-115 from Yunnan province. This accession has the highest 1000-seed weight among the diploid accessions. Cluster E contains tetraploid accessions Ya90-70 from Sichuan province and Ya01-101 from Guizhou province. The genetic similarity between the two accessions is 0.85. Two accessions have the similar morpho-agronomic characters to those of cluster A. Cluster F contains only the tetraploid accession Ya02-116 from the Yunnan province. In the field-test, accession Ya02-116 has a higher yield and a lower 1000-seed weight than other tetraploid accessions (Peng et al. 2007).

PCoA analysis

Principal coordinate analysis (PCoA) further validated the results of the cluster analysis. In the PCoA analysis, the first two components explained more

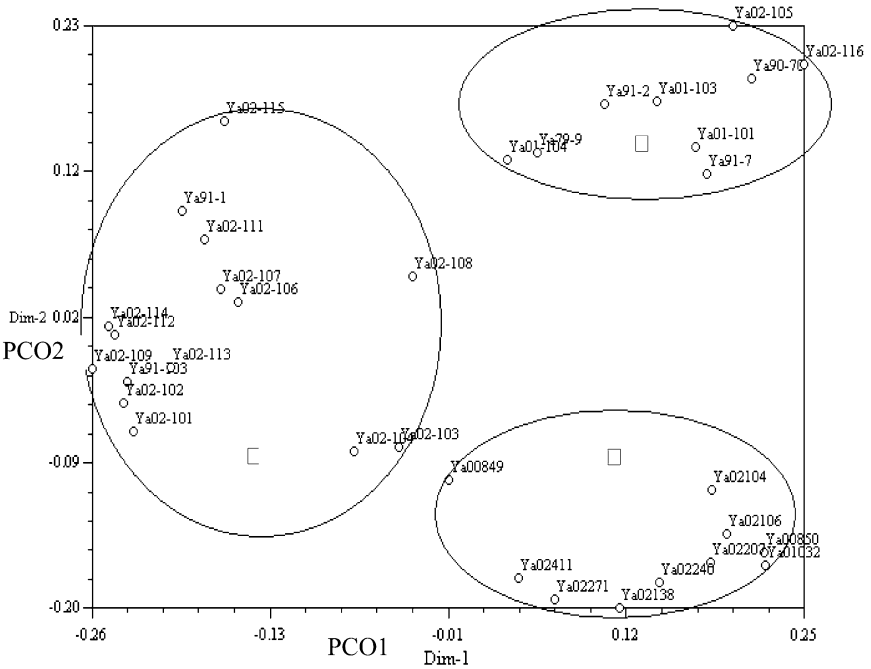

Fig. 2. Principal coordinate analysis for the first and second coordinates estimated for 400 AFLP markers using the genetic similarity matrix for 34 wild orchardgrass accessions.

than 44% of the variation in the estimates of genetic similarity. Altogether, three distinct groups were revealed by the first two principal coordinates (Fig. 2). Group I included all tetraploid accessions. The diploid accessions separated into two main groups that were mainly related to the place of distribution, i.e., one accessions from southwestern China were placed in group II, and the other accessions originating from Xinjiang were clustered in group III. Accordingly, the PCoA results clearly indicated that the tetraploid accessions were widely seperated from the diploid accessions and the diploid accessions from the same geographic locations or nearby regions tended to cluster into the same groups (Fig. 2).

Based on combined data from phenotypic and AFLP analyses, accessions like Ya02-116, Ya01-101 and Ya90-70 are morphologically and genetically potential for breeding purpose because of their higher yield and clear genetic variation.

DISCUSSION

Genetic diversity among 34 wild orchardgrass accessions, including 32 accessions from southwest and northwest China and two accessions from USA, was analyzed by means of AFLP markers. In this study, high level of polymorphism (80.5%) and a relatively low level of similarity coefficient between accessions (0.69–0.93) indicated a high level of genetic diversity in the wild orchardgrass germplasm. The range in genetic similarity coefficients from our study is consistent with the range for ISSR-based GS (0.61–0.93) for the 50 worldwide orchardgrass in the study by Zeng et al. (2006). In their study, the GS coefficients (0.75–0.92) for 21 wild Chinese orchardgrasses encompassed were higher than those from our study, but a lower GS coefficient compared with those from other countries was still detected. The reason for this might be that wild orchardgrass of China distributed in an extensive regions with diverse environmental condition. Therefore, the results of these two studies showed that the great genetic variation existed in the wild Chinese orchardgrass.

PCoA indicated that all tetraploid accessions were widely separated from the diploid accessions. The clustering of wild orchardgrass accessions based on AFLP polymorphisms was consistent with ploidy and variations found in morphological and agronomic characteristics (Peng et al. 2006). The results from cytogenetic and biochemic genetics of wild Chinese orchardgrass also revealed differences between the diploid and the tetraploid accessions (Shuai et al. 1998). Besides that, Sahuquillo and Lumaret (1995) reported similar findings working with morphological, allozyme and phenolic variation in subtropical group of orchardgrass. Lindner and Garcia

(1997) who investigated Galician orchardgrass also suggested that ploidy level is a significant factor in genetic differentiation of this species.

The tested diploid accessions from southwestern and northwestern China tended to cluster into different groups. Thus, the grouping of accessions was strongly linked with their geographic origin. This could be due to that large geographic discontinuities between southwest and northwest regions which favors gene isolation between populations. Additionally, in the phenotypic test, nine among ten accessions from Xinjiang were morphologically uniform because they failed to flower in the field plot, but only one (Ya02411) was distinct from other diploid cytotypes in developmental characteristics (Peng et al. 2006, 2007). The reason accounting for these results might be that accessions, of the same origin, could have developed different features due to environmental differences.

The diploid parentage of tetraploids in Dactylis has been best documented by Stebbins and Zohary (1959), Borrill (1977) and Lumaret (1988). By means of isozyme profiles and cpDNA restriction patterns, Lumaret (1984) and Lumaret et al. (1989) revealed a strong genetic similarity between the Galician diploids and tetraploids. These results were also in agreement with reports by Lumaret and Borrientos (1990) who assessed phylogenetic relationships between sympatric diploid and tetraploid plants of orchardgrass. In the present study, the greater variation was found among accessions within rather than among ploidy levels based on AMOVA analysis. The low percentage of variation (AMOVA: 12.02%) obtained by AFLP marker indicated a close genetic relationship between the wild Chinese diploid and tetraploid orchardgrass accessions from different geographic origins. Consequently, it is once confirmed that diploid and tetraploid orchardgrass are genetically very close.

In summary, from the present study it can be concluded that there was a close genetic relationship between diploid and tetraploid orchardgrass accessions and that great variation exists among accessions within ploidy level and geographical region. Genetic diversity and relationships of wild orchardgrass forms with different ploidy and geographic differentiation patterns, as indicated by this study, can be used in the development of germplasm collection, breeding and conservation. The results clearly indicate that comprehensive germplasm collection in major geographic regions such as southwest and northwest China is required to broaden the genetic base and sample the full extent of the available variation. Breeding strategies need to exploit the existing variation within the wild orchardgrss germplasm.

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