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Genetic variation and comparison of orchardgrass (*Dactylis glomerata* L.) cultivars and wild accessions as revealed by SSR markers

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ABSTRACT. Orchardgrass is a highly variable, perennial forage grass that is cultivated throughout temperate and subtropical regions of the world. Despite its economic importance, the genetic relationship and distance among and within cultivars are largely unknown but would be of great interest for breeding programs. We investigated the molecular variation and structure of cultivar populations, compared the level of genetic diversity among cultivars (Baoxing, Anba, Bote, and Kaimo), subspecies (*Dactylis glomerata* ssp *Woronowii*) and advanced breeding line (YA02-116) to determine whether there is still sufficient genetic diversity within presently used cultivars for future breeding progress in China. Twenty individuals were analyzed from each of six accessions using SSR markers; 114 easily scored bands were generated from 15 SSR primer pairs, with an average of 7.6 alleles per locus. The polymorphic rate was 100% among the 120 individuals, reflecting a high degree of genetic diversity. Among the six accessions, the highest genetic diversity was observed in Kaimo (H = 0.2518; I = 0.3916; P = 87.3%) and 02-116 had a lower level of genetic diversity (H = 0.1806; I = 0.2788; P = 58.73%) compared with other cultivars tested. An analysis

of molecular variance revealed a much larger genetic variation within accessions (65%) than between them (35%). This observation suggests that these cultivars have potential for providing rich genetic resource for further breeding program. Furthermore, the study also indicated that Chinese orchardgrass breeding has involved strong selection for adaptation to forage production, which may result in restricted genetic base of orchardgrass cultivar.

Key words: *Dactylis glomerata* L.; Cultivar; Simple sequence repeats; AMOVA

INTRODUCTION

Orchardgrass, or cocksfoot (*Dactylis glomerata* L.) is one of the most important cool-season forage grasses. It has been naturalized on nearly every continent and is a commonly used species for forage and hay production, owing to its good nutrition, high yield and good shade tolerance. Because of the importance of orchardgrass as a forage and hay grass, an understanding of species, population, and cultivar relationships will benefit plant breeding and enhance efforts to improve forage yield, quality, and other value-added traits. Compared with other field crops, grass breeding has a rather short history starting in the 1920s (Bolaric et al., 2005). For grass breeding, ecotype collections play an important role in broadening the genetic basis. Orchardgrass germplasm has been studied extensively in China over the past 20 years, and many natural populations and wild germplasms have been collected. Three cultivated varieties, including Gulin, Baoxing, and Chuandong, have been developed from wild materials recently, and five introduced varieties have been released. They have been widely utilized in cultivated pastures with high yield and good adaptability to the local environment, but a relevant question in this context is whether there is still sufficient genetic diversity within presently used cultivars for future breeding progress in China. Although previous orchardgrass diversity studies have shown a high level of genetic diversity among and within wild accessions (Zeng et al., 2008; Peng et al., 2008; Xie et al., 2010), the genetic relationship and distance among and within cultivars are largely unknown but would be of great interest for breeding programs. Therefore, further analysis on the genetic diversity of cultivars and a comparison with wild accessions may produce new insights and give a better understanding of the distribution of genetic diversity at the cultivar level.

Orchardgrass is a perennial outbreeding forage grass species, with a gametophytic self-incompatibility system, and therefore each cultivar is a heterogeneous population of genotypes. Molecular markers offer an efficient tool to investigate genetic diversity in plant populations. They have been successfully used to differentiate cultivars of outcrossing species, where the high level of diversity among individual plants can obscure cultivar variability (Benjamin et al., 2003; Bolaric et al., 2005). Compared with other molecular markers, microsatellites (simple sequence repeats, SSRs) are highly polymorphic, abundant and are accessible to other research laboratories via published primer sequences. They have become the marker of choice for genetic mapping, gene tagging, genetic diversity study, genomic and cDNA fingerprinting. Currently, SSR markers have been used to determine genetic diversity and relationships in many plant species (Zoghlami et al., 2009; Cheng and Huang, 2009;

Tahan et al., 2009). The objectives of this study were to investigate the molecular variation and structure of population cultivars, compare the genetic variation among cultivars, subspecies and an advanced breeding line, and to determine whether there is still a relatively high genetic diversity within orchardgrass cultivar for further breeding programs.

MATERIAL AND METHODS

Plant materials

A total of six accessions, including orchardgrass cultivars, advanced breeding line and subspecies, were chosen for analysis by means of SSR markers (Table 1). Seeds from each accession with a pretreatment at a low temperature were germinated on absorbent filter paper on Petri dishes with a temperature of 23°C. Germinated seeds were transplanted into a sand-peat mixture and the plants maintained in a greenhouse with an approximate temperature of 22°C and a 16-h photoperiod until they were 8 weeks old, then transplanted to field in the campus experimental garden at Sichuan Agricultural University, Ya'an, Sichuan (29° 58' N, 102° 58' E), and considered to be genotypes. The SSR analysis of accessions was performed on 20 randomly selected individual plants from each of the six accessions.

Table 1. Information on orchardgrass (*Dactylis glomerata* L.) accessions studied.

Accession name	Taxon	Country of origin	Year of release	No. of individuals	Background
YA02-116	*D. glomerata* L.	China	-	20	Wild material from Yunnan, China, advanced breeding line
PI538922	*D. glomerata* subsp *woronowii*	Russian	-	20	Wild accession was collected in 1986 in Azerbaijan and developed in Leningrad, Russian Federation
Baoxing	*D. glomerata* L.	China	1999	20	Developed from wild material in Baoxing county, China
Anba	*D. glomerata* L. cv. Amba	Denmark	2005	20	Introduced cultivar from Denmark
Bote	*D. glomerata* L. cv. Porto	Australia	2008	20	Introduced cultivar from Australia. It was derived from germplasm collected in Portugal
Kaimo	*D. glomerata* L. cv. Cambria	Spain	2001	20	Selected from ecotypes in northwestern Spain. Released by Barenbrug, USA

DNA extraction

DNA was extracted from fresh young leaves of 120 individuals representing six selected accessions by the cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1990). The quality and concentration of DNA were determined by comparing the sample with known standards of lambda DNA on 1% (w/v) agarose gels. The isolated genomic DNA was diluted to 10 ng/μL and stored at -20°C for use.

Primer selection and PCR amplification

One hundred SSR primer pairs for orchardgrass kindly provided by Honewei Cai, China Agricultural University, were first screened for PCR amplication. Fifteen polymorphic primer

pairs that generated clear, reproducible banding patterns were selected for further analysis. The effects of Mg^{2+}, dNTP, DNA template, primers, and DNA polymerase on the amplication were tested, and the determined optimal reaction system of SSR for orchardgrass was as follows: 50 ng template DNA, 1.5 mM Mg^{2+}, 1 U Taq DNA polymerase，240 μM dNTP, 0.4 μM primer in a total of 15-μL reaction volume. PCR amplification was performed using a PTC-200 Thermocycler (Bio-Rad Laboratories, CA, USA) under the following conditions: 5 min denaturation at 94°C, 35 cycles of 1 min at 94°C, 1 min at 48°-52°C, and 1 min at 72°C, and then a final extension of 10 min at 72°C and storage at 4°C. The optimal annealing temperature was determined by gradient PCR. Amplification fragments were separated on 6% denatured polyacrylamide gels [acrylamide-bisacrylamide (19:1), 1.0X TBE]. After electrophoresis, the gel was stained by $AgNO_3$ solution and photographed using the Gel Doc XR System (Bio-Rad Laboratories).

Data analysis

For the statistical analysis, the patterns at all SSR loci were scored for each polymorphic band as 1 for the band presence and 0 for the band absence. The resulting present/absent data matrix was analyzed using POPGENE v.1.31 (Yeh et al., 1999). The genetic identity (GI) and the genetic distance (GD) among six accessions were also computed using the same program. Genetic diversity was evaluated with the polymorphic information content (PIC) = $1 - \sum P^2_{ij}$, where P_{ij} is the frequency of the *j*th allele for the *i*th locus, summed across all alleles of the locus (Nei, 1973). The nonparametric analysis of molecular variance (AMOVA) program v 1.55 (Excoffier et al., 1992) was used as an approach to describe genetic structure among and within the populations. The input files for AMOVA were prepared with the aid of the DCFA1.1 program written by Zhang (2001). A dendrogram was constructed by the genetic identity matrices to display accession relationships using the unweighted pair group mean algorithm (UPGMA) of NTSYS-pc, version 2.10 (Rohlf, 1997).

RESULTS

Degree of polymorphism

Fifteen primer pairs were used to evaluate the genetic diversity of the six accessions (lines) (Table 2). Polymorphic bands, total bands, polymorphic rates (%), and PIC values per primer pair are presented in Table 3.

A total of 114 easily scorable bands were generated from 15 selected primer pairs, with an average of 7.6 alleles per locus, and a range of 4 (A02N22) to 11 (A01K14, A01E14) alleles. Only 9.6% of these markers were found in less than 20 individual plants, while the majority, 53%, were present in more than 60 plants (data not shown). Unique bands present in all the individuals of a particular cultivar but not present in any other individual of another cultivar were not observed in this study (Figure 1). The average polymorphic rate was 100% among 120 individuals within 6 accessions, reflecting the high degree of genetic diversity over all individuals studied. The PIC for SSRs varied from 0.51 for A02N22 to 0.90 for A01E14, with a mean of 0.81. The level of SSR variability differed among the accessions investigated. The polymorphic rate for a single accession ranged from 58.73 (02-116) to 87.3% (Kaimo), with an average of 68.12% (Table 4).

Table 2. Orchardgrass SSR primers used in this study.

Primer code	Primer sequence (5'-3')	SSR repeat	Amplicon size
A01F24	AAAATGTTTTATTCTCAGCCC TGCAAGATGGAATGCTCT	$(CA)_{11}$	175
A01I11	CATCGTAATGACTGCTAGTCC ACAGATCCATCGGTGGTT	$(CA)_{13}(TC)_{28}$	168
A01K14	AAGGATGGCCTGATCTTC GCAGAGGTCTTTCTCTTGG	$(CA)_{18}$	164
A02A10	AGGTTACCGATAGTAAGTGGG AGGGGATGGTTGGTTAGTAT	$(TG)_{16}$	130
A02J20	TCCAATGTTACACACATAGCA TGTGTGCGATTTTCTGTG	$(CA)_5(CA)_7(CA)_{10}$	208
A02N22	AAACATGTCGTGGTCGTC ATCATTTGTTATGCCGGTAG	$(CA)_{13}$	116
A03B16	TCTGGAATCTCTCTGAAATCA ATCTTGACCCTGATGTTCTG	$(CA)_{12}$	158
A03C05	TAAGAATCGATCCTCCCG ACCTTCTTCCACTCCGTC	$(CA)_{11}$	149
A03K22	AGACTCTAGGGTGGCACAC GTAGCACGCTAACGAGAGAT	$(CA)_{19}$	112
A04C24	AGCAACATATCTTACTGCAATG ATCAAACTCGAAAAGTTGTCA	$(CA)_{10}$	111
A04O08	AGAGGTTAGATGGATGTAGGC ATAGACCCATAGCATGTTGG	$(TG)_{10}$	136
A01E14	ACCCGTTTTCTATCTCCAG GTTCTAGCGTCGTGAGGG	$(CA)_{17}(CGCA)_3(CA)_{15}$	173
B01A05	GAGAGCGGCAGAGTTATTC AAAGGTCGATATCTCTATTCCA	$(GA)_8$	118
B01C15	GTCGATTGATGGTGTACGTA TCTAGTGCTACTTGTATGCACC	$(GA)_{17}$	116
B01E09	ACAACTCACAAACTCAAGAACA GTGGACTCGGAGGAGAAG	$(GA)_{27}$	123

Table 3. Amplification results from 15 primer pairs.

Primer code	Total number of bands	Polymorphism bands	Polymorphic rate (%)	Polymorphism information content
A01F24	5	5	100	0.77
A01I11	8	8	100	0.86
A01K14	11	11	100	0.87
A02A10	9	9	100	0.88
A02J20	9	9	100	0.82
A02N22	4	4	100	0.51
A03B16	5	5	100	0.74
A03C05	6	6	100	0.84
A03K22	7	7	100	0.86
A04C24	6	6	100	0.83
A04008	8	8	100	0.79
A01E14	11	11	100	0.90
B01A05	6	6	100	0.82
B01C15	9	9	100	0.78
B01E09	10	10	100	0.89
Total	114	114	-	-
Mean	7.6	7.6	100	0.81

Genetic variation based on SSR markers

AMOVA revealed a much larger genetic variation within accessions (65%) than between them (35%) (Table 5). To obtain a more detailed view of the distribution of genetic variation within the six accessions, the polymorphic rate (P), Nei's gene diversity (H) and

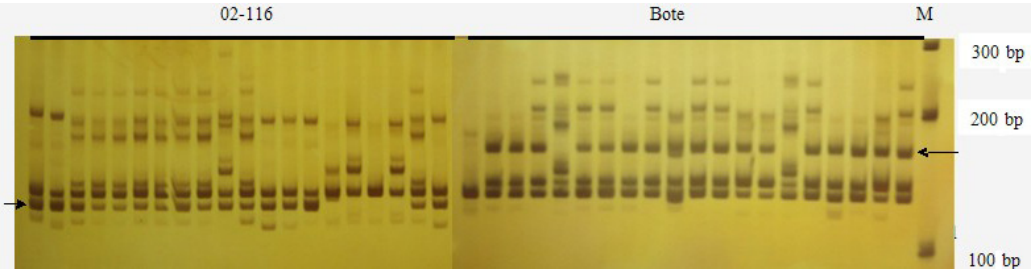

Figure 1. Example of DNA polymorphism detected in two orchardgrass accessions, 02-116 and Bote, with primer pair A03C05. The PCR products were separated on 6% denatured polyacrylamide gels. *Lane M* = Molecular weight marker, 100-bp ladder.

Shannon's information index of diversity (I) were estimated. Among the six accessions, the highest genetic diversity was observed in Kaimo (H = 0.2518; I = 0.3916; P = 87.3%), followed by Anba > PI538922 > Bote > Baoxing > 02-116 (Table 4).

Table 4. Genetic polymorphism indexes for six accessions of orchardgrass.

Code	H	I	PB	P (%)
02-116	0.1806	0.2788	74	58.73
Baoxing	0.1930	0.2952	77	61.11
Anba	0.2187	0.3406	94	74.6
Kaimo	0.2518	0.3916	110	87.3
Bote	0.1915	0.2939	79	62.7
PI538922	0.1964	0.3027	81	64.29
Mean	0.2053	0.3171	85	68.12

H = Nei's gene diversity; I = Shannon's information index of diversity; PB = polymorphic bands; P = polymorphic rate.

Table 5. Analysis of molecular variance (AMOVA) for orchardgrass.

Source of variation	d.f.	SSD	MSD	Variance component	Total variance
Variance among accessions	5.00	953.2	190.6	8.7	0.35
Variance within accessions	114.00	1869.1	16.4	16.4	0.65

DF = degrees of freedom; SSD = sum of squares; MSD = mean square deviation.

Phylogenetic relationships revealed by clustering of SSR markers

The values of the GI coefficient among accessions ranged from 0.8084 to 0.9273, with a mean of 0.8668. A dendrogram was constructed using data from UPGMA cluster analysis based on the genetic identity coefficient matrix for all the accessions. GD for the six accessions ranged from 0.0755 to 0.2126, with a mean of 0.1437 (Table 6). The lowest GD value (0.0755) was obtained between Kaimo and PI538922. The highest GD value (0.2126) was obtained between Baoxing and PI538922. Six accessions were clustered into three major groups (with a GI of 0.864) (Figure 2). Group one contained three accessions: 02-116, Baoxing and Anba, whereas group two included two accessions Kaimo and PI538922, and group three only contained Bote.

Table 6. Nei's unbiased measures of genetic identity and genetic distance.

Groups	02-116	Baoxing	Anba	Kaimo	Bote	PI538922
02-116	-	0.8947	0.8828	0.9089	0.8292	0.8371
Baoxing	0.1113	-	0.8938	0.8891	0.8310	0.8084
Anba	0.1247	0.1123	-	0.9001	0.8310	0.8244
Kaimo	0.0956	0.1175	0.1052	-	0.8900	0.9273
Bote	0.1873	0.1851	0.1851	0.1165	-	0.8555
PI538922	0.1778	0.2126	0.193	0.0755	0.1561	-

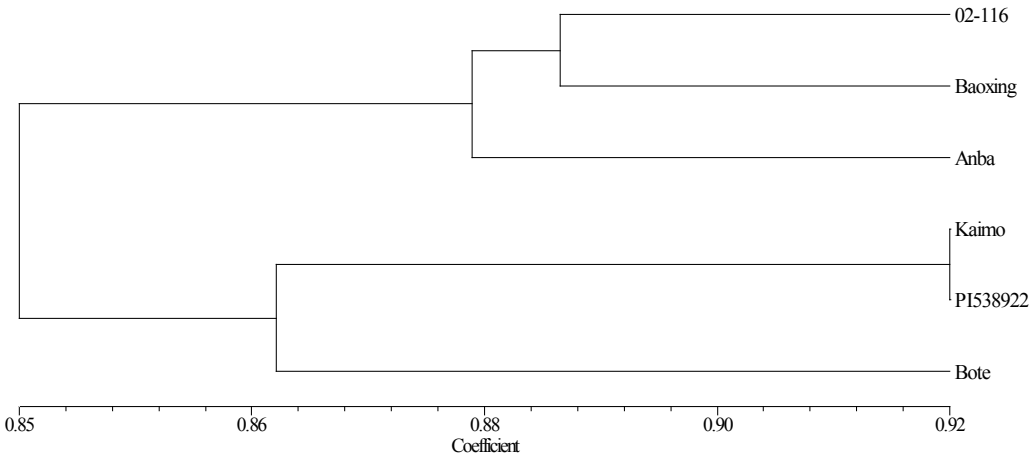

Figure 2. UPGMA-derived dendrogram of six orchardgrass accessions based on genetic identity (Nei, 1973). Accession designations refer to Table 1.

DISCUSSION

Based on our SSR data set, the polymorphic rate (P) over the six accessions was 100%. This showed that orchardgrass has a high level of genetic diversity, which was higher than reports of AFLP variation (P = 84.0%) (Peng et al., 2008) and ISSR variation (P = 86.3%) (Zeng et al., 2006), yet much higher than SRAP variation (P = 84.38%) (Zeng et al., 2008) in orchardgrass. Therefore, SSRs proved to be a high-resolution method for the detection of variation among and within orchardgrass accessions, suggesting that it was clearly possible to detect relatively more polymorphism by SSR markers.

The patterns of genetic variability within and among populations can be influenced by mutation, genetic drift, mating system, and selection. Typically, out-crossing species maintain relatively more of their genetic variation within populations than do self-pollinating species (Larson et al., 2004) and the variation is known to decrease with a reduced level of cross-fertilization (Charmet and Balfourier, 1994). As much as 65% of the variation was distributed within the populations, comparable with the values obtained from similar studies of other outbreeding grass species (Bolaric et al., 2005; Fjellheim and Rognli, 2005). The high level of intercultivar diversity detected in this study could be largely due to the strong outcrossing mechanism in this species, which is likely to increase the degree of polymorphism. This

observation also suggests that these cultivars have potential for providing further gain from selection within a breeding program. As for other outbreeding species, cultivar development is based on polycrosses among multiple parents to develop a synthetic population. In this study, the cluster analysis clearly demonstrates the existence of three distinct orchardgrass gene-pools among the accessions investigated. This information may assist plant breeders in their decisions of what germplasm to include in their breeding program. To broaden the existing breeding germplasm, it may be useful to include cultivars or ecotypes from major geographic regions and special eco-geographic environments such as tropic and frigid areas in order to sample the full extent of the available variation.

The genetic diversity of a species is affected by a number of evolutionary factors including mating system, seed dispersal, geographic range, as well as natural selection. Of these factors, breeding system is the main one that affects the diversity both among and within populations (Hamrick and Godt, 1990). In this study, the highest genetic diversity was observed in Kaimo (H = 0.2518; I = 0.3916; P = 87.3%), followed by Anba > PI538922 > Bote > Baoxing. The advanced breeding line, 02-116, had a lower level of genetic variation (H = 0.1806; I = 0.2788; P = 58.73%) compared with other cultivars tested in this study. In orchardgrass, breeding practice older or long-established cultivars were commonly based on natural populations, whereas newer cultivars were bred from a restricted number of clones, or earlier released cultivars. On the basis of this, there seems to be a general assumption that modern breeding methods will reduce genetic diversity (Clunies-Ross, 1995). In addition, newer cultivars have been subjected to a more intensive selection pressure, since they are synthetic populations based on the selection of a restricted number of superior genotypes (10-30) from a large (2000-4000) and diverse breeding population, following some form of progeny testing. Therefore, the breeding of cultivars has certainly involved strong selection for adaptation to forage pr uction, which may explain the restricted genetic base of the advanced breeding line (02-116). Orchardgrass breeding od needs a wide variety of breeding materials, of which introduced cultivars are an important source. In China, five introduced cultivars have been released recently, including Anba and Bote. Normally, seeds of introduced cultivars would have given rise to naturalized populations that may have, in turn, become the basis for development of new cultivars. Through this way, new genetic material could be introduced to increase or sustain the level of diversity in newer cultivars. On the other hand, natural pollination and crossing with local cultivars and wild accessions could affect the genetic diversity of introduced cultivars. This could explained why Anba has a high genetic similarity with Baoxing (GS = 0.8947) and 02-116 (GS = 0.8828). In short, the difference in genetic diversity of the cultivars analyzed indicates the effect of different methods of cultivar development.

Historically, cultivar identification and property rights protection have been based on morphological characteristics such as plant height, spike length, leaf width, flag leaf length, and flower color, but molecular markers are valuable tools for cultivar identification. An additional promising marker system is SSRs. Several results have shown that SSR is an appropriate technique for cultivar identification. But in our study, the UPGMA analysis conducted on all the individuals (data not shown) did not define a single cultivar, indicating that it would be difficult to use SSRs to identify orchardgrass cultivars. In addition, in this study, no marker could be identified that was exclusively found in all genotypes of one cultivar and absent in the others. This problem (cultivar identification) could be solved if the markers chosen were cultivar-specific and preferably linked to phenotypic traits used in the DUS (distinctness, uniformity, and stability) testing.

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