Identification of orchardgrass (*Dactylis glomerata* L.) cultivars by using simple sequence repeat markers

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ABSTRACT. The accurate identification of orchardgrass (*Dactylis glomerata* L.) cultivars is necessary to ensure purity for consumers, the effective utilization of cultivars, and to protect the intellectual property for breeders. Therefore, this study aimed to use SSR to construct DNA fingerprinting of orchardgrass cultivars. The genetic diversity of 32 orchardgrass cultivars originated from 21 countries, but grown in China, was assessed using a set of 29 SSR markers distributed across 9 linkage groups of the orchardgrass genome. A total of 229 bands were detected, with an average of 7.9 bands per marker. The average polymorphic rate for the species was 92.1%. The polymorphism information content ranged from 0.771 to 0.893.

The genetic similarity ranged from 0.55 to 0.84, which confirmed a high level of genetic diversity among orchardgrass cultivars. The unweighted pair-group method, in combination with the arithmetic mean algorithm (UPGMA) dendrogram and principal coordinate analysis, showed a separation of 6 major clusters among 32 cultivars. The number of distinguishable cultivars ranged from 3 to 23, with an average of 12.1 per primer. Moreover, 11 bands that showed stable and repeatable SSR patterns were amplified by A01E14, A01K14, and D02K13. These bands were used to develop the DNA fingerprints for 32 orchardgrass cultivars. In the DNA fingerprints constructed, each cultivar had a unique fingerprinting pattern that was easily distinguished from the others. These results indicate that the SSR marker was polymorphic, and reliable for use in potential large-scale DNA fingerprinting of orchardgrass cultivars.

Key words: *Dactylis glomerata* L.; Orchardgrass cultivar; DNA fingerprinting; Simple sequence repeats; Discriminatory power

INTRODUCTION

Dactylis L. is an allogamous, variable monospecific genus that contains several subspecies distinguished by morphology, chromosome number, and distribution. The main species of *D. glomerata* L. is referred to as orchardgrass or cocksfoot (Lindner and Garcia, 1997; Bushman et al., 2011), and is an economically important and extensively used cool-season perennial forage grass in China, along with many other countries that have temperate and cold climates (Sanada et al., 2010). Because of its high yield, good nutrition, adaptability, and shade tolerance, orchardgrass is widely used for grazing and hay production worldwide (Xie et al., 2012).

Knowledge about genetic diversity and genetic relationships among breeding materials has a great impact on crop improvement (Ganesh and Thangavelu, 1995). Orchardgrass germplasms are diverse, with a high level of genetic diversity, which provides important and valuable breeding materials and gene resources. Plant breeders tend to use clonal breeding, ecotype selection, and morphological mass selection to obtain excellent germplasms for breeding and animal husbandry (Casler et al., 2000). To date, 8 orchardgrass cultivars have been released, and many introduced cultivars have been utilized, in cultivated pastures with high yield and good adaptability to the local environments of China (Xie et al., 2010). With the increased use of orchardgrass cultivars, it is important to select the cultivar with the best fit for a specific service to increase the possibility of suitable performance for users. Therefore, the ability to rapidly and reliably identify a cultivar is becoming increasingly important.

Bushman et al. (2011) assessed the molecular diversity of 3 cultivars and several subspecies of orchardgrass. In addition, another study assessed the isozymes of orchardgrass populations in Europe (Lumaret, 1982). However, a method to identify individual cultivars of orchardgrass has yet to be developed, which would contribute toward maintaining the purity of cultivar utilization for consumers and effective intellectual protection for breeders.

The genetic diversity of crop species may be determined using morphological and agronomic characteristics, in addition to biochemical and DNA marker analysis (Liu, 1997). Orchardgrass is an allogamous species that has a strong self-incompatibility system (Xie et al., 2012), which guarantees that alleles in each cycle of mating are reshuffled to cause a high degree of genetic variation within populations (Hirata et al., 2011). Therefore, it is difficult to identify orchardgrass cultivars based on physiological and morphological traits, because these traits are subjective and quantitative in practice, limited in number, and affected by environmental conditions and the experience of evaluators (Roldán-Ruiz et al., 2001; Bolaric et al., 2005). To overcome these limitations, a number of molecular markers in orchardgrass have been employed for genetic diversity assessments, genetic mapping, and quantitative trait loci (QTL) analysis, because these traits are not influenced by variable environmental conditions with similar phenotype or plant phenology (Belaj et al., 2002). These markers include sequence-related amplified polymorphism (SRAP) (Zeng et al., 2008; Xie et al., 2011), random amplified polymorphic DNA (RAPD) (Kolliker et al., 1999; Tuna et al., 2004), amplified fragment length polymorphism (AFLP) (Peng et al., 2008), inter-simple sequence repeat (ISSR) (Zeng et al., 2006), and simple sequence repeats (SSR) (Xie et al., 2010; Bushman et al., 2011).

Of these molecular markers, SSR or microsatellites are highly polymorphic, abundant, reliable, and co-dominant. As a result SSR has been widely used for fingerprinting and the identification of various cultivars, including pear (Erfani et al., 2012), apple (Zhang et al., 2012a), and perennial ryegrass (Wang et al., 2009). However, additional genetic information about fingerprinting by SSR for allogamous forage grass species, such as orchardgrass, is still needed. Yet, it is not possible to use small amounts of genotype for identifying pure lines, cloning, or single crosses. Thus, conventional methods have been used for the effective identification of different species to date. Such methods include bulking or pooling strategies using 20-40 individuals to create a pool that represented a cultivar, with the removal of rare alleles from some individuals to form a cultivar (Marshall and Brown, 1975; Morell et al., 1995; Forster et al., 2001).

Recently, several orchardgrass cultivars originating from 21 countries, but which are grown in China, were collected for genotyping their DNA fingerprints. Therefore, the primary goals of this study were to: a) investigate the genetic diversity among these orchardgrass cultivars based on 29 highly polymorphic SSR primer pairs, b) examine the discriminatory power of these SSR markers for orchardgrass cultivars, and c) accurately identify, characterize, and establish DNA fingerprints for orchardgrass cultivars.

MATERIAL AND METHODS

Plant material

A total of 32 orchardgrass cultivars or lines were analyzed by SSR markers, of which 27 released cultivars were collected from 21 different countries, and represented most of the commercially available orchardgrass cultivars used in China, in addition to 5 new lines from China (Table 1). The seeds of each cultivar were germinated on absorbent filter paper in Petri dishes at a temperature of 25°C after a pretreatment at a temperature of 3°C. Germinated seeds were transplanted into a sand-peat mixture, and maintained in a

greenhouse at a temperature 23°C and 16-h photoperiod. Once the seedlings were 9 weeks old, they were transplanted to the field in the experimental station of Sichuan Agricultural University, Ya'an, China (29°58'N, 102°58'E).

Table 1. Description of 32 orchardgrass (*Dactylis glomerata* L.) cultivars used in this study.

Code	Name	Country of origin	Abbr.	Improvement status
1	Krasnodarskaya	Krasnodar territory, Russia	RU	Cultivar
2	Chinook	Romania	RO	Cultivar
3	Frode	Sweden	SE	Cultivar
4	Nika	Poland	PL	Cultivar
5	Georgikon	Hungary	HU	Cultivar
6	Weihenstephaner	Bavaria, Germany	DE	Cultivar
7	Asta	Lithuania	LT	Cultivar
8	Hawk	Iowa, United States	US	Cultivar
9	Oron	Canada	CA	Cultivar
10	Gippsland	Australia	AU	Cultivar
11	Akaroa	New Zealand	NZ	Cultivar
12	Gulin	Gulin, Sichuan, China	CN	Cultivar
13	Baoxing	Baoxing, Sichuan, China	CN	Cultivar
14	Chuandong	Daxian, Sichuan, China	CN	Cultivar
15	Amba	Denmark	DK	Cultivar
16	Porto	Italy	IT	Cultivar
17	Donata	Germany	DE	Cultivar
18	Grassland Wana	New Zealand	NZ	Cultivar
19	Cambria	Spain	ES	Cultivar
20	Baridana	Slovakia	SK	Cultivar
21	Athos	Luxembourg	LU	Cultivar
22	Aldebaran	Germany	DE	Cultivar
23	Endurance	United States	US	Cultivar
24	Sparta	United Kingdom	UK	Cultivar
25	Cristobal	France	FR	Cultivar
26	Intensiv	Czech Republic	CZ	Cultivar
27	Baraula	Netherlands	NL	Cultivar
28	01-103	Sichuan, China	CN	New line
29	02-116	Kunming,Yunnan, China	CN	New line
30	79-9	Lushan, Jiangxi, China	CN	New line
31	01175	Dushan, Guizhou, China	CN	New line
32	01472	Chongqing, China	CN	New line

DNA extraction

Leaf tissues were collected from young plants, and were frozen by liquid nitrogen for DNA isolation. Approximately 100 mg leaf tissue from 25 genotypes of each cultivar was extracted using the cetyltrimethyl ammonium bromide (CTAB) method (Doyle et al., 1990). DNA quality and concentration were measured by electrophoresis on 0.8% (w/v) agarose gel and NanoDrop 1000 ultraviolet and visible spectrophotometry (UV) (ND, NanoDrop Technologies, Inc. Wilmington, DE, USA). The genomic DNA was diluted to 10 ng/µL, and stored at 4°C until use.

Screening of SSR primer pairs

Twenty-nine primer pairs were used to evaluate the genetic diversity of the 32 culti-

vars, which were distributed across 9 linkage groups of the orchardgrass genome (Xie et al., 2010) (Table 2). Primers were screened from 88 SSR primer pairs, using a panel of 4 cultivars, i.e., Frode, Akaroa, Baoxing, and Amba. These primer sequences were kindly provided by Dr. Cai from Japan Grassland Farming and Forage Seed Association, Forage Crop Research Institute, Japan (Hirata et al., 2011).

Table 2. Orchardgrass SSR primers used in this study and the results detected in 32 orchardgrass cultivars.

Primers	LG	SSR Repeat motif	Forward primer (5'-3')	Reverse primer (5'-3')	EAS bp	AT °C	TB bp	PB bp	P (%)	PIC	DP
A03C05	1	$(CA)_{11}$	TAAGAATCGATCCTCCCG	ACCTTCTTCCACTCCGTC	149	52	9	9	100	0.868	12
A01I13	1	$(TG)_{23}$	ATGCTGTTTGATCACAGTCA	GTTGGACTGCCATTACTAGC	170	52	8	6	75	0.868	9
A01F24	1	$(CA)_{11}$	AAAATGTTTTATTCTCAGCCC	TGCAAGATGGAATGCTCT	175	52	6	5	83	0.827	8
B04H05	1	$(GA)_{15}$	AACAAGAAGGGAGGAAGAAC	TTGAGTTGCGTATGCATG	124	52	7	7	100	0.830	7
B03E14	1	$(GA)_{33}$	CAGCCTCCAATGTGATAGTT	ATATTTCCTCTTTCCATGATTG	174	52	5	5	100	0.778	4
B02N20	2	$(GA)_{28}$	CATATTGAGGAGACTGTCAGC	CAGACACACCAAGTTTGCTA	159	54	9	9	100	0.867	10
A01L14	2	$(TG)_{26}$	GCACAATGACACCAAATATG	ATCAGCATTGTGACCACC	173	52	8	8	100	0.863	14
A01L12	4	$(CA)_{10}$	GGCTCAATCCTTAGACACTG	ACGAGAAATCGTCGTATTGT	122	53	7	5	71	0.834	8
A02A10	4	$(TG)_{16}$	AGGTTACCGATAGTAAGTGGG	AGGGGATGGTTGGTTAGTAT	130	54	7	7	100	0.850	9
A01E02	5	$(TG)_{18}(GA)_8$	AGCTGTGGAGAAAAAAATGA	GATGCCATTAAGTTCAAAATG	119	52	5	5	100	0.771	5
A01B10	5	$(CA)_{13}(TC)_{28}$	TCTTCCTTGGAAAACATCAA	ACTTGCTTACACGGTATCATG	125	52	5	5	100	0.799	11
B04O13	5	$(GA)_{34}$	TTCAGGTACATGGCTTCTCT	ACGGCCTATAGATCAAGTCA	162	52	6	6	100	0.799	8
B06N03	6	$(TC)_{25}$	TCTCTATAGCCGCTCGTG	CTCCTTTCTCTTGCTCGG	125	54	5	5	100	0.780	3
A01K14	9	$(CA)_{18}$	AAGGATGGCCTGATCTTC	GCAGAGGTCTTTCTCTTGG	164	54	11	10	91	0.890	23
A03K22	1	$(CA)_{19}$	AGACTCTAGGGTGGCACAC	GTAGCACGCTAACGAGAGAT	112	55	9	8	89	0.876	21
B01E09	3	$(GA)_{27}$	ACAACTCACAAACTCAAGAACA	GTGGACTCGGAGGAGAAG	123	54	8	7	88	0.859	15
A03H11	3	$(CA)_{10}$	TCACACACATAACACGCAG	CGCGTGTATTTATTATCTTTCA	123	52	7	5	71	0.857	5
B01A02	8	$(GA)_{16}$	TTCTCCATTAAGCCTCCAG	CAGCGAGTCCTTGTCGAC	183	55	8	8	100	0.868	17
A01E14	-	$(CA)_{17}$ $(CGCA)_3(CA)_{15}$	ACCCGTTTTCTATCTCCAG	GTTCTAGCGTCGTGAGGG	173	55	9	9	100	0.861	18
A02B24	-	$(TG)_{30}$	GACGAGGCATGTTTGTTG	CTCTATAAAACCCATGAGCG	123	53	7	6	86	0.811	8
A02N22	-	$(CA)_{13}$	AAACATGTCGTGGTCGTC	ATCATTTGTTATGCCGGTAG	116	52	9	8	89	0.872	19
A03B16	-	$(CA)_{12}$	TCTGGAATCTCTCTGAAATCA	ATCTTGACCCTGATGTTCTG	158	53	8	7	88	0.873	13
A01I11	-	$(CA)_{13}(TC)_{28}$	CATCGTAATGACTGCTAGTCC	ACAGATCCATCGGTGGTT	168	54	9	8	89	0.880	13
B01C11	-	$(GA)_{19}(TG)_5$	GCCATGTAACCAGAATCCTA	TGTTTGTGCATAGATCAAGC	120	52	10	10	100	0.893	19
B01D10	-	$(TC)_{12}$	GGGAGATCTCAGTGGAGG	CCGTGATAACTCATAAACAGC	127	55	9	9	100	0.876	17
B01F08	-	$(TC)_{28}$	ATTAGTCCGTGTCTCCCAC	TTATCGAGACCTCCAGGAG	125	55	8	7	88	0.826	7
B05P14	-	$(GA)_{26}$	TGTGAGGGCTTGCTTAAG	ATTTGTTGCCATCACCAC	143	51	11	9	82	0.885	16
B05L12	-	$(GA)_{21}$	TTAGAGGTGAAAATTGATCACA	GTGCTTGGATTATGCTGG	157	51	10	8	80	0.879	19
D02K13	-	$(TA)_5(TCTA)_5$	TTGTTGTTCCGTTGCAAC	CGCAGGTTTCAATTTAATAGT	125	52	9	9	100	0.862	14
Total							229	210			
Mean							7.9	7.2	92.1	0.848	12.1

LG = linkage groups; EAS = expected amplicon size; AT = annealing temperature; TB = total bands; PB = polymorphic bands; P = polymorphic rate; PIC = polymorphism information content; DP = discriminatory power for number of cultivars.

PCR amplification and electrophoresis

The SSR-PCR program described by Xie et al. (2012) was followed, with minor revisions to the procedure. Each 15 μL PCR contained 50 ng genomic DNA, 0.4 μM primer, 1.5 mM $MgCl_2$, 240 μM dNTP, 0.75 U Taq Gold DNA polymerase, and 1 μL distilled water. PCR amplification was conducted on a PTC-200 Thermocycler (Bio-Rad Laboratories, Hercules, CA, USA), with the following program: 94°C for 5 min, 35 cycles at 94°C for 1 min, varying annealing temperature (Table 2) for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 10 min, and then a 4°C holding temperature. The PCR amplification products were separated on 6% denatured polyacrylamide gel electrophoresis (PAGE) using a program of 30 min at 200 V, followed by 2 h at 400 V. The resulting gel was stained by $AgNO_3$ solution and photographed with a digital camera (D3100, Nikon, Inc. Melville, NY, USA).

Data analysis

Unequivocal bands were scored as a data matrix for each locus (1 for present and 0 for absent). Nei and Li's (1979) pairwise similarities between cultivars was computed by using the NTSYS pc2.10 (Rohlf, 2000). A dendrogram was constructed based on the un-weighted pair-group method with arithmetic mean algorithm (UPGMA). The principal coordinate analysis (PCoA) based on the similarity matrix was computed from the same program. The polymorphic rate (P), number of total bands (TB), number of polymorphic bands (PB), and polymorphic information content (PIC) were used to evaluate genetic diversity. PIC was calculated as PIC = 1 - $\sum P^2_{ij}$, where P_{ij} is the frequency of the jth allele for the ith locus, summed across all alleles of the locus (Nei, 1973). The discriminatory power (DP) of each SSR marker was computed by the number of unique sequences required to identify a particular cultivar from the data matrix of all 32 cultivars. The SSR fingerprints of orchardgrass cultivars were constructed following the method reported by Weng et al. (2005). The samples were tested using PGI software (developed by Visual Basic 6.0) (Sun et al., 2005).

RESULTS

Polymorphism of SSR markers

The ability of TB, PIC, and discriminatory power (DP) to identify 32 orchardgrass cultivars was calculated (Table 2). PCR products were obtained for all 29 SSR markers from all cultivars. All of the 29 SSRs were polymorphic, and produced a total of 229 bands across the 32 cultivars, of which 210 bands (92.1%) were polymorphic. The size of the amplified fragments ranged from 79 bp (D02K13) to 292 bp (A01E14) for all SSR markers. The TB for each marker across different cultivars ranged from 5 (B03E14, A01E02, A01B10, and B06N03) to 11 (A01K14 and B05P14), with an average of 7.9 alleles per marker. The lowest and the highest PIC values were 0.771 and 0.893 for A01E02 and B01C11, respectively, with an average of 0.848 (Table 2).

## Genetic relationships based on SSR data

Dice similarity matrices of orchardgrass genotypes were calculated from SSR data. The pair-wise genetic similarity (GS) coefficients between cultivars ranged from 0.55 ('01-103' and 'Chinook') to 0.84 ('Baoxing' and 'Chuandong'), with an average of 0.67. The relationship among the 32 cultivars was further determined by UPGMA cluster analysis (Figure 1). Cluster analysis separated 32 cultivars into 6 groups at the GS = 0.69 level, according to their phylogenetic relationships. The cultivar 'Gippsland' and '01-103' were clustered into Groups I and III, respectively. Group II contained 4 cultivars; namely, 'Donata', 'Cristobal', 'Intensiv', and 'Baraula'. Group IV contained 7 cultivars; namely, 'Gulin', 'Baoxing', 'Chuandong', 'Porto', '79-9', '02-116', and '01175'. Within groups, there was also genetic distance between new lines, such as '02-116', and cultivars, such as 'Baoxing'. All of the cultivars in Group IV were from China. Group V contained 7 cultivars; namely, 'Frode', 'Weihenstephaner', 'Baridana', 'Aldebaran', 'Athos', 'Endurance', and 'Sparta'.

The rest of the cultivars belonged to Group VI, which was the largest. Similar results were obtained to UPGMA cluster analysis, with the PCoA also separating the 32 cultivars into six groups (Figure 2).

Cultivar identification and DNA fingerprinting

The number of cultivars distinguished by any SSR marker ranged from 3 (B06N03) to 23 (A01K14), with an average of 12.1 per primer (Table 2). Each amplified band was named depending on the primer that was used and the band size. For example, marker $A03C05_{143}$ was a 143 bp band amplified with primer A03C05. Some cultivars could be identified by a single SSR locus from each marker, i.e., $A03C05_{143}$ for 'Baoxing', $A01L14_{194}$ for 'Baraula', $A01K14_{108}$ for 'Intensiv', $A02A10_{144}$ for '01472', and $A03B16_{182}$ for 'Cristobal'. Moreover, some SSR loci could discriminate a small number of cultivars, e.g., $A03C05_{132}$ for 'Athos' and 'Aldebaran', $B04H05_{150}$ for 'Donata', 'Athos', and 'Cristobal', $A01K14_{121}$ for 'Krasnodarskaya', 'Amba', and 'Grassland Wana', and $B01F08_{170}$ for 'Hawk', 'Krasnodarskaya', and 'Cambria'. Furthermore, 10 primers had relatively strong discriminatory power, and were able to identify more than 15 cultivars, i.e., A01K14 (23), A03K22 (21), A02N22 (19), B01C11 (19), B05L12 (19), A01E14 (18), B01A02 (17), B01D10 (17), B05P14 (16), and B01E09 (15). In total, marker A01K14 had the largest values for DP (23) and TB (11), which indicated that this primer pair was the most efficient among the SSR markers tested in this study.

Based on the SSR analysis with 29 primers, 11 reproducible and reliable SSR bands were selected, and then used for DNA fingerprinting construction. The 11 selected
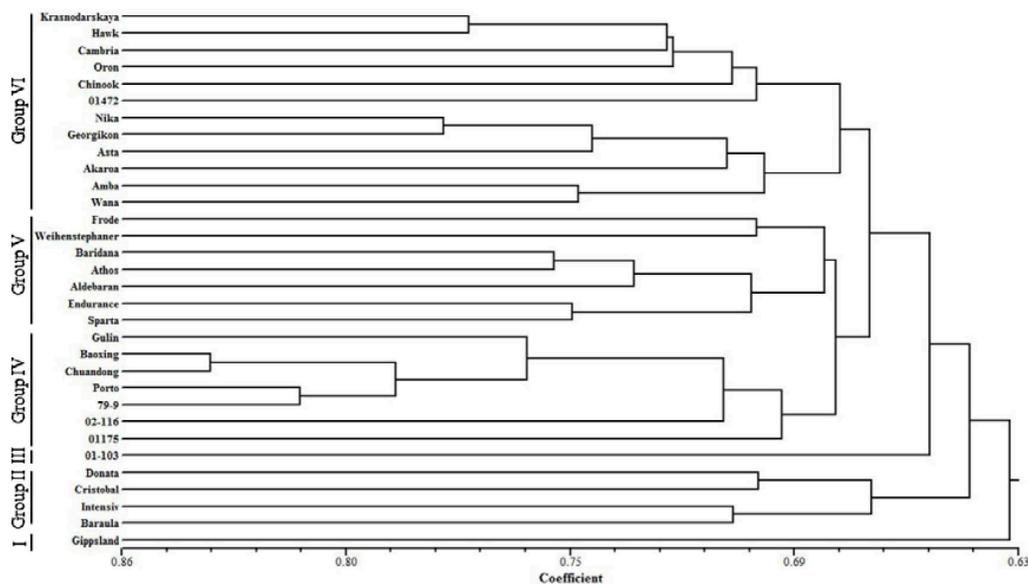

Figure 1. UPGMA dendrogram based on Dice's similarity coefficients among 32 orchardgrass cultivars generated from 29 SSR markers.

bands derived from the amplification products of A01E14, A01K14, and D02K13, and were numbered from 1 to 11. Then the DNA fingerprints of the 32 orchardgrass cultivars were generated (Figure 3).
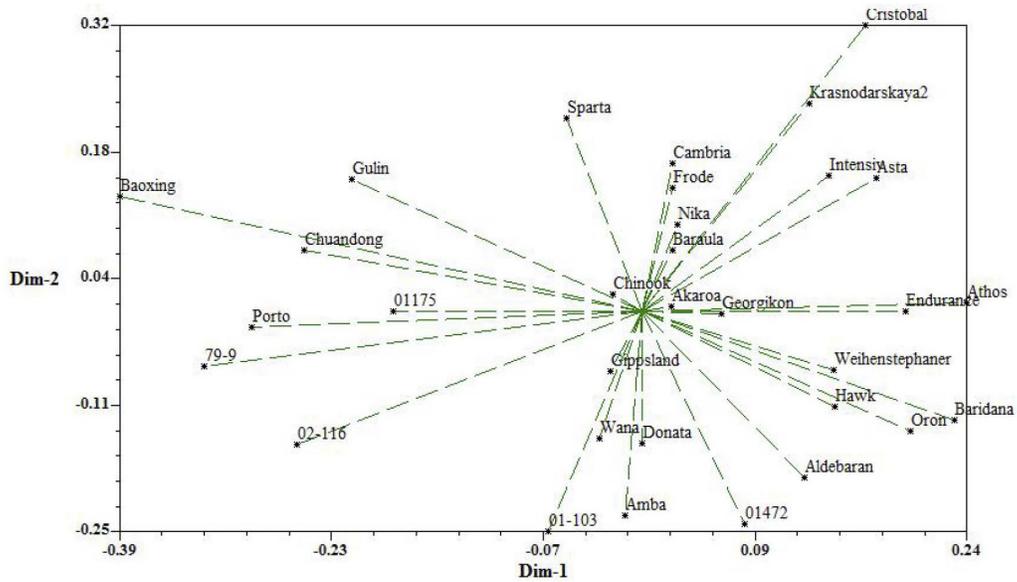
Figure 2. Principal coordinates analysis (PCoA) of 32 orchardgrass cultivars based on the genetic similarity coefficients derived from 29 polymorphic SSR markers.
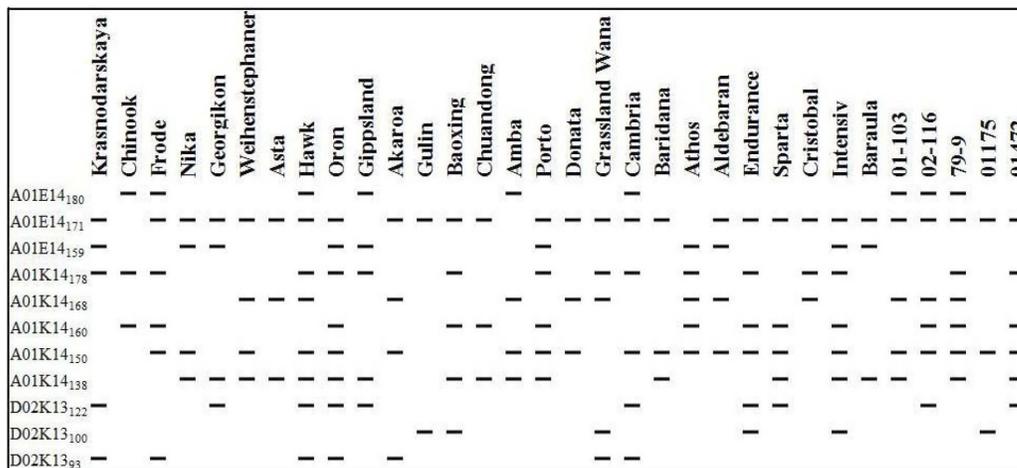
Figure 3. Molecular fingerprint of 32 orchardgrass (*Dactylis glomerata* L.) cultivars set up by 3 SSR markers. Numbers on the left side of the figure are the numbered 11 bands used for constructing the DNA fingerprints. Marker A01E14$_{180}$ was a 180-bp band amplified with primer A01E14. The black rectangles in the figure indicate that there are amplified bands of the orchardgrass cultivars on the site using the primers A01E14, A01K14 and D02K13.

The DNA fingerprints were converted into computer language expressed with 2 digitals, 1 and 0. Then the computerized DNA fingerprints were generated (Table 3). If the test sample was identical to 1 of the 32 examined cultivars, the computer showed the name of the orchardgrass cultivar. If the test sample was not identical to any of the 32 examined cultivars, the computer identified the sample as a new orchardgrass cultivar, and listed its similarities to each of the known 32 orchardgrass cultivars.

Table 3. Computerized DNA fingerprints of the 32 orchardgrass (*Dactylis glomerata* L.) cultivars.

Orchardgrass cultivars	Computerized DNA fingerprint	Orchardgrass cultivars	Computerized DNA fingerprint
Krasnodarskaya	01110000101	Donata	01001010000
Chinook	10010100000	Grassland Wana	01011000011
Frode	11010110001	Cambria	11010010101
Nika	01100011000	Baridana	01000011000
Georgikon	01100001100	Athos	00111110000
Weihenstephaner	01001011000	Aldebaran	01101010000
Asta	01001001000	Endurance	01010110110
Hawk	11011011101	Sparta	01000111100
Oron	01110111101	Cristobal	01011000000
Gippsland	10110001100	Intensiv	01110111010
Akaroa	01001010001	Baraula	01100001000
Gulin	01000000010	01-103	11001011000
Baoxing	01010101010	02-116	11001110100
Chuandong	01000101000	79-9	11011111000
Amba	10001011000	01175	01000010010
Porto	01110111000	01472	01010111100

DISCUSSION

Genetic diversity of orchardgrass

SSR markers have been widely used in genetic diversity analyses (Wang et al., 2009; Bushman et al., 2011; Erfani et al., 2012; Zhang et al., 2012a). In the current study, a total of 29 SSR primer pairs were used to estimate the genetic diversity of a collection of orchardgrass cultivars. The P value of the 32 orchardgrass cultivars studied was 92.1%, which was higher compared to that reported for expressed sequence tag-simple sequence repeat (EST-SSR) variation (P = 74.1%) (Xie et al., 2010), SRAP variation (P = 84.38%) (Zeng et al., 2008), AFLP variation (P = 84.0%) (Peng et al., 2008), or ISSR variation (P = 86.3%) (Zeng et al., 2006) in orchardgrass. The average allele of each marker was 7.9, which was higher compared to previous research on 74 accessions (6.3 alleles per locus) of *Dactylis* species (Xie et al., 2010). Therefore, the results of this study demonstrated the high polymorphism of SSR markers derived from orchardgrass cultivars.

The GS value was higher compared to that previously reported (GS = 0.44-0.70) (Bushman et al., 2011), confirming a high level of genetic diversity among orchardgrass cultivars. The UPGMA dendrogram and PCoA showed separation among the 32 cultivars, with most cultivars from the same geographic region being classified into the same group. Moreover, the diversity of cultivars provided an abundance of excellent material of potential use by plant breeders.

Relationship among PIC, TB, and cultivar identification

PIC and TB are widely used to analyze genetic diversity, because of their discrimina-

tory power and informative genotyping (Erfani et al., 2012; Hameed et al., 2012), which are related to the effectiveness of SSR markers for the identification of cultivars. Zhang et al. (2012b) selected 17 polymorphic SSR markers to assess differences in 19 mushroom cultivars, with the analyzed cultivars being efficiently differentiated using a combination of 3 polymorphic SSR markers with high PIC. In this report, 29 SSR primer pairs had a mean value of 0.848 (PIC) and 7.9 (TB) among 32 cultivars. The PIC in this study was higher compared to that reported by Xie et al. [2010 (PIC = 0.82), 2012 (PIC = 0.81)], confirming that SSR could be effectively used for studying orchardgrass cultivars.

TB could be used to differentiate markers The discernment of markers was associated with TB. For instance, A01K14 produced the highest TB values for 11, with the discriminatory power being high for 23 cultivars. In contrast, the lowest TB was obtained for B03E14 and B06N03, and the lowest number of identified cultivars was 4 and 3. The TB value provided a good reference for the fingerprinting of cultivars, but was not absolute. Higher TB values might identify fewer cultivars, such as B01F08. In contrast, lower TB values might identify more cultivars, such as B04O13. The PIC was not strongly correlated with the number of cultivars identified. For instance, compared with A01E14 (PIC = 0.861), A01I11 expressed a higher PIC value of 0.880, but identified fewer cultivars. In contrast, a higher TB from B01E09 and A01E14 could identify more cultivars compared to a lower TB from A03H11, based on a higher PIC value. This observation was consistent with the results obtained by Hameed et al. (2012), whereby PIC values serve as a reference for detecting genetic variability, but should be used in combination with other parameters for marker selection. The highest comprehensive scores of the 2 parameters for A01K14 (PIC = 0.890, TB = 11) could identify the largest number of orchardgrass cultivars. Therefore, a comprehensive understanding of the 2 parameters might contribute toward selecting highly identified efficient markers for orchardgrass fingerprinting.

Sample size selection and fingerprinting construction

Orchardgrass is a perennial allogamous forage grass species that is mostly cross-pollinated. It is auto-tetraploid, with a large genome (4312 Mbp) (Creber et al., 1994) and self-incompatibility system (Hirata et al., 2011). Compared to other forage material, the breeding of orchardgrass is hampered by its strong self-incompatibility system and severe inbreeding depression, which makes it difficult to produce inbred lines and construct fingerprinting (Xie et al., 2011). A series of molecule studies indicate that the conventional method of bulking could be effectively used for evaluating the diversity of cross-pollinated forage grass species (Marshall and Brown, 1975; Morell et al., 1995; Ghérardi et al., 1998; Forster et al., 2001; Bolaric et al., 2005), which requires a suitable sample size per population (Marshall and Brown, 1975). Moreover, more than 15 plants per population should be used for allogamous species (Morell et al., 1995). Furthermore, for each species and specific marker system, a certain sample size should be tested before conducting a diversity study (Bolaric et al., 2005). For example, a minimum sample size of 40 individuals per populations is required for the characterization of the allogamous tetraploid crop alfalfa (*Medicago sativa* L.) (Ghérardi et al., 1998), while 20 individuals are required for perennial ryegrass (*Lolium perenne* L.) cultivars (Bolaric et al., 2005). In this report, a sample size of 25 individuals per population was used for orchardgrass, with an average of 0.848, 7.9, and 92.1% for PIC, TB, and P, respectively.

These results were similar to that obtained in a previous study on *Dactylis* species (Xie et al., 2010), which used 25 individuals per cultivar, and obtained averages of 0.82 (PIC), 8.5 (TB), and 95.2% (P). These values were higher compared to a previous report by Xie et al. (2012), which used 20 individuals per population, and obtained values of 0.81 (PIC), 7.6 (TB), and 100% (P). These results support that the optimal sample size required for genetic diversity and fingerprinting of orchardgrass cultivars is about 25 individuals per population, based on SSR markers. However, further studies to confirm the minimum sample size of different molecule markers for orchardgrass are still needed.

The number of distinguishable cultivars ranged from 3 to 23, with an average of 12.1, which was higher compared to that reported by Delgado-Martinez et al. (2012) and Hameed et al. (2012). The use of minimum amounts of DNA bands and primers is an important principle for the construction of DNA fingerprints (Weng et al., 2005). In this report, we successfully constructed the fingerprints of 32 orchardgrass cultivars using 3 SSR markers (A01E14, A01K14, and D02K13). We found that each cultivar had a unique fingerprinting pattern, and could be easily distinguished from the other cultivars, indicating that SSR could contribute substantive information to the cultivar identification of orchardgrass. This finding supports the study by Delgado-Martinez et al. (2012), which successfully used 4 SSR markers to construct fingerprints for 77 olive accessions, corresponding to 25 cultivars from the Extremadura region of Spain. These results were also similar to Lee et al. (2012), who used 4 CAPS primers to generate co-dominant polymorphic banding patterns discriminating the Korean ginseng (*Panax ginseng* C. A. Meyer) cultivars from *P. quinquefolius* and *P. notoginseng*. In addition, Weng et al. (2005) used 3 out of 120 operon RAPD primers for the fingerprints of 27 *Porphyra* lines in China. However, the authors converted the specific RAPD molecular markers into SCAR, which indicated its practical application and utility as a good reference for further research. Different identification methods have been report by Tian et al. (2012), who used 9 SSR markers to identify 109 pear accessions, including 92 local Chinese accessions of *Pyrus bretschneideri* based on clusters directly obtained from the UPGMA dendrogram. Similar results were obtained by Madhou et al. (2013), who used 11 SSR loci to fingerprint and identify cultivars of 88 litchi accessions, based on the UPGMA dendrogram, contributing to the rapid identification of cultivars based on a high distances of the GS coefficient between cultivars. Overall, larger numbers of orchardgrass cultivars could be identified by the amplification of specific fragments from different primers.

Future prospects in orchardgrass fingerprinting

Cultivar identification is important for the protection and understanding of orchardgrass, which has a fundamental impact on the development of animal husbandry and agricultural practices. This research presented a method for the identification and fingerprinting of orchardgrass. Although, these figures were not absolute, they are expected provide a useful reference for the identification of orchardgrass cultivars. Hence, future research should focus on the collection and preservation of orchardgrass cultivars worldwide, to establish a cultivar fingerprint database for germplasms protection and control, in addition to enhancing the development of animal husbandry and agricultural practices.

In conclusion, the results of the present study showed that SSR-PCR analysis was polymorphic, feasible, and reliable for the identification of orchardgrass cultivars, and that

each cultivar could be easily distinguished from the others through genetic fingerprinting.

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