Analysis of diversity and relationships among orchardgrass (*Dactylis glomerata* L.) accessions using start codon-targeted markers

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ABSTRACT. Orchardgrass, or cocksfoot, is an important perennial forage grass worldwide. The comprehensive understanding of orchardgrass accessions will benefit germplasm collection and breeding progress, and it will enhance efforts to improve forage yield and quality. Therefore, 24 novel, simple, polymorphic, and reliable start codon-targeted (SCoT) markers were used to analyze the diversity and genetic relationships among 95 orchardgrass accessions. In total, 273 polymorphic bands were detected with an average of 11.4 bands per primer. The average polymorphic rate for the species was 83.4%, suggesting a high discriminating ability of the SCoT technique for orchardgrass. The molecular variance analysis revealed that 69.13 and 30.87% of variation resided within and among groups, respectively, demonstrating that the orchardgrass germplasms had a higher level of

genetic diversity within groups than among geographical regions and distributions. The distinct geographical divergence of orchardgrass was revealed between North America and Oceania. The unweighted pair-group method with arithmetic mean dendrogram revealed a separation of 7 main clusters between 95 accessions according to the geographical origin. Furthermore, each cluster was divided into subgroups mainly according to the origin of its state. The genetic divergence of orchardgrass might be influenced by the ecogeographical conditions, climatic types, breeding systems and gene flow with variations in cultures, bird migration, and breeder selection. These results could facilitate orchardgrass germplasm collection, management, and breeding worldwide.

Key words: *Dactylis glomerata*; Start codon-targeted markers; Genetic diversity

INTRODUCTION

Dactylis is an allogamous and variable monospecific genus that belongs to the Poaceae family (Lindner and Garcia, 1997) and consists of diploid (2n = 2x = 14), tetraploid (2n = 4x = 28), and hexaploid (2n = 6x = 42) populations and subspecies and the single species *Dactylis glomerata* is known as orchardgrass or cocksfoot (Bushman et al., 2011; Stewart and Ellison, 2011). Orchardgrass is an economically important and extensively used cool-season forage grass in China and in many other countries with temperate and cold climates (Sanada et al., 2010; Xie et al., 2012). It has been widely used as forage on nearly every continent because of its high yield, good nutrition, adaptability, and shade tolerance (Jafari and Naseri, 2007; Sanada et al., 2007; Xie et al., 2010). Orchardgrass is one of the top 4 perennial forage grasses grown in North America, Oceania, Europe, and Asia (Lindner and Garcia, 1997; Gauthier et al., 1998; Hirata et al., 2011), and it plays an important role in the livestock industry and in the sustainable development of environments worldwide.

Because of its economic importance as a perennial forage grass, a comprehensive understanding of genetic diversity and variation among cultivars and wild and cultivated accessions will benefit germplasm collection and breeding progress, and it will enhance efforts to improve forage yield, quality, and other value-added traits (Xie et al., 2010; Bushman et al., 2011). Genetic diversity in crop species can be determined using morphological and agronomic characteristics as well as biochemical and DNA marker analysis (Liu, 1997). However, physiological and morphological traits are subjective and quantitative in practice, and they can be affected by environmental conditions and the experience of evaluators (Roldán-Ruiz et al., 2001; Degani et al., 2003; Bolaric et al., 2005). In recent years, a number of molecular markers have been employed in orchardgrass for genetic diversity evaluation, genetic mapping, and quantitative trait locus analysis. These types of molecular techniques included random amplified polymorphic DNA (RAPD) (Kölliker et al., 1999; Tuna et al., 2004), amplified fragment length polymorphism (AFLP) (Reeves et al., 1998; Peng et al., 2008), inter-simple sequence repeat (ISSR) (Zeng et al., 2006), sequence-related amplified polymorphism (Zeng et al., 2008; Xie et al., 2011), simple sequence repeats (SSRs) (Hirata et al., 2011; Xie et al.,

2011, 2012), and expressed sequence tag-SSRs (Xie et al., 2010; Bushman et al., 2011). These marker systems are useful for biodiversity analyses, phylogenetic studies, germplasm management, cultivar identification, and other applications (Semagn et al., 2006; Luo et al., 2010). However, the investigation of genetic diversity by molecular markers for breeding purposes should be based on functionally characterized genes or targeted genes (Andersen and Lübberstedt, 2003) because these markers may reflect functional polymorphisms indirectly.

Of these molecular markers, start codon-targeted (SCoT) polymorphism is a novel, simple, highly polymorphic, and reliable gene-targeted marker technique that is based on a single primer amplification reaction, which was designed using a single primer according to the conservation of the ATG translation start site and flanking sequences in plant genes (Collard and Mackill, 2009; Luo et al., 2010; Guo et al., 2012). In addition, the SCoT technique is convenient for marker-assisted breeding because it effectively produces markers that are linked to traits. Moreover, SCoT markers can be used as an effective complementary method to ISSR and RAPD (Guo et al., 2012). During the last 4 years, this technique was successfully used in studies of genetic diversity in plants and showed high polymorphic information. However, little has been reported on the use of SCoT markers for genetic diversity analysis in orchardgrass.

Recently, 95 orchardgrass accessions from 21 countries and 7 geographical regions were collected to evaluate both the level of genetic diversity and the distribution of its variation in orchardgrass by SCoT markers. Therefore, the primary goals of this study were the following: a) examine the effectiveness of these SCoT markers for genetic diversity analysis of orchardgrass accessions, b) study genetic relationships among 95 orchardgrass accessions originating from various geographic regions, and c) investigate the genetic variation within and among geographical regions. These results could facilitate orchardgrass germplasm collection, management, and breeding worldwide.

MATERIAL AND METHODS

Plant materials and DNA extraction

Ninety-five orchardgrass accessions comprising cultivars and cultivated and wild materials collected from 21 countries and 7 geographical regions were analyzed by means of SCoT markers. They were obtained from the United States Department of Agriculture-Agricultural Research Service and Sichuan Agricultural University, China. Among these accessions, 43 Chinese [northern China (NC) and southern China (SC)] accessions were from Chongqing, Guizhou, Hubei, Jiangxi, Gansu, Sichuan, Xinjiang, and Yunnan; other accessions were from Europe [western Europe (WE), central Europe (CE), and northern Europe (NE)], North America (NA), and Oceania (OC), which represent the main distribution regions of orchardgrass worldwide (Table 1, Figure 1).

Each accession was represented by 25 plants, and 100 mg fresh young leaves from each plant was used to create a pool. Genomic DNA was extracted from the samples collected using the Plant Genomic DNA kit (Tiangen®, China) according to the manufacturer protocol. The DNA quality and concentration were confirmed by electrophoresis on 0.8% agarose gels and NanoDrop (ND) 1000 ultraviolet and visible spectrophotometry (ND Technologies, Inc., USA). The genomic DNA samples were diluted to 10 ng/μL and stored at 4°C for use.

Table 1. List of 95 orchardgrass (*Dactylis glomerata* L.) accessions.

Code	Accession No	Geographic source (Code)	Status	Code	Accession No	Geographic source (Code)	Status
1	01819	Denmark (NE)	Wild	48	01473	Australia (OC)	Wild
2	01820	Denmark (NE)	Wild	49	01475	Australia (OC)	Wild
3	01822	Denmark (NE)	Wild	50	PI285099	Australia (OC)	Cultivar
4	01824	Denmark (NE)	Wild	51	PI469234	New Zealand (OC)	Cultivar
5	79-14	Denmark (NE)	Wild	52	Wana	New Zealand (OC)	Cultivar
6	79-15	Denmark (NE)	Wild	53	01472	Chongqing, China (SC)	Cultivated
7	Anba	Denmark (NE)	Cultivar	54	2006-1	Chongqing, China (SC)	Wild
8	PI345603	Krasnodar, Russia (NE)	Cultivar	55	2006-5	Chongqing, China (SC)	Wild
9	PI381630	Sweden (NE)	Cultivar	56	Hongchiba	Wuxi, Chongqing, China (SC)	Wild
10	01993	Sweden (NE)	Wild	57	01175	Dushan, Guizhou, China (SC)	Wild
11	01994	Sweden (NE)	Wild	58	01-101	Bijie, Guizhou, China (SC)	Wild
12	01995	Sweden (NE)	Wild	59	01-102	Guizhou, China (SC)	Wild
13	01996	Sweden (NE)	Wild	60	02-102	Guizhou, China (SC)	Wild
14	01997	Sweden (NE)	Wild	61	02-103	Guizhou, China (SC)	Wild
15	01998	Sweden (NE)	Wild	62	02-104	Guizhou, China (SC)	Wild
16	01999	Sweden (NE)	Wild	63	79-9	Lushan, Jiangxi, China (SC)	Cultivated
17	02684	United Kingdom (NE)	Wild	64	01-103	Sichuan, China (SC)	Cultivated
18	Sibada	United Kingdom (NE)	Cultivar	65	02-105	Dazhou, Sichuan, China (SC)	Wild
19	Cristobal	France (WE)	Cultivar	66	02-106	Baoxing, Sichuan, China (SC)	Wild
20	Bote	Italy (WE)	Cultivar	67	02-107	Baoxing, Sichuan, China (SC)	Wild
21	Asuosi	Luxembourg (WE)	Cultivar	68	02-108	Baoxing, Sichuan, China (SC)	Wild
22	PI384017	Netherlands (WE)	Cultivar	69	02-109	Baoxing, Sichuan, China (SC)	Wild
23	00737	Netherlands (WE)	Wild	70	90-130	Maoxian, Sichuan, China (SC)	Wild
24	79-118	Netherlands (WE)	Wild	71	90-70	Kangding, Sichuan, China (SC)	Wild
25	PI311033	Romania (WE)	Cultivar	72	91-1	Hanyuan, Sichuan, China (SC)	Wild
26	Kaimo	Spain (WE)	Cultivar	73	91-103	Yuexi, Sichuan, China (SC)	Wild
27	Intensiv	Czech (CE)	Cultivar	74	Baoxing	Baoxing, Sichuan, China (SC)	Cultivar
28	96011	Germany (CE)	Wild	75	Chuandong	Daxian, Sichuan, China (SC)	Cultivar
29	PI260244	Bavaria, Germany (CE)	Cultivar	76	Gulin	Gulin, Sichuan, China (SC)	Cultivar
30	Denata	Germany (CE)	Cultivar	77	Yangping	Hongya, Sichuan, China (SC)	Wild
31	Jinniu	Germany (CE)	Cultivar	78	02-110	Yunnan, China (SC)	Wild
32	PI288998	Hungary (CE)	Cultivar	79	02-111	Yunnan, China (SC)	Wild
33	PI273738	Lithuania (CE)	Cultivar	80	02-112	Yunnan, China (SC)	Wild
34	PI305498	Poland (CE)	Cultivar	81	02-113	Yunnan, China (SC)	Wild
35	Dana	Slovakia (CE)	Cultivar	82	02-114	Qujing, Yunnan, China (SC)	Wild
36	02681	Canada (NA)	Wild	83	02-115	Yunnan, China (SC)	Wild
37	PI278699	Canada (NA)	Cultivar	84	02-116	Kunming, Yunnan, China (SC)	Cultivar
38	00595	United States (NA)	Wild	85	00947	Hubei, China (NC)	Wild
39	00937	United States (NA)	Wild	86	02122	Hubei, China (NC)	Wild
40	01076	United States (NA)	Wild	87	Tieluoping	Hubei, China (NC)	Wild
41	01436	United States (NA)	Wild	88	02746	Gansu, China (NC)	Wild
42	01476	United States (NA)	Wild	89	01032	Xinjiang, China (NC)	Wild
43	01821	United States (NA)	Wild	90	02068	Xinjiang, China (NC)	Wild
44	98-101	United States (NA)	Wild	91	02105	Xinjiang, China (NC)	Wild
45	98-102	Oregon, United States (NA)	Wild	92	02106	Xinjiang, China (NC)	Wild
46	PI578668	Iowa, United States (NA)	Cultivar	93	02138	Xinjiang, China (NC)	Wild
47	Yuanhang	United States (NA)	Cultivar	94	02240	Xinjiang, China (NC)	Wild
				95	02271	Xinjiang, China (NC)	Wild

NE = northern Europe; WE = western Europe; CE = central Europe; NA = North America; OC = Oceania; SC = southern China, and NC = northern China.

SCoT polymerase chain reaction (PCR) amplification

All SCoT primers were synthesized by the Shanghai Sangon Biological Engineering Technology & Services (China). Eighty primers were initially screened for polymorphism and reproducibility using 4 accessions, i.e., 01-101, 2006-5, Anba, and Baoxing. Each 15-μL amplification reaction consisted of 10 ng template DNA, 7.5 μL 2X Reaction Mix (Tiangen®, China), 0.4 μL 2.5 U/μL Golden DNA Polymerase (Tiangen®, China), 1.5 μL 10 μM primer, and 4.6 μL distilled water. PCR amplification was performed on a Peltier Thermal Cycler (DNA En-

gine®, Bio-Rad, USA) using the following program: 94°C for 3 min; 36 cycles of 94°C for 50 s, 50°C for 1 min, and 72°C for 2 min; a final extension at 72°C for 5 min; and a 4°C holding temperature. Amplified fragments were resolved on 1.8% agarose gels in 1X Tris-acetate-ethylenediaminetetraacetic acid buffer and stained with GelRed™ (10,000X in water). The banding pattern images were acquired under UV light using the Gel Doc XR system (Bio-Rad, USA).

Figure 1. Geographic map showing the location of 95 accessions of *Dactylis glomerata* from 21 countries and 7 geographical regions (NE, WE, CE, NA, OC, SC, NC). Numbers of cultivated or cultivar analyzed are shown within circles, and numbers of wild are shown outside of the circles, the yellow polka dot represent the collection site of *D. glomerata*. For abbreviations, see legend to Table 1.

Statistical analysis

The polymorphic information content (*PIC*) of each SCoT marker analyzed was calculated using the formula: $PIC = 1 - \sum P^2_{ij}$, where P_{ij} was the frequency of the *j*th allele for the *i*th locus, summed across all alleles of the locus (Nei, 1973). Reproducible bands were scored as absent (0) or present (1) for each of the SCoT markers. The POPGENE software version 1.31 (Yeh et al., 1999) was used to estimate the genetic diversity parameters grouped, percentage polymorphic loci (*PPL*), Nei's (Nei, 1973) gene diversity (*H*), Shannon's information index of diversity (*I*), and number of polymorphic loci (*NPL*) from the 1/0 matrices generated. Nei's unbiased measures of genetic identity (*GI*) and genetic distance (*GD*) among 7 geographical regions were also calculated using the same program (Nei, 1978). Cluster analysis based on the *GD* matrix among geographical regions was constructed using the NTSYS version 2.10 software (Rohlf, 2000). Analysis of molecular variance (AMOVA) was used to partition the total SCoT marker variation into the within and among geographical regions components (Excoffier et al., 1992). The input files for AMOVA were prepared by the DCFA1.1 program (Zhang, 2001). Nei and Li's (Nei and Li, 1979) pairwise similarities between 95 accessions were computed by the FreeTree program (Pavlícek et al., 1999), and a bootstrap analysis with 10,000 replicates was performed. A dendrogram showing the genetic relationships among accessions based on the unweighted pair-group method with arithmetic averages (UPGMA) was constructed using the Dendroscope 3 program (Huson and Scornavacca, 2012).

RESULTS

SCoT polymorphism and genetic diversity

The results of 24 primers that were used to screen SCoT markers of 95 orchardgrass accessions are summarized in Table 2. The SCoT primers were screened according to the methods described by Collard and Mackill (2009) and Luo et al. (2010). In prescreening assays with 4 orchardgrass accessions and 80 SCoT primers, clear reproducible polymorphic amplification products were generated by 24 primers, which were selected for genetic relationship analysis of orchardgrass in this study. Among the 95 orchardgrass accessions, 324 reliable bands were obtained from 24 SCoT primers. The total number of bands (*TNB*) per primer ranged from 6 to 19 the average *TNB* per primer was 13.5. The maximum *TNB* (19) was found for primers SCoT40 and SCoT61, whereas the lowest *TNB* (6) was obtained for SCoT10. Among the total obtained fragments, 273 bands were polymorphic with the average of 83.4% polymorphism, and the detected polymorphism per primer among the tested accessions ranged from 50% (SCoT11) to 100% (SCoT7, SCoT45, SCoT54, SCoT59, SCoT60, and SCoT65). The number of polymorphic bands (*NPB*) varied from 5 (SCoT10 and SCoT11) to 18 (SCoT65) with an average *NPB* of 11.4 per primer.

Table 2. Amplification results of *Dactylis glomerata* from 24 start codon-targeted (SCoT) primers.

Primer ID	Primer Sequence (5'-3')	%G/C	TNB	NPB	*P* (%)	*PIC*
6	CAACAATGGCTACCACGC	56	10	6	60.00	0.8973
7	CAACAATGGCTACCACGG	56	7	7	100.00	0.8276
8	CAACAATGGCTACCACGT	50	9	8	88.89	0.8796
9	CAACAATGGCTACCAGCA	50	9	8	88.89	0.8703
10	CAACAATGGCTACCAGCC	56	6	5	83.33	0.8307
11	AAGCAATGGCTACCACCA	50	10	5	50.00	0.8773
12	ACGACATGGCGACCAACG	61	10	6	60.00	0.8970
20	ACCATGGCTACCACCGCG	67	9	6	66.67	0.8621
23	CACCATGGCTACCACCAG	61	17	12	70.59	0.9325
26	ACCATGGCTACCACCGTC	61	18	16	88.89	0.9384
28	CCATGGCTACCACCGCCA	67	14	12	85.71	0.8777
29	CCATGGCTACCACCGGCC	72	17	11	64.71	0.9292
30	CCATGGCTACCACCGGCG	72	15	13	86.67	0.9170
36	GCAACAATGGCTACCACC	56	11	9	81.82	0.8964
40	CAATGGCTACCACTACAG	50	19	17	89.47	0.9402
44	CAATGGCTACCATTAGCC	50	16	12	75.00	0.9234
45	ACAATGGCTACCACTGAC	50	17	17	100.00	0.9313
54	ACAATGGCTACCACCAGC	56	14	14	100.00	0.9129
59	ACAATGGCTACCACCATC	50	15	15	100.00	0.9264
60	ACAATGGCTACCACCACA	50	14	14	100.00	0.9241
61	CAACAATGGCTACCACCG	56	19	14	73.68	0.9378
62	ACCATGGCTACCACGGAG	61	16	15	93.75	0.9247
63	ACCATGGCTACCACGGGC	67	14	13	92.86	0.9127
65	ACCATGGCTACCACGGCA	61	18	18	100.00	0.9362
Average			13.5	11.4	83.37	0.9043
Total			324	273	84.26	

TNB = total number of bands; NPB = number of polymorphic bands; *P* = polymorphic rate; *PIC* = polymorphic information content.

PIC varied from 0.8276 for SCoT7 to 0.9402 for SCoT40. The average value was 0.9043 for this species (Table 2). In order to obtain a detailed view of the genetic variation within different geographical regions, *H*, *I*, *NPL*, and *PPL* were estimated. The 4 genetic

diversity parameters for all accessions were 0.27, 0.41, 273, and 84.26%, respectively. The high values of the 4 genetic diversity parameters (NPL = 213; H = 0.2627; I = 0.3826; PPL = 65.74%) were found from WE, and the maximum values (NPL = 251; H = 0.2935; I = 0.4320; PPL = 77.47%) for orchardgrass were from Europe; the values were followed by values for orchardgrass from China (NPL = 213; H = 0.2376; I = 0.3530; PPL = 65.74%), and the lowest values (NPL = 116; H = 0.1297; I = 0.1931; PPL = 35.80%) were for orchardgrass from OC. Among the 7 geographical regions, the PPL ranged from 35.80% (OC) to 65.74% (WE) in the following order: WE > SC > NC > NE > CE > NA > OC (Table 3).

Table 3. Genetic diversity between 7 geographical groups of *Dactylis glomerata* detected by start codon-targeted (SCoT) analysis.

Groups	N	*NPL*	*H*	*I*	*PPL* (%)
Europe	35	251	0.2935	0.4320	77.47
Northern Europe (NE)	18	151	0.1623	0.2418	46.60
Western Europe (WE)	8	213	0.2627	0.3826	65.74
Central Europe (CE)	9	135	0.1504	0.2234	41.67
China	43	213	0.2376	0.3530	65.74
Northern China (NC)	32	176	0.1975	0.2932	54.32
Southern China (SC)	11	192	0.2174	0.3224	59.26
North America (NA)	12	117	0.1311	0.1941	36.11
Oceania (OC)	5	116	0.1297	0.1931	35.80
Total	95	273	0.2664	0.4050	84.26

N = number of accessions; *NPL* = number of polymorphic loci; *H* = Nei's gene diversity; *I* = Shannon's information index of diversity; *PPL* = percentage of polymorphic loci.

Cluster analysis

All of the 324 scored bands were used to calculate the genetic similarity (*GS*) among the 95 orchardgrass accessions. The *GS* ranged from 0.514 to 0.949 with an average of 0.715. The highest *GS* observed (0.949) was found between 00937 (United States) and 01821 (United States), whereas the lowest *GS* (0.514) was obtained between 01032 (China) and Dana (Europe). The *GD*s among the 7 geographical groups were estimated by Nei's (Nei, 1978) unbiased measure (Table 4). The *GD*s among the 7 geographical groups varied from 0.052 (NA and OC) to 0.3423 (CE and SC) with an average of 0.1554 (Table 4). The UPGMA dendrogram based on *GD* showed that group NA has the largest genetic distance with OC, and materials from the geographical regions of China (NC and SC) showed high genetic similarity and were grouped together (Figure 2).

Table 4. Nei's unbiased measures of genetic distance for *Dactylis glomerata*.

Groups	NE	WE	CE	NC	SC	NA	OC
NE	0.0000						
WE	0.0871	0.0000					
CE	0.2861	0.2585	0.0000				
NC	0.0608	0.1312	0.2516	0.0000			
SC	0.1160	0.1216	0.3423	0.1504	0.0000		
NA	0.0775	0.0795	0.3041	0.1053	0.0858	0.0000	
OC	0.0923	0.0777	0.3412	0.1425	0.0995	0.0520	0.0000

Underlined values represent the maximums and minimums of genetic distance values.
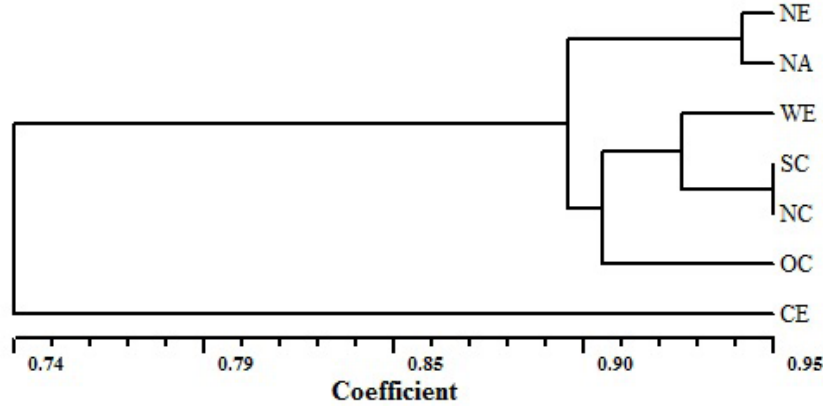
Figure 2. Cluster tree between the seven geographical regions of *Dactylis glomerata* based on Nei's (1973) genetic identity.

A dendrogram was obtained by UPGMA using the total number of amplified SCoT fragments and revealed the genetic relationships of the 95 accessions (Figure 3), which were grouped into 7 main clusters. The accessions were clustered mainly according to the geographic origin.
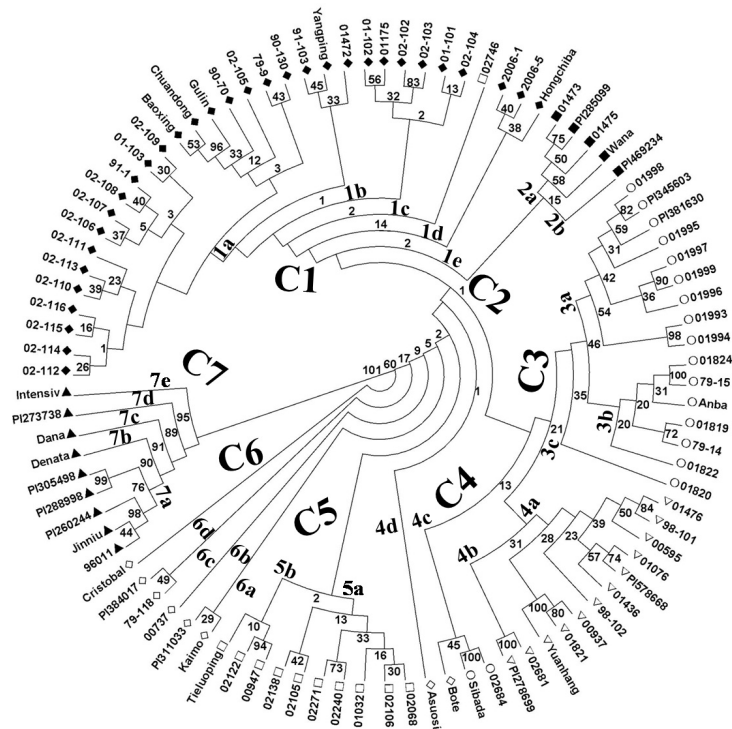

Figure 3. Dendrograms generated using UPGMA analysis, showing relationships between different *Dactylis glomerata* accessions using SCoT markers. Open square = northern China; filled diamond = southern China; open diamond = western Europe; open circle = northern Europe; filled triangle = central Europe; open triangle = North America; filled square = Oceania.

Cluster 1 (C1) included 33 accessions and could be further divided into 5 subgroups. Subgroup 1 (1a) was very large; the accessions from Yunnan (02-110, 02-111, 02-112, 02-113, 02-114, 02-115, and 02-116), Sichuan (02-105, 02-106, 02-107, 02-108, 02-109, 91-1, 01-103, 90-70, 90-130, Baoxing, Chuandong, and Gulin) and Jiangxi (79-9) formed one branch of subgroup 1. The accessions from Guizhou (01175, 01-101, 01-102, 02-102, 02-103, and 02-104) were in subgroup 3 (1c); subgroup 5 (1e) included accessions from Chongqing (2006-1, 2006-5, and Hongchiba); and subgroup 2 (1b) included accessions 91-103 and Yangping from Sichuan and 01472 from Chongqing. All of the accessions belonging to the SC geographical region except 02746, which belongs to the NC region, were grouped into subgroup 4 (1d). Cluster 2 (C2) divided all of the accessions from the OC region into subgroup 1 (2a) (01473, 01475, PI285099, and Wana) and subgroup 2 (2b) (PI469234). Cluster 3 (C3) included 16 accessions and 3 subgroups from the NE region. Subgroup 1 (3a) included accessions from Russia (PI345603) and Sweden (PI381630, 01993, 01994, 01995, 01996, 01997, 01998, and 01999). Accessions 01819, 01822, 01824, 79-14, 79-15, and Anba from Denmark were grouped into subgroup 2 (3b), and accession 01820 from Denmark was placed in subgroup 3 (3c). Cluster 4 (C4) included 16 accessions and could be further divided into 4 subgroups. The accessions from the United States (00595, 00937, 01076, 01436, 01476, 01821, 98-101, 98-102, PI578668, and Yuanhang) and Canada (02681 and PI278699) belong to the NA region and were placed in subgroup 1 (4a) and subgroup 2 (4b), respectively. Subgroup 3 (4c) included Sibada and 02681 from the NE region and Bote from the WE region. Asuosi (WE) was in subgroup 4 (4d). The accessions from the NC region were grouped in cluster 5 (C5), which was divided into subgroup 1 (5a) (00947, 02122, and Tieluoping from Hubei) and subgroup 2 (5b) (02746, 01032, 02068, 02105, 02106, 02138, 02240, and 02271 from Xinjiang). The accessions from the WE region (Cristobal, PI384017, 00737, 79-118, PI311033, and Kaimo) were grouped in cluster 6 (C6) and further divided into 4 subgroups. Cluster 7 (C7) included 9 accessions from the CE region (Intensiv, 96011, PI260244, Denata, Jinniu, PI288998, PI273738, PI305498, and Dana) and could be further divided into 5 subgroups (Figure 3).

AMOVA

AMOVA of the 5 orchardgrass groups revealed that 83.02 and 16.98% of the variation resided within and among groups (Europe, China, Oceania, and North America), respectively. AMOVA also revealed that 30.87 and 69.13% of the variation resided within and among the 7 regions (NE, WE, CE, NA, OC, SC, and NC), respectively (Table 5).

Table 5. Analysis of molecular variance (AMOVA) distribution for *Dactylis glomerata* in this study.

Source of variation	Sum of squares	Variance component	Percentage of variation	P values
Analysis among populations				
Among population	1133.33	12.64	30.87%	<0.001
Within populations	2490.58	28.30	69.13%	<0.001
Analysis among groups				
Among groups	525.82	6.96	16.98%	<0.001
Within groups	3098.10	34.05	83.02%	<0.001

DISCUSSION

SCoT polymorphism of orchardgrass

The use of isozymes technology is limited for agronomic trait evaluation, which could be affected by the growing environment and restricts their use for assessing genetic diversity (Xie et al., 2010). In contrast, molecular markers can provide abundant information and are not limited by the environment, which makes them better tools to characterize the genetic diversity among accessions. SCoT markers are designed from the short conserved region flanking the ATG start codon of all plant genes (Collard and Mackill, 2009). Previously, more than 80 primers were released for SCoT analysis (Collard and Mackill, 2009; Luo et al., 2010). Compared with other molecular markers, SCoT markers have emerged as an alternative system with several advantages: lower cost than AFLP, higher reproducibility than RAPD, and easier development of species-specific primers than SSR (Guo et al., 2012). Luo et al. (2010) showed that amplification products generated from the SCoT marker technique were associated with functional genes and their corresponding traits. Xiong et al. (2011) showed that SCoT markers could be utilized to identify DNA polymorphisms and fingerprint cultivars in domesticated peanut. Gorji et al. (2011) reported that SCoT markers were more informative for fingerprinting varieties than other markers based on the average percentage of polymorphism, *PIC*, and overall *I*. Therefore, SCoT markers have been extensively used in plants to characterize *GD* in a wide range of species (Collard and Mackill, 2009; Xiong et al., 2009, 2010, 2011; Luo et al., 2010; Gorji et al., 2011; Guo et al., 2012). As far as we know, this is the first *GD* analysis of orchardgrass using SCoT markers.

In this study, 24 SCoT primers were used to estimate the *GD* of orchardgrass accessions. Primers that produced only a few or many fragments were rejected, and those with poor reproducibility in repetitive experiments were not scored. Of the 324 scored fragments, 273 bands were polymorphic. The bands from 6 SCoT primers were 100% polymorphic, and the average polymorphic value was 83.4%. This value was lower than that reported by Guo et al. (2012) (P = 93.1%) in 64 grape varieties, but it was higher than that reported by Luo et al. (2010) (P = 76.19%) in mango accessions. The average *TNB* and *NPB* was 13.5 and 11.4, respectively, indicating the high discriminating ability of the SCoT technique for orchardgrass. The *PIC* values ranged from 0.8276 to 0.9402 with an average of 0.9043, indicating that these loci were highly informative. The *PIC* value is higher than those reported by Xie et al. [2010 (PIC = 0.77), 2012 (PIC = 0.81)] in orchardgrass, which indicated that SCoT markers could contribute substantial information to the genetics and breeding research of orchardgrass.

Among the 7 geographic regions, the genetic variation of orchardgrass from WE (P = 65.74%), SC (P = 59.26%), and NC (P = 54.32%) were higher than the values from other regions, i.e., NE (P = 46.60%), CE (P = 41.67%), NA (P = 36.11%), and OC (P = 35.80%). These results might be influenced by the number of samples and the complicated natural conditions of the geographic regions, and they indicated that orchardgrass from Europe and China might provide high *GD* for breeding and germplasm collection. In addition, the cluster analysis of the geographical groups showed a similarity between the groups from the same regions: the accessions from China (SC and NC) had a low distance and the best affinity relationship (Table 4). Differences in the climates and breeding systems might lead to differences in the genetic constitution of orchardgrass. In general, our results also agreed with previous research performed by Xie et al. (2010).

Cluster analysis

In this study, the *GS* values ranged from 0.514 to 0.949 with an average of 0.715 between 95 accessions, which was higher than the value that was reported by Bushman et al. (2011) (*GS* = 0.44-0.70). This confirmed a high level of genetic diversity among orchardgrass. The dendrogram obtained using UPGMA consisted of 7 major clusters (Figure 3), which were mainly separated according to the geographical origin. Each cluster was further divided into subgroups. However, a few accessions were not clustered into the group that included other accessions from its geographical region. For example, 02746 from Gansu, China, was from the NC region (C5) but was clustered with accessions from the SC region (C1), 02684 and Sibada from the United Kingdom belonged to the NE region (C3) but were clustered with the NA region (C4), Bote and Asuosi belonged to the WE region (C4) but were also clustered with the NA region (C4) (Figure 3). This clustering might be influenced by the allogamous characteristic of orchardgrass and the breeding system and gene flow among different geographical regions with variations in the culture, bird migration, and breeder selection.

Genetic variation based on SCoT markers

AMOVA for accessions is very important and contributes to the comprehensive understanding of germplasms and breeding (Excoffier et al., 1992). Large variation mainly existed within geographical groups; this variation pattern is in accordance with an ecotype study in fescue (Kölliker et al., 1999). We successfully used AMOVA to examine the distribution of variance within and among groups (Europe, China, Oceania, and North America) and regions (NE, WE, CE, NA, OC, SC, and NC). Both results (83.02 and 69.13%) indicated a large variation within geographical groups, which was consistent with results by Xie et al. (2010), who revealed 74.87% genetic variation within geographical regions using SSR markers. Additionally, Peng et al. (2008) used AFLP markers and detected 85.65% variation within geographical regions. The results demonstrated that the orchardgrass germplasms had a high level of *GD* within geographical regions than among geographical regions, which might play a guiding role in the management and breeding of orchardgrass.

Conservation implications

A study of the distribution and genetic variation of a species revealed the preconditions that are necessary to establish and implement effective and economical protection measures (Hamrick and Godt, 1996). It is important to recognize the *GD* and variation among and within accessions to choose the relevant strategy for the protection and understanding of orchardgrass, which has a great impact on the development of animal husbandry and agricultural practices. This research demonstrated that SCoT markers could present high *GD* and geographical differentiation for *D. glomerata*, which provided potency for further genetic analysis, germplasm collection, and conservation. *D. glomerata* is a perennial allogamous forage grass species. Effective measures to avoid natural pollination would contribute to the conservation and understanding of orchardgrass. In addition, more attention should be paid to the collection and preservation of orchardgrass germplasms worldwide from different ecogeographical regions, climatic types, and mountain ranges.

In conclusion, the results of this study suggested that the SCoT markers were a highly reproducible, efficient, and powerful tool to assess genetic relationships among orchardgrass accessions. This analysis revealed that *D. glomerata* had abundant *GD* and high variability within geographical regions.

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