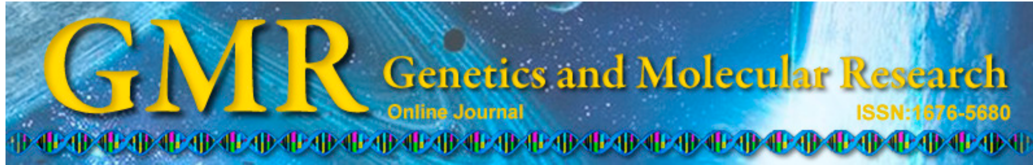

Genetic diversity and relationships in cultivars of *Lolium multiflorum* Lam. using sequence-related amplified polymorphism markers

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ABSTRACT. Sequence-related amplified polymorphism (SRAP) markers were used to analyze and estimate the genetic variability, level of diversity, and relationships among 20 cultivars and strains of annual ryegrass (*Lolium multiflorum* Lam.). Eighteen SRAP primer combinations generated 334 amplification bands, of which 298 were polymorphic. The polymorphism information content ranged from 0.4715 (me10 + em1) to 0.5000 (me5 + em7), with an average of 0.4921. The genetic similarity coefficient ranged from 0.4304 to 0.8529, and coefficients between 0.65 and 0.90 accounted for 90.00%. The cluster

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analysis separated the accessions into five groups partly according to their germplasm resource origins.

Key words: Genetic diversity; *Lolium multiflorum* Lam.; Sequence-related amplified polymorphism

INTRODUCTION

Sequence-related amplified polymorphism (SRAP) is recognized as a new useful molecular marker system based on its reproducibility, low cost, and little knowledge that is needed for target sequences in a species (Li and Quiros, 2001). Currently, SRAP has been successfully utilized to evaluate genetic diversity in species such as *Triticum dicoccoides* (Dong et al., 2010), *Trifolium repens* (Li et al., 2010), *Cynodon arcuatus* (Huang et al., 2013), *Elymus dahuricus* Turcz. (Chen et al., 2009), *Medicago sativa* Linn. (Castonguay et al., 2010), and *Panicum virgatum* (Huang et al., 2011).

Annual ryegrass (*Lolium multiflorum* L.) is one of the most important forage grasses in temperate regions of the world, including China. However, there is little information about the genetic diversity of *Lolium* (Guthridge et al., 2001). Previous studies have used random amplification of polymorphic DNA (Ma et al., 2013), while SRAP was used to identify the parent generation.

The goal of this study was to employ SRAP to analyze the genetic diversity of 20 varieties of annual ryegrass from different sources and to gain information about the selection of cultivars for its breeding programs.

MATERIAL AND METHODS

Plant material

Twenty (N = 20) annual ryegrass cultivars or new lines were obtained from several companies (Table 1).

Table 1. Varieties and sources of *Lolium multiflorum* L.

No.	Accession	Origin	Type	Voucher No.	No.	Accession	Origin	Type	Voucher No.
1	Tetragold	USA	Cultivar	227	11	Barspectra	USA	Strain	
2	Aubade	USA	Cultivar	023	12	Fasete	USA	Strain	
3	Splendor	USA	Cultivar	289	13	Green Gallery	USA	Strain	
4	Abundant	USA	Cultivar	366	14	Florida 4N	USA	Strain	
5	Ganxuan No. 1	Jiangxi, China	Cultivar	148	15	Angus No. 1	USA	Cultivar	367
6	Changjiang No. 2	Yaan, China	Cultivar	287	16	Jianbao	USA	Strain	
7	Diamond T	USA	Cultivar	302	17	Chenqu	USA	Strain	
8	Major	Denmark	Strain		18	Shangnong Tetraploid	Shanghai, China	Cultivar	152
9	Liaoyuan	Chengdu, China	Strain		19	Chuannong No. 1	Yaan, China	Strain	
10	Mufeng	USA	Strain		20	Gulf	USA	Strain	

Reagents

Ethylenediaminetetraacetic acid·2Na, Tris, boric acid, Taq polymerase, the Golden Easy polymerase chain reaction (PCR) system (Tiangen Biotech CO., Ltd., Beijing, China), agarose, ethidium bromide, mineral oil, urea, ammonium persulfate, acetic acid, silver nitrate, and sodium tetraborate were used in this study.

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DNA extraction

A total of 25 young fresh leaves from each of the 20 annual ryegrass plants were randomly collected and extracted using the DNeasy Plant Mini kit (Qiagen Inc, Valencia, CA, USA). The quality and concentration of the DNA were confirmed by electrophoresis on 0.8% agarose gels with the standardized lambda DNA size markers.

SRAP marker reaction system

The annual ryegrass SRAP marker reaction system was designed according to the protocol from Zheng et al. (2008) and Wang et al. (2008). A 20-μL system contained 10 μL Golden Easy PCR system (containing 500 μM dNTP, 20 nM Tris-HCl, 100 mM KCl, 3 mM $MgCl_2$, and other stabilizers and fortifiers), 4 μL 10 ng/μL DNA, 1 μL 10 μM each primer, and 0.2 μL 5 U/μL Taq DNA polymerase. The remaining volume was fulfilled with ddH_2O and covered with 20 μL mineral oil.

SRAP marker primers

Eighteen pairs of SRAP primers, which were selected from 80 combinations of 8 forward and 10 reverse primers, were used in the experiment (Table 2). These primer sequences were gained from previously related studies (Li and Quiros, 2001).

Table 2. Primer sequences used in sequence-related amplified polymorphism (SRAP) analyses of *Lolium multiflorum* L.

Forward primer sequence	Reverse primer sequence
me1 5'-TGAGTCCAAACCGGATA-3'	em1 5'-GACTGCGTACGAATTAAT-3'
me2 5'-TGAGTCCAAACCGGAGC-3'	em2 5'-GACTGCGTACGAATTTGC-3'
me3 5'-TGAGTCCAAACCGGAAT-3'	em4 5'-GACTGCGTACGAATTTGA-3'
me4 5'-TGAGTCCAAACCGGACC-3'	em5 5'-GACTGCGTACGAATTAAC-3'
me5 5'-TGAGTCCAAACCGGAAG-3'	em7 5'-GACTGCGTACGAATTCAA-3'
me6 5'-TGAGTCCAAACCGGTAA-3'	em8 5'-GACTGCGTACGAATTCTG-3'
me7 5'-TGAGTCCAAACCGGTCC-3'	em9 5'-GACTGCGTACGAATTCGA-3'
me10 5'-TGAGTCCAAACCGGTTG-3'	em10 5'-GACTGCGTACGAATTCAG-3'
	em14 5'-GACTGCGTACGAATTACG-3'
	em15 5'-GACTGCGTACGAATTTAG-3'

SRAP marker amplification

SRAP analysis protocols were constructed following the study by Li and Quiros (2001). The first five cycles were 1 min at 94°C for denaturing, 1 min at 35°C for annealing, and 1 min at 72°C for extension. Then, the annealing temperature was raised to 50°C for another 35 cycles, followed by an extension step for 10 min at 72°C, and then 4°C as a holding temperature. PCR products were separated on 6% denaturing polyacrylamide gels.

Data analysis

Only bands that were clearly scored through all the sampled materials were used in

this study. Expressed sequence tag-simple sequence repeat and SRAP fragments, which had the same mobility according to the molecular weight (bp), were scored manually for band presence (1) or absence (0). The presence/absence data matrix was statistically analyzed using the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc). A similarity matrix, which was constructed based on Dice's coefficient (Dice, 1945), was used to develop a dendrogram using the unweighted pair group method arithmetic average (UPGMA).

RESULTS

SRAP markers

Eighteen SRAP primer pairs produced a total of 334 DNA markers, of which 292 were polymorphic (representing 89.22% of all bands). The polymorphism information content ranged from 0.4163 (me5 + em7) to 0.4999 (me1 + em15) with an average of 0.4850 (Table 3), suggesting that the tested annual ryegrass had a high level of SRAP genetic diversity. Shangnong Tetraploid (No. 18) was distinctive as a result of primer pair me4 + em14 amplification (Figure 1).

Table 3. Amplification results from 18 SRAP primer combinations.

Primers	Total bands	Polymorphic bands	Percentage of polymorphic bands	Polymorphic information content
me1 + em15	19	18	90.00	0.4999
me2 + em2	14	10	71.43	0.4841
me2 + em15	16	13	81.25	0.4905
me3 + em5	16	12	75.00	0.4672
me3 + em9	16	15	93.75	0.4929
me3 + em10	15	15	100.00	0.4998
me3 + em15	18	16	88.89	0.4681
me4 + em1	19	16	84.21	0.4986
me4 + em2	21	20	95.24	0.4899
me4 + em5	19	17	89.47	0.4990
me4 + em14	18	17	94.44	0.4990
me5 + em4	15	10	66.67	0.4994
me5 + em7	27	25	92.59	0.4163
me5 + em10	11	9	81.82	0.4932
me6 + em5	24	22	91.67	0.4906
me6 + em8	19	17	89.47	0.4941
me7 + em10	23	19	82.61	0.4849
me10 + em1	23	21	91.30	0.4626
Total	334	292	-	-
Average	18.56	16.22	89.22	0.4850

Genetic relationships among different cultivars of annual ryegrass

Based on results of the SRAP markers, Green (No. 13) and Shangnong Tetraploid (No. 18) had the smallest genetic similarity coefficient (0.4304), indicating that they had the most distant genetic relationship, while Splendor (No. 3) and Abundant (No. 4) had the highest genetic similarity coefficients, which ranged from 0.65 to 0.90 (90.00%) (Figure 2), indicating that they had the closest genetic relationship.

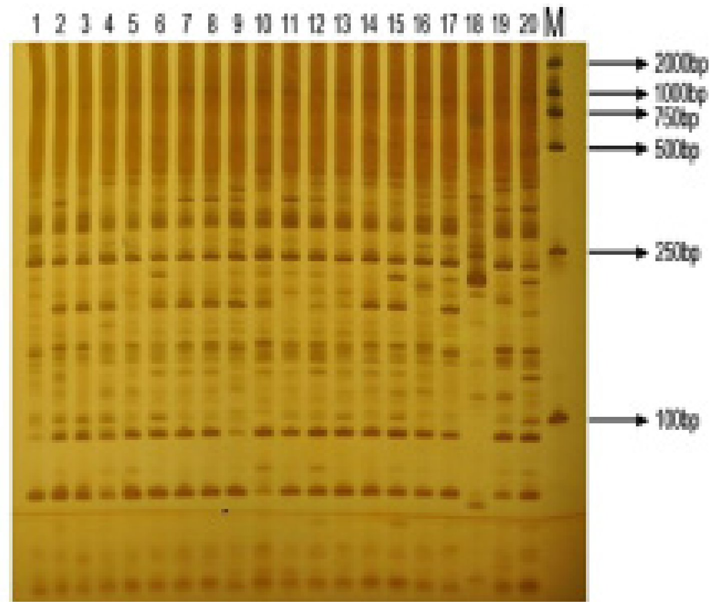

Figure 1. Polymerase chain reaction (PCR) amplification patterns by sequence-related amplified polymorphism (SRAP) primers me4 and em14 using DNA samples 1-20.

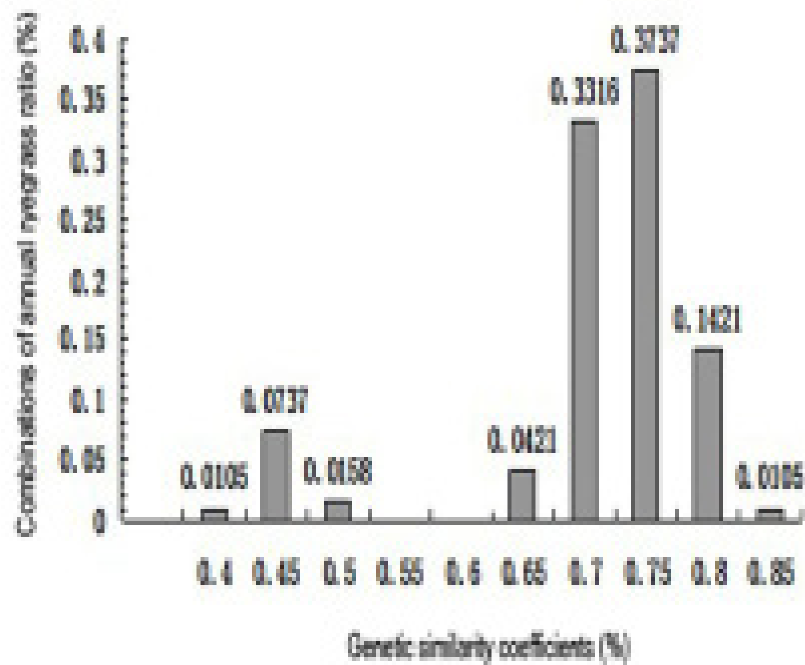

Figure 2. Distribution of genetic similarity coefficients of the material used in the study.

Cluster analysis

The UPGMA cluster analysis using the NTSYS program showed that five groups of annual ryegrass accessions were classified in a dendrogram according to a genetic similarity coefficient of 0.81 (Figure 3). Group I included four accessions: Tetragold (No. 1), Splendor (No. 3), Abundant (No. 4), and Barspectra (No. 11). Group II included five accessions: Ganxuan No. 1 (No. 5), Changjiang No. 2 (No. 6), Diamond T (No. 7), Major (No. 8), and Angus No. 1 (No. 15). Group III included six accessions: Mufeng (No. 10), Fasete (No. 12), Green Gallery (No. 13), Florida 4N (No. 14), Chuannong No. 1 (No. 19), and Gulf (No. 20). Group IV included four accessions: Aubade (No. 2), Liaoyuan (No. 9), Jianbao (No. 16), and Chenqu (No. 17). The accession Shangnong Tetraploid (No. 18) was the only one that was clustered into group V. The results of the cluster analysis were similar to those of the phenotypic characteristic analysis.

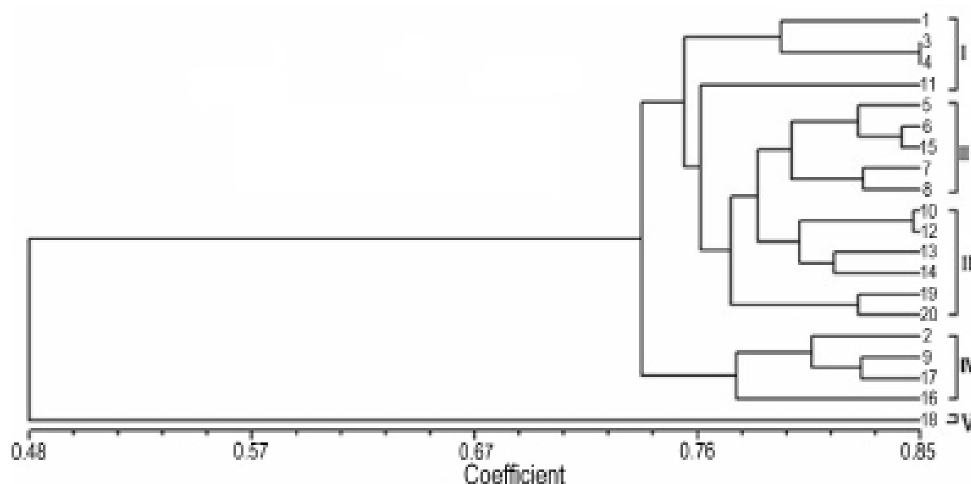

Figure 3. Dendrogram of 20 *Lolium multiflorum* L. accessions based on SRAP markers.

DISCUSSION

SRAP genetic diversity

High genetic diversity is fundamental to the survival of a species (Cota et al., 2011; Karakas et al., 2011; Medri et al., 2011). This genetic diversity is often associated with traits that enable a species to adapt, for example, expanding its distribution range and adapting to new environments. Scientific studies show that most extinct or endangered species have high genetic homozygosity or low genetic diversity (Rosas et al., 2011). Understanding the structural variations and the genetic diversity of the different populations of a species are important for studying evolutionary processes and for germ collection in conservation and breeding programs.

Our study showed that there was high genetic diversity among the 20 varieties studied [percentage of polymorphic bands (PPB) = 89.2%], which was comparable to the genetic di-

versity of other plants such as *Dactylis glomerata* L. (PPB = 84.38%) (Zeng et al., 2008), *Cynodon dactylon* (Linn.) Pers. (PPB = 87.29%) (Ling et al., 2010), and *Hemarthria compressa* R. Br. (PPB = 91.05%) (Fan et al., 2010).

Cluster analysis

The 20 tested annual ryegrasses were divided into five groups by germplasm resource origins using cluster analysis. Many factors affected the genetic structure of the population, such as genetic drift, gene flow, breeding methods, geographical distribution, and the size of individual sample groups (Slatkin, 1987; Schaal et al., 1998). Three reasons for the cluster divisions are as follows. First, most annual ryegrasses of China are obtained from the USA, which makes the original germplasm resources similar. Second, the number of samples was limited, and fewer germplasms were bred in China than in the USA. This led to a small proportion of distinctive bands from the Chinese germplasm resources, which might have a significant impact on the genetic diversity. Third, the varieties within a country may be from the same original parent; thus, the accessions with similar geographical distributions can be clustered together in a group. This study showed that 20 tested annual ryegrasses can be clustered by original germplasm resources and phenotypic characteristics. Therefore, breeding programs may need to involve varieties from different countries.

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