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SSRs transferability and genetic diversity of three allogamous ryegrass species

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ABSTRACT

Simple sequence repeat (SSR) markers are widely applied in studies of plant molecular genetics due to their abundance in the genome, codominant nature, and high repeatability. However, microsatellites are not always available for the species to be studied and their isolation could be time- and cost-consuming. To investigate transferability in cross-species applications, 102 primer pairs previously developed in ryegrass and tall fescue were amplified across three allogamous ryegrass species including *Lolium rigidum*, *Lolium perenne* and *Lolium multiflorum*. Their highly transferability (100%) were evidenced. While, most of these markers were multiple loci, only 17 loci were selected for a robust, single-locus pattern, which may be due to the recentness of the genome duplication or duplicated genomic regions, as well as speciation. A total of 87 alleles were generated with an average of 5.1 per locus. The mean polymorphism information content (PIC) and observed heterozygosity (Ho) values at genus was 0.5532 and 0.5423, respectively. Besides, analysis of molecular variance (AMOVA) revealed that all three levels contributed significantly to the overall genetic variation, with the species level contributing the least ($P < 0.001$). Also, the unweighted pair group method with arithmetic averaging dendrogram (UPGMA), Bayesian model-based STRUCTURE analysis and the principal coordinate analysis (PCoA) showed that accessions within species always tended to the same cluster firstly and then to related species. The results showed that these markers developed in related species are transferable efficiently across species, and likely to be useful in analyzing genetic diversity.

1. Introduction

The genus *Lolium* (ryegrasses) is a member of the Poaceae family, is native to Europe, the Near East and North Africa and consists of nine diploid species altogether with the somatic chromosome number of $2n = 2x = 14$ [1,2]. Of which, Italian ryegrass (*L. multiflorum*) and perennial ryegrass (*L. perenne*) are cultivated widely in the temperate climate areas of the world as forage and turf grasses [1]. *L. rigidum* is a winter annual grass originating from the Mediterranean region, and it is not only used as a cultivated fodder crop in some areas, mostly in Australia, adapting to drought and grazing pressure [3], but also an economically damaging crop weed [4] having evolved resistance to many different chemical herbicides [5,6]. The three above-mentioned species are generally self-incompatible species, and therefore each germplasm could be regarded as a heterogeneous population of genotypes [7]. In addition, the

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previous studies have reported that the *Lolium* and broad-leaved fescue (Section *Schedonorus*) are closely related, in particular to tall fescue (*Festuca arundinacea*), therefore, these grasses are commonly referred to as the *Lolium*/*Festuca* complex [8].

Simple sequence repeats markers (SSRs) have been proved to be an ideal tool for genotyping elite grass material, surveying genetic resources, map construction and genetic variability identification because they are codominant, multi-allelic, highly reproducible, abundant and are evenly distributed in the plant genome either in coding and noncoding regions [9,10]. While most of the strategies currently used to isolate SSR markers are both time- and cost-consuming, which limits the application of SSR in those genomes with less information [11]. Therefore, cross-species and cross-genus amplification of molecular markers is now a common strategy for the discovery of markers to use on the not so well studied species [12,13]. It has been reported that SSR markers has showed a high transferability across species or even in closely related genera [14], which originated from genomic libraries (genomic Simple Sequence Repeat, gSSRs) and/or derived from expressed sequence tags (ESTs) on the most important crops or model species, and thus are now frequently used on diversity, evolutionary and mapping studies in other related species [15–18].

At present, a large number of SSR markers have been developed for the *Lolium*/*Festuca* species, such as *L. perenne* [19], *L. multiflorum* [20] and tall fescue (*F. arundinacea*), a species closely related to *Lolium* [21–23]. However, SSR markers in *L. rigidum* has not been developed. Therefore, the main objectives of our present study are to: (1) assess the transferability of published *Lolium*/*Festuca* SSRs cross three *Lolium* species, including *L. rigidum*, *L. perenne* and *L. multiflorum*, (2) evaluate polymorphism and efficiency of the transferable SSR markers for the genetic diversity and relationship analyses among three ryegrass species, and (3) facilitate genetic studies and molecular breeding for ryegrasses programs.

2. Material and methods

2.1. Plant material

All plant materials including eight accessions from three important species of *Lolium* were obtained from the National Plant Germplasm System, USA (http://www.ars-grin.gov/) (Table 1). Seeds were germinated and the ploidy was identified as a diploid ($2n = 2x = 14$).

2.2. Genomic DNA extraction

Total genomic DNA was extracted from leaf tissues, with an average of eight genotypes per accession, by the cetyltrimethylammonium bromide (CTAB) protocol [24] using the Plant Genomic DNA Kit (TianGen Biotech, Beijing, China). The quality and concentration of the extracted DNA were determined by NanoDrop ND 2000 spectrophotometer (NanoDrop Technologies, Inc.) and 1% (w/v) agarose gels electrophoresis. The isolated genomic DNA was diluted to 10 ng/μL by 0.1 × TE buffer (1 mmol/L Tris-HCl, 0.1 mmol/L EDTA, pH = 8.0) and stored at −20 °C.

2.3. Primer selection and SSR–PCR amplification

A total of 102 SSR markers from different resources were used to genotype 64 individuals. Genic-SSR markers with the prefixes LMg [20], LP [25] and B3-B6 [21] were developed from annual ryegrass, perennial ryegrass and *Lolium*/*Festuca* complex of grasses, respectively. EST-SSR markers with prefix NFA [23] were developed from tall fescue. All SSR primers (Table 2) were synthesized by Shanghai Sangon Biological Engineering Technology and Services (Shanghai, China).

Each 15 μL amplification reaction consisted of 3.0 μL of template DNA (10 ng/μL), 0.8 μL primer (5 ng/μL), 0.3 μL of Taq polymerase (2.5 U/μL), 3 μL of sterile distilled water and 7.5 μL of 2 × Taq PCR Master Mix (Tiangen Biotech, Beijing, China). SSRs were amplified under the following PCR conditions: a touchdown PCR consisting of 95 °C for 5 min; 14 cycles of 94 °C for 1 min, (Tm + 2) ∼ (Tm-5)°C for 40 s decreasing by 0.5 °C/cycle and 72 °C for 1 min; and 22 cycles of 95 °C for 40s, (Tm-5)°C for 40 s and 72 °C for 5 min; followed by 72 °C for 5 min and 4 °C as the holding step. PCR amplified fragments were separated using electrophoresis on a 6% denaturing polyacrylamide gel (PAGE) and visualized after silver staining [26].

2.4. Statistical analysis

As a co-dominant nature of SSRs and *Lolium* species occur as diploid ($2n = 2x = 14$) [2], allelic bands were scored with genotype based on a molecular DNA marker (50 bp ladder) [27] and formed a "bp" typed original matrix to estimate the number, range and distribution of amplified alleles, and then to determine variation level in the accessions and species. Allele frequencies were computed using GenAlEx 6.5 [28] and polymorphism information content (PIC) was calculated according to Botstein's methods [29].

Table 1
Accessions of *Lolium* species used in the analysis.

Species	Code	Original ID	Origin	Breeding State	Sample size
Lolium multiflorum	LM1	PI 187220	Belgium	Uncertain	8
	LM2	PI 239486	Spain	Uncertain	9
	LM3	PI 241913	Italy	Wild material	8
Lolium perenne	LP1	PI 418725	France	Wild material	7
	LP2	PI 619018	Wales	Wild material	7
Lolium rigidum	LR1	PI 314447	Georgia	Wild material	8
	LR2	PI 422586	Morocco	Wild material	9
	LR3	PI 545666	Turkey	Wild material	8