Australian Journal of Crop Science AJCS
AJCS 9(7):666-670 (2015) ISSN:1835-2707

Analysis of nuclear DNA content and chromosome number for screening genotypes and crosses in Annual Ryegrass (*Lolium multiflorum* Lam.)

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Abstract

Lolium multiflorum Lam. (annual ryegrass) is a forage grows in temperate regions of the world. In South America, annual forms of *L. multiflorum* exist and have been used as forage for a long time in Argentina, Chile, Uruguay, and Brazil. This study determined the nuclear DNA content and chromosome number for screening genotypes and crosses in *L. multiflorum* from Brazil. Analyses to estimate nuclear DNA content by flow cytometry were performed in young leaves of four parental genotypes and eight offspring resulting from their crosses. To the chromosome counting, the slides were prepared by flame drying technique and Giemsa staining. The genotypes were classified in the following categories: diploid, triploid, and tetraploid with 14, 21 and 28 chromosomes, respectively. The average content of DNA in diploids ranged from 5.42pg to 5.85pg. A single triploid plant showed 8.30pg. In tetraploids, the average DNA content ranged from 10.21pg to 10.95pg. Thus, the combination of analyses by DNA quantification and chromosome counting prove to be efficient for screening and diagnosing ploidy level of parents and crosses in *Lolium.* This information may be used reliably for prior selection of genotypes for the breeding program.

Keywords: Annual ryegrass; cytogenetics; flow cytometry; plant breeding; polyploidy.

Introduction

The genus *Lolium* contains eight diploid species (2n = 2x = 14) and some polyploid cultivars obtained by chromosome doubling using colchicine (Polok, 2007; Integrated Taxonomic Information System, 2014). Among the species, *L. multiflorum* Lam., known as annual ryegrass, is a popular forage grass that is cultivated in the temperate regions of the world. In South America, annual forms of *L. multiflorum* have been known and used for a long time in Argentina, Chile, Uruguay, and Brazil (Humphreys et al., 2010). The species was probably introduced to southern Brazil in 1875 by Italian settlers, and it is widely cultivated in that region for both harvest and grazing (Carvalho, 2004; Flores et al., 2008; Mittelmann et al., 2010). Perennial, Italian and Westerwolths ryegrasses are natural cross-polinators with a high degree of self-incompatibility controlled by two or three loci with many alleles. In *L. multiflorum* at least 40 different alleles are likely to be present (Fearon et al., 1983). Some pseudo-self-compatibility can allow self-polination to produce a small amount of selfed seeds (Humphreys et al., 2010). Because of its importance to beef and milk production systems, especially in winter, *L. multiflorum* has been targeted for breeding. In addition to increasing productivity (leaves and dry matter) the intention is to develop cultivars with precocity, high initial vigor, good growth capacity (Fontaneli and Fontaneli, 2000), and adapted to different environmental conditions (Nunes et al., 2002). Likewise, genotypes with higher disease resistance (Nunes et al., 2002) and increased grazing tolerance are required (Corrêa et al., 2007).

Implementing a breeding program for annual ryegrass, in particular for establishing a germplasm collection, requires a large number of plants obtained from different environments. Therefore, the knowledge of available genetic variability requires complete information about germplasm, such as cytogenetics characterization. Cytogenetics analysis typically emphasizes the determination of chromosome number and ploidy. The referred determination allows genotypic discrimination and identification of similarities which assist in the planning of compatible crosses. These data are the link between appropriate use of in genetics resources characterization and breeding (Davide et al., 2009). Among various techniques for screening genetic material during planning and collection of germplasm, determining the amount of nuclear DNA via flow cytometry is a rapid and convenient method for assessing ploidy level in plants (Dolezel, 1997). Flow cytometry can also be useful during testing and pre-selections of genotypes as well as in hybridization and recombination. In addition, when parents differ in DNA content (Bennet and Leitch, 1995), this procedure can be used on a large scale to confirm both the ploidy level and the occurrence of crossing by comparing data between parents and offspring of young plants. Thus, quantification of nuclear DNA proves to be an important alternative to initial triage of numerous genotypes that comprise study collections of plant breeders, aiming to assist in the selection of genotypes with ploidy levels of interest. Subsequently, in cases where there is doubt, results obtained by flow cytometry may be confirmed by chromosome counts, which will provide accurate characterization of ploidy and

Table 1. Variation in nuclear DNA 2C content (v2C), mean nuclear DNA 2C content (m2C), ploidy (P), mean coefficient of variation per genotype (CV), and chromosome number (CN) plus number of fragments (f) in genotypes of *Lolium multiflorum*.

Genotypes	v2C (pg)	m2C (pg)	P	CV (%)	CN + f
Comum	5.33 to 5.64 (25)[1]	5.42a	2x	0.51	14 + (0 to 5f) (24)[2]
Avance	5.64 (1)	5.64a	2x	0.44	-
	9.99 to 11.89 (29)	10.91d	4x		28 + (0 to 4f) (13)
Barjumbo	10.19 to 11.65 (20)	10.87d	4x	0.43	28 + (3 to 8f) (16)
INIA Titan	5.42 to 5.80 (13)	5.65a	2x	0.49	14 + (0 to 3f) (4)
	9.79 to 10.96 (12)	10.37c	4x		28 + (0 to 10f) (10)
A41	5.56 to 5.95 (18)	5.76a	2x	0.51	14 + (1 to 2f) (4)
	9.96 to 10.57 (07)	10.21c	4x		-
A42	5.58 to 5.99 (05)	5.82a	2x	0.50	14 + 2f (1)
	9.91 to 11.32 (20)	10.57c	4x		-
A43	5.64 to 5.86 (10)	5.74a	2x	0.44	14 + (0 to 6f) (15)
	9.82 to 11.15 (15)	10.45c	4x		28 + (0 to 10f) (10)
A44	5.58 to 5.94 (8)	5.71a	2x	0.47	14 + (0 to 6f) (6)
A45	5.61 to 5.68 (3)	5.64a	2x	0.46	14 + (0 to 6f) (29)
	10.35 to 11.54 (17)	10.93d	4x		28 + (0 to 8f) (17)
A46	5.85 (1)	5.85a	2x	0.47	14 + (0 to 4f) (10)
	10.45 to 11.70 (24)	10.89d	4x		28 + (0 to 8f) (9)
A47	10.50 to 11.49 (13)	10.95d	4x	0.48	28 + (2 to 8f) (12)
ABARP	5.40 to 5.62 (3)	5.50a	2x	0.48	14 + (1 to 6f) (14)
	8.30 (1)	8.30b	3x		21 + (0 to 2f) (4)
	10.23 to 11.56 (21)	10.93d	4x		28 + (1 to 9f) (22)

* Means followed by the same letter do not differ by the Scott-Knott test at 5% probability, pg= picogram, DNA measure; Number of individuals. [2] Number of analysed metaphases.

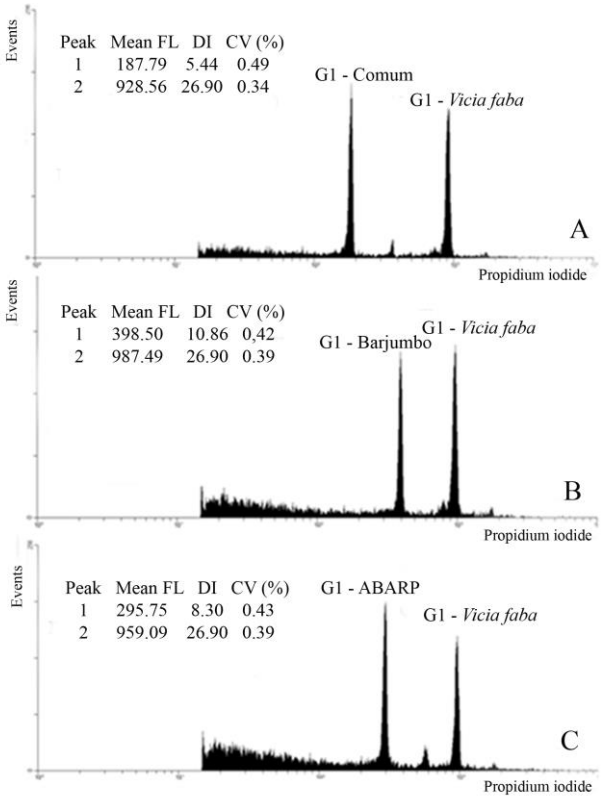

Fig 1. Flow cytometry histograms of relative fluorescence obtained after simultaneous analysis of nuclei isolated from the internal reference standard (*Vicia faba*) with 2C = 26.9pg DNA and *L. multiflorum*. Comum with 5.44pg DNA (**A**), Barjumbo with 10.86pg DNA (B), ABARP with 8.30pg DNA (**C**). Mean channel number (Mean FL), DNA index (DI: mean channel number of samples / mean channel number of reference standard) and coefficient of variation value (CV, %) of each peak are also given.

Table 2. Origin, number of tested plants (N) and expected ploidy (EP) in genotypes of *Lolium multiflorum*.

Genotypes	Origin	N	EP
Comum (male parental)	Local population	25	2x
Avance (female parental)	Cultivar introduced	30	4x
Barjumbo (female parental)	Cultivar introduced	20	4x
INIA Titan (female parental)	Cultivar introduced	25	4x
A41 (offspring)	INIA Titan x Comum	25	3x
A42 (offspring)	INIA Titan x Comum	25	3x
A43 (offspring)	INIA Titan x Comum	25	3x
A44 (offspring)	INIA Titan x Comum	08	3x
A45 (offspring)	Avance x Comum	20	3x
A46 (offspring)	Avance x Comum	25	3x
A47 (offspring)	Avance x Comum	13	3x
ABARP (offspring)	Barjumbo x Comum	25	3x

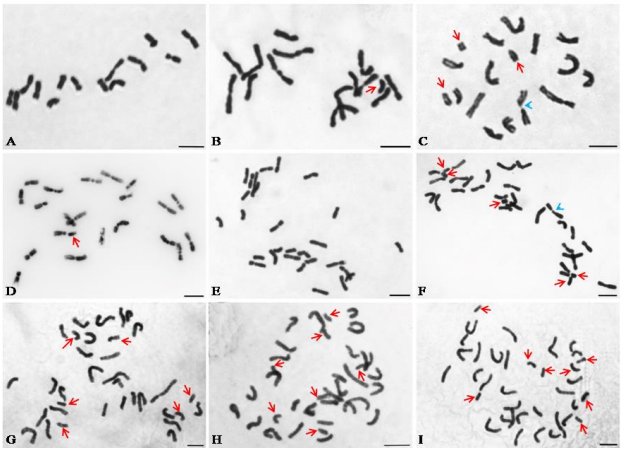

Fig 2. Chromosome number. 14 (A), 14 + 1 fragment (B), 14 + 3 fragments (C), 21 + 1 fragment (D), 28 (E), 28 + 5 fragments (F), 28 + 6 fragments (G), 28 + 7 fragments (H), 28 + 8 fragments (I). Red arrows indicate chromosomal fragments. Ends of blue arrows indicate chromosomal lesions. Bar = 5µm.

are particularly useful in identifying duplicate materials in the collection. This study determined the nuclear DNA content and chromosome number for screening genotypes and crosses in *Lolium multiflorum* Lam.

Results and Discussion

Flow cytometry

Several studies using flow cytometry for detection of ploidy for screening a large number of plants are becoming increasingly common in the literature because of fast results (Dolezel et al., 2007; Campos et al., 2009; Elling et al., 2010; Viehmannova et al., 2012; Zarrei et al., 2012).

With regard to nuclear DNA content, genotypes of *L. multiflorum* were categorized as diploid, triploid, and tetraploid in the present study (Table 1 and Fig. 1). The average content of DNA in diploids ranged from 5.42pg (genotype Comum) to 5.85pg (offspring A46). A single triploid plant (ABARP genotype, plant 15) showed 8.30pg. In tetraploids, the average DNA content ranged from 10.21pg to 10.95pg in offspring A41 and A47, respectively (Table 1).

Our results corroborate previously known estimates for DNA content of *Lolium*. Recent studies using flow cytometry reported that the average amount of nuclear DNA in diploid cultivar Prolog and in tetraploid cultivar Lubina was 5.25pg and 10.99pg, respectively (Kopecký et al., 2010). The present study found that the triploid plant had a nuclear DNA content of 8.30pg, which was close to the estimated 8.12pg. Smarda et al. (2008) estimated DNA content by flow cytometry to diploid *L. multiflorum* and obtained 5.44pg, that is, value very similar to those already reported. Screening results for DNA amount showed that cultivars Comum (diploid) and Barjumbo (tetraploid) had the expected values for all individuals. Regarding offspring A44 and A47, all plants possessed DNA content compatible with diploid and tetraploid level, respectively. The other genotypes were diploid and tetraploid, while ABARP offspring were of three different ploidies: diploid, triploid and tetraploid. Obtaining triploid offspring by using the chance hybrids technique occurred in small proportion (1/166) and may be due to the difficulty in maintaining this genotype, as it may show germination problems or even death of seedlings soon after germination. Thus, breeding techniques involving emasculation should be used to increase efficiency in obtaining triploid offspring. In our study, the occurrence of diploid and poliploid offspring in the same group of plants (A41, A42, A43, A45, A46, and ABARP) may be due to a probable mixture of seeds in the multiplication of parental seeds for commercial use, as it is extremely difficult to visually separate plants with different ploidies. This may have occurred with seeds of parents Avance and INIA Titan, which showed individuals with different ploidies. Furthermore, although ryegrass is a cross-pollinating species (Fearon et al., 1983), self-pollination may have occurred in a small amount of the examined plants thus contributing to the occurrence of offspring with different ploidies. This previously known information confirms the importance of using flow cytometry for both selection and certification of genotypes and detection of large scale crosses in *L. multiflorum*. Besides, it can help to confirm the crosses. The advantage of using flow cytometry is the possibility of assessing different types of young tissue in small fractions, as it is a fast, non-destructive method (Pereira et al., 2012). However, its use is limited because information is generated at the cell population level, which makes it impossible to evaluate the individual behavior of cells. Thus, the most reliable option would be cytogenetic analysis in mitotic metaphases, which makes it possible to accurately determine the chromosome number in individual cells.

Chromosomal counting

Chromosome counts included the identification of diploid genotypes (14 chromosomes and 14 chromosomes + 6 fragments), triploid genotype (21 chromosomes and 21 chromosomes + 2 fragments), and tetraploid genotypes (28 chromosomes and 28 chromosomes + 10 fragments) (Table 1

and Fig. 2). The Avance, INIA Titan, A41, A42, A43, A45, A46 e ABARP genotypes presented diploid and poliploid plants detected by flow cytometry and in most cases also by chromosome analysis (Table 1).

In *Lolium*, the formation of fragments is due to gaps and breakage at 45S rDNA site and it has already been reported as fragile sites (FS) in chromosomes of the genotypes of *L. perenne* (Huang et al., 2008; Rocha et al. (2015) and *L. multiflorum* (Huang et al., 2008; Bustamante et al., 2014). Bustamante et al. (2014) assessed mitotic metaphases with fluorochrome DAPI (4',6-diamidino-2-phenylindole) and FISH (fluorescence *in situ* hybridization) with 45S rDNA probe and observed formation of gaps and natural breaks in chromosomes of the genotypes Comum, Bar jumbo and ABARP, also evaluated in this study.

Of the 61 metaphases of *L. multiflorum* analyzed by Bustamante et al (2014), 95% showed fragments ranging from one to five in diploid plants; one to nine in tetraploid and two to four in triploid. Among the 36 metaphases and prometaphases analyzed in *L. perenne* cv. Ellet (diploid), only 2 showed preserved chromosome structure. The number of fragments ranged from one to six in this species (Rocha et al., 2015). The occurrence of FS in *Lolium* may be associated to several cytological effect on the transcriptional activity of the rDNA sites and variability in the number and position of these sites and of nucleoli (Rocha et al. 2015; Bustamante et al., 2014). Additionally, the FS may be associated with meiotic abnormalities like non-oriented chromosomes on the plate during metaphase I (63.3%), delayed chromosomes in telophase I (10.6%) and micronuclei in telophase I (0.54%) as noted in the Comum genotype (Freitas et al., 2013). However, these abnormalities should be restored because average values of pollen viability rates of the same genotypes used in this study (except A43 and A46 offspring) were higher than 93% (Nunes et al., 2012). A combination of chromosome counts and a quantification of DNA is very useful and it was also used for determining ploidy of interspecific hybrids between *Pennisetum purpureum* schum (2n=2x=28) *x P. glaucum* L (2n=2x=14) (Campos et al., 2009). Of the 200 seedlings treated with colchicine, 115 were characterized as mixoploid (57.5%), 68 as triploid (34%) and 17 as hexaploid (8.5%). Further confirmation of hexaploidy was performed in some individuals through stomatal morphology and chromosome counts.

Materials and Methods

Genetic materials

Analyses to estimate nuclear DNA content were performed in four parental genotypes of annual ryegrass and eight offspring resulting from crosses among them. It was analyzed between 8 and 30 plants per genotype, according to the plants availability, totaling 266 analyzed plants (Table 2). Genotypes were provided by the Active Germplasm Bank of Forage Grass and the Annual ryegrass breeding program of Embrapa Dairy Cattle/Embrapa Temperate Climate (Brazilian Research Institute), Juiz de Fora municipality, Minas Gerais State /Pelotas municipality-Rio Grande do Sul State, Brazil. Offspring were obtained by the chance hybrids technique, in which some parents are kept side by side in a greenhouse without emasculation, and flowering is synchronized by previous cuts in the material (Stelling, 1995).

Quantification of nuclear DNA

The estimated DNA content by flow cytometry was obtained from leaf tissue according to Dolezel and Bartos (2005). Approximately 20 - 30mg of young leaves per sample was used to assess the ploidy level. For each plant three repetitions were performed. The DNA amount of *Vicia faba* L was used as an internal standard reference (2C = 26.9 pg). Samples were crushed in a Petri dish containing 1 ml of cold buffer LB01 to obtain the nuclear suspension (Dolezel, 1997), to which 2.5µL of RNase was added and then stained with 25µL propidium iodide (1mg/mL). At least 10,000 nuclei were analyzed per sample. Histograms with coefficients of variation under 0.8% were obtained using a FACSCalibur cytometer (Becton Dickinson) with Cell Quest software (Becton, Dickinson and Company, San Jose, CA, USA) and then, average nuclear DNA contents were analyzed with WinMDI 2.8 software (2009). Based on average nuclear DNA contents, germplasm was grouped by ploidy within each genotype and subject to analysis of variance and Scott and Knott test ($p < 0.05$) with the Sisvar software (Ferreira, 2003).

Chromosome counts

Ploidy level estimates obtained by DNA quantification using flow cytometry were confirmed by chromosome analysis of, at least, one plant per genotype with the same ploidy. To obtain meristems, seeds were germinated at 15°C and emerging roots were harvested and then treated in iced water for 24h (Ksiazczyk et al., 2010), fixed in Carnoy (absolute ethanol: glacial acetic acid, 3:1) and kept at -4°C. The seeds of genotypes Avance diploid, A41 and A42 tetraploids were not viable. So, it was not possible to obtain roots and count chromosomes for them. Slides were prepared by flame drying (Dong et al., 2000) after enzymatic maceration in pectinase-cellulase (100U:200U) for 2h and 10min at 37°C and then stained with 2% Giemsa for approximately 7 min in coplin jar, protected from light and air dried (Guerra and Souza, 2002). The slides were observed under 1000-fold magnification using a bright field microscope (Zeiss AX10). The best metaphases were selected for chromosome counting.

Conclusions

The analyses for chromosome number confirmed the ploidy level determined by flow cytometry in the evaluated genotypes. Despite the occurrence of breaks in fragile sites, there were no changes in the DNA content of the genotypes tested. Analyses by DNA quantification and chromosome counting proved to be efficient for screening and diagnosis of ploidy level in parentals and crosses in genus *Lolium*.

Acknowledgements

To the National Council for Scientific and Technological Development - CNPq, the Foundation for Research Support of the State of Minas Gerais – FAPEMIG, the Coordination of Improvement of Higher Education Personnel - CAPES for financial support to research and scholarship grant, and the Brazilian Agricultural Research Corporation (EMBRAPA) for providing biological material.

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