RESEARCH

Distribution of rDNA in Diploid and Polyploid *Lolium multiflorum* Lam. and Fragile Sites in 45S rDNA Regions

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ABSTRACT

The distribution, number, and location of ribosome DNA (rDNA) regions on chromosomes were evaluated, and transcriptional activity was described in genotypes of Italian ryegrass (*Lolium multiflorum* Lam.), as well as in an offspring resulting from interbreeding. Nucleoli and nucleolar organizer regions were labeled with silver nitrate and fluorescent in situ hybridization (FISH) was performed with 5S and 45S rDNA as probes. Hybridization procedures were performed on slides previously stained with the Ag-NOR method. The 5S rDNA sites were highly conserved, while 45S rDNA sites had wide variability, even showing more than one site on the same chromosome. One of the genotypes had more than expected 45S rDNA sites. Approximately 95% of the cells at metaphases had at least one chromosome break/gap in the 45S rDNA site, resulting in chromosome fragmentation. Thus, 45S rDNA correspond to fragile sites in *L. multiflorum*. These events can affect genome organization and cause new chromosomal rearrangements which, along with some other factors, might be responsible for microevolutionary changes involved in differentiation and speciation. Not all 45S rDNA sites are transcriptionally active. Variation in both the number and size of nucleoli and mechanisms of nucleolar fusion were observed in *L. multiflorum.*

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Abbreviations: DAPI, 4'6-diamidino-2-phenylindole; DIG, anti-digoxigenin; FISH, fluorescent in situ hybridization; FITC, fluorescein isothiocyanate; NORs, nucleolar organizer regions; rDNA, ribosome DNA; SSC, saline solution citrate; TNB, tris/sodium chloride buffer; TRITC, tetramethyl rhodamine isothiocyanate.

The rDNA regions can represent up to 10% of plant genomes and are traditionally used as cytogenetic markers in evolutionary, phylogenetic, and taxonomic studies (Heslop-Harrison, 2000). As they are composed of tandem repeating units with highly conserved sequences, rDNA regions are easily identified by FISH (Roa and Guerra, 2012). The rRNA genes occur in loci named as nucleolar organizer regions (NORs). The active genes, in general, can be detected by a silver staining technique (Ag-NOR banding), which allows the visualization of NORs that were transcribed in the interphase (Hubbel, 1985).

The location of these sequences of rDNA in chromosomes has been widely used to both estimate karyotype similarity between species (Guerra, 2012) and to investigate evolutionary trends, such as in *Triticeae* (Castilho and Heslop-Harrison, 1995; De Bustos et al., 1996; Taketa et al., 1999). However, Guerra (2012) reported some restrictions regarding the use of 45S rDNA sites to identify chromosomal homologies due to the variability in their number and position found in various groups of plants such as *Allium*

Published in Crop Sci. 54:1–9 (2014).
doi: 10.2135/cropsci2013.05.0325

(Schubert and Wobus, 1985), *Triticeae* (Dubcovsky and Dvorak, 1995; Altinkut et al., 2006), *Aegilops* (Raskina et al., 2004a, 2004b), and *Oryza* (Shishido et al., 2000).

Intraspecific variation in these regions has also been described in *Lolium*. In diploid *L. perenne* L. ($2n = 2x = 14$), six to eight (Lideikyte et al., 2008) or five to seven 45S rDNA sites (Ksiazczyk et al., 2010) were described. In tetraploid cultivars of the same species, 9 to 16 (Lideikyte et al., 2008) or 12 to 15 sites (Ksiazczyk et al., 2010) were reported. In addition, there are descriptions of polymorphism in tetraploid *L. multiflorum* ($2n = 4x = 28$) with 11 to 14 sites (Lideikyte et al., 2008) and *L. rigidum* Gaundin ($2n = 2x = 14$) with 6 to 9 45S rDNA sites (Thomas et al., 2001). Also in *L. rigidum*, two sites syntenic of 45S rDNA were found in three metaphases, that is, they were located in the same chromosome (Thomas et al., 2001). Less frequently, some cultivars also showed variation in the 5S site (Lideikyte et al., 2008; Ksiazczyk et al., 2010).

Although other mechanisms, such as uneven genetic exchange, transposition, and gene conversion, also cause variation in number and location of 45S rDNA sites, the occurrence of fragile sites in 45S rDNA blocks described in *Lolium* (Huang et al., 2008; Huang et al., 2009) should be deemed a potential source of chromosome rearrangements. Fragile sites have been reported to occur frequently in human cells (Jones et al., 1995; Cimprich, 2003; Debatisse et al., 2012) and have been associated with precancerous chromosome gaps in the early stages of carcinogenesis but they were reported to occur in plants only recently (Huang et al., 2008).

In *Lolium*, these sites appear as breaks or gaps on the 45S rDNA sequence and may result in the formation of chromosomal fragments (Huang et al., 2008). In addition to promoting chromosomal rearrangement and instability and being possibly involved with fluctuation in the 45S rDNA region (Ruiz-Herrera and Robinson, 2007; Brown and O'Neill, 2010), fragile sites can affect the transcriptional activity of 45S rDNA (Huang et al., 2012). Thus, correlation between the number of 45S rDNA sites and either maintenance or lack of transcriptional activity should be investigated in more detail in genotypes with different ploidy levels, as well as in the progeny resulting from interbreeding.

This study investigated the occurrence of gaps and breaks in rDNA sites in chromosomes of diploid and polyploid genotypes of *L. multiflorum* and possible implications for transcriptional activity.

MATERIAL AND METHODS

Evaluations were conducted on seven individuals from two genotypes of Italian ryegrass (*Lolium multiflorum* Lam.) and progeny resulting from crosses between them (Table 1). The genotypes came from the Active Bank of Italian Ryegrass Germplasm and from the genetic improvement program of ryegrass at Embrapa Gado de Leite, Juiz de Fora, state of Minas Gerais/ Embrapa Clima Temperado, Pelotas, state of Rio Grande do Sul, Brazil.

Table 1. Genotypes, plants, origin, and ploidy of the *L. multiflorum* used the present study.

Genotypes	Plants	Origin	Ploidy
Comum (male parental)	2, 14, 19, and 32	Local population	2x
Barjumbo (female parental)	2, 11, and 33	Introduced cultivar	4x
ABARP (offspring)	14	Barjumbo × Comum	3x

The offspring was obtained with the "chance hybrids" technique, in which some parental plants are kept side by side in a greenhouse without emasculation, and flowering is synchronized by making cuts in the earlier material.

Roots from germinated seeds were collected and treated in ice water for 24 h (Ksiazczyk et al., 2010), fixed in Carnoy (absolute ethanol/glacial acetic acid, 3:1) and kept at −4°C. Slides were flame-dried (Dong et al., 2000) after enzymatic maceration in pectinase-cellulase (100U: 200U) for 2 h and 10 min at 37°C.

The silver nitrate staining procedure for nucleoli and NORs was based on Guerra and Souza (2002) and Huang et al. (2012). One drop of colloidal solution (2% gelatin, 2% formic acid) and two drops of 50% silver nitrate were added to the chromosome preparation. Slides were incubated in a moist chamber at 72°C for 2 to 7 min and washed with distilled water, then dehydrated in an ethanol series and observed under a bright field microscope (Zeiss AX10). Nucleoli and NOR stains were evaluated in 150 interphase cells and in at least five metaphases of each genotype.

We used 45S rDNA sequences (pTa 71 of *Triticum aestivum* L.) and 5S (D2 of *Lotus japonicus* Regel, a fragment about 400bp [Pedrosa et al., 2002]) as FISH-probes. The 5S and 45S probes were labeled, respectively, with digoxigenin and biotin, using nick-translation reaction. Chromosome preparation was denatured in 100 μL 70% formamide in saline solution citrate (SSC)2x at 85°C for 80 s. Subsequently, the slides were dehydrated in an ethanol series. The hybridization mixture (50% formamide, SSC2x, 10% dextran sulfate, and approximately 50 ng of each of the labeled probes) was denatured at 95°C for 8 min. The mixture was added to the chromosome preparation and hybridized for at least 16 h at 37°C in a moist chamber. Detection was performed with fluorescein isothiocyanate (FITC)-conjugated anti-digoxigenin (DIG) and avidin-conjugated tetramethyl rhodamine isothiocyanate (TRITC) in tris/ sodium chloride buffer (TNB) for 1 h at 37°C in a moist chamber. Slides were mounted in Vectashield (Vector Laboratories Inc., Burlingame, CA) with 4'6- diamidino-2-phenylindole (DAPI) and analyzed under an Olympus BX60 epifluorescence microscope. Images were digitized with cooled monochrome camera Axio Cam HRm (Zeiss) and processed with Axio Vision Release 4.8.2 software (Zeiss, 2012) and Adobe Photoshop CS3 (Adobe Systems, 2007).

Metaphases were analyzed for position and distribution of 5S rDNA and 45S rDNA sites, and the integrity of chromosome morphology. Chromosomal gaps were characterized by failures in the proximal region of the chromosome; in some cases, the chromosomes remained bound to the fragments by way of chromatin strands, thus not characterizing complete rupture of chromosomal arms. Breaks were characterized by

Table 2. Chromosome number (CN) and number of fragments (f), number of signals at 45S rDNA site (45S signs), number of breaks in 45S ribosome (rDNA) sites (NB), number of gaps at 45S rDNA sites (NG), number of 45S rDNA sites (45S), and number of 5S rDNA sites (5S) per somatic chromosome complement (2n) in *L. multiflorum* genotypes.

Genotypes	Number of metaphase cells	CN+f	45S signs	NB	NG	45S	5S
Comum 2	6	14 + 2 to 4f	10 to 13	2 to 4	0 to 2	7 to 8	2
Comum 14	7	14 + 2 to 5f	8 to 13	2 to 5	0 to 3	6 to 8	2
Comum 19	12	14 + 0 to 4f	6 to 10	0 to 4	0 to 2	6	2
Comum 32	14	14 + 1 to 5f	7 to 13	1 to 5	0 to 3	6 to 8	2
Mean				*2.49*	*0.77*	*6.64*	*2*
Barjumbo 2	12	28 + 3 to 6f	9 to 17	3 to 6	0 to 1	12	4
Barjumbo 11	4	28 + 4 to 8f	15 to 19	4 to 8	0 to 1	12 to 14	4
Barjumbo 33	2	28 + 1 to 9f	17 to 21	1 to 9	0 to 7	12 to 13	4
Mean				*4.88*	*0.83*	*12.17*	*4*
ABARP 15	4	21+ 2 to 4f	11 to 13	2 to 4	0 to 2	9 to 10	3
Mean				*2.75*	*0.5*	*9.75*	*3*

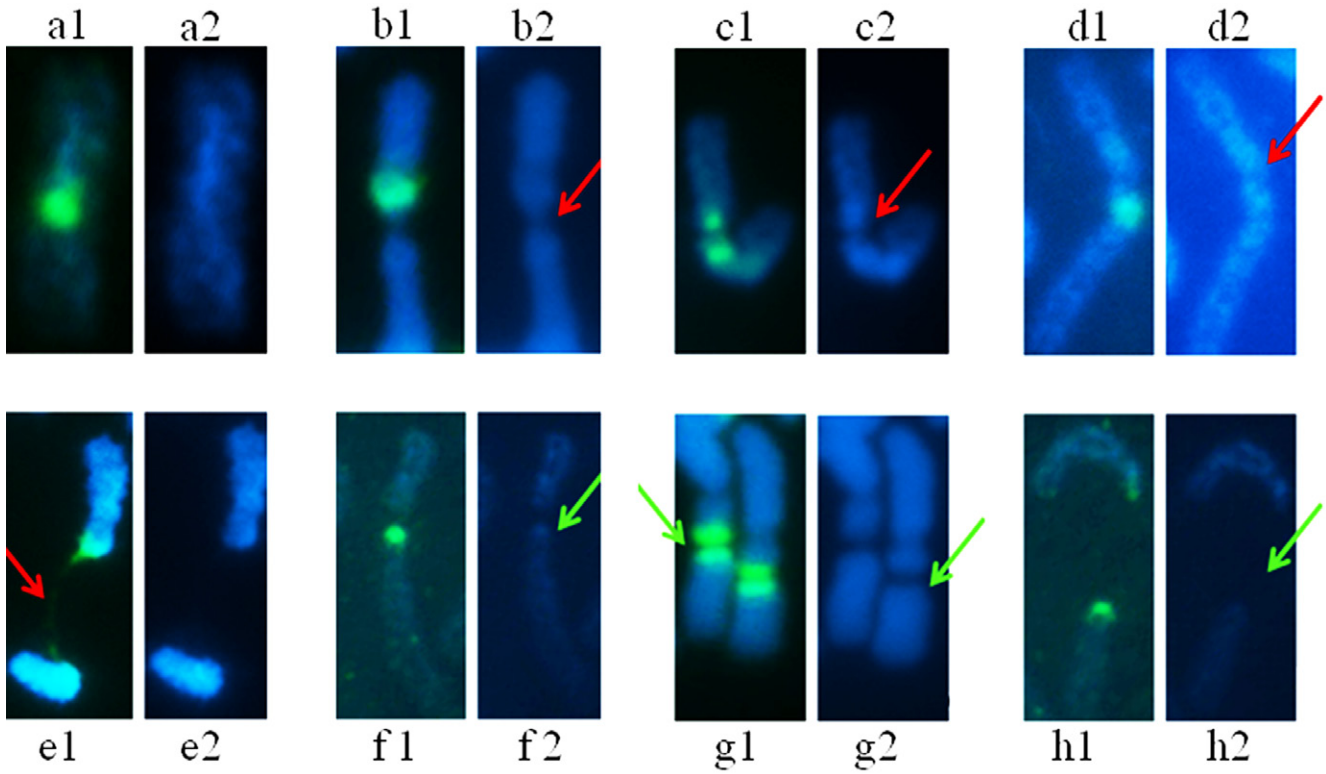

Figure 1. Characteristics of 45S ribosome DNA (rDNA) sites (green) in chromosomes of *L. multiflorum* found using fluorescent in situ hybridization (FISH) and DAPI, respectively. (a1 and a2) Intact chromosome showing one signal. (b1 and b2) Chromosome with a gap showing one signal at 45S site. (c1 and c2) Chromosome with a gap with two signals having the same fluorescence intensity. (d1 and d2) Chromosome with a gap with signals of different intensities. (e1 and e2) Chromatin filament at the chromosome with a gap binding the fragment to the chromosome. (f1 and f2) Chromosome with breaks and one signal of 45S. (g1 and g2) Chromosomes with a break and two signals with the same intensity. (h1 and h2) Chromosome with a break and two signals of different intensities. Red arrows represent gaps and green arrows represent breaks.

disruption or total separation of a chromosome region, thus forming fragments disengaged from the original chromosomes.

The term "signal" was used to identify the fluorescent marker detected by FISH in metaphase, which can be characterized as one signal corresponding to one intact site of rDNA or two signals, if the site of rDNA shows breaks or gaps.

RESULTS

Analysis in the mitotic metaphase with both conventional staining and DAPI showed variation in chromosome number between and within individuals of *L. multiflorum* genotypes (Table 2). Of the 61 metaphases analyzed, 95% showed an increase compared with the expected chromosome number. Using FISH with the 45S rDNA probe, we found that the apparent excess number of chromosomes in

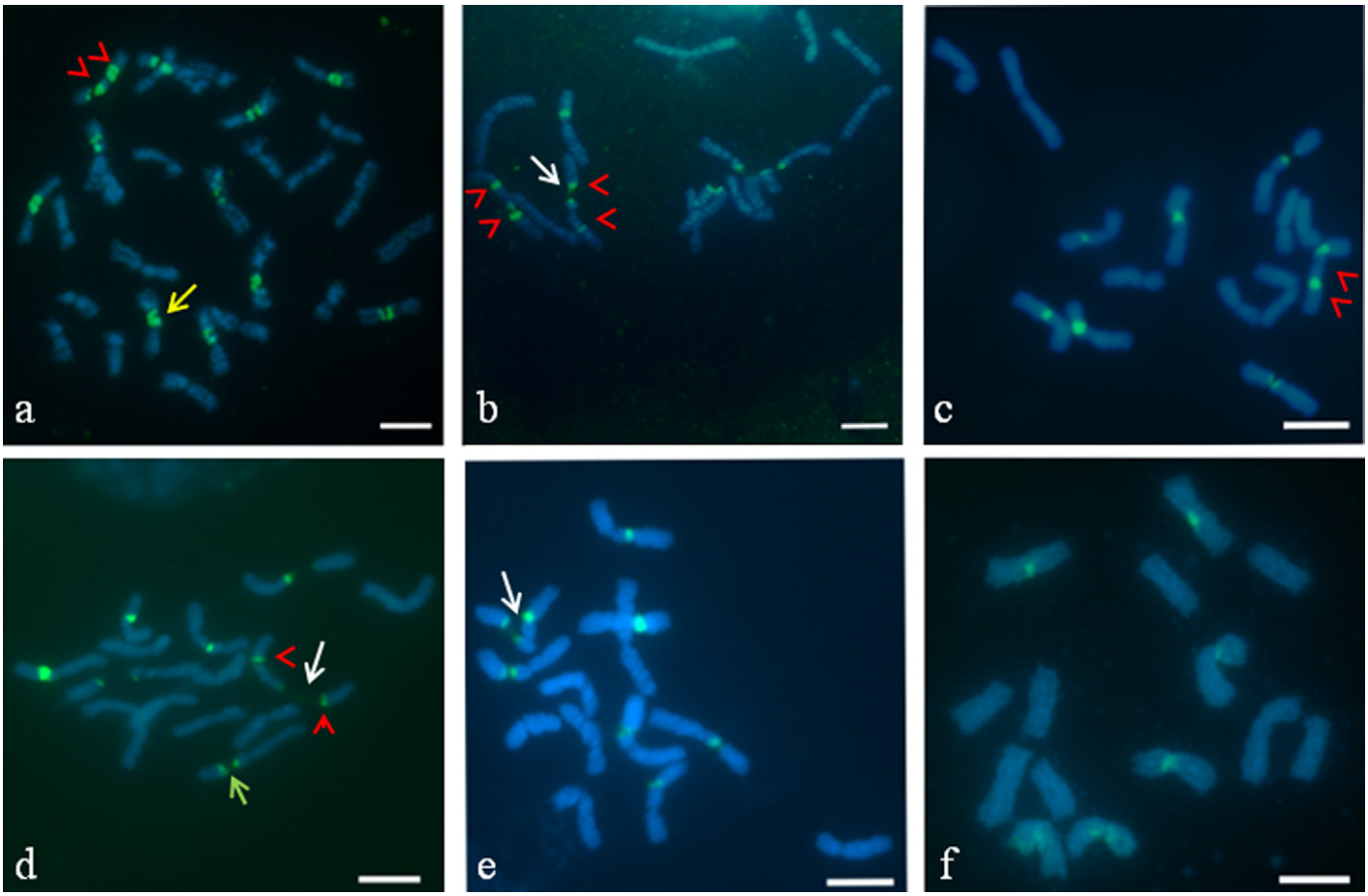

Figure 2. 45S ribosome DNA (rDNA) sites (green) detected with fluorescent in situ hybridization (FISH) in chromosomes of *L. multiflorum* genotypes. (a) Barjumbo-Plant 33: 12 labeled chromosomes, one with two 45S rDNA sites. (b) Comum-Plant 14: six labeled chromosomes, two with two 45S rDNA sites each. (c and d) Comum-plant 2: seven labeled chromosomes, one with two 45S rDNA sites. (e) Comum-plant 2: eight labeled chromosomes. (f) Comum-plant 19: six labeled chromosomes. Arrowheads indicate two 45S rDNA sites labeled in a single chromosome. The white arrow indicates a chromatin filament binding the fragment to the chromosome. The yellow arrow indicates a chromosome with a gap. The green arrow indicates a chromosome with a break. Bars 5 μm.

the genotypes results from the formation of fragments due to chromosomal breaks or gaps at the 45S rDNA site, which also had a variable number of signals (Fig. 1; Table 2).

The rDNA sites were analyzed in three situations: in intact chromosomes (Fig. 1a), in chromosomes with gaps (Fig. 1b–1e), and in chromosomes with breaks (Fig. 1f–1h). Intact chromosomes showed only one proximal sign of 45S rDNA (Fig. 1a). Chromosomes with gaps had signals occurring in either side (Fig. 1b) or both sides of the gap (Fig. 1c and 1d), with variation in size and fluorescence intensity of signals separated by the gap (Fig. 1d). In some cases, a thin chromatin fiber linked the fragment to the chromosome with the gap (Fig. 1e). Chromosomes with breaks showed signals at either end of chromosomes or fragments or both (Fig. 1f–1h), also showing variation in size and fluorescence intensity between the two signals separated by breaks (Fig. 1h). Thus, the chromosomes with gaps keep both parties connected while on chromosomes with breaks, no such connection between the fragment and the chromosome were observed.

The pattern of breaks and gaps was highly heterogeneous with variation even among metaphases in the same meristem. Some metaphases showed all intact chromosomes (Fig. 2f) while others showed breaks and gaps simultaneously at different frequencies (Fig. 2a to 2e).

Two 45S rDNA syntenic sites were found in both intact chromosomes (Fig. 2c) and in chromosomes with breaks or gaps (Fig. 2a, 2b, and 2d). In intact chromosomes, 45S sites were located on different arms of the chromosomes whereas in chromosomes with breaks or gaps, these sites were found in both the chromosome and its severed fragment. Signals detected at syntenic sites showed both equal or different intensities and sizes.

Comparison of silver-stained NORs and 45S rDNA signals showed partial correlation between them in the genotypes evaluated (Fig. 3). Not all 45S rDNA sites identified by FISH were marked by NOR.

Variation in the number of nucleoli in interphase nuclei was detected in all genotypes of *L. multiflorum* (Fig. 4), more often from one to two nucleoli in the Comum and ABARP genotypes, and two to four in the Barjumbo

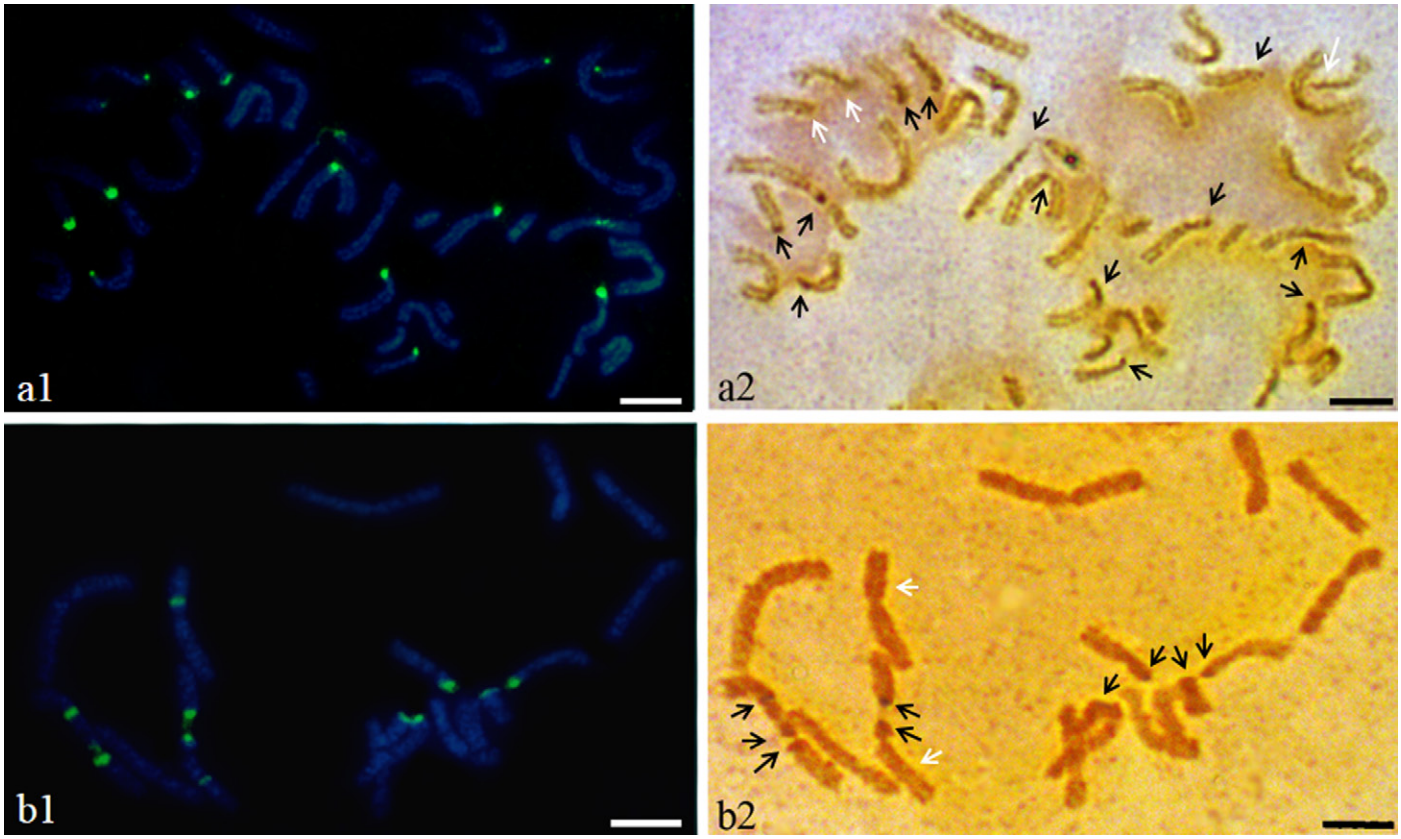

Figure 3. (a1 and b1) Mitotic metaphases showing 45S ribosome DNA (rDNA) sites (green) using fluorescent in situ hybridization (FISH). (a2 and b2) Ag-nucleolar organizer regions (NOR) banding. In a2 and b2, the tips of the black arrows show transcriptionally active sites, while the arrowheads white indicate inactive sites. Bars 5 μm.

genotype. No more than eight nucleoli were found in any genotype (Table 3). Among the seven and eight nucleoli detected in the interphase nuclei of Barjumbo-plant 33, there were micronucleoli, characterized by diameters smaller than 1 μm (Fig. 4g and 4h). In addition to the number variation, we found considerable heteromorphism and nucleolar fusion (Fig. 4).

No variability was found within any genotype of *L. multiflorum* in 5S rDNA sites. Diploid, triploid, and tetraploid genotypes showed two, three, and four sites, respectively. In both diploids and polyploids, all sites were located on the arms opposed to those containing 45S rDNA sites (Fig. 5).

DISCUSSION

As in the present study, Huang et al. (2008) found an apparent excess number of chromosomes in approximately 85% of metaphases in diploid *L. perenne* cultivar Player resulting from fragment formation due to chromosomal breaks or gaps at the 45S rDNA site. These authors also reported occurrence of breaks and gaps at the 45S rDNA site in tetraploid *L. multiflorum* cultivar Top One. Furthermore, these studies confirmed differences in fluorescence intensity of signals located on both sides of breaks by linescan curve analysis of fluorescence. In the present study, it was also possible to observe the difference in fluorescence intensity between signals located on breaks in images using FISH.

In a study of *L. perenne* using atomic force microscopy, Huang et al. (2009) detected one or more fine DNA fibers connecting fragment ends of one or both chromatids, caused by a break at the 45S rDNA fragile site. The authors suggest that failures in the complex folding of chromatin fibers at 45S rDNA fragile sites are responsible for breaks and gaps in vitro in *Lolium*. In our study, the 45S rDNA probe made it possible to visualize the binding of chromosomes to fragments via chromatin strands, which characterized the chromosome with gaps.

In plants, so far, breaks and gaps in 45S rDNA sites were reported only in *Lolium* species and were deemed similar to fragile sites described in human chromosomes (Huang et al., 2008, 2009), which occur with high frequency in lymphocytes, with about 200 regions already mapped (Cimprich, 2003; Debatisse et al., 2012). Fragile sites of human chromosomes are of interest for being targets of DNA damage in precancerous chromosome gaps, suggesting their association with the early stages of carcinogenesis.

Although we cannot rule out other mechanisms involved, breaks in fragile sites appear to be primarily responsible for the variability in number and position of 45S rDNA sites, which have been described for *Lolium* by other authors (Huang et al., 2008, 2009; Lideikyte et al., 2008; Ksiazczyk et al., 2010). For other plant groups, such as *Allium* (Schubert and Wobus, 1985), *Triticeae*

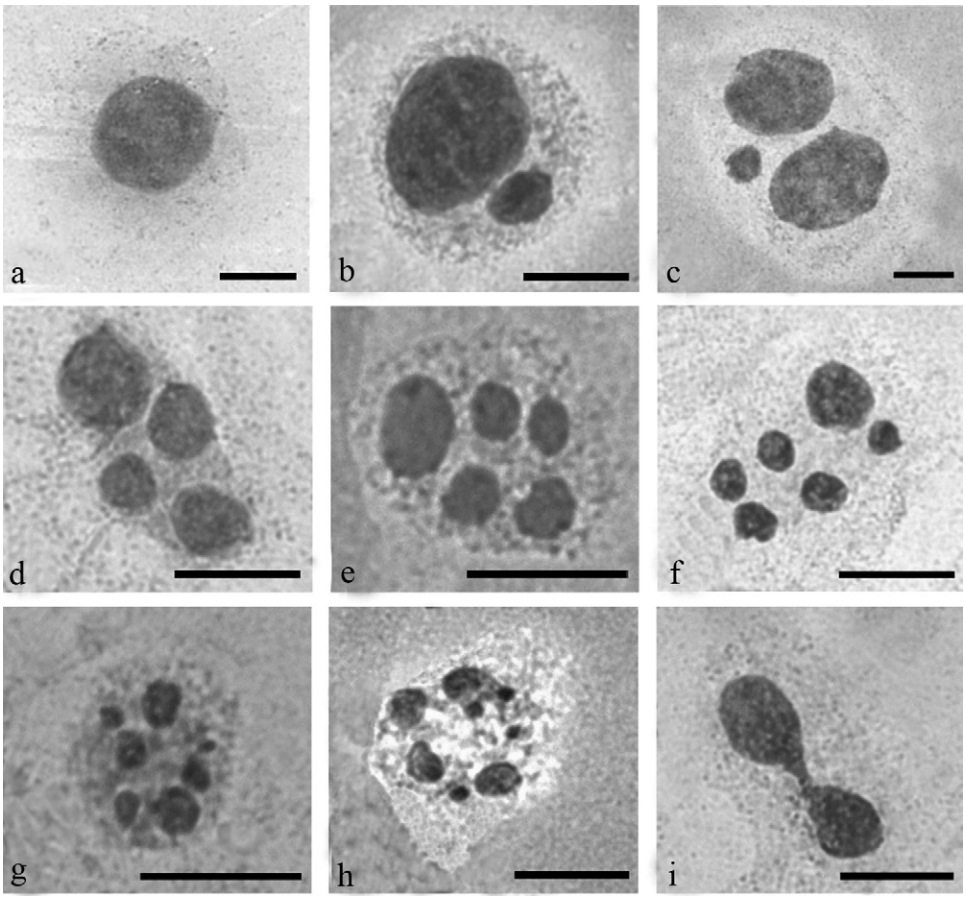

Figure 4. Number of nucleoli in *L. multiflorum.* (a) ABARP-plant 15: 1. (b) Comum-plant 32: 2. (c) Comum-plant 14: 3. (d) Barjumbo-plant 11: 4. (e) Barjumbo-plant 11: 5. (f) Barjumbo-plant 33: 6. (g) Barjumbo-plant 33: 7. (h) Barjumbo-plant 33: 8. (i) Comum-plant 32: Nucleolar fusion. Bars 10 µm.

Table 3. Percentage of interphase nuclei with nucleoli number ranging from one to eight in a total of 150 nuclei evaluated in *L. multiflorum* genotypes.

	Number of nucleoli							
Genotypes	1	2	3	4	5	6	7	8
Comum-plant 14	54.67	32.67	10.67	1.32	0.67			
Comum-plant 32	34.67	45.33	16.67	3.33				
Barjumbo- plant 11	7.33	25.33	28.68	27.33	10.00	1.33		
Barjumbo-plant 33	9.80	24.84	24.18	20.92	13.07	5.23	1.31	0.65
ABARP-plant 15	47.33	35.33	11.33	5.33	0.68			

(Dubcovsky and Dvorak, 1995; Altinkut et al., 2006), *Nemesia* (Datson and Murray, 2006), *Crotalaria* (Morales et al., 2012), *Passiflora* (Cuco et al., 2005), *Aegilops* (Raskina et al., 2004a, 2004b), and *Oryza* (Shishido et al., 2000), variation in the number and position of 45S rDNA blocks has been already reported. However, its occurrence was not associated with fragile sites but to other phenomena such as chromosomal rearrangements, genetic unequal exchange, transposition, and gene conversion.

Occurrence of 45S rDNA syntenic sites in four metaphases of the genotype Comum-plant 2 in this study was also reported in three metaphases of one genotype of *L. rigidum* by Thomas et al. (2001). This characteristic corroborates with the evidence that the 45S rDNA site in *Lolium* shows mobility and repositioning that may contribute to chromosome repatterning and are thus indicative of important mechanisms of chromosomal rearrangements.

Similar to results of most diploid genotypes in this study, Thomas et al. (1996) found six 45S rDNA sites in diploid *L. multiflorum*. However, seven and eight 45S sites (Fig. 2c and 2e, respectively) were found in the genotype Comum-plant 2 thus confirming the observations of intense rearrangements at this site and the resulting karyotypic changes in *Lolium*. Also, according to Thomas et al. (1996), twelve and nine 45S sites were expected in tetraploid and triploid genotypes, respectively, similar to the results of our study.

Signal mismatch of 45S rDNA in mitotic metaphases and NORs marked by silver staining in all genotypes confirms that not all sites labeled with FISH show transcriptional activity, since only silver-stained regions had previous gene activity in the interphase (King, 1980; Fel-Clair et al., 1998; Gallagher et al., 1999; Liu and Fredga, 1999).

In the case of chromosomes with breaks in which fragments or chromosomes were labeled with Ag-NOR (Fig. 3a2 and 3b2), probably one portion of the site remained active and the other became inactive. One

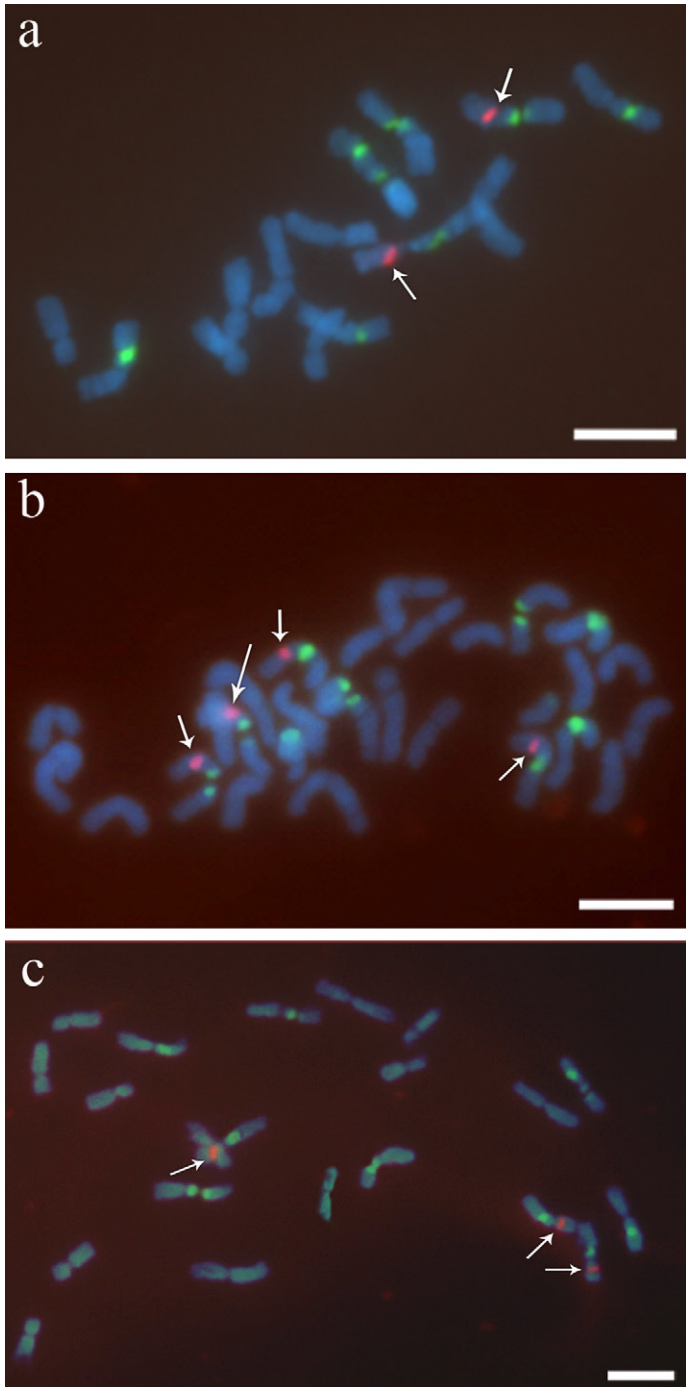

Figure 5. Location of 5S ribosome DNA (rDNA) sites (red) and 45S rDNA sites (green) detected with fluorescent in situ hybridization (FISH) in *L. multiflorum* genotypes. 5S rDNA sites: (a) Comum-plant 2: two sites. (b) Barjumbo-plant 2: four sites. (c) ABARP-plant 15: three sites. Arrows show 5S rDNA sites. Bars 5 μm.

possible explanation is that the absence or silencing of ribosomal genes by epigenetic mechanisms was triggered by the expression of fragile sites. The same rationale may be applied in the situation where only one of the two 45S rDNA sites in the same chromosome was labeled with silver (Fig. 3b2).

Transcriptional activity or inactivity related to the size of the 45S site, major/minor, was reported in oat and *Lysimachia mauritiana* L. (Winterfeld and Röser, 2007; Kono et al., 2011). In *L. perenne,* FISH signals in comparison to Ag-NOR banding in the same metaphase showed strong specific silver-stained signals colocated with breakage or gaps in decondensed rDNA sites. Conversely, intact regions in 45S rDNA sites showed either weak or no silver staining signals (Huang et al., 2012). The authors suggest that the differential susceptibility of 45S rDNA sites to Ag-NOR staining indicate that transcription by RNA polymerase I (Pol I) should prevent condensation of DNA during metaphase, resulting in brittleness of 45S rDNA sites.

Carnide et al. (1986) found six NORs in diploid *L. multiflorum*, where no breaks or gaps were reported, and the number of NORs corresponded to the number of secondary constrictions found in metaphase. Kopecky et al. (2010) also reported the occurrence of secondary constrictions near the telomere region in three chromosome pairs in diploid genotypes of *L. multiflorum*. Thus, in the present study, we expected nine and twelve NORs for triploid and tetraploid genotypes, respectively. However, the maximum number of nucleoli did not match the number of secondary constrictions in mitotic metaphase in *L. multiflorum*, since the maximum numbers found were eight and six nucleoli for Barjumbo-plant 33 and Barjumbo-plant 11 tetraploids, respectively. The other genotypes showed less than six nucleoli (Table 3). Mismatch may be due to spontaneous breaks at 45S rDNA sites in the genotypes, since breaks can interfere with nucleoli organization and sites inactivation. Furthermore, nucleolar fusion contributed to the fewer than expected number of nucleoli found.

Carnide et al. (1986) reported variation in size of NORs in metaphases of *Festuca arundinaceae* Schreb. and Festulolium (*F. arundinacea* x *L. multiflorum*). The size of nucleoli formed in association with a specific NOR is proportional to rRNA gene dosage and to level of gene activity (Warburton et al., 1976; Anastassova-Kristeva et al., 1977). Micronucleoli formation may be associated with rearrangements caused by breaks or gaps in the fragile sites as similarly described for barley, which showed translocations (Schubert and Künzel, 1990). Micronucleoli may indicate reduced transcriptional activity of NORs which underwent some type of rearrangement. The proportion of cells with different numbers of macronucleoli (diameter larger than 9 μm) found in *L. multiflorum* matches the pattern of nucleolar fusion described in barley by Nicoloff et al. (1977) and Schubert and Kunzel (1990).

With respect to 5S sites, Thomas et al. (1996) assessed seven taxa of the genus *Lolium* (*L. temulentum, L. remotum, L. persicum, L. rigidum, and L. rigidum* var. *rottbollioides, L. multiflorum, L. perenne,* and *L. rigidum* var. *rigidum*), identifying two sites for all taxa. Ksiazczyk et al. (2010) reported two 5S rDNA sites in the diploid cultivar Arka of *L. perenne* and four and five sites in the tetraploid cultivar Solen. Lideikyte et al. (2008) also found two 5S sites

in diploid cultivars (Bellem and Adin) of *L. multiflorum*. However, tetraploid cultivars (Catalpa, Lotos, and Meloa) showed three, four, and five sites, respectively.

In our study, 5S rDNA sites were stable in the genotypes, unlike 45S sites where breaks and gaps caused both chromosome fragmentation and occurrence of a greater number of sites than expected. This phenomenon may affect genome organization, probably resulting in new chromosomal rearrangements that will impact evolution, since new species arise through chromosomal rearrangements (Brown and O'Neill, 2010). In addition, fragile sites may generate chromosomal instability and are weak genome regions, which can undergo recombination events and contribute to karyotypic evolution (Ruiz-Herrera and Robinson, 2007).

Given the variability reported in 45S rDNA, the use of this site in evolutionary and taxonomic studies, as well as in construction of physical maps of chromosomes, should be conducted carefully at least for *Lolium*, since the site shows discontinuity in this position and cannot be considered a species-specific marker and cytogenetically stable.

CONCLUSIONS

There is considerable variability in the distribution and number of sites and signals of 45S rDNA, including the presence of more than one site in the same chromosome. In addition, the number of sites found was higher than expected.

The 45S rDNA site corresponds to a region of chromosome fragility, which is prone to breaks and gaps and to generation of chromosomal fragments in genotypes of *L. multiflorum*.

Not all 45S rDNA sites are transcriptionally active. There is high variability in the number and size of nucleoli, as well as occurrence of nucleolar fusion.

In addition, the 5S rDNA site is stable.

Acknowledgments

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

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