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Fluorescent *in situ* hybridization of the ribosomal RNA genes (5S and 35S) in the genus *Lolium*: *Lolium canariense*, the missing link with *Festuca*?

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Abstract

Inda, L.A. & Wolny, E. 2013. Fluorescent *in situ* hybridization of the ribosomal RNA genes (5S and 35S) in the genus *Lolium*: *Lolium canariense*, the missing link with *Festuca*? *Anales Jard. Bot. Madrid* 70(1): 97-102.

Two groups of taxa can be distinguished within the genus *Lolium* L. based on the pollination system, chromosome size, chromosomal location of nrDNA (5S and 35S (18S-5.8S-26S)) and nrDNA phylogeny. The first group includes self-pollinated taxa (*L. temulentum*, *L. persicum* and *L. remotum*), whereas the second group comprises cross-pollinated species (*L. perenne*, *L. multiflorum* and *L. rigidum*). Here we describe that the autogamous species have two 5S sites and four 35S sites in their genome. Two of the 35S sites are present in the chromosomes containing the 5S regions. The allogamous taxa possess two 5S rDNA sites and 6-10 35S sites per genome, depending on the species. Two of these regions (35S) may also be present in the chromosomes bearing 5S sites. Our study also demonstrates that *Lolium canariense* shows a distinctive pattern. It has two 5S and four 35S sites. In this case, the 35S loci are located in different chromosomes than the 5S. This cytogenetic pattern is consistent with that of *Festuca pratensis*. Thus, despite being allogamous, *Lolium canariense* does not entirely fit in either of the groups defined for the genus *Lolium*. The physical mapping of the nrDNA regions in *L. canariense* is different, and resembles that of *F. pratensis*, suggesting that this Macaronesian *Lolium* could be intermediate between *Festuca* and *Lolium*.

Keywords: *Festuca*, *Lolium*, *Lolium canariense*, *Festuca pratensis*, ribosomal DNA, FISH (Fluorescent *in situ* hybridization)

INTRODUCTION

Species of the genus *Lolium* L. (Poaceae; Pooideae) are grasses typically characterized by possessing a unique glume in each spikelet, except for the apical spikelet which has two glumes. This genus, native to Europe, temperate Asia and North Africa (Terrell, 1968) is currently spread across the planet. In his seminal work Terrell (1968) regrouped all the taxa into eight species: *L. perenne* L., *L. multiflorum* Lam., *L. rigidum* Gaud., *L. remotum* Schrank, *L. temulentum* L., *L. persicum* Boiss. & Hoh., *L. subulatum* Vis. (= *L. loliaceum* (Bory & Chaud.) Hand-Mazz.) and *L. canariense* Steud. Other species described subsequently are *L. edwardii* Scholz & Gaisberg, an endemism from El Hierro (Canary Islands) (Scholz & al., 2000) and *L. saxatile* Scholz & Scholz, native from of Fuerteventura and Lanzarote (Canary Islands) (Scholz & Scholz, 2005).

Lolium perenne, *L. multiflorum* L., *L. rigidum* and *L. canariense*, pollinated by the wind, are cross-fertilized species (alloga-

Resumen

Inda, L.A. & Wolny, E. 2013. Hibridación *in situ* con fluorescencia de los genes del ARN ribosómico (5S y 35S) en el género *Lolium*: ¿es *Lolium canariense* el eslabón perdido con *Festuca*? *Anales Jard. Bot. Madrid* 70(1): 97-102 (en inglés).

En trabajos previos se ha descrito que el género *Lolium* L. está formado por dos grupos de taxones basados en el tipo de polinización, tamaño de los cromosomas, localización cromosómica de los loci del ADN ribosómico [5S y 35S (18S-5.8S-26S)] y filogenia molecular basada en secuencias de ADN ribosómico. Los dos grupos son: especies autógamas (*L. temulentum*, *L. persicum* y *L. remotum*) y especies alógamas (*L. perenne*, *L. multiflorum* y *L. rigidum*). Aquí describimos que según la localización cromosómica de los loci ribosómicos, las especies autógamas tienen dos sitios 5S y cuatro sitios 35S; dos de las cuales coinciden en los mismos cromosomas que tienen el locus 5S. Las especies alógamas presentan dos sitios 5S y de seis a 10 sitios 35S dependiendo de la especie; dos de los cuales pueden coincidir con los cromosomas que tienen el locus 5S. Nuestro estudio muestra que *Lolium canariense* presenta dos sitios 5S y cuatro sitios 35S; estas regiones no coinciden en el mismo cromosoma, como sucede con la cercana *Festuca pratensis*. *F. pratensis* tiene dos sitios 5S y 35S en cromosomas diferentes. *L. canariense* no se circunscribe a ninguno de los dos grupos según la localización de sus loci ribosómicos. A pesar de ser una especie descrita como alógama, sus caracteres cromosómicos ribosómicos son diferentes. Por otro lado su parecido con *F. pratensis* parece indicar que tiene una posición intermedia, desde un punto de vista evolutivo, entre *Festuca* y los *Lolium*.

Palabras clave: *Festuca*, *Lolium*, *Lolium canariense*, ADN ribosómico, FISH (Hibridación *in situ* fluorescente).

mous). On the contrary, *Lolium remotum*, *L. temulentum* and *L. persicum* are self-fertilized species (autogamous). All these species are diploid ($2n = 14$), but among the cultivated species (known as English and Italian ryegrass, *L. perenne* and *L. multiflorum*) tetraploid can be found as well.

Thus, the genus *Lolium* can be divided into two groups based on the reproductive system (cross-pollinated and self-pollinated, Essad, 1954). The autogamous species have smaller chromosomes with a lower genome size than the allogamous species (Thomas, 1937; Naylor & Rees, 1958; Rees & Jones, 1967). Within the autogamous group, species can be distinguished based on their morphology (Kloot, 1983) and biochemical profiles (Bulinska-Radomska & Lester, 1985). Even so, discriminating between species within the allogamous group is not easy, neither morphologically (Vasek & Ferguson, 1963; Kloot, 1983) nor biochemically (Bulinska-Radomska & Lester, 1985, 1988). Some authors have therefore concluded that cross-pollinated species should not be

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Table 1. List of species studied, C-value (pg), geographic origin and collection of caryopses, chromosome number and number of ribosomal DNA signals detected.

Species	1 C-value (pg)	Origin	N. collection	Collection	2n	Signal rDNA	
						5S	35S
Autogamous species							
<i>L. temulentum</i>	2.86	Germany	PI 477121	USDA-USA	14	2	4
		India	PI 302664	USDA-USA	14	2	4
		Turkey	PI 545635	USDA-USA	14	2	4
<i>L. persicum</i>	3.18	Russia	PI 314446	USDA-USA	14	2	4
		Turkey	PI 545661	USDA-USA	14	2	4
<i>L. remotum</i>	3.03	Russia	PI 283613	USDA-USA	14	2	4
Allogamous species							
<i>L. multiflorum</i>	2.72	Europe	ABY- 2511	Aberystwyth Uni.-UK	14	2	6
		Egypt	PI 343155	USDA-USA	14	2	6
		Iran	PI 239804	USDA-USA	14	2	6
<i>L. perenne</i>	2.76	Europe	ABY-13866	Aberystwyth Uni.-UK	14	2	6
		Wales	PI 619001	USDA-USA	14	2	6
<i>L. rigidum</i>	2.75	Commercial			28	4	12
		Turkey	PI 545604	USDA-USA	14	2	8
<i>L. canariense</i>	2.13	Spain		J. Aibar-EPS Huesca	14	2	10
		Canary	PI 320544	USDA-USA	14	2	4
<i>F. pratensis</i>	3.23		ABY- 1593	Aberystwyth Uni.-UK	14	2	2

considered distinct species (Essad, 1954; Naylor, 1960; Bulinska-Radomska & Lester, 1985). The comparison of ribosomal DNA loci 18S-5.8S-26S- (hereinafter 35S) using fluorescence *in situ* hybridization carried out by Thomas et al. (1996) showed that the chromosomes of the autogamous species all had four sites (two *loci*) while the number of sites in the allogamous species varied depending on the species. All species showed two 5S sites (one *locus*).

More recent work based on the quantification of phenological characters did also separate *Lolium* into two groups, segregating again the autogamous and the allogamous species (Loos, 1993; Bennett & al., 2000 and Mirjalili & al., 2008). Furthermore, an analysis of the sequences of several nuclear (ITS) and plastid (*trnT-L*, *trnL-F*) regions of the species that make up the genus (Inda & al., unpublished) shows a clear divergence between the two groups of *Lolium*. However, *L. canariense* either appears separated from the two previous groups (allogamous and autogamous) or within the allogamous *Lolium* group.

In this paper we present the physical mapping of the two ribosomal regions studied (5S and 35S) in the chromosomes of seven *Lolium* species, and determine the cytogenetic relationship between *L. canariense* and other species of the genus. Previous studies had determined that the closest relative to *Lolium* were some members of the genus *Festuca* (Inda & al. 2008). Therefore, we also use this study to explore the putative relationships between the genus *Lolium* and *Festuca* L subgenus *Schedonorus* (P. Beauv.) Peterm. by comparing the relative position of the regions analyzed.

MATERIAL AND METHODS

The seeds used in the study were obtained from three different seed banks, the origin of their accessions is listed elsewhere (Table 1). The microscopic preparations were obtained following the protocol described by Hasterok & al.

(2006). Briefly, the seeds without paleas and lemmas were germinated on filter paper moistened with water for 4 days at 20-22 °C in darkness. Once germinated, the seeds were immersed in ice cold water for 24 hours. They were then fixed in a mixture of methanol / glacial acetic acid 3:1 (v / v) for 4 hours at room temperature and stored at -20 °C until use. The root tips were cut (1 cm) and washed several times with 0.01 M citrate buffer (pH 4.8). The cell wall was digested by incubating the samples in an enzyme mixture containing pectinase (Sigma P5146) 4% (v / v), cellulase (Calbiochem 21947) 1% (w / v) and cellulase (16419.02 Serva) 1% (w / v) for 2 hours at 37 °C. The meristem tip was removed from the rootlet in a solution of 45% acetic acid using a binocular microscope and two mounted needles. The meristem was then positioned between a slide and coverslip. The sample was fixed on the slide by freezing in dry ice. Chromosomes were located using a phase contrast microscope. The sample was fixed on the slide by freezing on dry ice. The detection of the two regions of the rDNA was performed using probes described as follows: i) the 5S region was obtained from a wheat pTa794 clone (Gerlach & Dyer, 1980) amplified by PCR and labelled with tetramethyl rhodamine-5-dUTP (Roche, USA); and ii) the region 35S region (including 18S-5.8S-26S) clone was obtained from an *Arabidopsis thaliana* clone (Jenkins & Hasterok, 2007) amplified and labelled with digoxigenin-11-dUTP using "nick translation" (Roche, USA).

The protocol followed to perform the *in situ* hybridization is described by Jenkins and Hasterok (2007). The conditions were as follows: samples were pre-treated to remove possible traces of RNA with RNase A (Sigma, R5503). They were then fixed with formaldehyde (1.1%) and dehydrated through an ethanol series (70%, 90%, 100%) at room temperature. The hybridization mixture (40 ml) with the labelled probes was denatured at 75 °C for 10 minutes and deposited on the chromosome preparation on a slide. The samples were denatured

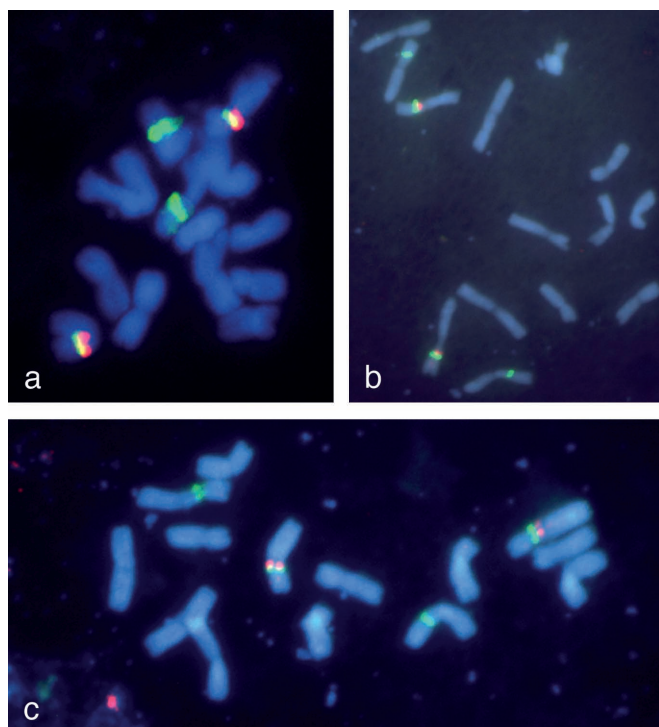


Fig. 1. FISH: 5S regions in red and 35S regions in green. *Lolium temulentum* ($2n = 14$): **a**, sample from Germany.; **b**, sample from Turkey; **c**, sample from India.

on a hot plate at 72 °C for 4.5 minutes, after which the hybridization was performed on slides overnight at 37 °C in a humid chamber. The preparations were washed to remove unbound labelled DNA with citrate buffer, sodium chloride and 10% formamide. For immunodetection of DNA and subsequent labelling with digoxigenin, the samples were first blocked with skimmed milk. Digoxigenin was then detected with anti-digoxigenin antibody labelled with fluorescein (FITC; Roche, USA) by incubating at 37 °C for one hour and a half. The samples were washed with citrate buffer, sodium chloride and Tween 20, dehydrated with ethanol (70, 90, 100%) and allowed to dry. The preparations were mounted with a drop of Vectashield / DAPI (Vector Laboratories, H-1000) and visualized using a fluorescence microscope (Olympus AX70).

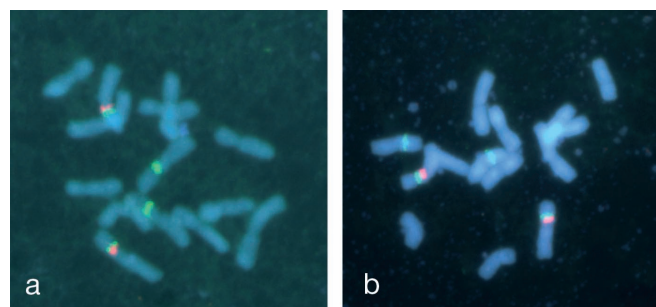


Fig. 2. FISH: 5S regions in red and 35S regions in green. *Lolium persicum* ($2n = 14$): **a**, sample from Russia.; **b**, sample from Turkey.

RESULTS

Comparative physical mapping in the genus Lolium

The microscopic preparations the autogamous species: *L. temulentum*, *L. persicum* and *L. remotum* (Figs. 1-3) revealed that all the samples analyzed were diploid ($2n = 14$) and characterized by the presence of two 5S sites and four 35S sites (Table 1, Figs. 1-3) located in an intercalary position close to the centromere. Regardless of the species and the origin of the samples, neither intraspecific nor interspecific variability was detected in the number and location of *loci* in the chromosomes. Thus, the 5S *locus* and one of the two 35S were seen in the same chromosome (2). The second 35S rDNA *locus* appeared in chromosome 3.

Among the allogamous species, *L. multiflorum*, *L. perenne*, *L. rigidum* and *L. canariense* (Figs. 4-7), all the samples surveyed showed diploid cytotypes ($2n = 14$), with the exception of one sample of commercial origin, *L. perenne*, whose chromosome number was $2n = 28$ (Table 1, Fig. 5c). The FISH pattern of the 5S and 35S rDNA *loci* in the species *L. perenne* and *L. multiflorum*, although unchanged in number with the exception of the tetraploid population of *L. perenne* (Table 1), was more heterogeneous regarding the location of the signals in the chromosomes. Thus, while diploid populations of *L. perenne* and *L. multiflorum* generally have an intercalary 5S rDNA *locus* in chromosome 3 (short arm) and three 35S rDNA *loci* intercalary in 1, 2 and 3 (long arm) chromosomes, in the Welsh population of *L. perenne* one chromosome of the homologous pair of chromosomes 3 (which in other locations has a 35S site and a 5S site) only contained the 5S site (Fig. 5b). The tetraploid sample had twice as many *loci* as the diploid populations: four 5S sites and 12 35S regions.

The number of 35S rDNA *loci* in the two studied populations of *L. rigidum*, as well as the *L. canariense*, differs from that found in earlier species (3 *loci*). Samples of *L. rigidum* from Turkey had four *loci* while those from Spain had five *loci* (Table 1). Both species had the 5S rDNA *loci* together with one of the 35S *loci* in the same chromosome (3). In the case of *L. canariense*, the pattern of 5S and 35S markings was the same as that found in self-pollinated species, although in this

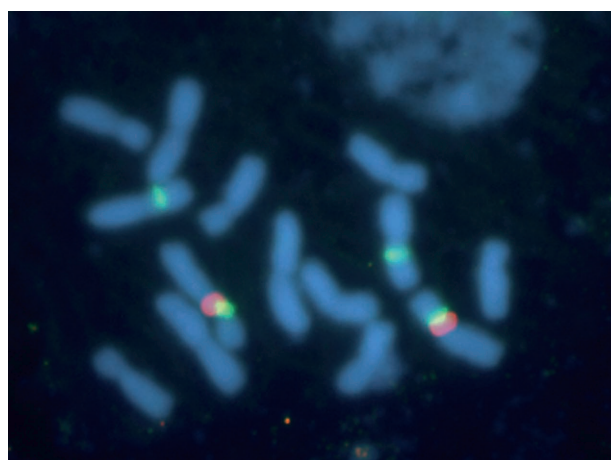


Fig. 3. FISH: 5S regions in red and 35S regions in green. *Lolium remotum* ($2n = 14$). Sample from Russia.

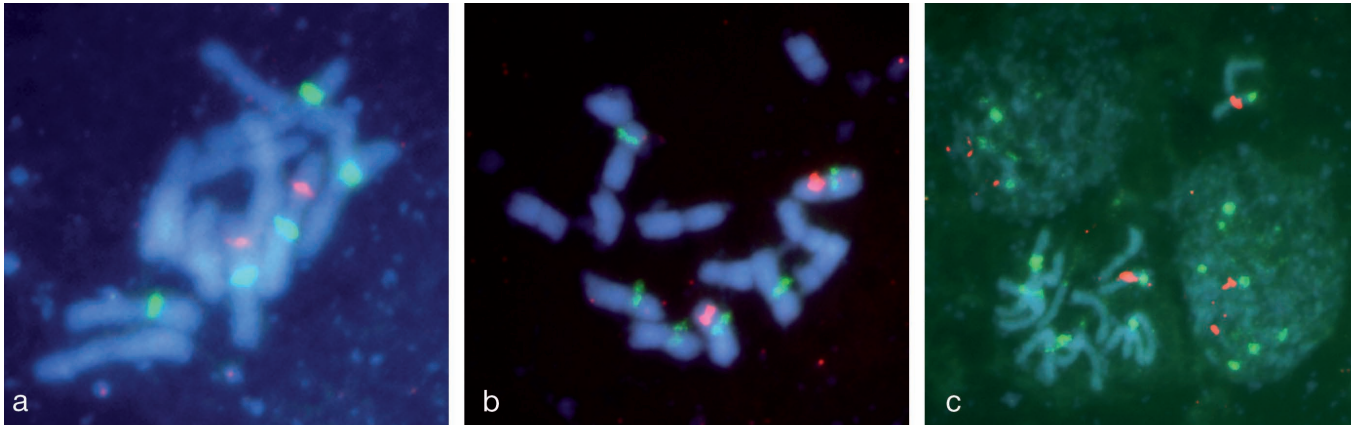


Fig. 4. FISH: 5S regions in red and 35S regions in green. *Lolium multiflorum* ($2n = 14$): **a**, sample from Europe; **b**, sample from Egypt; **c**, sample from Iran.

case none of the 5S and 35S labels failed to match in any chromosome (two 35S loci).

Comparative physical mapping in some species of broad-leaved Festuca species.

The study of the chromosomes from *F. pratensis* ($2n = 14$) (Fig. 8) shows a pattern which differs from that present in most species of the genus *Lolium*, with only one 5S and 35S locus present in different chromosomes. The fescues of sub-genus *Schedonorus* (P. Beauv.) Petrem.: *Festuca arundinacea* var. *atlantigena* (St.-Yves) Auquier ($2n = 8x$) and *F. arundinacea* var. *letournesiana* (St.-Yves) Torrecilla & Catalán ($2n = 10x$) showed eight 5S sites and eight 35S sites in different chromosomes in the first fescue (*F. arundinacea atlantigena*) and ten 5S zones and ten 35S sites in the second fescue (*F. arundinacea letournesiana*).

DISCUSSION

The relationship between the presence of multiple repeated loci and the amount of nuclear DNA (C-value)?

Allogamous species, which have more 35S ribosomal DNA loci, show slightly lower C values (2.7 pg) than autogamous species (2.86 to 3.18 pg) with repeated minor loci. *L. canariense* shows the lowest C value (2.13 pg) and *F. pratensis* the highest C value (3.23 pg), despite having only two 35S copies.

There is no relationship between the C-value and the number of copies of the 35S ribosomal DNA loci (Table 1) in any of these diploid species (Smarda & al., 2008; Bennet & Leith, 2012). No evolutionary trend indicating that an increase in the number of loci is associated to an increase the value of the amount of nuclear DNA has been observed among the diploid species. Likewise, the relationship between the level of polyploidy and the C or 1Cx values is evident among *Festuca* species, e.g. *F. arundinacea ssp. arundinacea* is a hexaploid and has a C-value of 8.49 while *F. gigantea* is hexaploid and has a C-value of 10.38 pg (Bennett & Leitch, 2012).

Phylogenetic relationships among species of Lolium and Festuca using mapping of the ribosomal DNA signals

The morphological characteristics of chromosomes can be used as taxonomic characters, and their study may clarify the relationship between the species. The location of specific chromosome sites (ribosomal marking pattern) has been used for this purpose. Thomas & al. (1996) published a paper in which they studied the position of the 35S and 5S regions in six species of the genus *Lolium*. In the autogamous species (*L. temulentum*, *L. remotum* and *L. persicum*) the 35S region was located in four chromosomes and the 5S appeared in two chromosomes. Each of these (5S) overlapped in a chromosome in which the other region (35S) was located. In the allogamous species (*L. perenne*, *L. multiflorum* and *L. rigidum*),

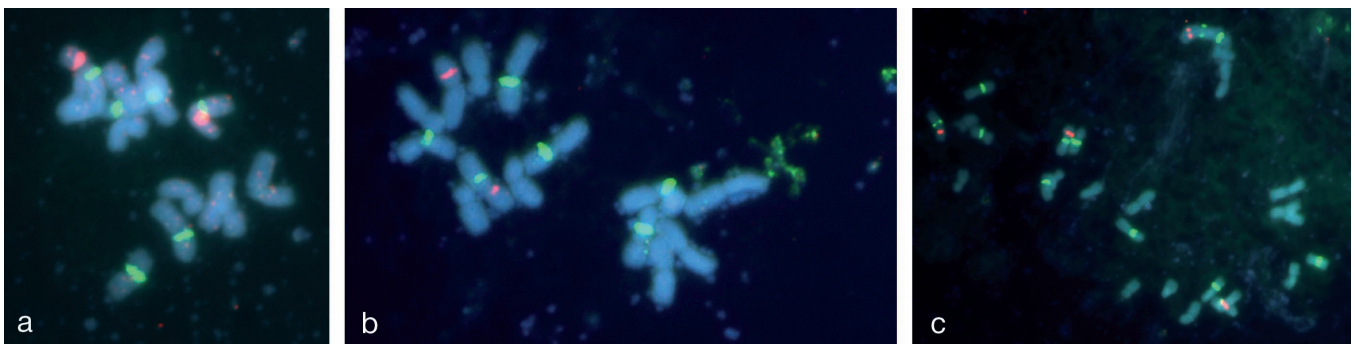


Fig. 5. FISH: 5S regions in red and 35S regions in green. *Lolium perenne* ($2n = 14, 28$): **a**, sample from Wales (UK); **b**, sample from Europe (2x); **c**, commercial sample (4x).

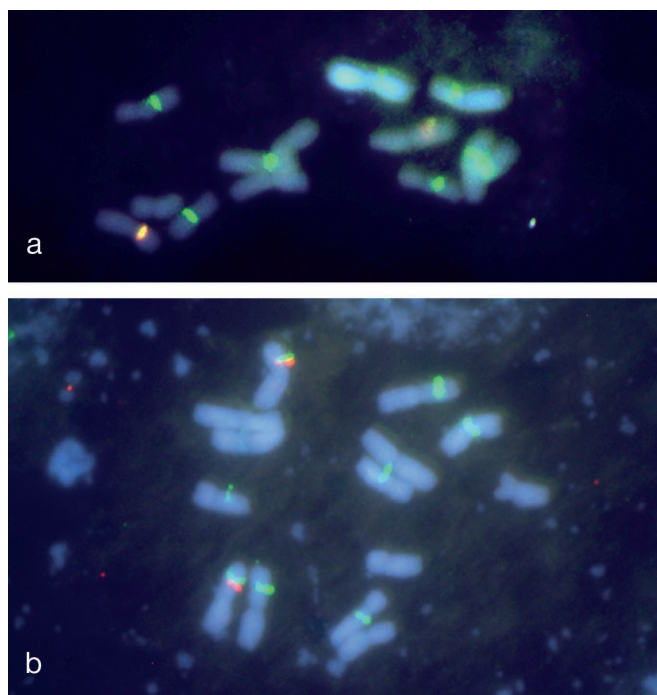


Fig. 6. FISH: 5S regions in red and 35S regions in green. *Lolium rigidum*: **a**, Sample from Spain.; **b**, sample from Turkey.

the 5S region was also located in two chromosomes, but the 35S regions varied with the species (7, 6 and 9 sites). Recently, Lideikyte & al. (2008) showed that within a species the number of loci signals may vary among cultivars. These previous studies did not include the Macaronesian taxon *Lolium canariense*. In recent phylogenetic studies based on nuclear and chloroplast sequences, the segregation into allogamous and autogamous lineages has remained, although *L. canariense* (which is allogamous) has been placed separately from these two groups (allogamous and autogamous) (Inda & al., 2008). Our data reveal that, in general, there is congruence between cytogenetic pattern of ribosomal DNA distribution and previous phylogenetic work, both supporting the segregation of the genus into two main groups according to their fertilization systems. However, *L. canariense*, considered allogamous, has a distribution of 5S and 35S loci (two 5S signals

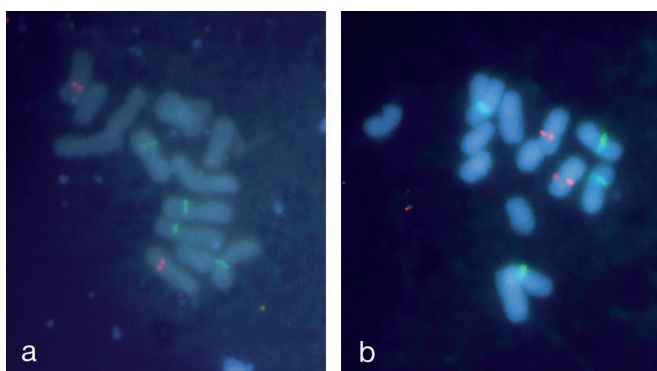


Fig. 7. FISH: 5S regions in red and 35S regions in green. *Lolium canariense* ($2n = 14$). Two different samples from Tenerife (Canary Islands).

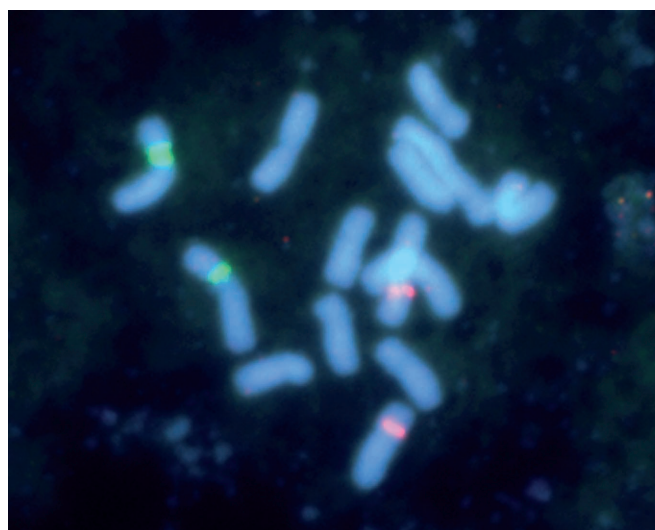


Fig. 8. FISH: 5S regions in red and 35S regions in green. *Festuca pratensis* Huds. ($2n = 14$). Sample from UK (ABY-1593).

and four 35S signals) different from that of other species of the genus, and although the number is similar to that of the autogamous pattern, their distribution in the genome differs from the rest of the genus.

Phylogenetic relationships among the genera *Festuca* and *Lolium* have been studied several times. Jenkin (1933) had already concluded that diploid species of *Festuca* and *Lolium* came from a common “prototype”. Other authors have assumed that *Lolium* had a common ancestor with *F. pratensis* based on its genetic structure (Xu & Sleper, 1994; Charmet & al., 1997; Torrecilla & Catalán, 2002). *F. pratensis* Huds. ($2n = 2x = 14$) separated from the ancestor of *Lolium* 2.8 million years ago, according to an analysis of the nuclear and plastid sequences (Inda & al., 2008). As our results show, *F. pratensis* has two 5S signals and two 35S signals in different chromosomes. This had already been described by Thomas & al. (1997), allowing them to differentiate between the genus *Lolium* and the genus *Festuca* from the presence of the two signals (5S and 35S) in the same chromosome (*Lolium*) or in different chromosomes (*Festuca*). Other species of the same subgenus *Schedonorus* (P. Beauv.) Peterm., such as *F. arundinacea* ssp. *atlantigena* (St.-Yves) Auquier ($2n = 8x$) seem to show eight 5S signals and eight 35S signals in different chromosomes. Similarly, *F. arundinacea* var. *letourneuxiana* (St.-Yves) Torrecilla & Catalán ($2n = 10x$) appears to show 10 5S signals and 10 35S signals in different chromosomes (figures not shown). Both species diverged before *F. pratensis* 5.9 million years ago (Inda & al., 2008) and show 5S and 35S ribosomal DNA signals in different chromosomes. With these results we could hypothesize that the union of the two regions in the same chromosome could have occurred between the appearance of *L. canariense*, which shows a pattern like those found in the genus *Festuca*, and the separation of the allogamous clade and the autogamous clade (about 2 million years ago). This could indicate that the divergence of *L. canariense* preceded the diversification of the other species of the genus *Lolium* into present autogamous and allogamous lineages.

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