Distribution patterns of rDNA loci in the Schedonorus-Lolium complex (Poaceae)

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Abstract

The Schedonorus-Lolium complex of the subtribe Loliinae (Poaceae) includes several economically important forage and turf grasses. This complex encompasses Lolium Linnaeus, 1753, Festuca Linnaeus, 1753 subgenus Schedonorus (P. Beauvois, 1824) Petermann, 1849 and Micropyropsis Romero Zarco et Cabezudo, 1983. New FISH results of 5S and 18S–26S rDNA sequences are presented for three species and the results are interpreted in a review of distribution patterns of 5S and 18S–26S rDNA sequences among other species in the complex. Micropyropsis tuberosa Romero Zarco et Cabezudo, 1983 (2n = 2x = 14) displayed a distribution pattern of rDNA sequences identical to that of F. pratensis Hudson, 1762, supporting a close phylogenetic relationship at the bottom of the phylogenetic tree. “Lolium multiflorum” Lamarck, 1779 accessions sourced from Morocco showed a different pattern from European L. multiflorum and could be a unique and previously uncharacterised taxon. North African Festuca simensis Hochstetter ex A. Richard, 1851 had a marker pattern consistent with allotetraploidy and uniparental loss of one 18S–26S rDNA locus. This allotetraploid has previously been suggested to have originated from a hybrid with Festuca glaucescens (Festuca arundinacea var. glaucescens Boissier, 1844). However, the distribution patterns of the two rDNA sequences in this allotetraploid do not align with F. glaucescens, suggesting that its origin from this species is unlikely. Furthermore, comparisons with other higher allopolyploids in the complex indicate that F. simensis was a potential donor of two sub-genomes of allohexaploid Festuca gigantea (Linnaeus) Villars, 1787. In the overall complex, the proximal locations of both rDNA markers were conserved among the diploid species. Two types of synteny of the two markers could, to a considerable extent, distinguish allo- and autogamous Lolium species. The ancestral parentage of the three Festuca allotetraploids has not yet been determined, but all three appear to have been sub-genome donors to the higher allopolyploids of sub-genus Schedonorus. Terminal locations of both the markers were absent from the diploids but were very frequently observed in the polyploids.
Keywords
Festuca, FISH, karyotype evolution, Lolium, rDNA locus evolution, species diversification

Introduction

Ryegrasses of the genus Lolium Linnaeus, 1753 with ten diploid species and fescues of the genus Festuca Linnaeus, 1753 subgenus Schedonorus (P. Beauvois, 1824) Petermann, 1849 are closely related and, together with Micropyropsis Romero Zarco et Cabezudo, 1983, form the “Schedonorus-Lolium complex”, belonging to the family Poaceae Barnhart, 1895, subtribe Loliinae Dumortier, 1829 (Inda et al. 2013; Cheng et al. 2016). Several of these Lolium and Festuca species, which are native to temperate regions of Europe, Asia and Africa, are widely used for forage and turf purposes in all major temperate regions of the planet. Micropyropsis tuberosa Romero Zarco et Cabezudo, 1983 (Romero Zarco and Cabezudo 1983) is the sole species of the genus and is diploid (Romero Zarco 1988).

Since the last major taxonomic revision of the genus Lolium by Terrell (1968), new species have been discovered and named, notably Lolium saxatile H. Scholz et S. Scholz, 2005 (Scholz and Scholz 2005) and Lolium edwardii H. Scholz, Stierstorfer et van Gaisberg, 2000 (Scholz et al. 2000). Although Festuca has over 500 diploid to dodecaploid species, subgenus Schedonorus is limited to approximately 20 species, most from Europe, W Asia or N Africa. However, the broad-leaved Festuca species from highland tropical Africa, including Festuca simensis Hochstetter ex A. Richard, 1851 have also been shown to be part of the Schedonorus-Lolium complex (Namaganda et al. 2006; Inda et al. 2014; Minaya et al. 2015).

Several molecular genetic analyses involving DNA markers have been successfully carried out for the phylogenetic reconstruction of subtribe Loliinae. It has been shown that the Schedonorus-Lolium complex represents a monophyletic group, with Lolium clearly differentiated from Festuca (Charmet et al. 1997; Gaut et al. 2000; Catalán et al. 2004; Namaganda et al. 2006; Hand et al. 2010; Inda et al. 2014; Minaya et al. 2015; Cheng et al. 2016). Fertile hybrids formed between Lolium and Festuca species show chromosome pairing and recombination but the chromosomes can be distinguished using genomic in situ hybridization (Humphreys et al. 1995).

Karyological differences featuring chromosome number, structure and morphology have long been used to infer the systematic status and the evolutionary history of species divergence. However, in some groups of species conventionally stained chromosome preparations do not clearly delineate structural differences among chromosomes or species karyotypes. Molecular cytogenetic mapping of specific DNA sequences through fluorescence in situ hybridization (FISH) can overcome such problems, and provide enhanced pictures of chromosome architecture, leading to clear karyotype and genome discrimination (Albert et al. 2010; Chester et al. 2010; Xiong and Pires...
Two different families of multicopy and highly conserved ribosomal RNA genes (rDNA), one coding for 5S and the other for 35S rRNA arrays are universally present in plants. Tandemly repeated blocks of these genes are located independently at particular chromosomal sites and provide species-specific markers (Roa and Guerra 2015). Each 35S rDNA unit carries 18S, 5.8S and 26S RNA genes along with two internal transcribed spacers (ITSs) and tandemly repeated blocks of these units form the nucleolar organizer regions (NORs) or secondary constrictions on chromosomes. FISH mapping of 5S and 35S rDNA sequences is widely used to compare the chromosomal structural changes of related species and to infer the karyoevolutionary variations that accompany species diversification (Fukushima et al. 2011; Lan and Albert 2011; Roa and Guerra 2012, 2015; Jang et al. 2013).

Species of the *Schedonorus-Lolium* complex all share $x = 7$ as the base chromosome number and all have very similar biarmed chromosome morphologies and symmetrical karyotypes. Therefore, conventional karyological information is of little value for evaluating evolutionary changes (Malik and Thomas 1966; Namaganda et al. 2006; Kopecký et al. 2010). Molecular cytogenetic mapping of 5S and 35S rDNA has detected variations in the distributional patterns of the two rDNA markers among diploids and polyploids in this complex (Thomas et al. 1996, 1997; Książczyk et al. 2010; Inda and Wolny 2013; Ansari et al. 2016; Ezquerro-López et al. 2017; Shafiee et al. 2020). Based on their report, Ezquerro-López et al. (2017) made a preliminary attempt to decipher the evolutionary relationships among *Festuca* species belonging to this complex.

In this study, we have mapped the chromosomal dispositions of 5S and 18S rDNA loci in five taxa, three of which were previously unmapped, and have discussed the evolutionary implications of the new results. Following this we have drawn together all the available information from disparate sources and have framed a more complete picture of rDNA chromosome patterns within the whole of this economically important complex. This is the first time such information has been integrated across numerous studies.

**Methods**

**Plant materials and chromosome preparations**

Seeds from five populations (Table 1) belonging to the *Schedonorus-Lolium* complex were accessed from the Margot Forde Forage Germplasm Centre at AgResearch Grasslands, Palmerston North and PGG Wrightson Seeds, Christchurch, New Zealand. *Lolium multiflorum* Lamarck, 1779 of Moroccan origin was designated MRCN to distinguish it from *L. multiflorum* material of European origin. Seeds were germinated and grown in a glasshouse. Somatic chromosome preparations were obtained from the meristematic tissue of actively growing root tips according to the flame-drying technique described earlier (Ansari et al. 1999, 2016). Good quality cytological preparations were selected after screening using phase contrast optics.
Fluorescence in situ hybridization (FISH)

The DNA probes used for FISH were pTr18S (GenBank accession number AF071069), a 1.8 kb fragment from Trifolium repens Linnaeus, 1753 containing almost the entire 18S rDNA sequence and pTr5S (GenBank accession number AF072692), a 596 bp DNA fragment encoding the T. repens 5S rRNA. 35S and 5S rDNA probes were directly labelled with fluorochromes Fluor-X-dCTP and Cy-3-dCTP (GE Healthcare, NZ), respectively by nick translation according to manufacturer’s specifications. Double target FISH using the above DNA probes, post-hybridisation washing and counterstaining of somatic chromosomes with DAPI were carried out as described earlier (Ansari et al. 1999). Chromosome preparations were mounted in Vectashield (Vector Laboratories). Fluorescence images were acquired using a Zeiss monochrome MRm CCD camera on a Nikon epifluorescence microscope Microphot-SA and were processed with an ISIS FISH Imaging System (MetaSystems, Germany). At least five good quality early to late metaphase cells from each plant were used for analysing hybridization signals.

Results

Results of double colour FISH mapping using 35S and 5S rDNA sequences as probes on pro-metaphase or metaphase chromosomes of Lolium perenne Linnaeus, 1753 (2n = 2x = 14) are given in Fig. 1. Six 35S rDNA signals representing three loci were located proximally on three pairs of chromosomes (Fig. 1a, b). One locus was on the short arm of one chromosome pair, and the other two displayed hybridization on the long arms of two pairs of chromosomes. One of the chromosome pairs with 35S on the long arm displayed co-localization of the single 5S rDNA locus proximally on the short arm. The chromatin housing 35S rDNA regions, representing GC-rich nucleolus organizer regions (NORs) or secondary constrictions, were frequently decondensed and sometimes stretched in our flame-dried somatic chromosome preparations. These loci are positioned pericentromerically, and the cloudy decondensed and stretched 35S rDNA FISH signals could be observed joining the two condensed parts of NOR-bearing chromosomes (Fig. 1a, b). L. multiflorum (2n = 2x = 14) of north European/Mediterranean origin produced rDNA FISH signals identical to the pattern observed for L. perenne (Fig. 1c, d).

Table 1. List of Schedonorus-Lolium complex taxa used in this study.

| Taxon                              | Identity and source of seed                          |
|------------------------------------|-----------------------------------------------------|
| Festuca simensis Hochstetter ex A. Richard, 1851 | BL 2043, Margot Forde Forage Germplasm Centre       |
| Lolium perenne Linnaeus, 1753      | Cv Impact, Margot Forde Forage Germplasm Centre     |
| Lolium multiflorum Lamarck, 1779   | B 3380, Margot Forde Forage Germplasm Centre        |
| Lolium multiflorum MRCN            | Cv. Barberia, PGG Wrightson Seeds                   |
| Micropyropsis tuberosa Romero Zarco et Cabezudo, 1983 | BZ 8319, Margot Forde Forage Germplasm Centre |

L. multiflorum (2n = 2x = 14) of north European/Mediterranean origin produced rDNA FISH signals identical to the pattern observed for L. perenne (Fig. 1c, d).
Figure 1. DAPI stained (grey scale) metaphase cells in the left column and the same cells in the right column displaying FISH mapping of 5S (red signals) and 35S rDNA sequences (green signals) in a, b L. perenne c, d L. multiflorum, European origin e, f L. multiflorum MRCN Moroccan origin g, h M. tuberosa i, j F. simensis. Dotted lines in a, c, e, g and i denote decondensed 35S rDNA chromatin.
In contrast to *L. perenne* and *L. multiflorum* of north European origin, *L. multiflorum* (*2n = 2x = 14*) of Moroccan origin displayed only two pairs of NORs (Fig. 1e, f), each pair located proximally on the long arm. One of these NOR-bearing chromosome pairs co-localised 5S sequences proximally on the short arm.

*Micropyropsis tuberosa*, 2*n = 2x = 14*, with a symmetrical karyotype, displayed one 5S and one 35S rDNA locus, each on separate chromosome pairs, and located proximally on the short arms (Fig. 1g, h). Co-localization of the two rDNA sequences on the same chromosome was not observed in *M. tuberosa*.

*Festuca simensis*, 2*n = 4x = 28*, displayed all biarmed chromosomes and a symmetrical karyotype. The eight FISH signals were distributed on separate chromosomes (Fig. 1i, j). One of the three pairs of 5S rDNA signals hybridized interstitially on the short arms. Each of the remaining two pairs of 5S signals were located distally in terminal regions, one in the short arm and the other in the long arm of two pairs of chromosomes. The only pair of 35S signals was located proximally on the short arms of a chromosome pair. Again, *F. simensis* did not show co-localization of the two rDNA sequences.

**Discussion**

We have mapped the diversity in the chromosomal locations of the two rDNA sequences for five taxa of the *Schedonorus-Lolium* complex. Three of these, *M. tuberosa*, *L. multiflorum* MRCN and *F. simensis*, were previously unmapped. The results for *L. perenne* and N European *L. multiflorum* agree with previous studies (Thomas et al. 1996; Ansari et al. 2016). The new results are discussed first and then rDNA chromosomal patterns across the complex are reviewed.

*Micropyropsis tuberosa* exhibited single 5S and 35S rDNA loci positioned proximally on separate chromosomes as was also the case for *F. pratensis* (Thomas et al. 1997). In phylogenetic reconstructions within the *Schedonorus-Lolium* complex based on ITS and plastid DNA sequences, the divergence of *M. tuberosa* preceded the basal split between the diploid lineages of *Festuca* and *Lolium* (Torrecilla and Catalán 2002; Catalán et al. 2004; Inda et al. 2008, 2014; Šmarda et al. 2008). The similar arrangement of single 5S and 35S rDNA loci in *M. tuberosa* and *F. pratensis* is consistent with the interpretation that this was the ancestral diploid *Schedonorus* arrangement before the *Lolium* split.

The “*L. multiflorum*” of Moroccan origin is typical of the main *Lolium* lineage in having more than one 35S rDNA locus. One of these 35S loci has a syntenic 5S locus on the opposite chromosome arm, in common with *L. perenne* and *L. multiflorum* of Eurasian origin. However, compared with Eurasian *L. multiflorum* the Moroccan taxon has one fewer 35S locus. The Moroccan “*L. multiflorum*” could be a new and unique N African taxon that has chromosomal affinities with the allogamous Eurasian *Lolium* species.

A previous cytological analysis of the tropical African broad-leaved fescue, *F. simensis*, showed it to be tetraploid (*2n = 4x = 28*) and AFLP fingerprinting revealed a close
Distribution patterns of rDNA loci in the *Schedonorus-Lolium* complex

phylogenetic relationship with European broad-leaved fescues, especially with hexaploid *F. gigantea*, (Namaganda et al. 2006). Nuclear and plastid DNA sequence studies also placed *F. simensis* in the *Schedonorus-Lolium* complex, close to *Lolium* (Inda et al. 2014). In this first molecular cytogenetics analysis of *F. simensis*, we have confirmed the tetraploidy, revealed a symmetrical biarmed karyotype and a distributional pattern of the two rDNA sequences consistent with allopolyploidy (Figs 1 and 2). In addition to two terminal 5S loci, on separate chromosomes, an interstitial 5S locus was observed on the short arm of a separate chromosome, a new location for this group of fescues. None of these 5S positions was consistent with the suggested close relationship with *Lolium*. On the other hand, the 35S rDNA locus was positioned proximally and could represent a link with a common ancestor to *Lolium*. Only one 35S locus was encountered in this allotetraploid, indicating uniparental loss during diploidisation. There are numerous examples of uniparental loss of 35S loci occurring in other allopolyploids (Ansari et al. 1999; Kotseruba et al. 2003, 2010; Williams et al. 2012; Kolano et al. 2016).

Based on a low-copy nuclear gene analysis, Minaya et al. (2015) suggested a Mediterranean origin of Afromontane *F. simensis* through hybridization between a diploid *F. glaucescens* and a *Lolium*-like diploid species. However, none of the distribution patterns of the two rDNA sequences in this allotetraploid align with *F. glaucescens* (*Festuca arundinacea* var. *glaucescens* Boissier, 1844). Instead, the distribution patterns are consistent with the possible involvement of *F. simensis* in the formation of 6x *F. gigantea* (Linnaeus) Villars, 1787. *Festuca pratensis* Hudson, 1762 is a putative diploid sub-genome donor of allohexaploid *F. gigantea* (Hand et al. 2010), but the sources of the other subgenomes remain unknown. We have noted a close similarity between the 5S and 35S patterns of allotetraploid *F. simensis* (present results) and *F. gigantea* (Thomas et al. 1997, Fig. 2). These species also show a close phylogenetic proximity based on DNA sequences (Namaganda et al. 2006; Inda et al. 2014). Hence, we infer that allotetraploid *F. simensis* could be a potential donor of the remaining two sub-genomes of allohexaploid *F. gigantea* (Fig. 2).

**rDNA locus patterns across the diploid *Schedonorus-Lolium* taxa**

All *Lolium* species, along with *M. tuberosa* and *F. pratensis* are natural diploids. The *Lolium* species, are evolutionarily more recent than the *Festuca* species based on DNA sequence phylogenies (Gaut et al. 2000; Catalan et al. 2004; Inda et al. 2014). All *Lolium* taxa studied so far, comprising eight of the ten extant species, displayed exclusively proximal chromosomal locations of both 5S and 35S rDNA sequences (Fig. 2). After the divergence from *Festuca*, the *Lolium* lineage invariably conserved the proximal locations of both the rDNA loci, but changes in the numbers and syntenic status of these loci apparently occurred later. The proximal localization of 5S rDNA in these diploids matches well with the general distribution pattern of this locus among angiosperms but contrasts with most Poaceae (Roa and Guerra 2015). The proximal mapping of 35S loci contrasts with more terminal localizations in the majority of angiosperms, including Poaceae (Roa and Guerra 2012; Garcia et al. 2017).
Figure 2. Schematic representation of the putative evolutionary lineages for chromosomes carrying 5S and 35S rDNA loci in the Schedonorus-Lolium complex. The numbers of marker and non-marker chromosomes are given inside the boxes. Red and black double circles represent 5S and 35S rDNA loci, respectively. *species in solid boxes were investigated during the present study; †synonym for L. rigidum var rotbloioides; ‡ synonym for F. arundinacea subsp. fenas (Lagasca y Segura) Bornmüller, 1928 (Ezquerro-López et al. 2017).
A single 5S rDNA locus (two FISH signals per cell) consistently occurred in all *Lolium* species. The number of 35S loci displayed has previously been noted as a distinguishing feature between *F. pratensis* (one locus) and *Lolium* species (more than one locus) (Thomas et al. 1996; Inda and Wolny 2013). All the *Lolium* taxa displayed increases in the number of 35S loci ranging from 2 to 5 (Fig. 2). Accordingly, there are two loci in *L. multiflorum* (Moroccan origin), *L. persicum* Boissier et Hohenacker, 1854, *L. temulentum* Linnaeus, 1753, *L. remotum* Schrank, 1789, *L. rigidum* var. *rottboellioideus* Heldreich ex Boissier, 1884 and *L. canariense* Steudel, 1855, three in *L. perenne* and *L. multiflorum* (European origin) to four or five in *L. rigidum* Gaudin, 1811. These results were consistent with those of angiosperms in general, where numbers of 5S sites vary considerably less than 35S sites (Lan and Albert 2011; Garcia et al. 2017).

The two types of rDNA loci can be located on the same chromosome (syntenic) or on separate chromosomes (non-syntenic) (Morales et al. 2012; Barros e Silva et al. 2013; Olanj et al. 2015). The Macaronesian *Lolium* species, *L. canariense*, has no synteny of 5S and 35S loci (Inda and Wolny 2013). However, the remaining *Lolium* taxa (including both geographical races of *L. multiflorum*) have synteny (Fig. 2). The syntenic patterns can be differentiated into two groups. In one (allogamous) group, the two types of rDNA sequences were located proximally on either side of the centromere of the same chromosome, as represented by *L. perenne* and both geographical forms of *L. multiflorum*. In the other (largely autogamous) group, represented by *L. persicum*, *L. temulentum*, *L. remotum*, and subspecies and races of *L. rigidum*, both types of rDNA sequences were adjacent on the same chromosome arm, with 35S always distal to 5S. *L. canariense* shows the diploid Micropyropsis-*F. pratensis* arrangement with proximally located 5S and 35S rDNA loci on separate chromosomes as well as an additional pair of 35S loci (a *Lolium* characteristic, Fig. 2). On this basis, Inda and Wolny (2013) have suggested that *L. canariense* could be the link between the *Festuca* and *Lolium* lineages.

**rDNA locus patterns among the polyploid *Festuca* species**

The data presented in Fig. 2, based on the present investigation as well as earlier reports and analyses of DNA sequences (Thomas et al. 1997; Hand et al. 2010; Inda et al. 2014; Minaya et al. 2015; Ezquerro-López et al. 2017), summarise the patterns among polyploid species in subgenus *Schedonorus*. All the species are allopolyploid (Cao et al. 2000; Hand et al. 2010; Inda et al. 2014; Minaya et al. 2015; Ezquerro-López et al. 2017) and show no changes in the basic chromosome number (*x* = 7) and no apparent changes in the ancestral karyotype.

The numbers of 5S loci range from two in the tetraploids, *F. mairei* St. Yves, 1922 and *F. glaucescens* to eight in decaploid *F. letourneuxiana* (*Festuca arundinacea* var. *letourneuxiana* (St. Yves) Torrecilla et Catalán, 2002) while 35S numbers ranged from one in tetraploid *F. simensis* to six in *F. letourneuxiana* (Fig. 2). Localisation of two 35S loci on the same chromosome, as in the tetraploids *F. mairei* and *F. glaucescens* (Thomas et al. 1997) is not frequently encountered in plants.
Seven of the eight *Festuca* polyploids had the 5S rDNA loci in the proximal region, either exclusively or in addition to other regions (Fig. 2). Terminal 5S loci were encountered in only three polyploid species and an interstitial 5S locus was found only in *F. simensis* (present study). In contrast, terminal 35S loci were more frequent. Five species mapped at least one 35S locus in the terminal region while four displayed exclusively terminal 35S loci (Fig. 2). Among these were tetraploids either with terminal 35S loci on each arm of one chromosome (*F. glaucescens*) or two 35S loci adjacent to each other on the same arm (*F. mairei*) (Fig. 2; Thomas et al. 1997). Three polyploids displayed exclusively proximal 35S hybridization signals including tetraploid *F. simensis* with only one 35S locus. The higher frequency of terminal 35S loci among the *Festuca* polyploids aligns well with the majority of angiosperms (Roa and Guerra 2012; García et al. 2017). None of the *Festuca* species in the *Schedonorus-Lolium* complex studied so far have a syntenic arrangement of 5S and 35S rDNA loci, except for hexaploid *F. corsica* Salm-Reifferscheid-Dyck, 1840 which displayed synteny only in heteromorphic form (Ezquerro-López et al. 2017).

Two allotetraploids, *F. mairei* and *F. glaucescens* have been suggested as the ancestral parents of allo-octoploid *F. atlantigena* (*Festuca arundinacea* subsp. *atlantigena* (St. Yves) Auquier, 1976) based on the formation of fertile interspecific hybrids between the two suggested ancestral parental species (Chandrasekharan and Thomas 1971) and FISH mapping of the two marker loci (Ezquerro-López et al. 2017). Six proximal 5S loci in the octoploid would reflect locus additivity from the ancestral parents while the elimination of one 35S locus may reflect genomic diploidisation. The ancestral parents of decaploid *F. letourneuxiana* could not be narrowed down by FISH mapping (Ezquerro-López et al. 2017). The allohexaploid species continental *F. arundinacea* Schreber, 1771 and *F. corsica* are hypothesised to share the same ancestral parents, viz., diploid *F. pratensis* and allotetraploid *F. glaucescens* (Humphreys et al. 1995; Thomas et al. 1997; Ezquerro-López et al. 2017; Fig. 2). Two distribution patterns of 5S and 35S rDNA sequences were observed in these allohexaploids, with differential losses of 35S loci and transpositions of both 5S and 35S loci. The display of two different trajectories of speciation in allopolyploids sharing the same lower-ploid ancestors has been proposed in other angiosperms (Bao et al. 2010; Weiss-Schneeweiss et al. 2012).

All four *Festuca* higher polyploids with putative parents reveal additivity of numbers of 5S loci, but, in three cases, losses of 35S loci, (Fig. 2). Diploidisation of polyploids may lead to the evolutionary loss of repetitive sequences and duplicate copies of genes (Renny-Byfield et al. 2013). Older polyploids often, but not always, show losses of copies of 35S rDNA genes and, in allotetraploids, uniparental losses of 35S loci are common (Leitch et al. 2008; Pellicer et al. 2010; Roa and Guerra 2012; Weiss-Schneeweiss et al. 2013; García et al. 2017). Although there were positional shifts involving both 5S and 35S types, the results were consistent with the general observation for angiosperms that 5S loci are less variable than 35S loci (Lan and Albert 2011; Garcia et al. 2017).

The three allotetraploids (*F. simensis*, *F. mairei* and *F. glaucescens*), as the putative sub-genome donors to the allohexaploid and octoploid species, provide a novel exam-
ple of sequential allopolyploidisation. The putative progenitors of all three allotetraploids remain unknown. However, nuclear and chloroplast DNA sequence analyses (Hand et al. 2010), supported by FISH mapping (Thomas et al. 1997) indicate that a diploid sub-genome is shared between \textit{F. mairei} and \textit{F. glaucescens}. The tetraploid species that became the sub-genome donors for higher ploidy fescues had terminal 5S and 35S loci that were largely conserved in the derivative species (Fig. 2). Among the \textit{Schedonorus-Lolium} complex diploids studied so far, none have shown terminal localization of either marker, and neither were their DNA sequences consistent with them having been progenitors of these tetraploids (Hand et al. 2010). Harper et al. (2004) speculated on the basis of molecular cytogenetic findings, that diploid \textit{F. scariosa} Lagasca y Segura ex Willkomm, 1861, belonging to the sub-genus \textit{Scariosae} outside the \textit{Schedonorus-Lolium} complex, was a potential ancestral parent for allotetraploid \textit{F. mairei}. The likelihood of involvement of diploid sub-genome donor species from outside the \textit{Schedonorus-Lolium} complex should be further explored using molecular and cytogenetic methods, including genomic \textit{in situ} hybridization.

The variations in numbers of 35S sites in \textit{Lolium} and the post-polyploidisation changes in the \textit{Festuca} species have apparently occurred without any obvious changes in the symmetrical bi-armed karyotype that is a consistent feature of the \textit{Schedonorus-Lolium} complex. Such lability in the absence of obvious structural changes might be attributable to paracentric chromosome rearrangements and/or the activity of transposable elements (Datson and Murray 2006; Raskina et al. 2008; Lan and Albert 2011; Barros e Silva et al. 2013; Weiss-Schneeweiss et al. 2013; Kolano et al. 2015).

**Conclusion**

This report has extended the distributional data on the rDNA sequences to seven of the ten known \textit{Lolium} species and has added \textit{F. simensis} to the list of seven polyploid fescue species already characterised. It has also explored the distribution patterns of rDNA loci within the \textit{Schedonorus-Lolium} complex and considers some possible evolutionary trends. While these patterns can be used to deduce relationships among the higher polyploid \textit{Festuca} species, the diploid progenitors of the allotetraploid species remain unidentified and enigmatic.

**Author contributions**

HAA designed the study with AVS and WMW. HAA performed the experiment, analysed the data and wrote the manuscript with co-writing from WMW. NWE isolated the DNA and labelled all the probes for FISH. AVS and NWE provided significant help in improving the manuscript. All authors read and approved the final manuscript.
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References

Albert PS, Gao Z, Danilova TV, Birchler JA (2010) Diversity of chromosomal karyotypes in maize and its relatives. Cytogenetic and Genome Research 129: 6–16. https://doi.org/10.1159/000314342

Ansari HA, Ellison NW, Bassett SA, Hussain SW, Bryan GT, Williams WM (2016) Fluorescence chromosome banding and FISH mapping in perennial ryegrass, Lolium perenne L. BMC Genomics 17: e977. https://doi.org/10.1186/s12864-016-3231-z

Ansari HA, Ellison NW, Reader SM, Badaeva ED, Friebe B, Miller TE, Williams WM (1999) Molecular cytogenetic organization of 5S and 18S–26S rDNA loci in white clover (Trifolium repens L.) and related species. Annals of Botany 83: 199–206. https://doi.org/10.1006/anbo.1998.0806

Bao Y, Wendel JF, Ge S (2010) Multiple patterns of rDNA evolution following polyploidy in Oryza. Molecular Phylogenetics and Evolution 55: 136–142. https://doi.org/10.1016/j.ympev.2009.10.023

Barros e Silva AE, dos Santos Soares Filho W, Guerra M (2013) Linked 5S and 45S rDNA Sites are highly conserved through the subfamily Aurantioideae (Rutaceae). Cytogenetic and Genome Research 140: 62–69. https://doi.org/10.1159/000350695

Cao M, Sleper, DA, Dong F, Jiang J (2000) Genomic in situ hybridization (GISH) reveals high chromosome pairing affinity between Lolium perenne and Festuca mairei. Genome 43: 398–403. https://doi.org/10.1139/g99-129

Catalán P, Torrecilla P, López Rodriguez JA, Olmstead RG (2004) Phylogeny of the festucoid grasses of subtribe Loliinae and allies (Poeae, Pooidae) inferred from ITS and trnL-F sequences. Molecular Phylogenetics and Evolution 31: 517–541. https://doi.org/10.1016/j.ympev.2003.08.025

Chandrasekharan P, Thomas H (1971) Studies in Festuca. 5. Cytogenetic relationships between species of Bovinae and Scariosae. Zeitschrift für Pflanzenzüchtung 65: 345–354.

Charmet G, Ravel C, Balfourier F (1997) Phylogenetic analysis in the Festuca-Lolium complex using molecular markers and ITS rDNA. Theoretical and Applied Genetics 94: 1038–1046. https://doi.org/10.1007/s001220050512

Cheng Y, Ma X, Zhou K, Humphreys MW, Zhang XQ (2016) Phylogenetic analysis of Festuca-Lolium complex using SRAP markers. Genetic Resources and Crop Evolution 63: 7–18. https://doi.org/10.1007/s10722-015-0324-5
Chester M, Leitch AR, Soltis PS, Soltis DE (2010) Review of the application of modern cytogenetic methods (FISH/GISH) to the study of reticulation (polyploidy/hybridisation). Genes 1: 166–192. https://doi.org/10.3390/genes1020166

Datson PM, Murray BG (2006) Ribosomal DNA locus evolution in Nemesia: transposition rather than structural rearrangement as the key mechanism? Chromosome Research 14: 845–857. https://doi.org/10.1007/s10577-006-1092-z

Ezquerro-López D, Kopecký D, Inda LA (2017) Cytogenetic relationships within the Maghrebian clade of Festuca subgen. Schedonorus (Poaceae), using flow cytometry and FISH. Anales Jardín Botanico de Madrid 74: 1–9. https://doi.org/10.3989/ajbm.2455

Fukushima K, Imamura K, Nagano K, Hoshi Y (2011) Contrasting patterns of the 5S and 45S rDNA evolutions in the Byblis liniflora complex (Byblidaceae). Journal of Plant Research 124: 231–244. https://doi.org/10.1007/s10265-010-0366-x

Garcia S, Kovařík A, Leitch AR, Garnatje T (2017) Cytogenetic features of rRNA genes across land plants: analysis of the Plant rDNA database. Plant Journal 89: 1020–1030. https://doi.org/10.1111/tpj.13442

Gaut BS, Tredway LP, Kubik C, Gaut RL, Meyer W (2000) Phylogenetic relationships and genetic diversity among members of the Festuca-Lolium complex (Poaceae) based on ITS sequence data. Plant Systematics and Evolution 224: 33–53. https://doi.org/10.1007/BF00985265

Hand ML, Cogan NO, Stewart AV, Forster JW (2010) Evolutionary history of tall fescue morphotypes inferred from molecular phylogenetics of the Lolium-Festuca species complex. BMC Evolution Biology 10: e303. https://doi.org/10.1186/1471-2148-10-303

Harper JA, Thomas ID, Lovatt JA, Thomas HM (2004) Physical mapping of rDNA sites in possible diploid progenitors of polyploid Festuca species. Plant Systematics and Evolution 245: 163–168. https://doi.org/10.1007/s00606-003-0110-2

Humphreys MW, Thomas HM, Morgan WG, Meredith MR, Harper JA, Thomas H, Zwierzykowski Z, Ghesquiere M (1995). Discriminating the ancestral progenitors of hexaploid Festuca arundinacea using genomic in situ hybridization. Heredity 75: 171–174. https://doi.org/10.1038/hdy.1995.120

Inda LA, 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 Jardín Botánico de Madrid 70: 97–102. https://doi.org/10.3989/ajbm.2329

Inda LA, Sanmartín I, Buerki S, Catalán P (2014) Mediterranean origin and Miocene-Holocene Old World diversification of meadow fescues and ryegrasses (Festuca subgenus Schedonorus and Lolium). Journal of Biogeography 41: 600–614. https://doi.org/10.1111/jbi.12211

Inda LA, Segarra-Moragues JG, Müller J, Peterson PM, Catalán P (2008) Dated historical biogeography of the temperate Loliinae (Poaceae, Pooideae) grasses in the northern and southern hemispheres. Molecular Phylogenetics and Evolution 46: 932–957. https://doi.org/10.1016/j.ympev.2007.11.022

Jang TS, Emadzade K, Parker J, Temsch EM, Leitch AR, Speta F, Weiss-Schnareweiss H (2013) Chromosomal diversification and karyotype evolution of diploids in the cytologically diverse genus Prospero (Hyacinthaceae). BMC Evolutionary Biology 13: e136. https://doi.org/10.1186/1471-2148-13-136
Kolano B, Siwinska D, McCann J, Weiss-Schneeweiss H (2015) The evolution of genome size and rDNA in diploid species of Chenopodium s.l. (Amaranthaceae). Botanical Journal of the Linnean Society 179: 218–235. https://doi.org/10.1111/boj.12321

Kolano B, McCann J, Orzechowska M, Siwinska D, Temsch E, Weiss-Schneeweiss H (2016) Molecular and cytogenetic evidence for an allotetraploid origin of Chenopodium quinoa and C. berlandieri (Amaranthaceae). Molecular Phylogenetics and Evolution 100: 109–123. https://doi.org/10.1016/j.ympev.2016.04.009

Kopecký D, Havráňková M, Loureiro J, Castro S, Lukaszewski AJ, Bartoš J, Kopecka J, Dolezel J (2010) Physical distribution of homoeologous recombination in individual chromosomes of Festuca pratensis in Lolium multiflorum. Cytogenetics and Genome Research 129: 162–172. https://doi.org/10.1159/000313379

Kotseruba V, Gernand D, Meister A, Houben A (2003) Uniparental loss of ribosomal DNA in the allotetraploid grass Zingeria trichopoda (2n = 8). Genome 46: 156–163. https://doi.org/10.1139/g02-104

Kotseruba V, Pistrick K, Blattner FR, Kumke K, Weiss O, Rutten T, Fuchs J, Endo T, Nasuda S, Ghukasyan A, Houben A (2010) The evolution of the hexaploid grass Zingeria kochii (Mez) Tzvel. (2n = 12) was accompanied by complex hybridization and uniparental loss of ribosomal DNA. Molecular Phylogenetics and Evolution 56: 146–155. https://doi.org/10.1016/j.ympev.2010.01.003

Ksia, czyk T, Taciak M, Zwierzykowski Z (2010) Variability of ribosomal DNA sites in Festuca pratensis, Lolium perenne, and their intergeneric hybrids, revealed by FISH and GISH. Japanese Journal of Genetics 51: 449–460. https://doi.org/10.1007/BF03208874

Lan T, Albert VA (2011) Dynamic distribution patterns of ribosomal DNA and chromosomal evolution in Paphiopedilum, a lady’s slipper orchid. BMC Plant Biology 11: e126. https://doi.org/10.1186/1471-2229-11-126

Leitch IJ, Hanson L, Lim KY, Kovarik A, Chase MW, Clarkson JJ, Leitch AR (2008) The ups and downs of genome size evolution in polyploid species of Nicotiana (Solanaceae). Annals of Botany 101: 805–814. https://doi.org/10.1093/aob/mcm326

Malik CP, Thomas PT (1966) Karyotypic studies in some Lolium and Festuca species. Caryologia 19: 167–196. https://doi.org/10.1080/00087114.1966.10796216

Minaya M, Díaz-Pérez A, Mason-Gamer R, Pimentel M, Catalán P (2015) Evolution of the beta-amylase gene in the temperate grasses: non-purifying selection, recombination, semiparalogy, homeology and phylogenetic signal. Molecular Phylogenetics and Evolution 91: 68–85. https://doi.org/10.1016/j.ympev.2015.05.014

Morales AG, Aguiar-Perecin MLR, Mondin M (2012) Karyotype characterization reveals an up and down of 45S and 5S rDNA sites in Crotalaria (Leguminosae-Papilionoideae) species of the section Hedriocarpea subsection Macrostachyae. Genetic Resources and Crop Evolution 59: 277–288. https://doi.org/10.1007/s10722-011-9683-8

Namaganda M, Lye KA, Friebe B, Heun M (2006) AFLP-based differentiation of tropical African Festuca species compared to the European Festuca complex. Theoretical and Applied Genetics 113: 1529–1538. https://doi.org/10.1007/s00122-006-0400-5

Olanj N, Garnatje T, Sonboli A, Vallès J, García S (2015) The striking and unexpected cytogenetic diversity of genus Tanacetum L. (Asteraceae): A cytometric and fluorescent
in situ hybridisation study of Iranian taxa. BMC Plant Biology 15: e174. https://doi.org/10.1186/s12870-015-0564-8

Pellicer J, Garnatje T, Hidalgo O, Tagashira N, Vallès J, Kondo K (2010) Do polyploids require proportionally less rDNA loci than their corresponding diploids? Examples from Artemisia subgenera Absinthium and Artemisia (Asteraceae, Anthemideae). Plant Biosystems 144: 841–848. https://doi.org/10.1080/11263504.2010.522783

Raskina O, Barber JC, Nevo E, Belyayev A (2008) Repetitive DNA and chromosomal rearrangements: speciation-related events in plant genomes. Cytogenetics and Genome Research 120: 351–357. https://doi.org/10.1159/000121084

Renny-Byfield S, Kovarik A, Kelly LJ, MacAs J, Novak P, Chase MW, Nichols RA, Pancholi MR, Grandbastien M, Leitch AR (2013) Diploidization and genome size change in allopolyploids is associated with differential dynamics of low- and high-copy sequences. Plant Journal 74: 829–839. https://doi.org/10.1111/tpj.12168

Roa F, Guerra M (2012) Distribution of 45S rDNA sites in chromosomes of plants: structural and evolutionary implications. BMC Evolutionary Biology 12: e225. https://doi.org/10.1186/1471-2148-12-225

Roa F, Guerra M (2015) Non-random distribution of 5S rDNA sites and its association with 45S rDNA in plant chromosomes. Cytogenetics and Genome Research 146: 243–249. https://doi.org/10.1159/000440930

Romero Zarco C (1988) Números cromosomáticos de plantas occidentales, 472–486. Anales del Jardín Botanico de Madrid 45: 273–279.

Romero Zarco C, Cabezudo B (1983) Micropyropsis, género nuevo de Gramineae. Lagascalia 11: 94–99.

Scholz H, Stierstorfer C, Gaisberg MV (2000) Lolium edwardii sp. nova (Gramineae) and its relationship with Schedonorus sect. Plantynia Dumort. Feddes Repertorium 111(7–8): 561–565. https://doi.org/10.1002/fedr.20001110722

Scholz S, Scholz H (2005) A new species of Lolium (Gramineae) from Fuerteventura and Lanzarote (Canary Islands, Spain). Willdenowia 35: 281–286. https://doi.org/10.3372/wi.35.35208

Shafiee P, Amini F, Mirzaghaderi G, Mortazavian SMM, Noori SAS (2020) Karyotype analysis in six species of Lolium and Festuca (Poaceae). Cytologia 85: 281–288. https://doi.org/10.1508/cytologia.85.281

Šmarda P, Bureš P, Horová L, Foggi B, Rossi G (2008) Genome size and GC content evolution of Festuca: ancestral expansion and subsequent reduction. Annals of Botany 101: 421–433. https://doi.org/10.1093/aob/mcm307

Terrell EE (1968) A taxonomic revision of the genus Lolium. Technical Bulletin 1392. United States Department of Agriculture, Washington.

Thomas HM, Harper JA, Meredith MR, Morgan WG, King IP (1997) Physical mapping of ribosomal DNA sites in Festuca arundinacea and related species by in situ hybridization. Genome 40: 406–410. https://doi.org/10.1139/g97-054

Thomas HM, Harper JA, Meredith MR, Morgan WG, Thomas ID, Timms E, King IP (1996) Comparison of ribosomal DNA sites in Lolium species by fluorescence in situ hybridization. Chromosome Research 4: 486–490. https://doi.org/10.1007/BF02261775
Torrecilla P, Catalán P (2002) Phylogeny of broad-leaved and fine-leaved *Festuca* lineages (Poaceae) based on nuclear ITS sequences. Systematic Botany 27: 241–251.

Weiss-Schneeweiss H, Emadzade K, Jang TS, Schneeweiss GM (2013) Evolutionary consequences, constraints and potential of polyploidy in plants. Cytogenetic and Genome Research 140: 137–150. https://doi.org/10.1159/000351727

Weiss-Schneeweiss H, Blöch C, Turner B, Villaseñor JL, Stuessy TF, Schneeweiss GM (2012) The promiscuous and the chaste: frequent allopolyploid speciation and its genomic consequences in American daisies (*Melampodium* sect. *Melampodium*; Asteraceae). Evolution 66: 211–228. https://doi.org/10.1111/j.1558-5646.2011.01424.x

Williams WM, Ellison NW, Ansari HA, Verry IM, Hussain SW (2012) Experimental evidence for the ancestry of allotetraploid *Trifolium repens* and creation of synthetic forms with value for plant breeding. BMC Plant Biology 12: e55. https://doi.org/10.1186/1471-2229-12-55

Xiong Z, Pires JC (2011) Karyotype and identification of all homoeologous chromosomes of allopolyploid *Brassica napus* and its diploid progenitors. Genetics 187: 37–49. https://doi.org/10.1534/genetics.110.122473

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