MARCO TULIO MENDES FERREIRA

CYTOGENETICS OF NATIVE SPECIES OF *Festuca*, CROSSOVER ANALYSES AND CHROMATID INTERFERENCE IN FESTULOLIUM

LAVRAS – MG
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Orientadora: Profa. Dra. Vânia Helena Techio
Co-orientador: Dr. David Kopecký

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CITOGENÉTICA DE ESPÉCIES NATIVAS DE *Festuca*, ANÁLISE DE CROSSING-OVER E INTERFERÊNCIA CROMATÍDICA EM FESTULOLIUM

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Bertolt Brecht (adaptado)

RESUMO

Ryegrasses (*Lolium* L.) são gramíneas ideais para uso como forragem animal na agricultura de pastagens temperadas, no entanto, seu uso é restrito por serem suscetíveis à estresses bióticos e abióticos. *Festuca* L., por sua vez, apresenta espécies mais resistentes, porém com desempenho inferior quando se trata de provisão de forragem. O complexo *Lolium-Festuca* tem fascinado citogeneticistas e melhoristas há mais cem anos por algumas razões. Em primeiro lugar, devido à natureza incomum e fácil com que híbridos intergenéricos (Festulolium) são obtidos e por apresentarem recombinação homeóloga, característica também presente em híbridos de *Allium* L.. Em segundo lugar, pela importância econômica, principalmente por unirem as melhores qualidades de ambos os gêneros: a alta produção de forragem do *Lolium* e a resistência de *Festuca*. Análises citogenéticas realizadas com hibridização *in situ* genômica (GISH) possibilitaram discriminar visualmente os genomas parentais, cromossomos/segmentos introgredidos e acompanhar o comportamento de ambos ao longo das gerações. Esta técnica, geralmente combinada com a hibridização *in situ* fluorescente (FISH), permite a descrição da composição do genoma híbrido, fornecendo informações valiosas para a produção de novas cultivares, seleção de material nas fases iniciais de programas de melhoramento e caracterização de espécies nativas selvagens. Apesar dos inúmeros estudos citogenéticos já realizados, o complexo ainda é um campo fértil para análises pioneiras, tais como para espécies nativas do Brasil e no esclarecimento de fenômenos genéticos ainda não totalmente elucidados, como, por exemplo, a *crossover/chromatid interference*. Nesse sentido, o presente trabalho está dividido em dois manuscritos, cujos objetivos foram: construir e caracterizar os cariótipos de duas espécies nativas do Brasil, *Festuca ulochaeta* Steud. e *Festuca fimbriata* Ness., utilizando FISH com sondas de rDNA 35 e 5S; evidenciar a existência de interferência cromossômica e entre cromátides no crossing-over por meio de GISH em híbridos de Festulolium (*L. multiflorum* Lam. x *F. pratensis* Huds.). Os resultados com o primeiro estudo, mostraram que ambas as espécies nativas apresentaram 42 cromossomos e um par de sítios de rDNA 5S. Para o sítio de rDNA 35S, foram identificados dois pares em *F. fimbriata* e um par em *F. ulochaeta*. A análise das características cromossômicas indica que essas espécies possuem cariótipo simétrico e possível origem alopoliploide. No segundo artigo, por meio da GISH em células em anáfase I de híbridos interespecíficos (*L. multiflorum* × *F. pratensis* e *Allium cepa* × *A. Roylei*), foi constatada a ação do fenômeno da *crossover interference*. Na análise foi possível quantificar o envolvimento de duas a quatro cromátides em crossing-over duplos e, naqueles que envolveram o número máximo de cromátides, a frequência foi de 64%, quando o esperado seria de apenas 25%. Também foi possível fornecer a medida física da distância da interferência que cobriu de 30 a 40% do comprimento relativo do braço cromossômico e evidenciar que o centrômero atua como uma barreira para a propagação da interferência.

Palavras-chave: Poaceae. *Lolium*. Híbridos intergenéricos. Hibridização *in situ*. Cariótipo. Homeologia cromossômica.

ABSTRACT

Ryegrasses (*Lolium* L.) are ideal grasses for use as animal fodder in temperate pasture farming, however, their use is restricted as they are susceptible to biotic and abiotic stresses. *Festuca* L., in turn, presents more resistant species, but with lower performance when it comes to the provision of forage. The *Lolium-Festuca* complex has fascinated cytogeneticists and breeders for over a hundred years for some reasons. Firstly, due to the unusual and easy nature with which intergeneric hybrids (Festulolium) are obtained and have homeologous recombination, feature also present in hybrids of *Allium* L.. Secondly, due to its economic importance, mainly because it combines the best qualities of both genus: the high production of Lolium forage and the resistance of Festuca. Cytogenetic analyzes carried out with genomic in situ hybridization (GISH) made it possible to visually discriminate parental genomes, chromosomes/introgressed segments and monitor the behavior over generations. This technique, usually combined with fluorescent in situ hybridization (FISH), allows the composition description of the hybrid genome, providing valuable information for the production of new cultivars, selection of material in the early stages of breeding programs and characterization of wild native species. Despite the countless cytogenetic studies already carried out, the complex is still a fertile field for pioneering analyzes, such as for native species of Brazil and in the clarification of genetic phenomena not yet fully elucidated, such as, for example, chromosomal/chromatid interference in crossing-over. Therefore, the present work is divided into two manuscripts, which the objectives were: to build and characterize the karyotypes of two species native to Brazil, *Festuca ulochaeta* Steud. and *Festuca fimbriata* Ness., using FISH with 35 and 5S rDNA probes; to evidence the existence of chromosomal/chromatid interference by means of GISH in Festulolium hybrids (*L. multiflorum* Lam. x *F. pratensis* Huds.). The results with the first study were that both native species had 42 chromosomes and a pair of 5S rDNA sites. For the 35S rDNA site, two pairs were identified in *F. fimbriata* and one pair in *F. ulochaeta*. The chromosomal characteristics indicates that these species have a symmetrical karyotype and possible allopolyploid origin. In the second manuscript, through GISH in anaphase I cells of interspecific hybrids (*L. multiflorum* × *F. pratensis* and *Allium cepa* × *A. Roylei*), the action of the interference phenomenon in crossing-over observed. In the analysis it was possible to quantify the involvement of two to four chromatids in double crossovers and, in those that involved the maximum number of chromatids, the frequency was 64%, when the expected would be only 25%. It was also possible to provide the physical measure of the interference that covered 30 to 40% of the relative length of the chromosomal arm and that the centromere acts as a barrier for the propagation of the interference.

Keywords: Poaceae. *Lolium*. Intergeneric hybrids. In situ hybridization. Karyotype. Chromosomic homeology.

SUMÁRIO

1 INTRODUÇÃO GERAL

A citogenética é a área que estuda a estrutura, as propriedades dos cromossomos e seu papel na hereditariedade (KANNAN; ZILFALIL, 2009). Enquanto ciência ela surgiu no início do século XX a partir de trabalhos pioneiros realizados após 1880 por inúmeros cientistas que, baseando-se nos pressupostos de Gregor Mendel, observaram o comportamento dos cromossomos e determinaram que estes eram os portadores dos genes. Theodor Boveri foi o responsável por fornecer evidências citológicas concretas de que os cromossomos eram realmente os vetores do material hereditário e, entre 1902 e 1903, juntamente com Walter Sutton propuseram, de forma independente, a Teoria Cromossômica da Herança (MARTINS, 1999; SCHULZ-SCHAEFFER J, 1980). Desde então, inúmeros estudos associativos entre as áreas de citologia e genética foram incentivados, podendo ser este momento, portanto, considerado como o do nascimento da citogenética.

Somente a partir da década de cinquenta, após a obtenção de metáfases com cromossomos não sobrepostos e com melhor morfologia, que outras técnicas de citogenética clássica, tais como os bandamentos, foram desenvolvidas (KANNAN; ZILFALIL, 2009). Estas técnicas foram de grande importância, pois possibilitaram uma contagem cromossômica mais confiável e uma análise mais detalhada de sua morfologia, comportamento e alterações numéricas (KANNAN; ZILFALIL, 2009; ROONEY, 2001; SILVA; SOUZA 2013). As primeiras técnicas de citogenética molecular em animais e plantas foram desenvolvidas doravante os trabalhos de Pardue e Gall (1969), que possibilitaram a localização de moléculas híbridas de DNA-DNA em preparações citológicas, técnicas recentemente conhecidas como hibridização *in situ* fluorescente (FISH) e hibridização *in situ* genômica (GISH) (SILVA; SOUZA, 2013). Ambas permitiram maiores avanços nos estudos dos cromossomos e ampliaram o campo das pesquisas em citogenética.

A FISH trouxe um refinamento na análise dos cromossomos, possibilitou a construção de cariótipos mais confiáveis, a localização de genes de cópia única e de sequências repetitivas em tandem ou dispersas (HARRISON; HESLOP-HARRISON, 1995; HESLOP-HARRISON et al., 1991; JIANG, 2019). A GISH por sua vez, permitiu um maior entendimento das relações de parentesco e homologia entre genomas de espécies e híbridos (BRAMMER et al., 2007; 2009). Ambas as técnicas tiveram e ainda tem um papel de destaque nos estudos citogenéticos voltados para o conhecimento da organização/evolução das plantas pertencentes ao complexo *Lolium-Festuca* (L-F) e existem algumas razões para isso (HUMPHREYS; ZWIERZYKOWSKI, 2020).

Primeiro, porque as plantas do complexo formam híbridos intergenéricos e interespecíficos e nestes, os cromossomos apresentam recombinação homeóloga; segundo, porque algumas das gramíneas pertencentes ao complexo, como, por exemplo, o *Lolium perenne* L. (Lp - 2n=2x=14), *Lolium multiflorum* Lam. (Lm - 2n=2x=14), *Festuca arundinacea* Schreb. (Fa - 2n=6x=42), *Festuca pratensis* Huds. (Fp - 2n=2x=14) e *Festuca glaucescens* Hegetschw. (Fg - 2n=4x=28), apresentam um papel de destaque na agricultura de países com clima temperado (HUMPHREYS; ZWIERZYKOWSKI, 2020). O pareamento homeólogo e a frequente recombinação foi também evidenciada entre espécies de *Allium* e é um fenômeno explorado em programas de melhoramento que visam, por exemplo a introgressão de *Allium fistulosum* L. em *A. cepa* L. mediado por *A. roylei* Stearn. (KHRUSTALEVA; KIK, 2000).

Além das espécies já citadas, o gênero *Lolium* apresenta algumas outras espécies diploides e *Festuca* conta com mais de 600 outras espécies (HODKINSON, 2018) com representantes diploides (2x) à dodecaploides (12x) (KOPECKÝ; LUKASZEWSKI; DOLEZĚL, 2008; ŠMARDA; STANČÍK, 2006;), distribuídas globalmente, inclusive, com ocorrência de espécies hexaploides no Brasil (DUBCOVSKY, 1989; SCHIFINO; ESSI; DA SILVA, 2018{http://floradobrasil.org.br.; LONGHI-WAGNER, 1987; STANČÍK; PETERSON, 2007; WINGE, 1982; ZULOAGA et al., 1994), como, por exemplo, *Festuca ulochaeta* Steud. e *Festuca fimbriata* Ness.. O conhecimento da diversidade cariotípica dessas duas espécies nativas é de particular interesse, pois pode revelar mecanismos (cito)genéticos que contribuíram para a diversificação do gênero nas regiões tropicais e subtropicais Sul-americanas, assim como já foram descritos para outras plantas de interesse comercial e híbridos do complexo *Lolium-Festuca*.

Dentre os inúmeros trabalhos que fizeram uso da hibridização *in situ* (ISH) no complexo, Thomas e colaboradores, em 1994, foram pioneiros ao utilizarem sondas de DNA em híbridos diploides de Lm x Fp e confirmarem frequentes recombinações homeólogas entre os cromossomos das espécies parentais. Humphreys e colaboradores (1995), utilizando sondas de Fp e Fg, confirmaram a constituição genômica de *F. arundinacea* (2n=6x=42) como sendo FpFpFgFgFgFg. A FISH ratificou os parentais de Fa a partir do número e posição dos sítios de rDNA 35S e 5S (THOMAS et al., 1997). Pašakinskiene et al. (1998), por meio da GISH, identificaram a presença de cromatina de Lm em cromossomos de Fa e especularam o papel de Lm na evolução de Fa após sua alopoliploidização e diversificação de seus genitores. A alta frequência de recombinação intergenérica, possibilitada pela homeologia entre os cromossomos das espécies do complexo L-F, torna a ISH ferramenta atrativa para o melhoramento via

introgressão com a posterior detecção por meio de mapeamento físico de caracteres de interesse (KOPECKY; LUKASZEWSKI; DOLEŽEL, 2008). Isso só é possível graças a uma característica marcante nestas espécies: apresentam DNA suficientemente diferentes ao ponto de serem completamente identificados/diferenciados na GISH, porém semelhantes ao ponto de permitirem o pareamento e a disjunção na meiose (KOPECKY; LUKASZEWSKI; DOLEŽEL, 2008; ZWIERZYKOWSKI et al., 1998). Essa característica singular existente entre os híbridos do complexo, possibilita a identificação dos pontos de recombinação entre os genomas homeólogos e a sua distribuição ao longo dos cromossomos e cromátides (KOPECKÝ et al., 2010). Tamanha resolução favorece observações citológicas e permite identificar e mensurar fenômenos como a *crossover/chromatid interference* e sua possível ação através do centrômero, que, na maioria dos organismos, é estudada a partir de mapas genéticos associados a modelos matemáticos (TEUSCHER et al., 2000).

A característica citada anteriormente associada a relativa facilidade de estabelecimento das técnicas de hibridização *in situ* no complexo, possibilitaram a publicação de vários trabalhos envolvendo as espécies e os híbridos intergenéricos, o que mostra a importância da citogenética no estudo dos cromossomos das espécies do complexo L-F. Apesar dos inúmeros trabalhos já realizados, o complexo ainda é um campo fértil para pesquisas dessa natureza (FERREIRA et al., 2018; KOPECKÝ et al., 2019; MAJKA et al., 2019; ZWYRTKOVÁ et al., 2020), permitindo estudos pioneiros com sondas ribossomais em espécies nativas pouco estudadas ou fenômenos genéticos não completamente esclarecidos, como, por exemplo, a interferência cromossômica e cromatídica no crossing-over. Assim sendo, o presente trabalho está dividido em dois manuscritos, cujos objetivos foram: construir e caracterizar os cariótipos de duas espécies nativas do Brasil, *Festuca ulochaeta* Steud. e *Festuca fimbriata* Ness., utilizando a FISH com sondas de rDNA 35 e 5S; evidenciar a existência da *crossover/chromatid interference* por meio da GISH em híbridos Festulolium (*L. multiflorum* x *F. pratensis*).

2 REFERENCIAL TEÓRICO

2.1 Caracterização geral do complexo *Lolium-Festuca*

Lolium e *Festuca* são gêneros representantes da família Poaceae, pertencentes à subfamília Pooideae e da tribo Poeae (JAUHAR, 1993). *Lolium* é um gênero que apresenta representantes perenes e anuais, todas diploides naturais com 2n=2x=14 (CLAYTON; RENVOIZE, 1986) e algumas cultivares poliploides obtidas a partir da duplicação cromossômica induzida (PEREIRA et al., 2014; POLOK, 2007). As mais importantes espécies dentro do gênero *Lolium* são conhecidas como azevém anual ou italiano (*L. multiflorum*) e o azevém perene (*L. perenne*) (JAUHAR, 1993). Estas são mundialmente conhecidas por apresentarem alto teor proteico e serem capazes de suprir a escassez forrageira, característica do período de inverno nos países de clima temperado (POLOK, 2007; YAMADA et al., 2005). Apesar do elevado potencial para fornecimento de forragens com alto valor nutritivo, os azevéns são susceptíveis a invernos rigorosos e as elevadas temperaturas no verão (HUMPHREYS; ZWIERZYKOWSKI, 2020).

O azevém perene e o italiano são proximamente relacionadas a *F. pratensis* (2n=2x=14), conhecida como festuca do prado ou *meadow fescue* e com *F. arundinacea* (2n=6x=42), popularmente chamada de festuca alta ou *tall fescue* (PAŠAKINSKIENĖ; JONES, 2003), ambas da seção Bovinae Fr.. *Festuca*, apresenta mais 600 espécies (HODKINSON, 2018), que variam desde representantes diploides (2n=2x=14) a dodecaploides com 2n=12x=84 (*F. summilusitana* Franco & Rocha Afonso), distribuídas por quase todos os continentes, principalmente na região holártica da Eurásia, América do Norte e em zonas temperadas do hemisfério Sul (KOPECKÝ; LUKASZEWSKI; DOLEZEL, 2008; ŠMARDA; STANČÍK, 2006; WATSON; DALLWITZ, 1992). Dentre todas essas, Jauhar (1993) considera *F. arundinacea* e *F. pratensis* como as mais importantes para a agricultura devido a tolerância à seca e ao estresse fisiológico causado por invernos rigorosos.

Em relação as *Festuca* de origem sul-americanas, poucos são os estudos citogenéticos existentes, os quais são relacionados à contagem cromossômica, determinação de nível de ploidia e estudos meióticos. Os trabalhos citológicos de Dubscovsky e Martinez são referências para estas gramíneas (DUBCOVSKY; MARTINEZ, 1987, 1988, 1991b, 1992), visto que eles descreveram o número cromossômico de aproximadamente 30 espécies. Dentre estas destacam-se duas nativas do Brasil, *Festuca ulochaeta* e *Festuca fimbriata*, com 2n=6x=42 cromossomos

(DUBCOVSKY; MARTÍNEZ, 1992; SCHIFINO; WINGE, 1982), as quais estão distribuídas nos Estados de Minas Gerais, Paraná, Rio de Janeiro, Rio Grande do Sul, São Paulo e Santa Catarina (STANČÍK; PETERSON, 2007). Um estudo mais aprofundado dessas espécies se faz necessário, principalmente, para o conhecimento da diversidade local e caracterização de seus papéis biológicos, como, por exemplo, o de restauração ecológica (BARBOSA et al., 2015), mas também por possivelmente constituírem fontes potenciais de genes úteis a serem explorados pelos programas de melhoramento. Por conta da ausência de espécies diploides no continente Sul-americano (DUBCOVSKY; MARTINEZ, 1991), estudos filogenéticos e de homologia genômica - GISH - com as festucas cultivadas, principalmente as diploides, se fazem necessários.

Os estudos apontam que a homeologia cromossômica entre algumas espécies do complexo *Lolium-Festuca* é grande o que levou os pesquisadores a concluírem que elas poderiam possuir origem monofilética (WIESNER et al., 1995). Até a década de 90, os estudos taxonômicos e filogenéticos ficaram restritos a análises citológicas e cruzamentos interespecíficos (TERREL, 1966). Destes cruzamentos, os programas de melhoramento de plantas desenvolveram um híbrido chamado Festulolium, que apresenta as melhores qualidades de ambos os gêneros: a alta produção de forragem do *Lolium* e a resistência ao estresse ambiental da *Festuca* (DINELLI et al., 2004; HUMPHREYS; ZWIERZYKOWSKI, 2020). A habilidade para produzir híbridos intergenéricos entre *Lolium* e *Festuca* e o sucesso comercial do Festulolium estimularam novas pesquisas sobre comportamento e estrutura desses genomas. Tais estudos revelaram uma extraordinária plasticidade dos genomas dos híbridos, principalmente pela habilidade de recombinação e pareamento dos cromossomos homeólogos (KOPECKÝ; LUKASZEWSKI; DOLEZEL, 2008).

2.2 Origem dos híbridos intergenéricos Festulolium

O primeiro híbrido Festulolium obtido envolveu o cruzamento entre *L. perenne* e *F. pratensis* (JENKIN, 1933). Apesar do sucesso na hibridação intergenérica, a produção comercial das cultivares de Festulolium aconteceu mais tardiamente e as gerações foram obtidas principalmente por dois processos. O primeiro por anfiploidia, realizado na Europa, promoveu o intercruzamento de plantas autotetraploides (LEWIS; TYLER; CHORLTON, 1973). Nesse cruzamento, o genoma de *Lolium* spp. foi combinado com o de *F. pratensis,* de forma a permitir o pareamento dos cromossomos e também estabilizar a composição destes cromossomos entre

gerações (GHESQUIÈRE; HUMPHREYS; ZWIERZYKOWSKI, 2010). O segundo processo, realizado nos Estados Unidos, foi obtido por introgressão de *Lolium* spp. em *F. arundinacea* (6x) (BUCKNER; BURRUS; BUSH, 1977).

De acordo com Ghesquière, Humphreys e Zwierzykowski (2010), na União Europeia, todos as cultivares híbridas formadas do cruzamento com *F. arundinacea* (6x) foram colocados na lista nacional de *Festuca* (festuca alta ou *tall fescue*), enquanto que as plantas identificadas como Festulolium eram formadas restritamente pelo cruzamento entre *L. multiflorum* e *F. pratensis*. Recentemente, a Comissão Europeia estendeu a definição de Festulolium, de forma a incluir todos os híbridos originados do cruzamento de espécies de *Lolium* e *Festuca*, independente do número cromossômico e se os híbridos foram retrocuzados intencionalmente com as espécies parentais (GHESQUIÈRE; HUMPHREYS; ZWIERZYKOWSKI, 2010; HUMPHREYS; ZWIERZYKOWSKI, 2020).

O melhoramento via anfiploidia é conseguido pelo acúmulo dos efeitos fenotípicos das espécies parentais. Isto é arranjado ao se tentar manter os conjuntos genômicos dos pais intactos nos híbridos e também assegurando a sua transferência de forma equilibrada para qualquer geração subsequente (GHESQUIÈRE; HUMPHREYS; ZWIERZYKOWSKI, 2010). Mesmo sendo a técnica até então, mais aplicada para a produção de Festulolium, a anfipolidia promoveu um alto grau de desequilíbrio nas gerações avançadas do híbrido anfidiploide (2n=4x=28) oriundo do cruzamento de *Lolium* spp. com *F. pratensis* (KOPECKÝ et al., 2006; PAŠAKINSKIENĖ; JONES, 2003; ZWIERZYKOWSKI et al., 2006). Esse processo ocorre pois a herança dissômica não é completamente obtida em nenhuma cultivar híbrida anfiploide de Festulolium e, consequentemente, o balanço genético pode mudar ao longo das gerações (KOPECKÝ et al., 2006; ZWIERZYKOWSKI et al., 1998; 2006). Como consequência, um grande esforço foi feito para atingir resultados satisfatórios com a técnica de melhoramento genético via introgressão (KOSMALA et al., 2007).

A introgressão se tornou uma técnica de melhoramento com aplicação na produção de Festulolium ao ser entendido que os híbridos poliploides são pontes entre as espécies parentais. A estabilidade e o grau de fertilidade não são fatores determinantes, desde que os híbridos F1 sejam férteis suficientes para conseguirem transferir genes com as características de interesse durante os sucessivos retrocruzamentos (GHESQUIÈRE; HUMPHREYS; ZWIERZYKOWSKI, 2010). Vários são os trabalhos que evidenciaram que ao retrocruzar o híbrido (fêmea) com o parental (macho), por uma ou duas gerações, o genoma da espécie recorrente é altamente reestabelecido, não comprometendo a fertilidade ou a estabilidade do

fenótipo (HUMPHREYS et al., 2005; KOPECKÝ; LUKASZEWSKI; DOLEŽEL, 2008; ZWIERZYKOWSKI et al., 1999). A incorporação de técnicas baseadas em marcação GISH, FISH e outras cromossomo-específica, permitiram e auxiliaram o monitoramento da transferência de genes a partir de um cruzamento intergenérico (KOSMALA et al., 2007).

As primeiras cultivares de Festulolium foram desenvolvidas no Institute of Grassland and Environmental Research (IGER), Aberystwyth, Reino Unido (LEWIS et al., 1973), a partir dos cruzamentos de *L. perenne* x *F. pratensis* (cultivar Prior) e *L. multiflorum* x *F. pratensis* (cultivar Elmet). Posteriormente, nos EUA, Dr. Buckner desenvolveu a cultivar Kenhy após o retrocruzamento do híbrido de *L. multiflorum* x *F. arundinacea*, com *F. arundinacea* (KOPECKÝ; LUKASZEWSKI; DOLEŽEL, 2008). A alta produtividade dessa cultivar fez com que ela fosse utilizada sozinha por um longo período ou em associação com uma cultivar pura de *F. arundinacea* (BUCKNER; BURRUS; BUSH, 1977). Por conta deste pioneiro sucesso, nos anos seguintes mais de 30 cultivares de Festulolium foram registradas ao redor do mundo, concentrando-se, principalmente, na Europa central (FOJTÍK, 1994; ZWIERZYKOWSKI et al., 1998). Apenas uma cultivar anfiploide de Festulolium foi registrada no continente Sul-americano, especificamente na Argentina, oriunda do cruzamento entre *L. multiflorum* (4x) com *F. pratensis* (4x) (HUMPHREYS; ZWIERZYKOWSKI, 2020).

Até 2010, das 42 cultivares citadas na literatura como Festulolium, pelo menos uma vez, 24 são registradas na lista de 2009 da Organisation for Economic Co-operation and Development (Organização de Cooperação e Desenvolvimento Econômico - OCDE, Paris-França). Vinte e três cultivares, todas tetraploides (2n=4x=28), foram produzidas via anfiploidia, basicamente pelo cruzamento recíproco entre *L. multiflorum* e *F. pratensis*. Outras 19 cultivares de Festulolium foram produzidas via introgressão, que, à exceção da cultivar Matrix, são todas tetraploides (2n=4x=28) (GHESQUIÈRE; HUMPHREYS; ZWIERZYKOWSKI, 2010). O último levantamento do número de cultivares de Festulolium, feito em 2017, aponta para mais de 50 híbridos registrados e utilizados ao redor do mundo (KOPECKÝ et al., 2017).

Ghesquière, Humphreys e Zwierzykowski (2010) afirmaram que o híbrido F1 do cruzamento entre *Festuca* e *Lolium* pode ser obtido facilmente. Dentre as técnicas mais aplicadas, destaca-se aquela que emascula qualquer um dos genitores. Normalmente o azevém é utilizado como fêmea, e as inflorescências emasculadas são ensacadas com inflorescências de *Festuca*, neste caso a espécie polinizadora. Depois de 10-16 dias após a polinização, há o resgate *in vitro* dos embriões híbridos. Os autores ainda afirmam que espécies de *Lolium* quando

intercruzadas com *Festuca* diploides ou poliploides podem produzir F1 híbrida com baixa fertilidade gametofítica e baixa, ou nenhuma, fertilidade esporofítica, o que acaba impedindo o seu uso nos programas de melhoramento.

A limitação da F1 pode ser superada por meio da duplicação artificial dos cromossomos via boqueadores mitóticos, por exemplo colchicina. Híbridos diploides, após a duplicação com colchicina, produzem alotetraploides, restaurando a fertilidade. Outro caminho é a prévia duplicação dos parentais diploides (autotetraploidizados), os quais são usados nos cruzamentos, produzindo híbridos férteis em F1 (KOPECKÝ; LUKASZEWSKI; DOLEŽEL, 2008). A fertilidade do híbrido F1, de acordo com a ploidia dos genitores de diferentes espécies e em diferentes cruzamentos, pode ser visualizada na tabela 1.

Tabela 1: Fertilidade do híbrido F1 em relação ao nível de ploidia das espécies parentais.

Parent 1 (ploidy)	Parent 2 (ploidy)	Fertility of F1 hybrid progeny
Lolium (2x)	*F. pratensis* (2x)	sterile
Lolium (2x)	*F. pratensis* (4x)	sterile
Lolium (4x)	*F. pratensis* (2x)	fertile
Lolium (4x)	*F. pratensis* (4x)	fertile
Lolium (4x)	*F. arundinacea* (6x)	fertile
Lolium (2x)	*F. arundinacea* (6x)	male sterile, low female fertile
Lolium (4x)	*F. glaucescens* (4x)	fertile
Lolium (4x)	*F. mairei* (4x)	fertile

Fonte: KOPECKÝ; LUKASZEWSKI; DOLEŽEL, 2008

2.3 Citogenética molecular das principais espécies de *Lolium-Festuca* e do híbrido Festulolium

Dentre os trabalhos citológicos desenvolvidos até a década de 90, destaca-se o trabalho de Malik e Thomas (1966) por ter sido pioneiro na apresentação do cariótipo de espécies do complexo *Lolium-Festuca*. No gênero *Lolium*, tanto para *L. perenne* quanto para *L. multiflorum*, foram descritos três pares de cromossomos submetacêntricos com constrições secundárias (regiões organizadoras do nucléolo – RONs). Além destes, foi identificado um par metacêntrico (maior em relação aos outros pares) e três pares menores (submetacêntricos) (MALIK; THOMAS, 1966). Rocha et al. (2015) relataram sete sinais de rDNA 35S colocalizados com bandas CMA^+ e um par de 5S em *L. perenne*. O número de sítios 35S em *L. multiflorum* é

altamente variável, com quatro, cinco e seis sítios identificados, além de relatos de sítios sintênicos (BUSTAMANTE et al., 2014; FERREIRA et al., 2018).

Festuca, por sua vez, apresenta uma maior variação cariotípica associada a poliploidia e distribuição geográfica do gênero (MALIK; THOMAS, 1966). *Festuca pratensis* apresenta quatro pares de cromossomos com constrições secundárias. Os sítios de rDNA 35S foram contabilizados nas cultivares diploides (2n=2x=14) e autotetraploides (2n=4x=28) dessa espécie por Książczyk et al. (2010). Em todos os acessos diploides foram encontrados dois sinais de rDNA 35S no par cromossômico 2, na constrição secundária. A cultivar autotetraploide apresentou quatro sinais de rDNA 35S nos quatro homólogos do cromossomo 2, também localizados na constrição secundária (KSIĄŻCZYK et al., 2010). Para algumas cultivares de *F. arundinacea* (2n=6x=42) foram descritos até seis pares de constrições secundárias e quatro RONs, as quais foram detectadas por meio da coloração com nitrato de prata (CARNIDE et al., 1986) e confirmadas pela FISH com sonda de rDNA 35S (THOMAS et al., 1997).

As diferenças nos números de sítios ribossomais e a similaridade morfológica torna laboriosa a discriminação dos cromossomos de *Lolium* e *Festuca* nos híbridos intergenéricos, dificultando, por exemplo, o estudo do comportamento meiótico e identificação de mudanças estruturais pós-hibridação em Festulolium (KOPECKÝ; LUKASZEWSKI; DOLEŽEL, 2008). A homeologia entre os genomas das duas espécies impede a determinação de um padrão da heterocromatina constitucional via bandamento C, diferentemente do que acontece em outros híbridos nos quais o comportamento dos cromossomos é conhecido com precisão (JAUHAR, 1993). Somente em 1994, a partir dos trabalhos realizados por Thomas e colaboradores, com o a aplicação da técnica de GISH, a dinâmica dos cromossomos de Festulolium começou a ser desvendada. Nesse trabalho, os autores, ao utilizarem o DNA genômico de *F. pratensis* no cultivar híbrido e nos genitores (*L. multiflorum* x *F. pratensis*), demonstraram que os genomas de *L. multiflorum* e *F. pratensis* eram diferenciados com base nas suas sequências repetitivas dispersas. A partir desse trabalho também foi possível, pela primeira vez, confirmar a homeologia entre os cromossomos destas duas espécies e, consequentemente, a ocorrência de recombinação.

A aplicação das técnicas de GISH e FISH constituiu um marco no estudo dos híbridos interespecíficos de *Lolium* e *Festuca* possibilitando um melhor entendimento sobre a estrutura dos cromossomos, comportamento, origem e a relação de parentesco entre eles (KSIĄŻCZYK et al., 2014; THOMAS et al., 1994). De acordo com Kopecký, Lukaszewski e Dolezel (2008),

a GISH é uma técnica que pode ser usada para selecionar os híbridos potencialmente úteis no início de um programa de melhoramento, assim como auxiliar no registro de uma nova cultivar.

Após o sucesso obtido pela GISH no Festulolium e a identificação de cromossomos recombinantes, em 1998, Zwierzykowski et al. analisaram as proporções dos genomas parentais nos híbridos tetraploides derivados dos cruzamentos recíprocos entre *L. multiflorum* e *F. pratensis*, em quatro populações. Estas foram selecionadas visando melhor performance agronômica. Todas elas eram férteis, produtivas e apresentavam certa estabilidade e uniformidade (ZWIERZYKOWSKI et al., 2008). Entre as 25 plantas testadas, foi possível perceber que as proporções dos genomas parentais nos híbridos variaram de 49,2% a 66,7% em favor do genoma do *Lolium* e também dentro e entre as populações. A predominância do genoma do *Lolium* sobre o de *Festuca* também foi relatada por outros trabalhos, em diferentes cultivares (CANTER et al., 1999; KOPECKÝ et al., 2006; KOPECKÝ et al., 2017). Kopecký e colaboradores (2006), também utilizando a GISH, descreveram a composição do genoma de 19 cultivares anfiploides de Festulolium. Os autores evidenciaram uma variação considerável na proporção do genoma de *Lolium* entre os híbridos, desde pequenas porções, por exemplo na cultivar Elmet, até um alto predomínio na cultivar Spring Green.

A análise da constituição genômica por meio da GISH, em sucessivas gerações do cruzamento entre *L. perenne* e *F. pratensis*, foi realizada por Zwierzykowski et al. (2011) nos híbrídos tetraploides. Os autores constataram uma alteração gradual na proporção a favor do genoma de *Lolium* entre as gerações F2 e F7, estabilizando apenas entre as gerações F7 e F8. Em 2012, em outro trabalho, a partir da seleção ao acaso das plantas em 4 gerações, foi confirmado a alteração gradual a favor do genoma de *Lolium* no avançar das gerações, porém de forma mais lenta (ZWIERZYKOWSKI et al., 2013).

2.4 Por dentro da meiose em híbridos Festulolium diploides e tetraploides

Apesar do consenso de que os cromossomos de espécies aparentadas não pareiam e, portanto, não recombinam na meiose, em algumas espécies isso é possível. Na natureza existem algumas excessões a regra do não pareamento homeólogo entre híbridos, por exemplo em *Allium* L. e *Lilium* L. (MAJKA et al., 2020). Em *Allium* o pareamento homeólogo tem sido explorado nos programas de melhoramento a partir da obtenção de híbridos via introgressão. Khrustaleva e Kik (2000) obtiveram plantas estáveis e identificaram uma alta frequência de bivalentes (82.6%) na metáfase I oriunda do cruzamento de *A. cepa* x (*A. fistulosum* x *A. roylei*)

por meio da GISH, com pontos de recombinação identificados ao longo dos cromossomos dos três genomas envolvidos no cruzamento.

Em Festulolium o pareamento dos cromossomos genitores - azevéns e festucas - é também altamente permissivo, sendo quase completo ou completo na meiose I (JAUHAR, 1975; MAJKA et al., 2020). O pareamento em híbridos intergenéricos diploides (2n=2x=14) de *L. perenne* x *F. pratensis* e *L. multiflorum* x *F. pratensis* é regular, ou seja, sete bivalentes são formados na meiose I, sendo a frequência de quiasmas um pouco menor que em seus parentais isoladamente (JAUHAR, 1975). Mesmo com o pareamento regular, estes híbridos apresentam anteras indeiscentes e, portanto, são macho estéreis. Em alguns casos, híbridos F1 podem expressar baixa fertilidade gametofítica, possibilitando o retrocruzamento com as espécies parentais (ZWIERZYKOWSKI et al., 2008).

A utilização de híbridos F1 tetraploides (2n=4x=28) de *Lolium* x *Festuca*, obtidos por anfiploidia ou introgressão, foi de extrema importância para os programas de melhoramento que visavam combinar as características complementares de ambos os gêneros (THOMAS; HUMPHREYS, 1991; ZWIERZYKOWSKI et al., 2008). O sucesso na obtenção de híbridos anfiploides tetraploides férteis possibilitou uma certa estabilidade no genoma, aumentando a sua produção e presença no mercado (CASLER et al., 2001; KOPECKÝ et al., 2006). O cenário ideal para estes híbridos seria aquele em que a integridade do genoma se mantivesse ao longo das gerações, graças ao pareamenento homólogo preferencial ao homeólogo, à segregação regular e consequentemente a herança dissômica, mas isso tem se mostrado difícil de conseguir (ZWIERZYKOWSKI et al., 2006), uma vez que os conjuntos cromossômicos de *Lolium* e *Festuca* se pareiam e se recombinam em frequências significativas.

Estudos relatam uma alta frequência de bivalentes e meiose regular em híbridos tetraploides. Thomas e Thomas (1973) evidenciaram que a conformação cromossômica média por meiócito em híbridos *L. perenne* (4x) x *F. pratensis* (4x) foi de 1.33 I + 11.95 II + 0.21 III + 0.55 IV. De forma semelhante Zwierzykowski et al. (2008) identificaram uma maior prevalência de bivalentes em 6 híbridos FpFpLpLp com conformação média por célula de 1.13 I + 11.11 II + 0.32 III + 0.72 IV. Nesse último estudo, a frequência de bivalentes variou de 10.80 a 12.23 por célula. Do total de 180 células mãe do grão de pólen analisadas, 39 apresentaram 14 II, destas, 27 apresetaram somente bivalentes intragenômicos (7 II LpLp e 7 II FpFp) e nas 12 células restantes, somente de 2 a 4 II intergenômicos (LpFp) foram observados.

Baseado no que foi dito anteriormente, a existência de um crossing-over intergenômico em híbridos Festulolium aponta ou para a existência de um sistema de controle de pareamento laxo, possibilitando a recombinação entre sequências de DNA altamente divergentes, ou que as sequências envolvidas na recombinação dos genomas nesses híbridos são altamente conservadas (KOPECKÝ; LUKASZEWSKI; DOLEŽEL, 2008). O fato é que nessas plantas existe uma extensa recombinação meiótica e, consequentemente, uma grande variabilidade genética na progenie. De acordo com Majka et al. (2020), em alguns híbridos Festulolium o pareamento e recombinação homeóloga é extremamente frequente e em algumas combinações de espécies, alcançando praticamente os níveis de pareamento homólogo.

Kopecký et al. (2008), em um trabalho muito interessante, estudaram a frequência do pareamento cromossômico homeólogo em linhagens de substituição monossômicas e dissômicas de *F. pratensis* em *L. multiflorum* tetraploide. Eles mostraram que a frequência do pareamento homeólogo foi relativamente menor que o pareamento homólogo ao acaso em linhagens dissômicas. Por outro lado, a análise do pareamento nas linhagens monossômicas permitiu evidenciar o pareamento perfeito entre os cromossomos homeólogos. Mais tarde, em 2010, Kopecký et al. reportaram a alta frequência desse pareamento nas regiões intersticiais e distais dos cromossomos e *coldspots* em áreas pericentroméricas, centroméricas, subteloméricas e teloméricas.

2.5 *Crossover/chromatid interference*

Durante a meiose o reconhecimento entre os cromossomos é um fator determinante que permite o alinhamento e o pareamento dos mesmos. Somente precedida a formação do complexo sinaptonêmico, uma estrutura protéica que serve como escafóide para os fatores de recombinação promotores do crossing-over, que a sinapse pode prosseguir (YOUDS; BOULTON, 2011). Na maioria dos organismos, a recombinação meiótica se inicia a partir da formação programada de quebras na dupla fita do DNA (DSBs) pela proteína SPO11 em associação com outras proteínas (LAM; KEENEY, 2015). De acordo com Wang e Copenhaver (2018), o processamento das DSB pela proteína Exo1 produz fitas simples de DNA com extremidades 3’ livres que, com auxílio das recombinases DMC1 e RAD51, fazem a busca entre os cromossomos homólogos ou as cromátides irmãs na tentativa de encontrar um molde para reparar a quebra – processo também chamado de invasão da fita simples. Essa invasão resulta em uma estrutura conhecida por D-loop. A extremidade 3’ se torna alvo para a síntese de DNA

associada a recombinação, que extende a D-loop e expõe sequências que podem se anelar a segunda extremidade 3' localizada no lado oposto da quebra original. Ainda de acordo com os autores, a síntese de um DNA adicional utilizando a segunda extremidade 3', seguido de ligação, forma uma estrutura intermediária chamada de junções de Holliday duplas (dHj). O resultado da resolução das dHj se dá, principalmente, em *crossovers* (CO), embora *noncrossovers* (NCO) também sejam teoricamente possíveis (WANG; COPENHAVER, 2018). O reparo da DSB utilizando a cromátide irmã resulta em moléculas de DNA idênticas, uma vez que aquelas envolvidas no reparo não apresentam variações em suas sequências (LAM; KEENEY, 2015). Eventos de NCO podem resultar em conversão gênica.

Os COs podem ser classificados como sensíveis (Tipo I) ou insensíveis (Tipo II) a um fenômeno chamado de *crossover interference* (WANG; COPENHAVER, 2018). Apesar do elevado número de DSBs durante a meiose o número total de CO é bem pequeno, variando de um por cromossomo a um por braço cromossômico (HUNTER, 2015). A formação de, pelo menos, um CO por par de homólogo é um pré-requisito para a correta disjunção na meiose – "crossing-over obrigatório" (*crossover assurance*) (KRISHNAPRASAD et al., 2015). De acordo com Hunter (2015), o CO obrigatório e a CO *interference* ditam o limite superior e inferior do número de possíveis CO.

Na literatura, inúmeros estudos apontam para uma não uniformidade na distribuição da recombinação gênica nos cromossomos. Regiões genômicas que apresentam uma maior frequência relativa de recombinação são chamadas de *hot spots*, enquanto que aquelas que possuem baixas frequências são chamadas de *cold spots* (LIU; LIU; HUANG, 2016). Estas regiões já foram mapeadas em inúmeras espécies, na verdade, em quase todos os organismos eucariotos que foram profundamente investigados, regiões com alta e baixa probabilidade de ocorrência de crossing-over (CO) foram identificadas (BUARD; DE MASSY, 2007; MÉZARD, 2006; GILL et al., 1996; KURATA et al., 1994;), incluindo *Lolium* e *Festuca* (KING et al., 2002).

Na maioria dos organismos eucarióticos existe um complexo mecanismo conhecido como *crossover interference*, que determina a posição e o número de recombinações possíveis (SUN et al., 2017). Essa interferência genética foi descrita pela primeira vez a mais de cem anos em *Drosophila melanogaster* (MULLER, 1916; STURTEVANT, 1913). Muller (1916), naquele momento, apontou que a ocorrência de um CO interferiria na formação de um segundo evento envolvendo o mesmo par de homólogos. Desde então, o número de estudos aumentou substancialmente e o que se sabe hoje é que existe uma relação intrínseca entre as taxas de CO

interference com as diferenças de sexo (PETKOV et al., 2007), estresses físicos (AGGARWAL et al., 2015) e a idade do organismo (WANG et al., 2016), da mesma forma em que tais fatores já foram descritos para variações na frequência e distribuição dos CO (FRANCIS et al., 2007; LENORMAND; DUTHEIL, 2005; SINHA; HELGASON, 1969).

Diferentes modelos tentam explicar/medir a interferência. O modelo estatístico gama tem sido utilizado para modelar as distâncias entre eventos de CO por meio da disposição linear do cromossomo (MCPEEK; SPEED, 1995), mas seus parâmetros podem ser influenciados por mudanças nos padrões de CO que não estão relacionados à interferência (ZHANG et al., 2014). Na tentativa de melhorar esta estatística, foi desenvolvido um modelo que busca integrar a captura de interferência em associação a um processo exponencial, permitindo o estabelecimento aleatório de um CO sem a ocorrência de interferência (HOUSWORTH; STAHL, 2003).

Existem dois modelos teóricos que visam explicar o mecanismo da interferência: o modelo de tensão mecânica e o modelo de polimerização. O primeiro se baseia no fato de que eventos de CO liberam estresse a uma determinada distância ao longo do eixo do cromossomo, o que impede novos CO nas proximidades (BÖRNER; KLECKNER; HUNTER, 2004). O segundo é baseado no fato de que uma vez que a estrutura do CO é estabelecida ao longo do complexo sinaptonêmico, ela tem a mesma chance por unidade de tempo para iniciar um evento de polimerização bidirecional. Essas unidades podem ser consideradas como nódulos de recombinação tardia que podem ser vistos no paquíteno (KING; MORTIMER, 1990). Além da abordagem genética, a CO *interference* também tem sido caracterizada por marcadores citológicos (SUN et al., 2017), o que permite identificar nos bivalentes as posições do CO, como quiasmas, nódulos de recombinação tardia e localização de proteínas envolvidas no CO por imunofluorescência (BERCHOWITZ; COPENHAVER, 2010). Tais abordagens, porém, não conseguem quantificar/identificar as diferentes cromátides envolvidas no crossing-over, portanto não fornecem evidências para um outro fator associado a interferência, mais especificamente a *chromatid interference*.

Ao contrário da CO *interference*, pouco se sabe sobre a regulação e envolvimento das quatro cromátides de um bivalente em um CO duplo (*double crossover* - DCO). Esse último mecanismo é conhecido como *chromatid interference* (CI) e é o responsável por enviesar a distribuição de múltiplos CO a favor de cromátides não irmãs específicas (ZHAO et al., 1995). Três diferentes conformações de DCO podem resultar da combinação das quatro cromátides presentes no par de cromossomos homólogos: ambos os CO envolvem as mesmas cromátides

não irmãs (2 cromátides – uma possibilidade), DCO compartilham apenas uma cromátide em comum (3 cromátides - duas possibilidades) ou DCO envolvem diferentes pares de cromátides não irmãs (4 cromátides – uma possibilidade) (SARENS; COPENHAVER; STORME, 2021). Na ausência de CI estas três conformações ocorrem ao acaso resultando em uma proporção de 1:2:1 para DCO. Em situações em que a CI é positiva o que se espera é que ocorra uma maior proporção de DCO envolvendo as 4 cromátides. Por outro lado, CI negativa ocorre quando o mesmo par de cromátides tem maior probabilidade de estar envolvido no DCO (ZHAO et al., 1995).

Relatos de *chromatid interference* fraca ou ausente já foram descritos para *Saccharomyces cerevisiae, Neurospora crassa, Aspergillus nidulans, Arabidopsis thaliana, Zea mays* e humanos (LINDEGREN; LINDEGREN, 1942; STRICKLAND, 1957; HAWTHORNE; MORTIMER, 1960; ZHAO et al., 1995; COPENHAVER et al., 1998; CHEN et al., 2008; HOU et al., 2013; LI et al., 2015). A presença da CI, positiva ou negativa, pode afetar a composição dos gametas a partir do enviesamento das cromátides envolvidas no DCO e, portanto, na frequência desses eventos, o que traz um grande impacto na elaboração dos mapas genéticos e ensaios de QTL. Modelos mais robustos estão disponíveis e procuram contabilizar a ocorrência de DCO (PERKINS, 1949; HALDANE, 1919; KOSAMBI, 1944), no entanto essas funções matemáticas assumem a completa ausência da CI e integram a distribuição ao acaso de 1:2:1 para estes eventos. Admitir isso, no entanto, pode trazer imprecisões no cálculo da distância genética real. Na presença de CI negativa, por exemplo, espera-se uma maior ocorrência de DCO que envolvem o mesmo par de cromátides, diminuindo as chances de se detectar estes eventos e como resultado trazendo uma subestimação da real distância genética (SARENS; COPENHAVER; STORME, 2021).

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SEGUNDA PARTE – ARTIGOS

ARTIGO 1 - IDENTIFICATION OF RIBOSOMAL SITES AND KARYOTYPE ANALYSIS IN *Festuca ulochaeta* STEUD. AND *Festuca fimbriata* NESS., GRASSES NATIVE TO BRAZIL

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Identification of ribosomal sites and karyotype analysis in *Festuca ulochaeta* Steud. and *Festucafimbriata* Ness., grasses native to Brazil

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Abstract

Festuca L. has more than 600 perennial species described, which makes it the largest genus within the family Poaceae. In Brazil, only two native species of *Festuca* have been described, for which cytogenetic studies need to be strengthened: *Festuca ulochaeta* and *Festuca fimbriata*. The aim of this study was to characterize the karyotypes of *F. ulochaeta* and *F. fimbriata* based on the mapping of rDNA sites. The FISH was performed with 35S and 5S rDNA probes. Both species have 42 chromosomes, of which 36 were metacentric and six were submetacentric. *Festuca fimbriata* has two pairs of 35S rDNA sites, one located on the metacentric pair 4, in an interstitial position, and one at the submetacentric pair 14 in the proximal position. *Festuca ulochaeta* has one pair of 35S rDNA in interstitial-proximal position in the metacentric pair 3. Both species showed 5S rDNA sites only on chromosome pair 21 in the terminal position of the short arm. The analysis of the chromosomal characteristics indicates that these species have a symmetrical karyotype and allopolyploid origin.

Keywords Poaceae · Polyploidy · FISH · Ribosomal DNA

Introduction

The family Poaceae contributes significantly to the diversity of Angiosperms, with about 11,506 species distributed in 768 genera [1], among which stands out *Festuca* L. Currently 610 species are classified in this genus, which makes it the largest within the family [1, 2]. Representatives of the genus *Festuca* are perennial and annual and are distributed on almost all continents, mainly in Eurasia, North America and temperate zones of the southern hemisphere, except for Antarctica [3]. Specifically, in South America, about 150 native species are distributed from the Andes to the sea level [4, 5].

The genus *Festuca* presents a basic number of chromosomes × = 7, with species ranging from diploid (2 ×) to dodecaploid (12 ×) [6, 7], which shows the significant role of polyploidy in its evolution, especially thenaturally occurring allopolyploids in most species of the genus [8]. This high diversity of ploidy levels has been shown to be an important taxonomic character in the characterization, organization and distribution of *Festuca* species [6]. Despite diversity, studies have focused on species of economic interest, notably *Lolium pratense* (Huds.) Darbysh. (≡ *Festuca pratensis* Huds.; 2n = 2 × = 14) and *Lolium arundinaceum* (Schreb.) Darbysh. (≡ *Festuca arundinacea* Schreb.; 2n = 6 × = 42), both species are tolerant to abiotic stresses [9]. The proximity between *Lolium* and *Festuca*, both morphological and genomic, and the chromosomal homoeology among species [10, 11] prompted taxonomists to erect Festulolium [12], which have important characteristics, such as: high forage production and resistance to biotic and abiotic stresses [13].

The diploid species of *Festuca* are found mainly in Europe, which is considered the center of origin and primary diversification of the genus, whereas in other continents, and in Latin America, diploid species are rare or only polyploids are found [14]. The second possible center ofdiversification is in South America, with the South American Andes being an important location for this process [14, 15]. According to Stančík and Peterson [15], the distribution of the species does not occur homogeneously in South America, where the highest diversity of species (about 50%) is found between

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central Peru and northern Argentina. In the South American continent, most studies were concentrated in Argentina, Bolivia, Chile and Peru [14, 16–21, 23]. In Brazil, two native species have been described so far, *Festuca ulochaeta* Steud. and *Festuca fimbriata* Ness. [15, 18, 22, 24–26], which are distributed in the states of Minas Gerais, Paraná, Rio de Janeiro, Rio Grande do Sul, São Paulo and Santa Catarina [15].

There are few cytogenetic studies involving these two native species of *Festuca*, with chromosome counts which confirmed 2n = 6 × = 42 for both [14, 24] and flow cytometry data in collection of seeds. Based on the DNA content, Šmarda and Stančík [6] indicated *F. ulochaeta* as tetraploid, which does not coincide with the information on the chromosome number presented previously for the species. These contradictory results reinforce the need for further cytogenetic studies.

One of the most used tools by cytogeneticists is karyotyping combined with molecular cytogenetic techniques [27], specifically fluorescent in situ hybridization (FISH) with 35S and 5S rDNA probes. Ribosomal genes (rDNAs) are highly repetitive in the eukaryotic genome and have their sequences conserved throughout the course of evolution [28, 29]. These characteristics are important for taxonomic and phylogenetic studies [30]. When observed that the distribution of the rDNA sites is species-specific, it can be used as a reference point in the identification of ectopic recombination, inversions, translocations and transposition events [31].

The assembly of the karyotype of these two species may reveal cytogenetic mechanisms that have contributed to the diversification of the genus in tropical and subtropical regions and evolutionary trends. The aim of this study is to characterize, for the first time, the karyotype of *F. fimbriata* and *F. ulochaeta* based on the mapping of rDNA sites.

Materials and methods

Plant material, pre-treatment, fixation and chromosome preparation

The tillers were collected at two sites: *F. ulochaeta* at Aparados da Serra National Park, Itaimbezinho - municipality of Cambará do Sul, State of Rio Grande do Sul, Brazil (S29 09′33.0″ WO50 04′45.6) and *F. fimbriata* in municipal- ity of Bom Jesus, State of Rio Grande do Sul, Brazil (S28 36′02.3″ WO50 24′29.7″). The vouchers were stored in the ICN Herbarium of the Federal University of Rio Grande do Sul; *L. Nogueira da Silva 1081* (ICN) for *F. fimbriata* and *L. Nogueira Silva 1085* (ICN) for *F. ulochaeta*. The species were identified using the keys published in Longhi- Wagner [25] and the plants were kept in a greenhouse at the Department of Biology of the Federal University of Lavras. (UFLA). Five plants of each species were used for chromosome preparation.

To obtain C-metaphases, roots were treated with 2.5 mM colchicine at 4 °C for 24 h, fixed in Carnoy (ethyl alco- hol–acetic acid, 3:1), kept for 3 days at room temperature and then stored at − 4 °C. Fixed roots were placed in a mix of enzymes containing cellulase Onozuka R10 (0.7%), cellulase Sigma-Aldrich (0.7%), pectolyase Sigma-Aldrich (1%) and cytohelicase Sigma-Aldrich (1%) at 37 °C, for 1 h and 26 min. Subsequently to the technique of cell dissociation and air drying [32] the FISH technique with 35S and 5S rDNA probes was applied to the best C-metaphase chromosome preparations.

Fluorescent in situ hybridization (FISH)

FISH was performed with 35S rDNA probes indirectly labeled with biotin (pTa 71 *Triticum aestivum* L.) and with 5S rDNA probes directly labeled Texas Red (pTa 794 *Triticum aestivum* L.), via nick translation reaction. The slides were denatured in 70% formamide in 2 × SSC for 1 min and 40 s at 85 °C and immediately transferred to a cooled alcoholic series to stop denaturation and dehydrate the preparation. The hybridization mixture (50% formamide, 2 × SSC, 10% dextran sulfate and approximately 40 ng/μL of the 35S and 5S rDNA probes) was denatured for 8 min. at 95–98 °C and immediately transferred to ice. Hybridization was done at 37 °C for 48 h in a humid chamber. Detection occurred only for the 35S rDNA probe from the use of anti-biotin-FITC secondary antibody for 1 h at 37 °C in a humid chamber. The slides were mounted on Vectashield with DAPI and evaluated.

Assembly of karyotypes and ideograms

KaryoType Software [33, 34] was used to obtain chromosome measurements using five metaphases of each species. The following morphometric data were obtained: short arm (SA) and long arm (LA) chromosomal lengths; chromosomal total length (TL = SA + LA); total length of the haploid set (TLH = ΣLTi); arm ratio (AR = L/S); centromeric index (CI = c/LTi × 100); and relative length (RL = LTi/ ΣLTi × 100). Based on the position of the centromere, the chromosome morphology was established using the AR values, as proposed by Levan et al. [35]. The position of the ribosomal sites on the chromosomes was defined from the classification proposed by Roa and Guerra [36] and the karyotypic asymmetry was defined according to the parameters of Stebbins [37]. The karyotypes were assembled using Adobe Photoshop® software and the ideograms were provided by KaryoType software [33, 34].

Results

Karyotype analysis

Festuca fimbriata and *F. ulochaeta* have 42 chromosomes, with a karyotype formula 2n = 36 m + 6 sm (Figs. 1, 2). The chromosome numbers confirm that both species are hexaploid. However, the chromosome morphology and pattern of distribution of ribosomal sites suggests the assembly of karyograms into chromosome pairs, since six groups of homomorphic chromosomes were not detected as expected for autopolyploid species (Figs. 1c, 2c). Exact discrimination of chromosomes/genomes can only be made based on a higher number of markers on the chromosomes.

The chromosomes of *F. fimbriata* ranged from 4.37 to 6.61 μm, with the pairs 14, 15 and 18 as submetacentrics, whereas those from *F. ulochaeta* ranged from 3.74 to 6.08 μm, with pairs 10, 17 and 19 classified as submetacentrics (Tables 1, 2). The ratio of the length of the largest chromosome to the smallest chromosome was 1.57 for *F. ulochaeta* and 1.42 for *F. fimbriata*, that is, less than 2, as well as the arm ratio for 100% of the chromosomes in both species, allows classification of asymmetry of the karyotypes as 1A, following the proposal of Stebbins [37]. According to the karyotype data, all chromosomes of *F. fimbriata* presented greater chromosome length and, consequently, higher TLH when compared to *F. ulochaeta* (Tables 1, 2).

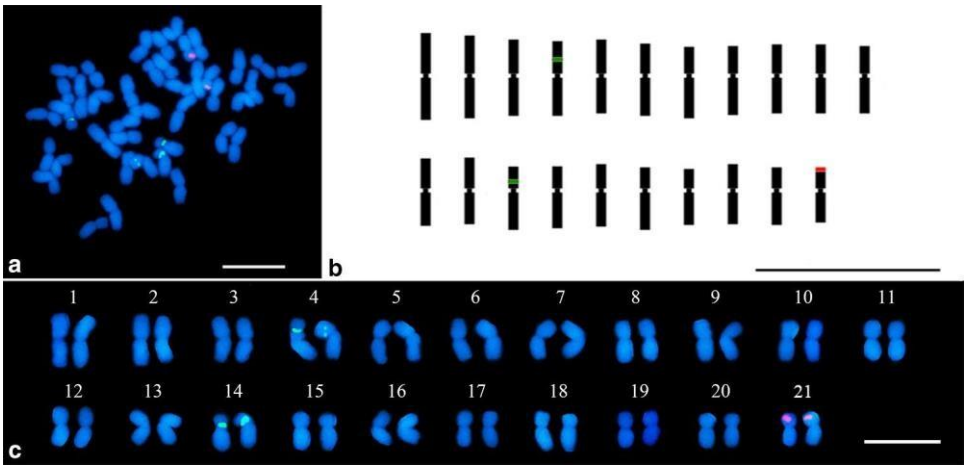

Fig. 1 Metaphase (**a**); Ideogram (**b**) and Karyogram (**c**) of *Festuca fimbriata* from FISH with rDNA probes. A- chro- mosomes in blue counterstained with DAPI, 35S rDNA (green fluorescence) and 5S rDNA (red fluorescence) sites. Bar: 10 μm. (Color figure online)

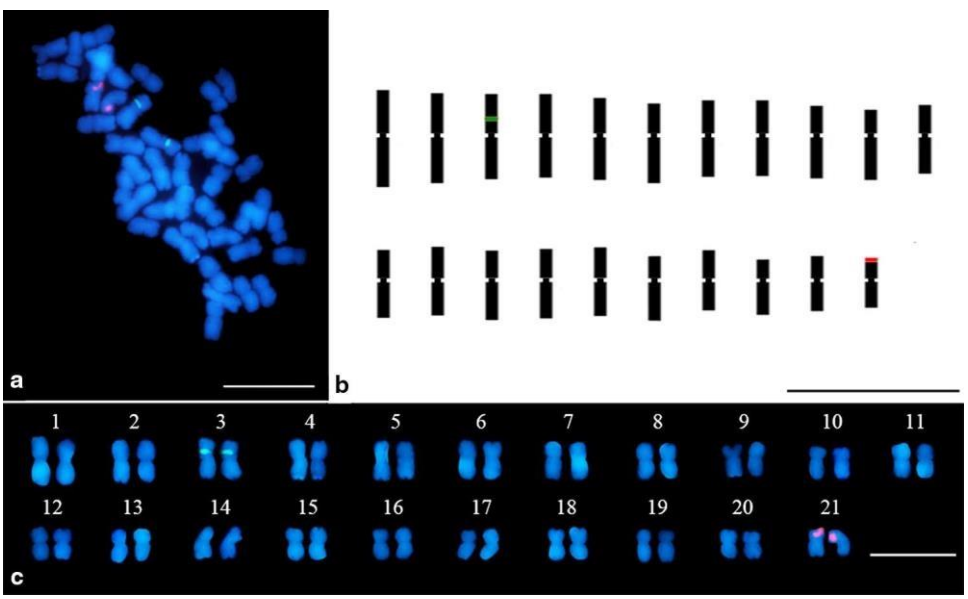

Fig. 2 Metaphase (**a**); Ideogram (**b**) and Karyogram (**c**) of *Festuca ulochaeta* from FISH with rDNA probes. A- chro- mosomes in blue counterstained with DAPI, 35S rDNA (green fluorescence) and 5S rDNA (red fluorescence) sites. Bar: 10 μm. (Color figure online)

Table 1 Karyotype data of *Festuca fimbriata*

Chromosome pair	TL (µm)[a]	AR[b]	Type	Chromosome pair	TL (µm)	AR	Type
1	6.61	1.15	m	12	5.05	1.26	m[c]
2	6.01	1.11	m	13	5.00	1.31	m
3	5.87	1.34	m	14	5.13	1.72	sm*
4	5.87	1.11	m*	15	4.91	1.77	sm[d]
5	5.73	1.19	m	16	4.82	1.37	m
6	5.66	1.28	m	17	4.52	1.38	m
7	5.53	1.31	m	18	4.46	1.75	sm
8	5.38	1.20	m	19	4.45	1.34	m
9	5.33	1.28	m	20	4.45	1.30	m
10	5.15	1.23	m	21	4.37	1.38	m**
11	5.13	1.31	m	CTLH[e]	109.54		

[a]Total chromosome length

[b]Arm ratio

[c]Metacentric chromosomes

[d]Submetacentric chromosomes

[e]Total length of the haploid set

[*,**]Chromosomes carrying the 35 and 5S rDNA sites, respectively

Table 2 Karyotype data of *Festuca ulochaeta*

Chromosome pair	TL (µm)[a]	AR[b]	Type	Chromosome pair	TL (µm)	AR	Type
1	6.08	1.11	m[c]	12	4.39	1.28	m
2	5.55	1.15	m	13	4.25	1.16	m
3	5.31	1.04	m*	14	4.24	1.29	m
4	5.28	1.15	m	15	4.21	1.26	m
5	5.10	1.34	m	16	4.20	1.30	m
6	4.89	1.26	m	17	4.18	1.72	sm[d]
7	4.81	1.24	m	18	3.89	1.18	m
8	4.57	1.29	m	19	3.88	1.70	sm
9	4.44	1.25	m	20	3.75	1.25	m
10	4.44	1.77	sm	21	3.74	1.20	m**
11	4.42	1.35	m	CTLH[e]	95.73		

[a]Total chromosome length

[b]Arm ratio

[c]Metacentric chromosomes

[d]Submetacentric chromosomes

[e]Total length of the haploid set

[*,**]Chromosomes carrying the 35 and 5S rDNA sites, respectively

Identification of 35S and 5S rDNA sites

In *Festuca fimbriata* two pairs of 35S rDNA were identified. One at the metacentric pair 4, in an interstitial-proximal position of the short arm and one in proximal position of the submetacentric pair 14, also in the short arm (Fig. 1). In contrast, *F. ulochaeta*, presented only one pair of 35S rDNA in an interstitial-proximal position of the short arm of the metacentric pair 3 (Fig. 2). Both species showed 5S rDNA sites only on chromosome pair 21 in terminal position in the short arm (Figs. 1, 2).

Discussion

The identification of ploidy level by chromosome counting for South American *Festuca* species has been reported in several studies using classical cytogenetic techniques [15,

17, 22, 24, 25, 38–40]. Nevertheless, among the numerous world-known species [4, 5, 16, 20, 41, 42], only about 25 South American species had their chromosome numbers described [6], including *F. ulochaeta* and *F. fimbriata*, hexaploids native to Brazil, with 2n = 42 chromosomes [14], data confirmed in this study. Although the species have thesame chromosome number, *F. fimbriata* presented higher TLH than *F. ulochaeta* and this trend correlated with the genome size, since *F. ulochaeta* has 1C = 5.417 and 1Cx = 1.80 pg and *F. fimbriata* 1C = 5.79 and 1Cx = 2.23 pg (unpublished data).

Karyotypic analysis by physical mapping by FISH for the two species revealed differences for the 35S rDNA sites and conservation in the number and position of the 5S rDNA site. In *F. fimbriata*, the location of four secondary constrictions, presumably nucleolar organizer regions (NORs), in the interstitial region of the short arms of the chromosomes was described in Dubcovsky [22], without reference to the chromosomes bearing these markers. For *F. ulochaeta*, Dubcovsky [22], described two to six secondary constrictions.

The divergence between the numbers of NORs described for *F. ulochaeta* and the number of rDNA sites recorded in our study is attributed to the greater refinement of the FISH technique, allowing precise identification of heterochromatic blocks of rDNA. For example, Malik and Thomas [43] studied the karyotype of 17 *Festuca* species and identified a large number of secondary constrictions, higher than the number of 35S rDNA sites reported by Thomas et al. [44] for three species.

Identification of 35 and 5S rDNA sites for the two South American *Festuca* species had not been reported so far. The location of the 35S rDNA sites on the short arm of chromosomes in *F. ulochaeta* and *F. fimbriata* is also a common feature in angiosperms in general [36]. Lima-de-Faria [45] demonstrated that in grasses, 91% of the 97 plants analyzed had one or two pairs of chromosomes with satellites also in the short arm. The presence of only one pair of 5S rDNA sites, as well as for the species studied here, was also identified in 54.1% angiosperm species listed by Roa and Guerra [46], and in these, 58.7% of the 5S rDNA sites were located on the short arm of the chromosomes. The high percentage of species with only one pair of 5S rDNA suggests that these sites have a lower dispersion capacity than the 35S rDNA sites [46]. This interspecific numerical constancy of the 5S rDNA sites was reported for diploid and polyploid representatives of *Festuca* [44] and in closely related diploid species of *Lolium* [47–49].

The pattern of distribution of ribosomal sites in chromosomal pairs observed in *F. fimbriata* and *F. ulochaeta* was reported for other allohexaploid species of *Festuca*. *Lolium arundinaceum* (2n = 6 × = 42) is thought to have originated from crossing *L. pratense* (2n = 2 × = 14) and *F. glaucescens* Hegetschw. (2n = 4 × = 28) [7]. It hasthree pairs of 5S rDNA sites (two proximal on the short arm and one terminal on the long arm) and four 35S rDNA sites located on the short arm, two proximal, found in *L. pratense*, and two on terminal position, as well found in *F. glaucescens*. *Festuca glaucescens* has three pairs of 35S rDNA sites. In addition to a terminal pair in common with *L. arundinaceum*, *F. glaucescens* has two other pairs located in terminal position in both arms of the same chromosome [44]. The hypotheses raised by the authors are that *F. glaucescens* acquired a new site after the formation of the original allohexaploid hybrid or that these two sites, which were not visualized in *L. arundinaceum*, were deleted from the genome in the hybrid species [44].

The proposed allopolyploid origin for *F. fimbriata* and *F. ulochaeta* is plausible when considering the morphometric differences between the chromosomes, the distribution of the rDNA sites and the fact that the chromosomes do not group into a set of six homomorphic ones, which is expected in autopolyploidy. The structure of the ideograms is similar to that observed for other species of South American hexaploids of *Festuca*, which, in addition to the polymorphism of the chromosomes, shows a low or absent multivalent frequency in meiosis [16, 21–23, 40]. This same behavior was described for other genera of temperate grasses, such as *Bromus* L. [50], *Poa* L. [51] and *Briza* L. [52].

The karyotypes of *F. fimbriata* and *F. ulochaeta* were classified as symmetrical according to criteria of Stebbins [37]. This symmetry was observed in 12 other hexaploid species of South American *Festuca* [40], and in other species of Eurasian *Festuca*. Both species studied presented a karyotype formula 2n = 6 × = 36 m + 6sm, similar to four of the 14 species studied by Dubcovsky and Martínez [40]: *F. thermarum* Phil., *F. scabriuscula* Phil., *F. acanthophylla* E. Desv. and *F. ventanicola* Speg.. Among these species, *F. acanthophylla* also presented submetacentric chromosomes in the pair 14 as for *F. fimbriata* and in pairs 10 and 19, as for *F. ulochaeta*.

Despite the karyotypic similarities between *F. fimbriata* and *F. ulochaeta* with *F. acanthophylla*, we still cannot confirm common ancestry among them. The absence of reports of diploid species of *Festuca* in South America [40] suggests a possible ancestry for species restricted to Eurasia [6, 14, 53]. New cytogenetic studies using genomic in situ hybridization (GISH), for example, may help to determine the parents of *F. fimbriata*, *F. ulochaeta* and other species native to the Southern Hemisphere and to establish relations of homology with the Eurasian species.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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ARTIGO 2 - DIRECT EVIDENCE FOR CROSSOVER AND CHROMATID INTERFERENCE IN MEIOSIS OF TWO PLANT HYBRIDS (*Lolium multiflorum*×*Festuca pratensis* AND *Allium cepa*×*A. roylei*)

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RESEARCH PAPER

Direct evidence for crossover and chromatid interference in meiosis of two plant hybrids (*Lolium multiflorum*×*Festuca pratensis* and *Allium cepa*×*A. roylei*)

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Abstract

Crossing over, in addition to its strictly genetic role, also performs a critical mechanical function, by bonding homologues in meiosis. Hence, it is responsible for an orderly reduction of the chromosome number. As such, it is strictly controlled in frequency and distribution. The well-known crossover control is positive crossover interference which reduces the probability of a crossover in the vicinity of an already formed crossover. A poorly studied aspect of the control is chromatid interference. Such analyses are possible in very few organisms as they require observation of all four products of a single meiosis. Here, we provide direct evidence of chromatid interference. Using *in situ* probing in two interspecific plant hybrids (*Lolium multiflorum*×*Festuca pratensis* and *Allium cepa*×*A. roylei*) during anaphase I, we demonstrate that the involvement of four chromatids in double crossovers is significantly more frequent than expected (64% versus 25%). We also provide a physical measure of the crossover interference distance, covering ~30–40% of the relative chromosome arm length, and show that the centromere acts as a barrier for crossover interference. The two arms of a chromosome appear to act as independent units in the process of crossing over. Chromatid interference has to be seriously addressed in genetic mapping approaches and further studies.

Keywords: Centromere, chromatid interference, crossover interference, homoeologous chromosome, hybrid, meiosis, recombination.

Introduction

Meiotic division is an integral part of sexual reproduction; it maintains stable chromosome numbers over generations. Chiasmata, the cytological expression of crossing over, form mechanical connections between homologous chromosomes during the first meiotic division and hence are essential elements of an orderly reduction of the chromosome number. As

such, crossovers are under strict genetic control, for both their number and distribution. This control is exercised via positive crossover interference which, in essence, has a suppressive effect on the formation of another crossover in the vicinity of an already established crossover. On the other hand, a mechanism of crossover assurance guarantees that each pair of homologues gets at least one, the so-called obligatory crossover (Jones, 1984; John, 1990; Hillers, 2004; Jones and Franklin, 2006). A single crossover between two homologues ensures their normal be- haviour in meiosis. The number of potential sites of crossing over, represented by double strand breaks (DSBs), is always far greater than the number of eventual crossovers, and is presumably regulated genetically and epigenetically at the whole-genome level (Wang and Copenhaver, 2018; Modliszewski *et al*., 2018). Because of the tight control, the number of cross- overs per bivalent varies between one and three for a majority of species, and only loosely correlates with chromosome length, offering more evidence for the regulation of crossover numbers (Mather, 1938; Fernandes *et al*., 2018; Otto and Payseur, 2019).

Crossover interference was observed and described already in the very first genetic mapping experiments in *Drosophila melanogaster* (Sturtevant, 1913; Muller, 1916) and is defined as a situation where the formation of one crossover reduces the probability of another crossover in its vicinity. Its existence can be readily verified in any genetic mapping exercise because the frequency of double crossovers between any sufficiently close loci on a chromosome is always lower than the product of frequencies of independent crossovers in these segments. The frequency of double crossovers is lower the closer the monitored intervals are to each other. As a consequence, crossover events are not randomly distributed along chromosomes (Portin, 2012; Zickler and Kleckner, 2016). Generally, the extent of crossover interference decreases with distance, understood in genetic and not physical terms. In polyploids such as wheat, genetic lengths of homoeologous chromosomes (their genetic maps) are essentially the same despite large differences in their length/DNA content, demonstrating that double crossovers, when they occur, are formed much more closely physically in shorter chromosomes than in longer chromosomes. Instances of negative crossover interference have been observed frequently in lower organisms such as fungi, but at times also in certain chromosome variants in *Drosophila*, barley, and maize (Auger and Sheridan, 2001; Esch and Weber, 2002). Whether negative interference exists in higher organisms with normal chromosome structure is debatable (Säll and Bengtsson, 1989). Widespread strong negative interference was invoked in genetic mapping in tetraploid wheat (Peng *et al*., 2000) but, with genetic maps roughly twice as long as can reasonably be expected based on chiasma frequencies and mapping in very similar populations (Lukaszewski and Curtis, 1993), marker scoring errors appear as an equally plausible explanation. Similarly, the issue of the independence of the two arms of a chromosome for crossover formation has not yet been resolved satisfactorily, and crossover interference across the centromere remains a point of dispute, with some evidence pointing in opposite directions (Laurie and Hultén, 1985; Zhao *et al*., 1995a; Berchowitz and Copenhaver, 2010).

The mechanism of crossover interference remains unclear, but probably involves some crossover-discouraging signal or substance that spreads along the chromosome arm. The mechanical stress model of crossover interference is based on the presumption that the stress drives formation of a crossover, but in doing so the local area is then relieved and there is not enough stress to drive formation of a second crossover nearby (Börner *et al*., 2004; Kleckner *et al*., 2004). The polymerization model assumes that once the crossover structure is attached along the synaptonemal complex, it has the same chance per unit time to initiate a bidirectional polymerization event. The structures responsible for it are presumably the late recombination nodules which can be observed in pachytene (King and Mortimer, 1990). Crossover interference clearly involves the synaptonemal complex as it is not transmitted via synaptonemal complex discontinuities, such as synaptic partner exchange points in translocation heterozygotes, or across separated centromeres in double ditelocentric lines (reviewed in Dawe, 1998). Interestingly, there appear to be two pathways leading to crossovers, one interference sensitive and the second interference insensitive (Osman *et al*., 2011; Mercier *et al*., 2005). In plants, the former is more abundant and accounts for ~80–85% of total crossovers (Berchowitz *et al*., 2007).

A bivalent in the first meiotic division is composed of two pairs of sister chromatids. Additional crossovers in a chromo- some (or a chromosome arm) bring up the issue of chromatid choice for each event. It is generally accepted that for any given crossover, the chromatid choice is random, and hence a crossover involving two non-sister chromatids does not affect the choice of non-sister chromatids for another crossover in the same chromosome (Zhao *et al*., 1995a). This is, however, an assumption with very limited experimental support, particularly in higher organisms. Direct tests of chromatid interference require analyses of all four products of any given meiosis. This condition is met only in a very few instances, all in lower organisms, such as *Aspergillus* spp. where chromatid interference does not appear to operate (Whitehouse, 1958). In higher organisms, re-analysis of experimental data from several organisms (Zhao *et al*., 1995b) and mathematical modelling of genetic mapping data suggest that chromatid interference may exist (Teuscher *et al*., 2000), but no direct observation has been possible thus far.

Crossover and chromatid interference can be analysed in several different ways. Generally, the former can be studied directly, and copious raw data for such analyses are generated in each genetic mapping effort, especially when mapped loci can be directly placed on the DNA sequence assemblies. Chromatid interference, on the other hand, in a great majority

of organisms can be studied only indirectly, such as by mathematical modelling (Teuscher *et al.*, 2000) mentioned above. However, recent technological advances offer a chance to study both phenomena directly by cytological observation in meiosis. One such approach is to visualize sites of crossovers in meiotic cells with fluorescently labelled probes, for example for the MLH1 mismatch repair protein (Barlow and Hultén, 1998; Phillips *et al.*, 2013; Anderson, 2014). This approach, however, may lack the resolution of individual chromatids. Another way is the analysis of the anaphase I chromosomes in hybrids where the DNA sequence divergence between the parental genomes permits chromosome painting, hence direct visualization of the crossover points. Thus, it is possible not only to score the overall level and distribution of crossovers in individual chromosome arms, chromosomes, and entire genomes, but also to study their distribution among chromatids. Another advantage of this approach is that the results are not biased by potential gametophytic selection, which may distort the ratios of recombinant and parental chromosomes transmitted to the progeny. Its weakness is in the selection of parents: these need to be sufficiently different at the DNA sequence level to enable unambiguous discrimination of homologues by chromosome painting, and yet sufficiently close genetically to provide for regular chromosome pairing and disjunction. Such hybrids do exist among plant species. Here we focus on the F_1 hybrids between representatives of *Lolium* and *Festuca*, known for a high (87–97%) metaphase I chromosome pairing and crossing over (Jauhar, 1975; Kopecký *et al.*, 2008). Scoring crossovers in anaphase I, where individual chromatids are clearly visible, offers direct evidence not only for crossover interference but also for chromatid interference and possible effects across the centromere. Cytogenetic stocks from our past experiments (Kopecky *et al.*, 2008) made it possible to analyse the physical attributes of crossing over in individual chromosomes and their arms, while high-density chromosome genetic maps (Kopecky *et al.*, 2010) made it possible to relate these observations to the DNA sequence across the entire genome. Similar observations were made on wide hybrids in the genus *Allium* (*A. cepa*×*A. roylei*) displaying frequent pairing and recombination between homoeologous chromosomes (Khrustaleva and Kik, 2000).

Materials and methods

Plant material and chromosome preparations

The frequency of possible types of crossover configurations during meiosis was evaluated in diploid F_1 hybrids of *Festuca pratensis*×*Lolium multiflorum*. Chromosome substitution lines of *F. pratensis*/*L. multiflorum* hybrids were used to estimate the frequency and distribution of double crossovers for all seven *F.pratensis* chromosomes individually. The tetraploid monosomic substitution lines ($2n=4x=28$; 27L+1F) were developed in previous studies (Kopecký *et al.*, 2008, 2010). Plants with the same substituted *F.pratensis* chromosome were intercrossed and their progenies were germinated and individually analysed. Similarly, the F_1 hybrids of *A. cepa*×*A. roylei* (Scholten *et al.*, 2016) were intercrossed and their progenies were individually screened by genomic *in situ* hybridization. Individual anthers confirmed to be in meiotic anaphase I stage and root tips were fixed in Carnoy's solution (absolute ethanol/glacial acetic acid, 3:1 v/v) at 37 °C for 7 d and microscope preparations were made according to Masoudi-Nejad *et al.* (2002).

Genomic in situ hybridization

Genomic *in situ* hybridization analyses were done on mitotic and meiotic chromosome spreads according to Kopecký *et al.* (2008). Total genomic DNA (gDNA) of *L. multiflorum* and *A. cepa* was used as blocking DNA and total gDNAs of *F. pratensis* and *A. roylei* were labelled with digoxigenin (DIG) using the DIG-Nick Translation Kit (Roche Applied Science, USA) according to the manufacturer's instructions, and used as probes for grass and onion hybrids, respectively. The probe/blocking DNA ratio was ~1:150. Signal detection was made with anti-DIG–fluorescein isothiocyanate (FITC) conjugate (Roche Applied Science). Chromosomes were counterstained with DAPI in Vectashield (Vector Laboratories, Oberkochen, Germany). Chromosome analysis was done under an Olympus AX70 microscope equipped with epifluorescence and a SensiCam B/W camera. Images were captured with Micro Image and processed with Adobe Photoshop v.6 software.

Scoring and statistical treatment

All statistical analyses were done in R 3.4.3 (R Core Team, 2019). For meiotic analyses of diploid F_1 hybrids of *L. multiflorum*×*F. pratensis*, we evaluated exclusively the anaphase I stage. Each cell has seven pairs of chromosomes. Each arm was evaluated separately. Each pair of homologous arms displayed either no crossover or one of the possible crossover exchanges: single crossover or double crossover with two, three, or four chromatids involved. Theoretically, with no chromatid interference, the proportion of the three crossover types should be 1:2:1. We statistically treated the observed frequencies for this ratio using the multinomial test from the 'stats' library (R Core Team). Proportions of different numbers of crossovers on one arm between chromosomes with one and two crossovers on the other arm were compared using the two-sided Fisher's exact test. For calculation, the function Fisher.test with *P*-values computed by Monte Carlo simulations based on 2000 replicates from the 'stats' library was used.

To determine the positions of the crossover events among the progenies of onion and grass hybrids, we measured the lengths of the introgressed segments and the lengths of both arms of recombined chromosomes using ScionImage software. Calculations of the distances (in megabases) between two crossovers on one arm were done based on the length of individual *F.pratensis* chromosomes (Kopecký *et al.*, 2010). The difference in distribution of different crossover types was evaluated by comparing their distributions along chromosome arms divided into 10 segments (bins) of 10% of their length. Two empirical distributions were compared using the function ks.boot in the 'Matching' library in R. The function uses a bootstrap version of the Kolmogorov–Smirnov test, providing accurate coverage even when the distributions being compared are not entirely continuous and ties occur in the dataset (Sekhon, 2011).

Results

Frequency of crossovers in anaphase I configurations in Lolium×Festuca *hybrids*

Using *in situ* probing with labelled total gDNA, we monitored crossover events involving individual chromatids in each chromosome of 96 meiocytes of *L. multiflorum*×*F. pratensis* ($2n=2x=14$; seven *L. multiflorum* chromosomes + seven

F. pratensis chromosomes) F_1 hybrids in anaphase I of meiosis. All crossovers involved chromosomes of *L. multiflorum* and *F. pratensis* and were therefore exclusively homoeologous. In total, there were 672 chromosome pairs and, subsequently, 1344 pairs of chromosome arms. Single crossovers were observed in 308 chromosome pairs and two crossovers (each on one arm) in 66 chromosomes pairs, while double crossovers (two crossovers in one arm) were observed in 133 chromosome pairs. Chromosome pairs with double crossovers in one arm and a single crossover in the other arm were observed in 26 cases. Double crossovers on both arms were observed in only six chromosome pairs. The remaining 133 pairs of chromosomes (19.8%) had no detectable crossovers.

Direct evidence of chromatid interference

Each pair of chromosomes or chromosome arms involves two pairs of sister chromatids. When two crossovers occur, two, three, or four chromatids can be involved (Fig. 1). With a random choice of chromatids, a 1:2:1 ratio is expected for two, three, or four chromatid involvement. In total, 171 pairs of chromosome arms with double crossovers were observed (133 + 26 + 6×2) in *Lolium*×*Festuca* hybrids. Four chromatids were involved in 109 double crossovers (63.7%), three chromatids in 36 (21.1%), and two chromatids in 26 (15.2%) (Fig. 2). This is significantly different from the theoretical ratio of 1:2:1 for random choice of chromatids (χ^2=137.9, df=2, P<0.001). This is based on a genome-wide analysis as identification of individual chromosomes was not possible in this experimental system.

Crossover interference extends over ~30% of a chromosome arm length

Among the progenies of monosomic single chromosome substitution lines of *F. pratensis* into *L. multiflorum* (27 *Lolium* chromosomes and one *Festuca* chromosome) for each of the seven *Festuca* chromosomes (referred to as 'grasses'), we scored the frequencies of single and double crossovers following their transmission to progeny. The same analysis was done in the F_2 generation of the *Allium cepa*×*A. roylei* hybrids (referred to as 'onions'). Single chromosome substitution lines of grasses enabled scoring of crossovers in individual chromosomes; in onion hybrids, only a genome-wide analysis was possible. Among the progenies, three types of recombined chromosomes were observed: (i) single exchanges per chromosome; (ii) double exchanges per chromosome, one in each arm; and (iii) double exchanges in one arm. It should be mentioned that the segregation of chromosomes in anaphase I and sister chromatids in anaphase II separates the products of double crossovers. Consequently, only those double crossovers involving two of four chromatids and one half of those with three chromatids can be tracked in the progeny and are considered in the subsequent analysis. Conversely, all four products (chromatids) of a double crossover where all four chromatids are involved appear in the progeny as single events and, thus, are placed in the category of single crossovers.

Out of 629 and 328 recombined chromosomes of grasses and onions, respectively, the majority demonstrated single crossovers (63.8% and 65.8%), followed by two crossovers, one on each arm (23.2% and 21.7%) and double crossovers, sometimes with single or double crossover on the other arm (13.0% and 13.1%), respectively. The proportions of chromosomes in each of the three categories varied for individual *Festuca* chromosomes (Table 1).

Using progenies of single chromosome substitution lines, it was possible not only to estimate the frequencies of double crossovers for each of the *Festuca* chromosomes, but also to estimate the mean distances in megabases between adjacent crossovers in each chromosome (Fig. 3; Table 2). The frequencies of double crossovers differed markedly between the short and long arms. In general, the short arms had considerably lower frequencies of double crossovers compared with the long arms. In the shortest arm of the *F. pratensis* karyotype, that of chromosome 5, we did not observe any products of double crossover (involving two or three chromatids). With the obligatory chiasma and the positive crossover interference, this is a typical distribution as affected by the chromosome arm length. It was clearly evident in wheat during the genetic mapping of the physical attributes of chromosomes (Lukaszewski and Curtis, 1993), and was confirmed by ultra-high-resolution analysis (Jordan *et al.*, 2018).

On average, two adjacent crossover events on one arm of a single chromatid were separated by ~97 Mb. However, this distance showed considerable variation from one chromosome arm to another. The shortest distance between two adjacent crossovers in an arm was ~26 Mb. There was no correlation between chromosome/chromosome arm length (CL), the frequency of double crossover (FdCO), and the average distance (AD) between two crossovers on one arm (double crossovers involving two or three chromatids, but not four) as revealed by Spearman correlation coefficient (for arms, n=14: CL versus FdCO r=0.42, P=0.149; CL versus AD r=0.19, P=0.535; FdCO versus AD r= –0.15, P=0.635; for chromosomes, n=7, CL versus FdCO r= –0.43, P=0.333; CL versus AD r= –0.29, P=0.535; FdCO versus AD r= –0.20, P=0.670).

The distribution of single and double crossovers differed in both grass and onion hybrids (Fig. 4). The distribution of single crossovers in both hybrids showed similar patterns, with the highest frequency in the intervals from 20% to 40% of the arm length from the telomeres, and with a significant decline in frequencies nearing the centromeres (proximal 30% of the arm). Relatively low frequencies were also evident in the most distal 10% of the arm (next to telomeres). However, this may be an artefact of the method used, as short terminal segments from the non-probe parent may be undetectable by *in situ* probing with labelled DNA (Lukaszewski *et al.*, 2005). On the other hand, in grasses, double crossovers had the highest frequency in the intervals of 10–20% (the first crossover) and

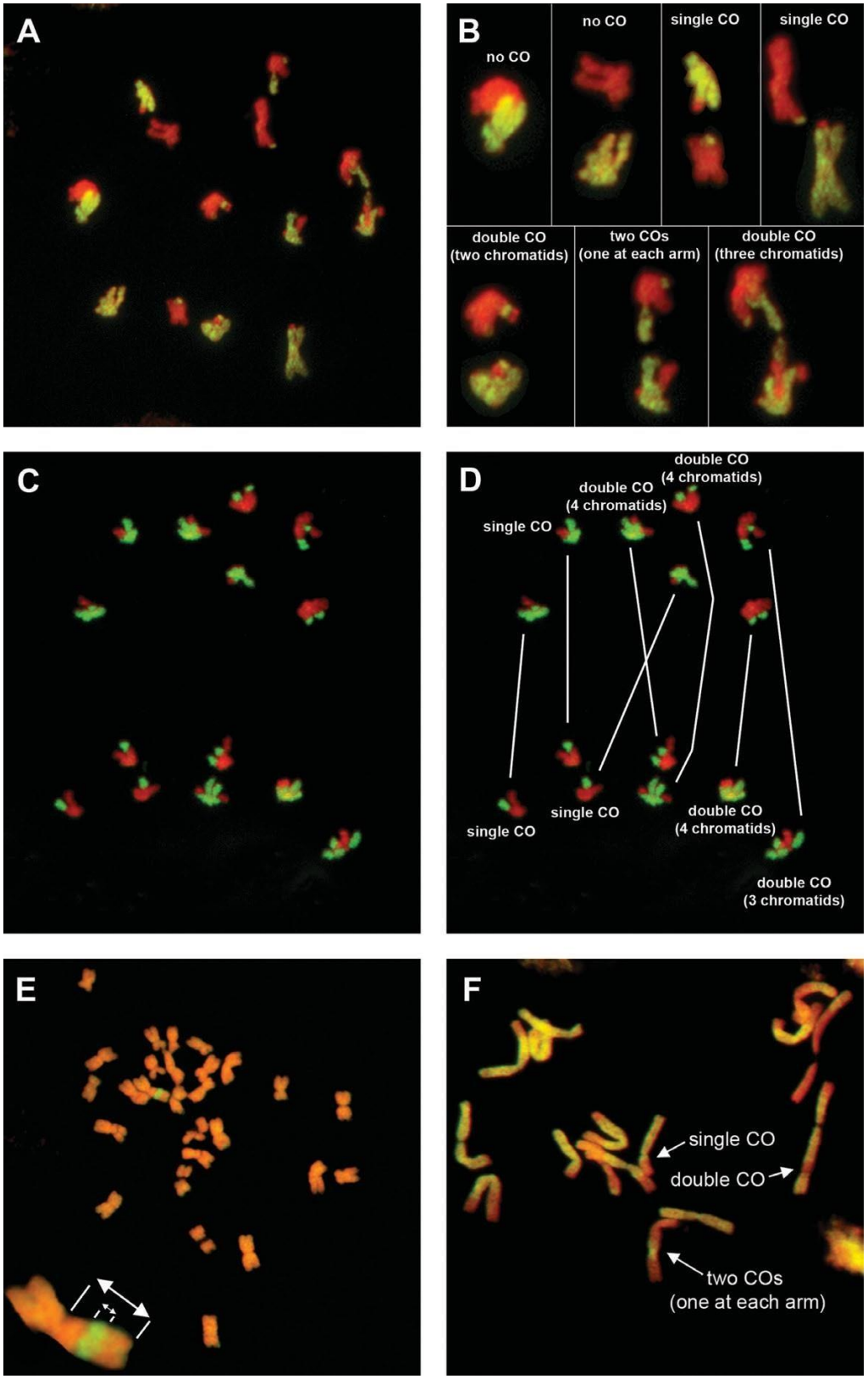
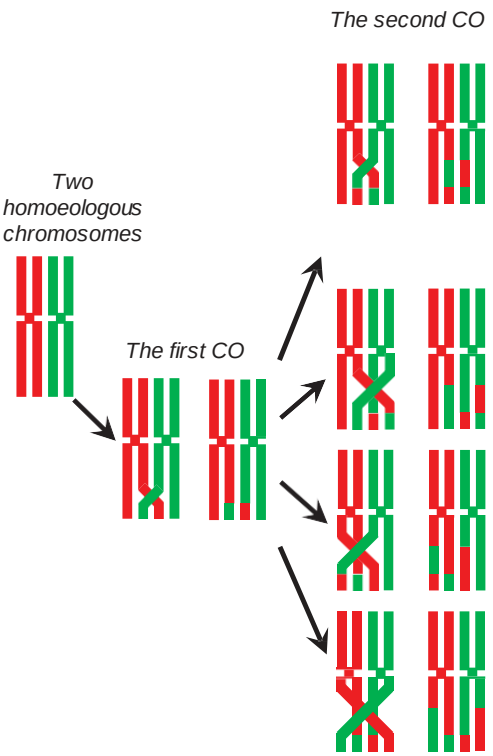


Fig. 1. Crossovers in grasses and onions visualized by genomic *in situ* hybridization. Various types of crossovers can be seen directly during anaphase I in F_1 hybrids of *Lolium multiflorum*×*Festuca pratensis* (A–D), in the mitotic cell of the progeny of *F. pratensis*/*L. multiflorum* single chromosome substitution lines (E), and in the mitotic cell of the progeny of F_1 *Allium cepa*×*A. roylei* hybrids (F). Insets provided in the upper right figure (B) are enlargements of the pairing partners in the metaphase I plate (A) with the description of crossover type. Similarly, white lines indicate pairing partners in the duplicated figure (D) of the metaphase plate (C). Total gDNA of *F. pratensis* and *A. roylei* was labelled with digoxigenin (green/yellow colour) and sheared DNA of *L. multiflorum* and *A. cepa* was used as blocking DNA (red pseudocolour).



No. of chromatids involved in CO	Observed numbers (frequency in%)	Theoretical numbers (frequency in%)
2	26 (15.2)	43 (25)
3	36 (21.1)	85 (50)
3		
4	109 (63.7)	43 (25)

Fig. 2. Theoretical and observed numbers and frequencies of double crossover types in diploid F_1 *L. multiflorum*×*F. pratensis* hybrids. Two, three, and four chromatids can be involved in a double crossover with theoretical proportions of 1:2:1 (assuming no chromatid interference).

Table 1. Numbers or chromosomes with and without homoeologous crossovers and frequencies (in parentheses) of different crossover types among the progeny of single chromosome substitution lines of *F. pratensis* into *L. multiflorum* (individually for each chromosome)

Fp/Lm chromosome	No crossover	Single crossover (%)	Two crossovers, one on each arm (%)	Double crossover (%)
1	5	63 (63)	21 (21)	16 (16)
2	7	59 (57)	24 (23)	21 (20)
3	4	58 (62)	25 (27)	10 (11)
4	6	56 (70)	18 (23)	6 (8)
5	3	62 (78)	10 (13)	7 (9)
6	2	26 (40)	27 (42)	12 (18)
7	6	67 (77)	10 (11)	10 (11)
Average		**(64)**	**(23)**	**(13)**

40–50% (the second crossover) of the arm measured from telomeres.This indicates that crossover interference in grasses acts up to a distance of ~30% of the average arm length. A similar crossover distribution was observed in onions, even though no clear frequency peaks were evident. Once we calculated the frequencies of the theoretical and actual appearance of double crossovers for all combinations of intervals (an interval equalled 10% of the physical arm length), the positive cross-over interference was observed between intervals 0–10% and 10–20%, 10–20% and 20–30%, 10–20% and 30–40%, 20–30% and 30–40%, 20–30% and 40–50%, 20–30% and 50–60%, 30–40% and 40–50%, and between 30–40% and 50–60% (data not shown). On average, two crossovers in one arm were separated by 36% and 41% of the arm length (ranging from 13% to 78% and from 9% to 83%) in onions and grasses, respectively (Fig. 4). This supports the indication that in both hybrids, crossover interference acts at a physical distance of 30–40% of an average chromosome arm. This does rule out the possibility that the crossover interference is governed by mechanisms modulated by the absolute physical distance between two crossovers.

In general, the average frequency of chromosomes with double crossovers in grasses was 13% (Table 1).The highest frequency of such events was ~20% for chromosome 2, which also had the lowest average distance between two adjacent crossovers (77 Mb) (Table 2), and the lowest was 8% for chromosome 4, which is the longest chromosome in the genome (Kopecký *et al.*, 2010) and seems to have strong crossover interference (average length

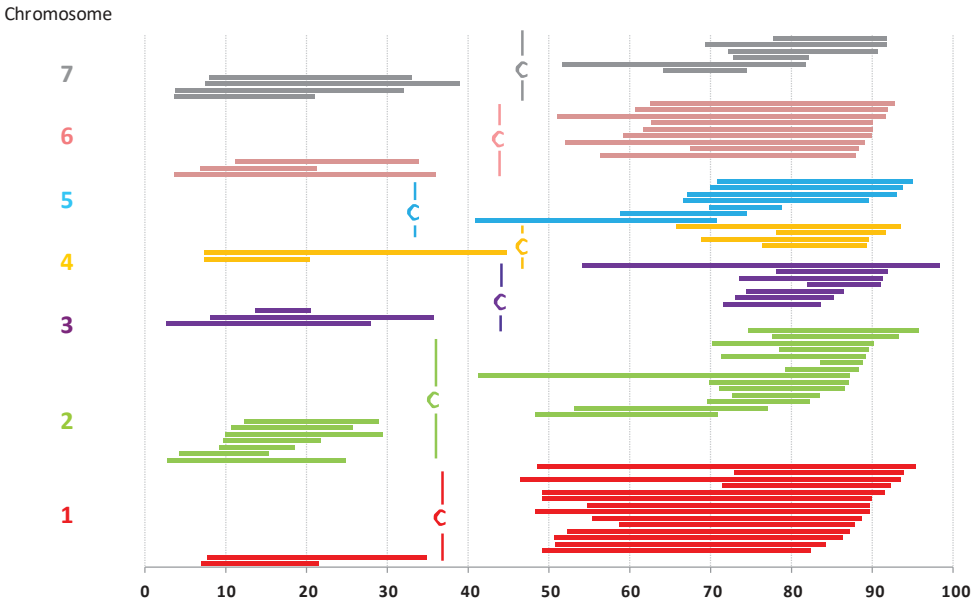


Fig. 3. The distribution of double crossovers along individual chromosomes of *F. pratensis*/*L. multiflorum*. Short arms are on the left and long arms on the right. The coloured lines represent the recombined segments in all chromosome arms (different colours). The position of the centromere in each chromosome is indicated by a vertical line with letter 'C'.

Table 2. Average distances between two events spanning double crossover for individual *F.pratensis*/*L. multiflorum* chromosomes/chromosome arms

Fp/Lm chromosome	Length(μm[a])	1C (Mb[a])	Average intercrossover distance and the range (in parentheses; in Mb)
1	4.67	373	122
1S	1.71	137	77 (55–100)
1L	2.96	237	129 (74–168)
2	6.07	485	77
2S[b]	2.42	194	72 (49–99)
2L	3.64	291	80 (26–147)
3	6.25	499	90
3S[c]	2.92	233	97 (35–134)
3L	3.33	266	87 (45–221)
4	6.79	543	112
4S	3.18	254	139 (70–209)
4L	3.61	289	99 (62–149)
5	5.04	403	79
5S	1.76	140	–
5L	3.29	263	79 (43–102)
6	4.93	394	107
6S	1.97	158	85 (54–118)
6L	2.95	236	115 (84–147)
7	6.05	484	97
7S	2.90	231	123 (89–145)
7L	3.16	252	80 (42–134)
Average			**97.2**

[a] 1C=molecular size of one copy of individual chromosome/chromosome arm; adopted from Kopecký *et al.* (2010).
[b] Chromosome arm with 5S rDNA.
[c] Chromosome arm with 45S rDNA.

between two events spanning a double crossover was 112 Mb) (Table 2). On the other hand, chromosome 1 is the shortest in the *F. pratensis*/*L. multiflorum* genomes (Kopecký *et al.*, 2010) but it showed the highest average distance between adjacent crossovers. This suggests that the interference distance in the system studied here may be chromosome specific and not genome wide.

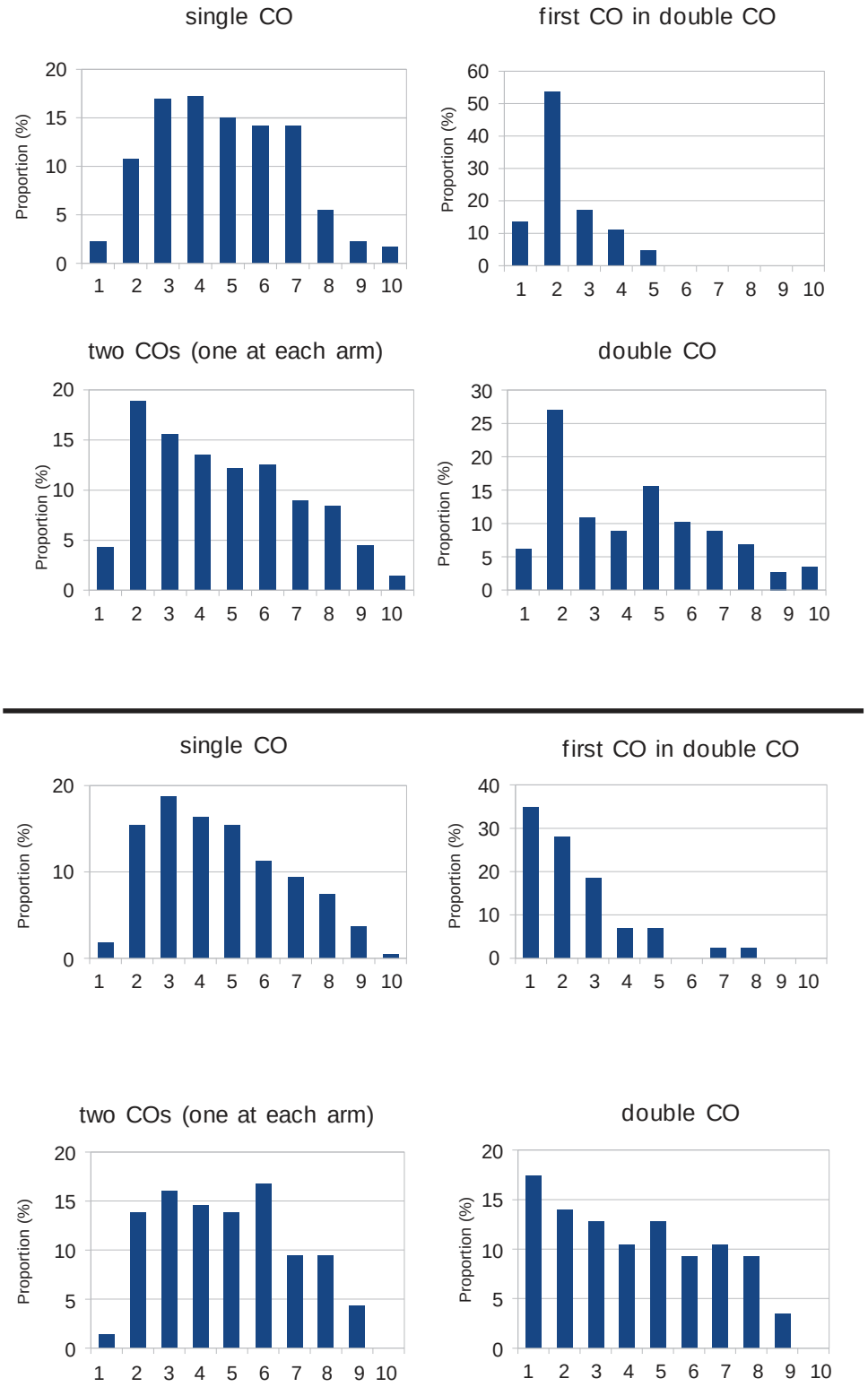


Fig. 4. The frequency and distribution of crossovers based on their types in grass (upper part) and onion (lower part) hybrids. The *x*-axis represents a chromosome arm (from the telomere on the left to the centromere on the right) divided into bins of 10% of relative arm length.

There was a significant difference in the distribution of single crossovers along the chromosome arms and of the first crossover when two crossovers were present on an arm, both in grasses (Kolmogorov–Smirnov test, D=0.542, bootstrap $P<0.001$) and in onions (D=0.455, bootstrap $P<0.001$)

(Fig. 4). When two crossovers were present, the first crossovers were much more distal, with a peak at 10–20% of the arm length, and dropped rapidly towards the centromere. In grasses, the first crossovers of double crossover arms were limited to the distal half of the arms. A similar distribution

Table 3. Average positions of crossovers based on their abundance (in % of the arm length, measured from the telomere)

Type of crossover	Average position of crossover (% of arm from telomere)	
	Onions	Grasses
Single crossover	41.32	43.5
Two crossovers (one on each arm)	43.85	40.6
Single crossover with double crossover on the other arm	46.49	38.6

Table 4. The relationship between the number of crossovers in one chromosome arm and the number of crossovers in the other arm

	Grasses			
No. of crossovers onone arm	Number of crossovers on the other arm			
	0	1	2	3
0		308	133	0
1	308	66	26	0
2	133	26	6	0
3	0	0	0	0
	Onions			
No. of crossovers onone arm	Number of crossovers on the other arm			
	0	1	2	3
0		215	31	2
1	215	57	9	1
2	31	9	0	0
3	2	1	0	0

was observed in onions, with a gradual decrease from the telomere toward the centromere. It is evident that second crossovers on an arm are possible only when the first cross- over is sufficiently distal.

Crossover interference does not act across the centromere

To test if crossover interference acts across the centromere, or if the two arms of a chromosome are independent for crossover formation, we performed three comparisons between the expected values and the experimental data. If the interference acts across the centromere, the following should apply. (i) The distribution of single crossovers and of two crossovers (each on one of the arms) should differ, with the latter located more distally towards the telomeres. Indeed, we observed that the distribution of single crossovers per chromosome and two crossovers per chromosome with one at each arm differed significantly in grasses (Kolmogorov–Smirnov test, D=0.101, bootstrap P=0.002), but not in onions (D=0.079, bootstrap P=0.334). In grasses, however, single crossovers were distributed primarily in the interstitial parts of the chromosome arms, while two cross- overs were more spread over the arms and were localized relatively frequently outside the interstitial regions. In on- ions, single crossovers and two crossovers were spread over the arms, except for a rapid decline in the centromeric and pericentromeric regions. (ii) When a double crossover is formed on one arm, the distribution of crossovers on the other arm should be more distal. Although there were not enough such cases for statistical tests, the average positions of crossovers were similar between a single crossover, two crossovers (one on each arm), and a single crossover with a double crossover on the other arm (Table 3). (iii) With two crossovers in one arm, there should be fewer crossovers in the other arm as compared with a single crossover in the first arm. No difference was observed in the distribution of crossovers in the second arm when comparing one and two crossovers in the first arm in both grasses (Fisher's exact test, P=0.415) and onions (Fisher's exact test, P=0.754) (Table 4). These three tests indicate that in grasses and in onions, crossover interference does not act across the centromere; two arms of a single chromosome appear to be independent for establishment of the crossover.

Discussion

This study was performed on wide hybrids and so observations and results may not be fully representative of strictly homologous chromosome pairing and recombination. Wide hybrids made direct observations possible and offered a unique chance of studying the immediate effects of crossing over as early as anaphase I, but at the same time they might have skewed the results in some unpredictable direction. However, these hybrids show surprisingly high regularity of meiotic chromosome pairing and segregation (Jauhar, 1975; Kopecky *et al.*, 2008), suggesting that the results may be of wider significance. On the other hand, there is a chance that the distribution of crossovers, as judged by the exchange points in recombined chromosomes, may be wider than what could be expected based on the chiasma distribution in the parental species (Karp and Jones, 1983). It also must be pointed out that data on the physical distribution of crossovers along chromosomes and chromosome arms do carry an inherent error. There are differences in genome sizes of the parental species of both hybrids, and these translate into differences in chromosome lengths. Whether these differences are evenly distributed along all chromosomes is very far from clear. For that reason, most of the analyses are based on relative length values.

Chromatid interference

The ability to distinguish parental chromatin by chromosome painting allowed us to analyse the frequency and distribution

of crossovers directly during the meiotic division, immediately after bivalent separation, and ensured that all products of every individual meiosis could be scored (Fig. 1). This avoided any possible bias associated with uneven chromosome transmission and gametic or zygotic selection. These direct observations were supplemented with earlier observations of the structure of chromosomes among the progeny.

Ever since Barbara McClintock's demonstration of the relationship between chiasmata and crossing over (Creighton and McClintock, 1931), chiasmata alone indicated that crossovers were not distributed evenly along chromosomes. This has been demonstrated in many organisms and in several different ways (reviewed in Otto and Payseur, 2019). The pattern of crossovers is affected by genetic and presumably epigenetic systems, with a general preference for distal location, and perhaps by the chromatin structure, which makes some parts of the chromosomes inaccessible to crossing over, regardless of the position on the telomere–centromere axis (Lukaszewski, 2008; Lukaszewski *et al.*, 2012; Higgins *et al.*, 2014).Within this general pattern, if more crossovers are formed in an arm, their frequencies and distribution tend to form a sinusoid pattern, reflecting the presence and strength of the positive crossover interference (Muller, 1916; Mather, 1938).

Of the two components of genetic interference, crossover interference can be easily scored in any genetic mapping experiment. The second component, chromatid interference, is far more difficult to observe. It informs if the chromatids involved in the first crossover affect the choice of chromatids for a second crossover on the same chromosome. Chromatid interference can only be reliably and directly scored when all four products of a single meiosis are available, and this happens infrequently. Consequently, the current knowledge of chromatid interference stems from statistical models, and the general assumption is that chromatid interference does not exist. Geneticists generally assume its absence while studying recombination and creating genetic maps (Strickland, 1958; Broman and Weber, 2000; Broman *et al.*, 2002).

Early studies on *Neurospora crassa*, *Saccharomyces cerevisiae*, and *Aspergillus nidulans* indicated that the choice of chromatids for second crossovers may not be random (Lindegren and Lindegren, 1942; Hawthorne and Mortimer, 1960). Similarly, the model of Teuscher *et al.* (2000), based on data from a study on mouse and two studies on *Drosophila*, suggested that chromatid interference may play an important role in meiosis. Similarly, weak chromatid interference was deduced in maize, human oocytes, and *Caenorhabditis elegans* (Hou *et al.*, 2013; Li *et al.*, 2015; Li *et al.*, 2018). Our study offers clear and unambiguous evidence for a strong positive chromatid interference: the second crossover in an arm was formed more frequently between chromatids not involved in the first crossover (Fig. 2). In other words, all four chromatids of two homoeologous chromosomes were involved in double cross- overs much more frequently than expected (64% versus25%) and the most abundant class expected for random chromatid selection, that with three chromatids involved, was seriously under-represented, accounting for only 21% of cases versus 50% of those theoretically expected. Thus, our results provide strong support for the model of Teuscher (2000) and deliver interesting results for further studies on crossover formation and recombination in plants. They also point out a need to consider chromatid interference in genetic mapping. We envisage that newly developed visual assays for yeasts and the model plant Arabidopsis (Berchowitz and Copenhaver, 2008), which enable scoring all four products of meiosis, could increase our understanding of chromatid interference in the near future.

The molecular mechanism underlying chromatid interference is unknown. However, a study on *C. elegans* suggests that an orthologue of the breast cancer susceptibility gene 1 (*BRCA-1*) may be involved in this pathway.Numbers of double crossovers with two chromatids involved were reduced and numbers of single crossovers were elevated in the *brc-1*; *zim-1* mutant compared with the *zim-1* single mutant. This indicates that BRCA-1 may counteract chromatid interference under meiotic dysfunction, such that more crossovers with the same two chromatids involved occur (Li *et al.*, 2018). Some studies also pointed to the role of Tel1, a protein kinase that responds to DNA damage, in both crossover and chromatid interference (Zhang *et al.*, 2011; Garcia *et al.*, 2015; Cooper *et al.*, 2016). According to Fowler *et al.* (2018), Tel1 is activated when the first formation of DSBs occurs in a clustering hotspot, promoting the phosphorylation and inactivation of some components of the DSB-forming complex, which prevents the formation of a second DSB in the vicinity. In the absence of Tel1, multiple breaks are formed, increasing the frequency of crossovers. The clustering of DSB hotspots can involve each homologous chromosome separately (two chromatids) or both together (four chromatids), and in species where clustering involves the homologues pair the interference is stronger (Smith and Nambiar, 2020). As such, cluster gathering of four chromatids may explain the chromatid interference found in our study.

Crossover interference

Apartfrom a fewexceptions,such as *A. nidulans* and *S. pombe* discussed above,crossover interference is a widespread phenomenon in eukaryotes (Strickland,1958;Snow,1979),but its strength and spatial efficiency differ among species. In roundworm *C. elegans*, the absence of double crossovers may be taken as an indication of complete crossover interference (Meneely *et al.*, 2002). Similarly, crossover interference appears to be extremely strong in mouse (Broman *et al.*, 2002). In other species, it may be strong in short intervals: it is almost complete in *D. melanogaster* and very strong in *Neurospora crassa* (Foss *et al.*, 1993).

Similar to genetic studies based on screening of progeny to assess the crossover interference, our approach did not allow

incorporation of a double crossover where four and three chromatids were involved. Thus, it provides detailed characterization of crossover interference arising exclusively from double crossovers involving two chromatids. In grasses and onions, we never observed double crossovers at a distances shorter than 9% and 12% of the arm length, respectively. Using genome-wide numbers, this translates to ~37 Mb and 241 Mb (Labani and Elkington, 1987; Doležel *et al*., 1992; Kopecký *et al*., 2010). As the distance from the initial crossover increases, the interference strength weakens and chances for a second crossover increase. Crossover interference is effective up to 15–20 Mb in *Drosophila*, corresponding to about one half of a chromosome arm, and up to 0.9 Mb in *Neurospora*, corresponding to ~30% of an average arm length (Foss *et al*.,1993). Similarly, Lawrie *et al*. (1995) observed crossover interference extending over ~68% and 77% of a chromosome length in mouse autosomes 5 and 15, corresponding to about one-third of the arms. Complete crossover interference over distances equalling 25–30% of a chromosome arm has been confirmed in *Chorthippus branneus* (Jones, 1987). In budding yeast, Malkova *et al*. (2004) detected interference overdistances of about a quarter of the total length of a chromosome arm.

The examples listed above indicate that the effectiveness of crossover interference is surprisingly similar among the species tested, and it extends up to about one-third of a chromo- some arm. Even though we worked with wide hybrids, the results follow the same pattern: in both cases, crossoverinterference acts efficiently up to ~30–40% of the chromosome arms (Fig. 3). The same range was observed in the B genome of wheat (Lukaszewski and Curtis, 1993). Interestingly, it appears that it is the relative distance along the chromosome arm and not the physical or genetic distance that matters. The two hybrids used in our study differ significantly in their genome sizes and lengths of chromosomes in megabases: an average chromosome in onions is ~2 Gb in length, while it is ~0.4 Gb in grasses (Doležel *et al*., 1992; Kopecký *et al*., 2010), corresponding to a physical crossover interference distance of ~350 Mb and 100 Mb, respectively (Table 2). Mean interchiasma distances in mouse and *S. cerevisiae* chromosomes gave the impression of being correlated with chromosome lengths so that the mean interchiasma distance was greater in longer chromosomes (Lawrie *et al*., 1995). The possible mechanism explaining this phenomenon involving a conformational chain reaction leading to the allosteric blocks of recombination in neighboring regions in a time-dependent manner has been described by Kaback *et al*. (1999). This indicates that the processes underlying crossover interference maybe evolutionarily conserved. Weshould note that despite the general mechanism of crossover interference acting at the whole-genome level, there are exceptions where crossover interference has variable intensities and distributions in different regions of a chromosome,suchasobserved in budding yeastand in *Populus euphratica* (Malkova *et al*., 2004;Wang *et al*., 2019).

As in other cases studied earlier (see John, 1990), we observed that second crossovers on an arm are predominantly formed only when the first crossovers are sufficiently distal (Fig. 4). This may be related to the progress of synapsis, from the telomere toward the centromere, with the distal crossover (presumably formed first) leaving enough room on the arm for crossover interference strength to drop before the end of the recombination process, hence increasing the opportunity for an additional crossover to form. The role of the centromere as a barrier for crossover interference remains unclarified(reviewed in Nambiar *et al*., 2019). The question is: are the two arms of a chromosome independent in crossover formation or can a crossover on one arm interfere with crossovers on the other arm? The current evidence is unclear. No positive interference was observed across the centromere in *N. crassa* (Strickland, 1961; Perkins, 1962). On the other hand, in the grasshopper, Colombo and Jones (1997) provided evidence that interference did act across the centromere, and the level of interference in the region spanning centromeres appeared to be no different from that seen in any other chromosome segment. Similarly, the chiasmata numbers on two arms of a single chromosome do not appear to be independent in humans (Broman and Weber, 2000) or in species such as *Culex* and *Paeonea* (Callan and Montalenti,1947; Harte, 1956). In this study, we did not observe any significant effect of crossovers in one arm on the crossover frequency and distribution in the other arm, implying that in our material crossover interference may not extend across the centromere (Tables 3, 4). However, crossover distribution might have also played a role here: with predominantly distal crossovers, the distance across the centromere in most cases would be greater than the interference distance.

In conclusion, our study clearly illustrates that chromatid interference does operate in higher Eukaryotes, along with crossover interference. Both play an important role in meiotic division, the latter acting over a distance corresponding to 30–40% of the physical length of a chromosome arm. In materials studied here, the centromere appears to act as an effective barrier for crossover interference and thus two arms of a chromosome seem to act as independent units for crossovers. We are aware that our study is based on observations of crossovers between homoeologous chromosomes in wide hybrids and these may not completely correspond to interactions between perfectly homologous chromosomes. However, meiotic chromosome pairing in these hybrids is essentially normal (for illustrations of meiosis in the *Lolium*×*Festuca* hybrid see https://olomouc.ueb.cas.cz/ getattachment/Research-groups/Kopecky-group/Meiosis- With-Labeled-Parental-Genomes.pdf.aspx?lang=en-US). Moreover, all crossovers must meet certain DNA criteria, both for the substrate length and for the level of homology (Shen and Huang, 1986, 1989; Datta *et al*., 1997) and there is no reason to suspect that the crossovers observed here

violate them. Given the recent progress of technology, we envisage that it will be possible to monitor both homologous and homoeologous crossovers in the near future using tetrad analysis based on the *quartet1* (*qrt1*) mutation, haplotype-specific chromosome painting as shown in maize, or the DeepTetrad technology currently available only for *Arabidopsis thaliana* (Copenhaver *et al.*, 2000; Martins *et al.*, 2019; Lim *et al.*, 2020).

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Author contributions

Conceptualization:AJL, DK; data curation: MTMF,MG,AJL, DK; formal analysis:MG,MD,DK;funding acquisition:JD,DK;investigation: MTMF, KP, MK, AJL, DK; methodology: AJL, DK; project administration: DK; resources: OS,VHT,AJL, DK; supervision:VHT, JD,AJL, DK; validation: AJL, DK; visualization: MTMF,MG,KP,AJL, DK; writing—original draft preparation: MTMF, AJL, DK; writing—review and editing: OS, VHT, JD, AJL, DK.

Data availability

All data supporting the findings of this study are available within the paper.

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