Genome Recombination in Intergeneric Hybrids Between Tetraploid *Festuca pratensis* and *Lolium multiflorum*

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Recombination of homoeologous genomes was studied using in situ hybridization of the total genomic DNA in four F_8 populations selected from reciprocal intergeneric hybrids of tetraploid ($2n = 4x = 28$) *Lolium multiflorum* with *Festuca pratensis.* The populations were selected for agronomic performance as forage grasses. They are fertile and productive and have satisfactory levels of stability and uniformity. The proportion of the total genome length occupied by the *L. multiflorum* chromatin among the 25 plants analyzed ranged from 49.2% to 66.7% and significant differences within and among the populations were found. Apart from the consistently higher proportion of *L. multiflorum* chromatin, no other patterns of introgression were apparent. This suggests that despite considerable taxonomic distance, the chromosomes of the two genera are genetically equivalent and almost perfect mixing of the genomes is possible.

Members of the *Lolium-Festuca* complex of grasses, and especially the four agriculturally important species—*Lolium multiflorum* Lam. (Italian ryegrass), *L. perenne* L. (perennial ryegrass), *Festuca pratensis* Huds. (meadow fescue), and *F. arundinacea* Schreb. (tall fescue)—offer a range of agronomically complementary characteristics (Breese et al. 1981; Jauhar 1993). The ryegrasses provide forage of high nutritional value, while the fescues have better adaptive characteristics: persistency, winter hardiness, and drought resistance (Thomas and Humphreys 1991). It has been a goal of many forage grass breeding programs to combine these characteristics into agronomically superior commercial cultivars.

Despite being members of two different genera, and sometimes with large differences in the nuclear DNA content and chromosome length (Rees et al. 1966; Seal and Rees 1982), chromosomes of *Lolium* and *Festuca* species show considerable pairing affinity. In many instances, metaphase I pairing in the intergeneric hybrids of these two taxa, including diploids ($2n = 2x = 14$), is complete or nearly so (Jauhar 1975; reviewed in Jauhar 1993). This means that the desirable characteristics of two species or genera can be combined not only through the development of amphidiploids but also via chromosome and genome recombination.

Recombination of genomes of related species (homoeologous genomes) has been postulated as a means of genome evolution in polyploids (Zohary and Feldman 1962). Such recombination may occur by independent assortment of homoeologues alone, or in combination with crossing over. Assuming full compensation and lack of structural chromosome aberrations, independent assortment of homoeologous chromosomes belonging to two genomes of seven chromosomes each can theoretically produce 2^7 balanced genomes with different chromosome constitutions. Any pairing and crossing over of homoeologues not only increases the probability of recovery of such new genomes but also dramatically extends the range of chromosome variants appearing in the progeny. Recombination of genomes involving segregation of complete chromosomes and homoeologous crossing over was documented in tetraploid wheat-rye hybrids (Lukaszewski et al. 1987).

There has never been much doubt that in the *Lolium-Festuca* complex intergeneric introgressions of chromatin are taking place. Chromosome pairing affinity in interspecific and intergeneric hybrids is high (Jauhar 1975, 1993), and morphological and physiological characteristics have been introgressed from one species into another in breeding programs (Ghesquiere et al. 1996; Humphreys 1989; Humphreys and Ghesquiere 1994). However, until recently no estimates of the extent of such

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introgression at the genome or chromosome level were possible. In situ hybridization techniques using total genomic DNA were found capable of discriminating the ancestral genomes in the polyploids of *Festuca,* and chromosomes and chromosome segments in their hybrids with *Lolium* (Humphreys and Pasakinskiene 1996; Humphreys et al. 1995; Thomas et al. 1994). Thomas et al. (1994) demonstrated that numerous introgressions of *F. pratensis* chromatin were present in the backcross derivatives of the *L. multiflorum* × *F. pratensis* hybrids, but estimates of the actual proportions of the parental chromatin present were not made. In this study we present evidence on the extent of recombination of related genomes in advanced breeding populations selected from reciprocal tetraploid hybrids of *F. pratensis* with *L. multiflorum.*

Materials and Methods

The plant material in this study consisted of three F_8 populations selected directly from tetraploid ($2n = 4x = 28$) hybrids of *F. pratensis* × *L. multiflorum* (designated FL-I, FL-II, and FL-V) and one F_8 population selected from a reciprocal hybrid, *L. multiflorum* × *F. pratensis* (designated LF-6). The intergeneric F_1 hybrids and the progress of selection were described in detail elsewhere (Zwierzykowski et al. 1993; Zwierzykowski and Rybczynski 1981). Briefly, in the first three to four generations selection was primarily for fertility and meiotic stability. Starting from the F_3 generation, selection was applied for agronomic characteristics, including plant morphology, yield, protein content, digestability, and soluble carbohydrate content. In each cycle of selection, the selected clones were included in an intermating population and the resulting progenies were screened again.

All four populations are in the final stages of state trials in Poland, and two of them, FL-I and FL-V, are being considered for release as commercial cultivars of forage grasses under the provisional names Felopa and Sulino, respectively. All four lines are maintained as free mating populations with isolation. The populations have a satisfactory level of uniformity; the level of variation for morphological characteristics and aneuploid frequency does not exceed those typical of standard cultivars of tetraploid ryegrass or fescue.

Seed samples of the four lines were germinated on moist filter paper. Several seedlings of each line were planted in pots in the greenhouse. Root tips from the germinated seed and from the plants growing in the greenhouse were collected into ice water, refrigerated for up to 40 h, and fixed in a mixture of three parts absolute alcohol to one part glacial acetic acid for a few h at room temperature and refrigerated until used. Root tips from individual plants were squashed in a drop of 45% acetic acid on the HCl-cleaned slides, frozen on dry ice, coverslips removed, and the preps stored at −18°C until used. Leaf samples of the parental species, *L. multiflorum* cv Tur ($2n = 14$) and *F. pratensis* cv Skawa ($2n = 14$), were collected from four to five plants from each accession and DNA was extracted by a modification of the selective precipitation method of Murray and Thompson (1980).

All preparations of all plants tested were screened using total genomic DNA of *L. multiflorum* as the probe and *F. pratensis* DNA as a block. In three separate runs with five slides each, on preparations of LF-6 and FL-1, the roles of the probe and block were reversed: the total genomic DNA of *F. pratensis* was used as a probe and the DNA of *L. multiform* as a block. Probe DNA was mechanically sheared to 5–10 kb fragments by repeated passing through a hypodermic needle and labeled with digoxigenin-11-dUTP by nick translation (ENZO Diagnostics, Inc.). Block DNA was sheared by the same technique and used at a ratio of 1:100 (probe:block).

The in situ DNA hybridization technique used was that of Schwarzacher et al. (1994) with several modifications. The preparations were fixed in 4% paraformaldehyde, washed three times in 2× SSC, dehydrated through an alcohol series, and air dried. Chromosomal DNA was denatured with 70% formamide in 2× SSC at 70°C for 2 min. The hybridization mixture consisted of 50% (v/v) deionized formamide, 10% (w/v) dextran sulfate, SSC (20× stock) at 2×, labeled probe DNA (about 10 ng/slide), unlabelled block DNA (about 1 μg/slide), and salmon sperm DNA (at a final concentration of 15 μg/ml). The hybridization mixture was denatured by boiling for 10 min and was held on ice for 5 min. Fifteen microliters of the hybridization mixture was added to each slide. Hybridization was carried out overnight at 37°C in a moist chamber. Several washes were performed, with the most stringent being in 20% formamide in 0.1× SSC at 42°C. Sites of hybridization were detected using antidigoxigenin:fluorescein (Boehringer-Mannheim). The signal was amplified using antidigoxigenin, mouse Ig, and anti-mouse Ig-digoxigenin (Boehringer-Mannheim). The chromosomes were counterstained with propidium iodide (5 μg/ml in buffer-containing antifade).

Hybridized preparations were screened under a fluorescent microscope using appropriate filter sets. Complete cells with adequate detail were photographed using Kodak Elite II or Kodak Ektachrome film, or scanned using a Bio-Rad MRC600 confocal scanner attached to a Nikon Optiphot-2 microscope. Chromosome measurements were taken either from images of slides/negatives scanned into the computer or were taken directly from the computer screen images obtained in the confocal microscope. In both approaches, the total length of the chromosomes in a cell were measured, as well as the total length of chromosomes and chromosome segments fluorescing yellow-green (for the hybridization sites of the FITC-labeled probe DNA) and orange-red (for the block DNA). Additionally the number of translocation breakpoints was determined for each scanned cell. Four to eight plants per population with one to six (most often three) cells per plant were analyzed.

Results and Discussion

A total of 72 cells from 25 plants were analyzed in the four populations, with the number of plants per population ranging from four to eight (Table 1). Among the analyzed plants six were aneuploid: four plants had 29 chromosomes and two had 27 chromosomes. Considering the sample size, this aneuploid frequency does not appear substantially different from an 11.7% aneuploid frequency among 615 plants of the F_6 generation of the *F. pratensis* × *L. multiflorum* hybrids (Zwierzykowski et al. 1993) and is well within the range of the aneuploid frequencies found in populations of autotetraploids of the two parental species (Nagata and Okabe 1978; Simonsen 1975).

The proportion of the total genome length contributed by *L. multiflorum* was always greater than that contributed by *F. pratensis* (Table 1; Figure 1) and ranged from 50.8% to 66.7%, except for a single observation made in one cell of an FL-I plant where it was 49.2%. Conversely the contribution of *F. pratensis* ranged from 33.3% to 49.2%, with one exception. Analysis of variance of the proportions of the genome contributed by *L. multiflorum* showed that the differences among plants within the populations and among populations were statistically significant ($P >$

Table 1. Percentage of the total length of somatic chromosomes of tetraploid F_8 derivatives of hybrids of *Festuca pratensis* with *Lolium multiflorum* contributed by *L. multiflorum*

Population	Plant 1	2	3	4	5	6	7	8	Mean
FL-I	65.7 (5)	66.2 (3)	61.7 (4)	63.5 (5)	64.1 (5)	59.4 (1)	50.8 (1)	49.2 (1)	62.8
FL-II	54.1 (3)	53.7 (4)	55.3 (4)	56.3 (1)					54.8
FL-V	57.1 (3)	53.2 (1)	60.5 (3)	58.7 (3)	52.7 (3)	57.4 (4)			56.6
LF-6	62.9 (6)	61.8 (3)	64.6 (5)	55.5 (1)	61.3 (1)	66.0 (1)	66.7 (1)		62.7

Numbers of cells analyzed for each plant in parentheses.

.01, $P > .05$, respectively). Among the populations, FL-I and LF-6 had higher and FL-II and FL-V had lower proportions of *L. multiflorum* chromatin.

In situ hybridization experiments where the probe and block DNA were reversed did not reveal substantial differences in the proportions of chromatin contributed by both parental species. Using *L. multiflorum* DNA as a probe, three cells of an FL-I plant (plant 4, Table 1) averaged 64.1% of the chromatin of that species, whereas in the reverse hybridization, two cells measured of the same plant averaged 62.6% chromatin of *L. multiflorum.* Reverse hybridization in LF-6 (plant 1, Table 1) gave proportions of *L. multiflorum* chromatin of 63.2% (ranging from 58.5% to 70%) and 61.5% for five cells and one cell, respectively. Such consistency of the results obtained in the reverse hybridization experiments implies that nonspecific hybridization of the probe DNA was not a factor in the experiments and that actual contribution of chromatin from the two parental species was detected.

A part of the observed variation in the contribution of the parental chromatin may be explained by experimental error. In most instances, lower proportions of chromatin of the "block" species and higher levels of chromatin from the "probe" species were observed in cells with highly contracted chromosomes. It would appear that the halo effect of the bright fluorescing label attached to the probe DNA introduces a measurement error. As a result, the contribution of the chromatin from the probe species (generally *L. multiflorum*) is probably overestimated. The degree of overestimation depends on the intensity of the detection signal and the level of chromosome contraction. A very bright signal and highly condensed chromosomes would give proportionately greater error than a low-level signal in less contracted chromosomes.

The number of detected translocation breakpoints per cell ranged from 22 to 38 and per chromosome from 0 to 7. The number of chromosomes without any detectable chromatin from the other parent was low and never exceeded 8 out of 28 possible. A majority of chromosomes in each cell analyzed were hybrid chromosomes composed of segments of chromatin from both parents.

Although no explicit analysis was performed, it was clear that the number of the detected translocation breakpoints was inversely related to the level of chromosome condensation. Small introgressions were difficult to detect in highly contracted chromosomes and only large blocks of chromatin from the parental species could be visualized (Figure 1). Observations of less condensed chromosomes, either in early metaphase or in late prophase clearly showed that the blocks of chromatin contributed by the parental species ranged in length from entire chromosomes uniformly labeled in the same color to very short segments detectable only as dots of bright fluorescence on the red-orange background of the propidium iodide counterstain. Therefore we assume the presence of even shorter introgressed stretches of chromatin that could not be detected with the technique. If such short introgressions were indeed present, we observed only a fraction of all translocation breakpoints present.

In light of numerous earlier observations of various hybrid *Lolium-Festuca* derivatives, the presence of intergeneric introgressions in the populations analyzed in this study was not surprising. Clear morphological, biochemical, and physiological indications of such introgressions were observed in commercial cultivars selected from such hybrids (Ghesquiere et al. 1996; Jauhar 1993). In situ hybridization experiments using total genomic DNA only confirmed this (Humphreys and Pasakinskiene 1996; Humphreys et al. 1995; Pasakinskiene et al. 1997; Thomas et al. 1994). Unusual and surprising was the extent of genome recombination observed here, considering that the study populations are direct descendants of intergeneric hybrids and have been subjected to several cycles of rigorous selection for a range of agronomic characteristics, including fertility, stability, uniformity, and productivity. At the whole genome level, there was almost complete mixing of chromatin from the two genera, with only somewhat higher than random contribution of *L. multiflorum.*

The techniques used in this and other studies of the same nature do not have the resolving power to address the issue of preferential selection for retention or elimination of certain blocks of chromatin which may operate at the chromosome level or below. However, the presence of such selection does not appear likely. Many attempts were made in this study to karyotype individual plants or populations, but only in a few cells was it possible to identify a few pairs of chromosomes, but never quartets, which appeared identical based on the length, arm ratio, and the DNA hybridization pattern. Presumably these were pairs of homologues. However, since the analyzed populations were tetraploid, they should have had either two pairs of homologues or four homoeologues present in each group. DNA hybridization patterns appeared to be a reliable method of chromosome identification because characteristic patterns were easily identifiable among different cells of the same plant, even among different hybridization runs (Figure 1A,B). On the other hand, of the easily identifiable patterns, none were repeated among different plants from the same line. The satellited chromosomes were easily identifiable in most cells. The hybridization patterns on these chromosomes differed within and among plants (Figure 1C) and populations. This suggests that there might have been no preferential fixation of certain blocks of chromatin from one or the other parent.

Introgressions of a range of agronomically desirable characters from one species into another in polyploids within the *Lolium-Festuca* complex (Humphreys and Pasakinskiene 1996), controlled introgression of designated chromosome arms and smaller chromosome segments at the diploid level (King et al. 1997; Pasakinskiene et al. 1997), and what appears to be random mixing of chromatin observed in this study suggest very strongly that homoeologous chromosomes within the complex

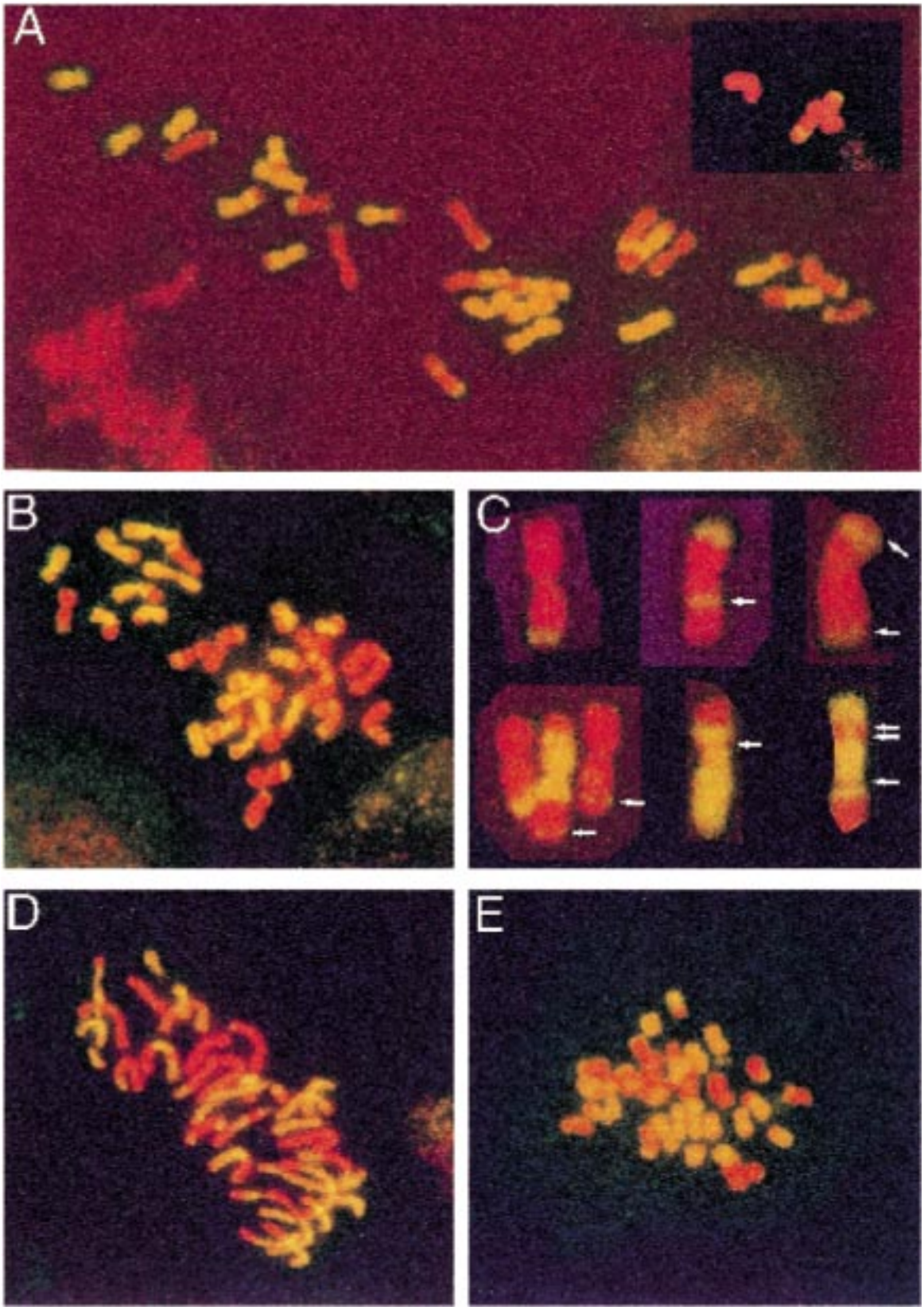

Figure 1. In situ hybridization using total genomic DNA to mitotic chromosomes of two plants of line FL-V selected from an intergeneric hybrid *F. pratensis* × *L. multiflorum.* DNA of *L. multiform* was used as a probe (FITC-labeled yellow-green); DNA of *F. pratensis* was used as a block (orange-red). A, B, C, D are from plant 6, E is from plant 1. Both plants are aneuploid with 29 chromosomes. **(A)** Three chromosomes belonging to this cell which were outside of the camera field were inserted electronically into the photo. Different levels of chromosome contraction allow different resolution of the introgression detail. Long metaphase **(A,C)** and prophase **(D)** chromosomes show considerable detail; only large blocks of parental chromatin are visible on highly condensed chromosomes **(E)**. At least 10 different chromosomes with similar length, arm ratios and color patterns can be identified on A and B, but homologous pairs are not identifiable. **(C)** Enlarged selected chromosomes from A, showing small introgressions (arrowed) of chromatin from one parent into blocks of chromatin of the other parent.

are almost perfectly equivalent genetically (see also Jauhar 1975) and, for all practical purposes, are interchangeable. Yet these chromosomes are sufficiently differentiated at the DNA level for easy and precise labeling using total genomic DNA. This poses intriguing questions about the mechanisms controlling chromosome pairing within the complex. Much lower levels of chromosomal differentiation in other grasses, such as *Aegilops, Triticum,* and *Secale,* present much more difficult obstacles to chromosome pairing and gene flow between species and genera.

The observations made in this study on the extent of genome recombination in the *Lolium-Festuca* complex suggest that with seemingly complete interchangeability of chromatin between genera, any variation introduced across the taxonomic boundary may not be different from any allelic variation present in an outcrossing population. Once the hybridization or sterility barriers are broken, the entire genetic pool of the two genera appears accessible to manipulation. This should facilitate the development of superior commercial stocks of forage grasses with far greater adaptability and range of characteristics than that possible in the conventional breeding approaches.

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