Research Paper

Utilization of flow cytometry for festulolium breeding (*Lolium multiflorum* (2x) × *Festuca arundinacea* (6x))

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Festulolium is a hybrid between *Festuca* and *Lolium* species that has valuable agronomic traits from both grass species. The purpose of our breeding program is to produce hexaploid festulolium that introduces tolerance to summer depression into Italian ryegrass (*Lolium multiflorum*) by crossing it with tall fescue (*Festuca arundinacea*). However, we found the DNA ploidy of hexaploids was not stable and was reduced in successive generations. We aimed to find out how to obtain stable high-ploidy festulolium. F1 hybrids of *L. multiflorum* and *F. arundinacea* were produced. The F3 generation was produced from putative hexaploid F2 individuals by open pollination. The F4 to F6 generations were obtained by polycrossing. The DNA ploidy levels of F2 to F6 individuals were estimated by flow cytometry. Cytological characteristics of the F5 and F6 individuals were investigated by FISH and GISH. The DNA ploidy level of hexaploid festulolium was reduced and stabilized at almost the same level as a tetraploid. Seed fertility was inversely correlated with an increase in ploidy level. GISH revealed no preferential *Lolium* transmission. FISH with a telomere probe revealed that counting the exact number of chromosomes in festulolium was difficult. DNA ploidy level was strongly correlated with the number of chromosomes.

Key Words: *Festuca arundinacea*, festulolium, flow cytometry (FCM), fluorescence *in situ* hybridization (FISH), genomic *in situ* hybridization (GISH), *Lolium multiflorum*, DNA ploidy.

Introduction

Festuca and *Lolium* are valuable grass genera with complementary characteristics that are regarded as useful agronomic attributes. In general, *Lolium* species, represented by *L. multiflorum* and *L. perenne*, are known for their palatability, digestibility and rapid establishment. However, they lack resilience against abiotic stresses, which is a feature of *Festuca* species such as *F. arundinacea* and *F. pratensis*. Both genera are taxonomically close enough to hybridize even in natural populations, and this has spurred plant breeders to produce hybrids of *Festuca* and *Lolium* species, called festulolium, which combine their complementary characteristics (Yamada *et al.* 2005). *L. multiflorum* features more vig-

orous growth than *L. perenne* and is well suited for use as hay or silage. When introducing traits of *Festuca* into *Lolium*, *F. arundinacea* has the advantages of more vigorous growth and higher tolerance to summer depression than *F. pratensis*. The history of festulolium breeding programs for developing germplasm adapted to Japanese conditions is not long; only three Japanese festulolium cultivars exist. The first Japanese festulolium cultivar, 'Tohoku 1', was released in 2009 (Yonemaru *et al.* 2011). The second cultivar, 'Icarus', and the third, 'Nakei 1', were released in 2011 and 2014, respectively (Ueyama *et al.* 2014). Additional festulolium cultivars are required in Japan, which has a variety of climatic conditions. The purpose of our breeding program is to produce festulolium that is resistant to summer depression and is adapted to the Tohoku district of Japan by hybridizing diploid *L. multiflorum* and hexaploid *F. arundinacea*.

Although hybrids of *L. multiflorum* $(2n = 2x = 14) \times$ *F. arundinacea* $(2n = 6x = 42)$ are male sterile and show

low female fertility, it is possible to restore seed fertility by chromosome doubling or backcrossing (Crowder 1953). An important factor in breeding of festulolium cultivars is the maintenance of genomic stability, which contributes to adequate seed fertility and insures the transmission of desired characteristics to subsequent generations (Eizenga *et al.* 1991). In the amphiploidy approach, however, attempts to obtain meiotic stability have failed. For example, Kleijer (1987) observed a decrease in chromosome number among progeny of 56-chromosome amphiploid hybrids derived from *L. multiflorum* (2x) × *F. arundinacea* (6x) following colchicine treatment, which might cause genomic instability.

A major problem in festulolium is genomic instability among generations. The first trend in investigation of the instability was meiotic analysis to reveal the cause of sterility of festulolium (Ahloowalia 1965, Dijkstra and De Vos 1975, Jauhar 1975). Since Thomas *et al.* (1994) reported that genomic *in situ* hybridization (GISH) could discriminate between *Lolium* and *Festuca* in a festulolium genome, GISH has been employed by many researchers to investigate the genomic constitution of festulolium (Pašakinskienė and Jones 2005). Canter *et al.* (1999) found *Lolium* genome dominance in the genome of the amphiploid festulolium (4x) 'Prior', produced by hybridization between *L. perenne* × *F. pratensis*. Zwierzykowski *et al.* (2011) reported the reduction of *Festuca* chromatin in advanced generations of *F. pratensis* (4x) × *L. multiflorum* (4x).

From a breeder's point of view, plants with high ploidy could be expected to generate genetic variation in successive generations. A gene dosage effect could be expected too. Guo *et al.* (1996) reported that gene expression, with some exceptions, increases as ploidy level rises from haploid to tetraploid in maize. Moreover, other advantages of being polyploid, such as heterosis, gene redundancy and loss of self-incompatibility, could be expected (Comai 2005). These ploidy effects could contribute to festulolium breeding. Changes in ploidy levels have been highly influential in the history of crop improvement (Udall and Wendel 2006). Diploid, triploid, tetraploid, and other polyploid states occur naturally in hybrids of *Lolium* and *Festuca* (Berg *et al.* 1979); therefore, plants with high ploidy can be obtained from hybrids without any chemical treatment, such as colchicine treatment. The highest ploidy of festulolium germplasm is octoploid in 'KY-2N56' (Pedersen *et al.* 1990), which is the only octoploid germplasm. Six hexaploid cultivars are registered in the world. Hexaploid 'Felina', 'Hykor', 'Korina', and 'Lesana' were produced by backcrossing with *F. arundinacea* (6x) in the Czech Republic (Fojtik 1994). 'Kenhy' and 'Johnstone' were derived from an initially octoploid amphiploid *L. multiflorum* (2x) × *F. arundinacea* (6x) in the USA (Buckner *et al.* 1977, 1983, Ghesquière *et al.* 2010). Since there is only one registered octoploid, the hexaploid level seems a reasonable target high-ploidy level for our breeding program.

The chromosome number is basic cytogenetic information that indicates genomic stability. However, counting the number of chromosomes is not suitable for investigating a large number of materials. Flow cytometry (FCM) is a useful tool to determine ploidy level of plants (DeLaat *et al.* 1987, Ochatt 2008). Though an accurate ploidy level can be determined only by chromosome observation, FCM has the advantage of ease of measurement. This feature contributes to its use in investigating ploidy levels in large numbers of samples of festulolium. Following Suda *et al.* (2006), the ploidy level estimated by FCM is described with the prefix "DNA".

The objective of this study was to obtain hexaploid hybrids between *L. multiflorum* (2x) × *F. arundinacea* (6x), and to assess the genomic stability of the hybrids at the DNA ploidy level by FCM, the genomic constitution by FISH and GISH, and the relationship between fertility and ploidy.

Materials and Methods

Overview of the study strategy

The overall study strategy is illustrated in **Fig. 1**. Ploidy levels in the F2 to the F6 generations were analyzed by FCM, seed fertility of the F3 generation by an X-ray apparatus, and genomic constitution in the F5 and the F6 generations by molecular cytogenetic methods.

Plant materials

Diploid *L. multiflorum* 'Hataaoba' ($2n = 2x = 14$, maternal parent) was crossed with the hexaploid *F. arundinacea* 'Gloria' ($2n = 6x = 42$, paternal parent). The generation 2 was obtained from open-pollinated F1 individuals. Hereafter, generation n is described as Fn generation to easily recognize the generation of individuals. Three approximately hexaploid progeny of the F2 generation were screened by FCM. The F3 generation was produced by open pollination as the F2 individuals were male sterile. The breeding field also contained diploid and tetraploid *L. multiflorum* being cultivated as pollen parents. Eight putative hexaploid F3 individuals were screened from 96 F3 individuals. The F4 generation was obtained by polycrossing among eight F3 individuals and the F5 generations by polycrossing 14 F4 individuals. Three groups of the F6 generation were obtained by polycrossing among five, seven and three F5 individuals that were classified according to their DNA ploidy level.

L. multiflorum 'Hataaoba' clone Ha1 ($2n = 2x = 14$) and *F. arundinacea* 'Gloria' clone 83301 ($2n = 6x = 42$) were used as internal standards to estimate the DNA ploidy level of the hybrids by FCM. Tetraploid festulolium 'Tohoku 1' was used as a reference for tetraploid festulolium.

Analysis of seed fertility

Approximately 200 seeds per F3 individuals were collected and seed fertility was tested by an X-ray apparatus (Softex, type TV-25-1, Tokyo, Japan), which can be used to observe the internal structure of seeds. Seed fertility was
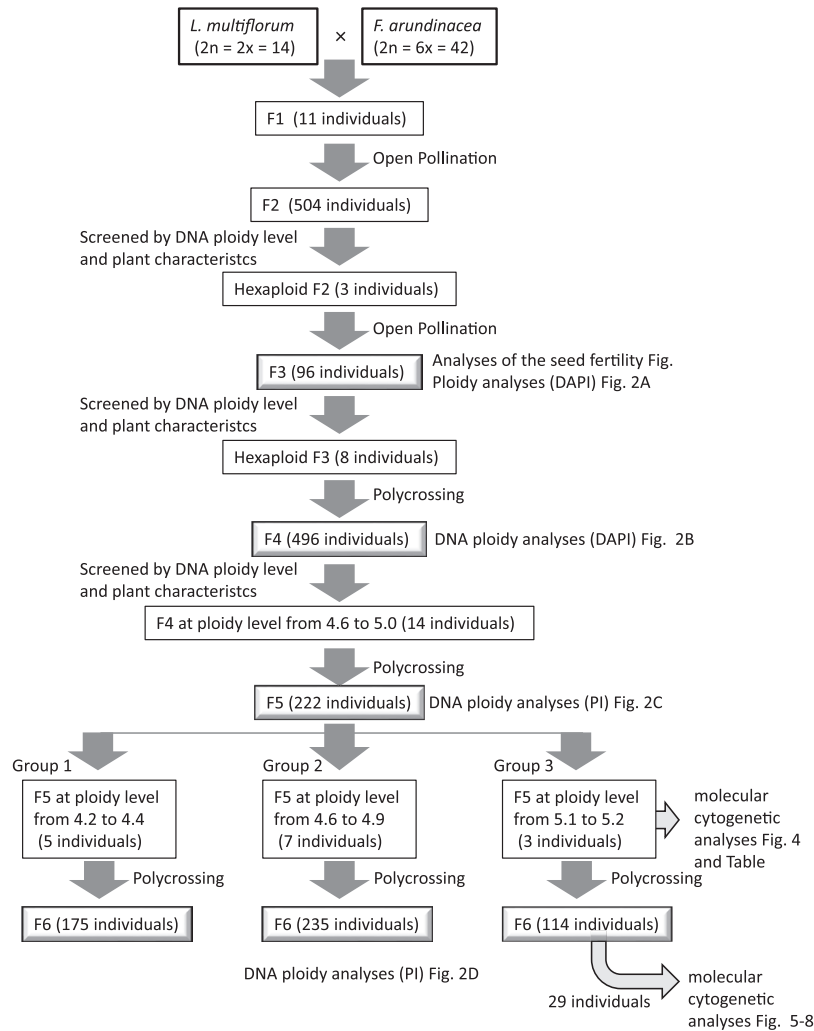
Fig. 1. Experimental scheme in this study.

judged by the existence of an endosperm and was calculated based on the ratio of fertile to all tested seeds.

Estimation of DNA ploidy level by FCM

DNA ploidy levels in F2 to F4 individuals were estimated with a Partec PA ploidy analyzer (Partec, Münster, Germany) and ploidy of the F5 and the F6 individuals was estimated with an Attune cytometer (Applied Biosystems LLC, Foster City CA, USA). The Partec PA ploidy analyzer can analyze only the fluorescence intensity of 4',6-diamidino-2-phenylindole (DAPI), which preferentially stains AT clusters in the minor groove (Kubista *et al.* 1987), meaning that the results are susceptible to error due to differences in relative AT/GC content of samples. The Attune cytometer can analyze the fluorescence intensity of propidium iodide (PI), which equally stains all four nucleotides, allowing a more accurate estimate of DNA content.

Portions of leaves (1–2 cm^2) of individuals from each generation and of *L. multiflorum* 'Hataaoba' or *F. arundinacea* 'Gloria', used as an internal standard, were placed together

in a Petri dish. The leaves were chopped with a razor blade in nucleus isolation buffer (0.2 M Tris-HCl, pH 7.5, 4 mM MgCl$_2$, 0.5% Triton X-100) (Pfosser *et al.* 1995). After chopping, the suspension of nuclei was passed through a 50 μm mesh nylon filter to remove residual leaf pieces. For the F3 and the F4 individuals, DAPI solution (nucleus isolation buffer supplemented with 5 μg/ml DAPI) was added to the suspension and mixed thoroughly. For the F5 and the F6 individuals, PI solution (nucleus isolation buffer supplemented with 100 μg/ml PI) was added to the suspension and mixed thoroughly. DNA ploidy level was determined by comparing the fluorescence peak of an F3, F4, F5 or F6 individual with the internal standard using the formula:

$$\text{DNA ploidy level} = [(\text{Sample peak mean})/(\text{Standard G1 peak mean})] \times 2$$

When *F. arundinacea* 'Gloria' was used and the peak of 'Hataaoba' overlapped the sample peak, the DNA ploidy levels were corrected based on *L. multiflorum* 'Hataaoba'.

The DNA ploidy levels of 96 F3 individuals, 496 F4 individuals and 222 F5 individuals were analyzed. Additionally, 175, 235 and 114 individuals of three F6 groups were analyzed.

Analysis of genomic constitution by molecular cytogenetic analysis

FISH using a telomere probe, GISH, and image analysis were performed according to Akiyama *et al.* (2010) with slight modifications. Genomic DNA of *L. multiflorum* and *F. arundinacea* was directly labeled with fluorescein-12-dUTP (PerkinElmer Co., Billerica, MA, USA) and Texas Red-5-dUTP (PerkinElmer Co.), respectively, by a nick translation kit (Roche, Basel, Switzerland). For the telomere probe, *Arabidopsis* telomere repeat sequences (Richards and Ausubel 1988) were PCR labeled with biotin-16-dUTP (Roche) and detected by Streptavidin DyLight 549 (Vector Laboratories, Burlingame, CA, USA) as the first layer of signal amplification, biotinylated anti-streptavidin (Vector Laboratories) as the second layer and again with Strepta-vidin DyLight 549 as the third layer. Chromosome spreads were observed under an Olympus BX61 fluorescence microscope (Olympus Optical Co., Ltd., Tokyo, Japan) equipped with a DP72 CCD camera (Olympus Optical Co., Ltd.). Based on the GISH results, the ratio of the *Festuca*-specific genomic component to the total festulolium genome (referred to as the f ratio) was analyzed using intensity values in DAPI images by Adobe Photoshop CS6 Extended (Adobe Systems Inc., San Jose, CA, USA) on a Windows 7 platform (Microsoft Corporation, Redmond, WA, USA). At least 6 chromosome spreads were analyzed to determine the f ratio in each sample.

Results

Selection of putative hexaploids from F2 and F3 generations

DNA ploidy levels of 504 F2 individuals were estimated from the peak ratio relative to the internal standard, *L. multiflorum* 'Hataaoba'. Our flow cytometric system with the Partec PA ploidy analyzer estimated the DNA ploidy level of *F. arundinacea* 'Gloria' to be approximately 5.6. Based on this value, three F2 individuals that had DNA ploidy levels of five or more and sufficient seed fertility were selected as putative hexaploid parents. Likewise, DNA ploidy levels of the obtained 96 F3 individuals were estimated; eight of them were selected as putative hexaploid parents. The distribution pattern of DNA ploidy level in the F3 individuals is shown in **Fig. 2A**. DNA ploidy levels of the F3 individuals ranged from 3.9 to 6.6, with a mean of 4.7 ± 0.6. The most frequent value was 4.1.

Relationship between seed fertility and DNA ploidy in the F3 generation

The seed fertility of 96 F3 individuals was examined. **Fig. 3** shows the relation between seed fertility and DNA ploidy levels. The mean value for seed fertility was 0.24.
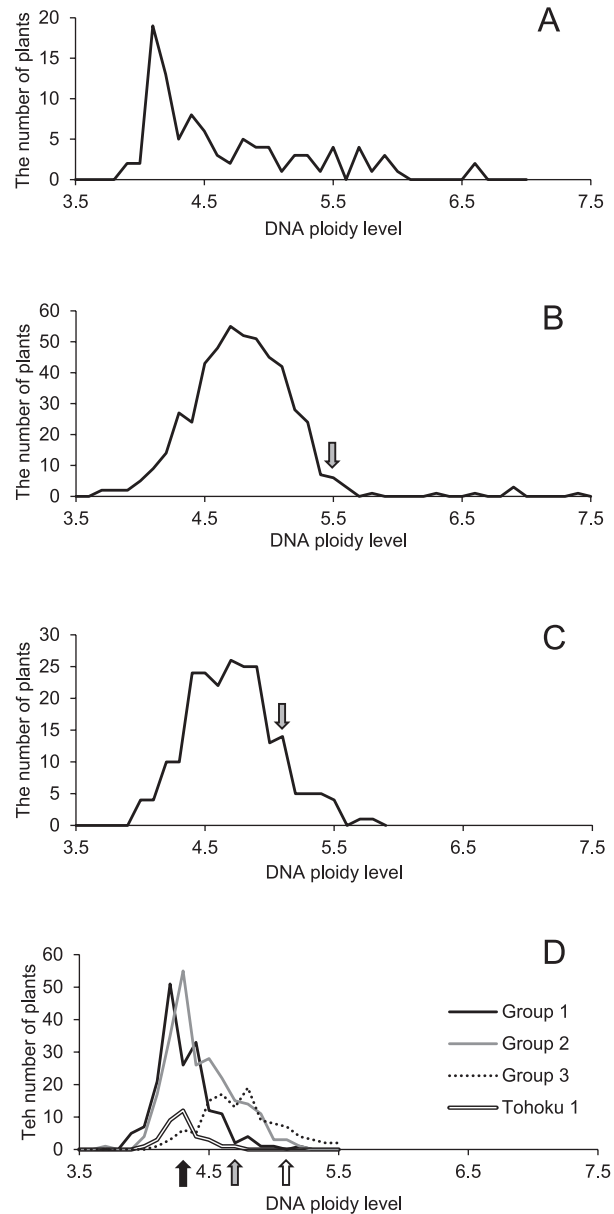

Fig. 2. Distribution patterns of DNA ploidy levels. The ploidy level is the ratio of genome size of individuals and *L. multiflorum* 'Hataaoba' (2x). A–D are the F3–F6 generations, respectively. Arrows indicate mean DNA ploidy levels of parents. In D, solid arrow, gray arrow and open arrow indicate mean DNA ploidy levels of parents of group 1, 2 and 3, respectively.

There were 46 plants with higher fertility than the mean value, and about 85% of these (39/46 plants) had DNA ploidy levels of 3.9–4.4. Pearson's correlation coefficient was −0.63 and significant at the 0.01 level. This result suggests that individuals with low DNA ploidy tended to have high fertility.

Distribution of DNA ploidy in the F4 generation

The distribution pattern of DNA ploidy level in the F4 is shown in **Fig. 2B**. DNA ploidy of the F4 generation ranged
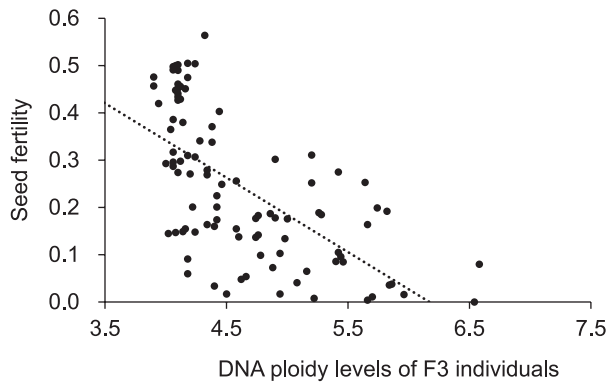
Fig. 3. Relationship between DNA ploidy levels of 96 F3 individuals and their seed fertility. Seed fertility was defined as the number of fertile seeds per tested batch of 200 seeds.

from 3.7 to 7.4, with a mean of 4.8 ± 0.4. Most F4 individuals (480/496) had lower DNA ploidy than the mean DNA ploidy level (5.5) of the F3 parents. DNA ploidy of 50% of the F4 individuals ranged from 4.6 to 5.0. The difference between the DNA ploidy levels of the eight F3 parents and 496 F4 individuals was statistically significant (Student's t test, $P < 0.01$). Fourteen F4 individuals showed better growth in early fall and early spring, and their DNA ploidy as assessed by DAPI staining ranged from 4.6 to 5.0 (mean 4.8). These individuals were selected as parents of the F5 generation.

Distribution of DNA ploidy in the F5 generation

DNA ploidy levels of the F5 generation were estimated based on measurement of PI intensity with the Attune cytometer. DNA ploidy levels of the 14 F4 parents of the F5 individuals were reanalyzed in a way similar to the analysis of the F5 generation. The mean DNA ploidy was 5.1, which was higher than the value estimated by DAPI staining (4.8). The distribution pattern of DNA ploidy in the F5 generation is shown in **Fig. 2C**. DNA ploidy of the F5 generation ranged from 4.0 to 5.8, with a mean of 4.6 ± 0.3. Most F5 individuals (201/222) had lower DNA ploidy than the mean DNA ploidy level of the F4 parents, 5.1. The difference between the DNA ploidy levels of the 14 F4 parents and 222 F5 individuals was statistically significant (t test, $P < 0.01$).

Distribution of DNA ploidy in the F6 generation

F5 individuals were sorted into three groups according to their DNA ploidy levels, group 1 (DNA ploidy range 4.2–4.4, mean 4.3), group 2 (DNA ploidy range 4.6–4.9, mean 4.7) and group 3 (DNA ploidy range 5.1–5.2, mean 5.1), consisting of five, seven, and three individuals, respectively. They were polycrossed within each group, and the DNA ploidy levels of the progeny obtained and tetraploid festulolium 'Tohoku 1' were estimated based on PI intensity similarly to the analysis for the F5 generation. The results are shown in **Fig. 2D**.

For the F6 progeny of group 1 (named F6-G1), 175 indi-

viduals were analyzed. DNA ploidy ranged from 3.8 to 5.1, with a mean of 4.2 ± 0.2. Although the range of DNA ploidy was wider than for the parents, the mean and the peak of the distribution was similar. The difference between the DNA ploidy levels of the individuals and their parents was not statistically significant (t test, $P > 0.8$).

For the F6 progeny of group 2 (named F6-G2), 235 individuals were analyzed. DNA ploidy ranged from 3.7 to 5.1, with a mean of 4.4 ± 0.3. Most individuals (190/235) had lower DNA ploidy than the mean DNA ploidy level of the parents, 4.7. The difference between the DNA ploidy levels of the individuals and their parents was statistically significant (t test, $P < 0.05$).

For the F6 progeny of group 3 (named F6-G3), 114 individuals were analyzed. DNA ploidy ranged from 4.1 to 5.5, with a mean of 4.7 ± 0.3. Most individuals (104/114) had lower DNA ploidy than the mean DNA ploidy level of the parents, 5.1. The distribution pattern was similar to that of the F5 generation (**Fig. 2C**). The difference between the DNA ploidy levels of the individuals and their parents was statistically significant (t test, $P < 0.05$).

For Tohoku 1, 34 individuals were analyzed. DNA ploidy ranged from 4.0 to 4.6, with a mean of 4.2 ± 0.1. The difference between the DNA ploidy of F6-G1 and Tohoku 1 was not statistically significant (t test, $P > 0.8$). The DNA ploidy of F6-G2, G3 and Tohoku 1 differed significantly (t test, $P < 0.01$), respectively.

FISH and GISH analysis for F5 parents and F6-G3

Chromosomal characteristics of 29 individuals belonging to F6-G3 and their parents were analyzed by FISH and GISH (**Table 1**). GISH and FISH results for F5 individual No. 3 are shown in **Fig. 4** as an example. Many chromosomes that were recombinant between *L. multiflorum* and *F. arundinacea* were observed. The percentage of recombinant chromosomes out of the total number of chromosomes in F5 parents was 42.86–55.88%, with a mean of 49.58%, and in F6-G3 individuals was 38.71–72.73%, with a mean of 60.68%, so the number of recombinant chromosomes tended to increase in F6 individuals. The number of chromosomes was counted based on not only chromosome morphology but also the number of telomere signals. In many chromosome spreads, satellite chromosomes were observed (**Fig. 4**, arrows). Small host chromosomes that were difficult to distinguish from satellite chromosomes by morphology were also observed (**Fig. 4D**, arrowheads). One had a recombination between *L. multiflorum* and *F. arundinacea* (**Fig. 4A**, arrowhead), indicating a change in chromosome size due to recombination.

Two of the three F5 parents had 34 chromosomes, and the third had 35. The number of chromosomes in F6-G3 ranged from 27 to 35, with a mean of 30.59, indicating genome reduction in F6-G3 resulting from chromosome deletion. The distribution pattern of the number of chromosomes in F6-G3 is shown in **Fig. 5**, which resembles a normal distribution. The relationship between the number of

Table 1. Characteristics of chromosomes in F6-G3 progeny and their parents

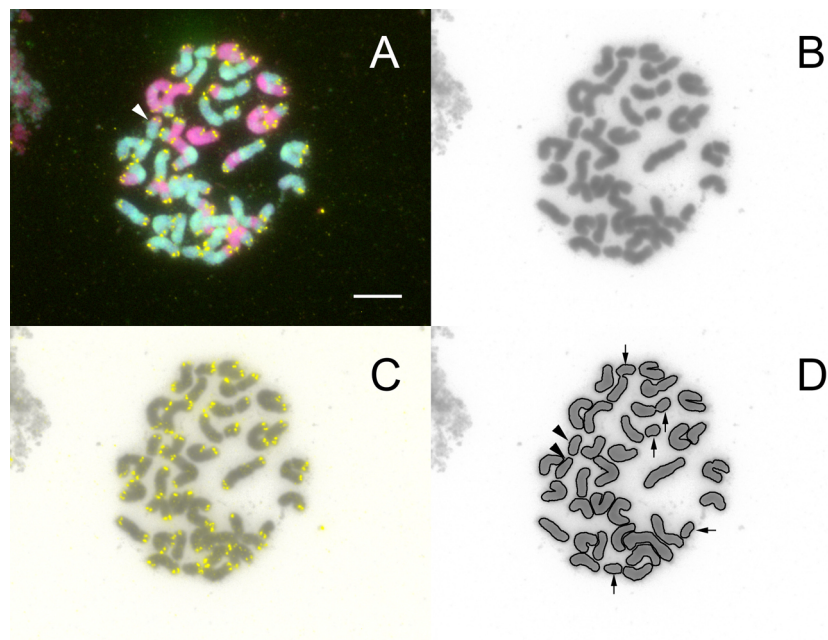|	Plants	N	f ratio (%)	S.D.	The number of chromosomes	Ploidy level by FCM	The number of *Festuca* chromosomes	The number of recombinant chromosomes	The numebr of *Lolium* chromosomes	Ratio of recombinant chromosome in total chromosomes (%)
F5	1	9	28.39	1.84	35	5.24	5	15	15	42.86
	2	5	28.00	2.28	34	5.05	2	17	15	50.00
	3	11	27.41	1.39	34	5.09	2	19	13	55.88
Mean			27.93		34.33	5.13	3.00	17.00	14.33	49.58
F6	1	15	29.87	2.13	30	4.55	2	16	12	53.33
	2	12	31.10	1.61	35	5.47	2	19	14	54.29
	3	10	33.57	2.39	28	4.64	1	20	7	71.43
	4	11	26.21	1.84	27	4.16	3	16	8	59.26
	5	15	34.05	2.18	30	4.43	3	21	6	70.00
	6	11	26.36	2.59	32	4.89	1	19	12	59.38
	7	46	28.49	2.22	30	4.56	1	21	8	70.00
	8	7	24.14	1.42	33	5.04	3	19	11	57.58
	9	7	22.59	2.29	28	4.45	1	16	11	57.14
	10	25	27.69	2.00	29	4.62	0	19	10	65.52
	11	13	23.22	1.94	32	4.94	1	19	12	59.38
	12	11	23.37	1.56	30	4.52	1	19	10	63.33
	13	9	25.28	2.41	30	4.43	3	14	13	46.67
	14	11	27.70	2.97	31	4.58	0	20	11	64.52
	15	8	32.22	2.43	32	4.77	1	21	10	65.63
	16	6	30.96	1.46	30	4.49	4	15	11	50.00
	17	20	26.48	1.97	31	4.71	0	19	12	61.29
	18	10	30.24	3.29	29	4.86	2	17	10	58.62
	19	16	27.74	1.62	28	4.16	1	19	8	67.86
	20	16	30.29	2.77	31	4.61	5	12	14	38.71
	21	8	21.42	1.21	31	4.74	0	18	13	58.06
	22	15	34.33	1.75	33	4.95	1	24	8	72.73
	23	7	22.63	0.90	35	5.30	0	24	11	68.57
	24	14	25.11	2.02	33	4.47	1	17	15	51.52
	25	7	30.91	1.87	29	4.42	1	20	8	68.97
	26	7	25.69	0.86	31	5.08	0	20	11	64.52
	27	10	25.02	1.90	27	4.06	1	17	9	62.96
	28	9	26.46	2.26	29	4.39	2	15	12	51.72
	29	10	28.52	1.05	33	4.99	1	22	10	66.67
Mean			27.64		30.59	4.66	1.45	18.55	10.59	60.68

Fig. 4. GISH and FISH results for a representative F5 individual (festulolium No. 3). (A) Bound GISH probes in the *Lolium* and *Festuca* genomes appear green and red, respectively. FISH probe bound to telomeres appears yellow. Arrowhead indicates a recombination on a satellite or host chromosome (B) Inverted DAPI image. (C) Inverted DAPI image with yellow telomere signals. (D) Outlined chromosomes in inverted DAPI image. Arrows indicate satellite chromosomes. Arrowheads indicate either satellite or host chromosome, with identification unclear. Scale bar = 10 μm.
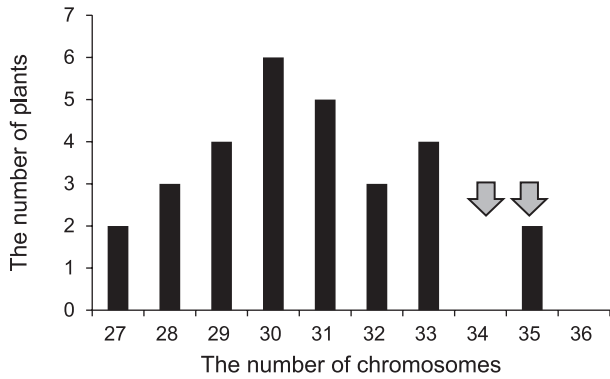
Fig. 5. Distribution pattern of the number of chromosomes in F6 individuals of group 3. Arrows indicate the number of chromosomes of parents.
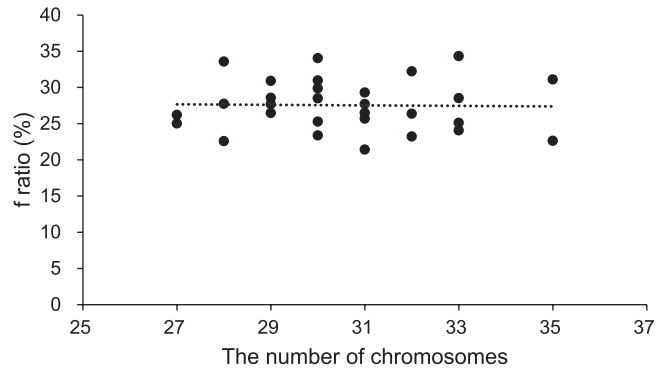

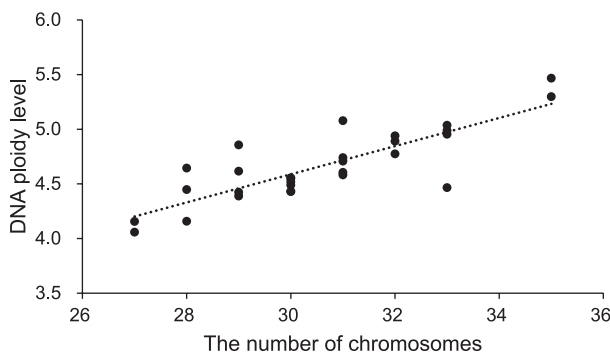**Fig. 8.** Relationship between the number of chromosomes and the f ratio in F6 individuals of group 3.

Fig. 6. Relationship between the number of chromosomes and DNA ploidy levels in F6 individuals of group 3.
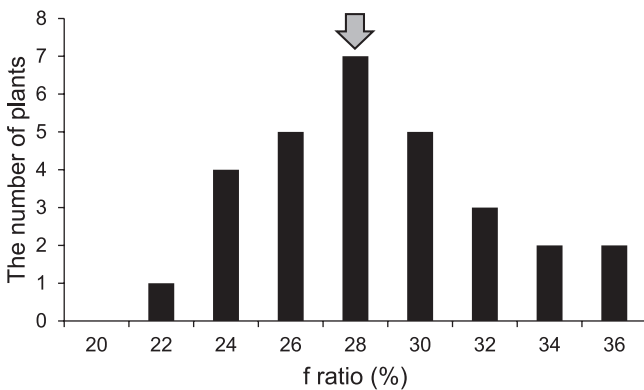

Fig. 7. Distribution pattern of the f ratio in F6 individuals of group 3. Arrow indicates mean f ratio of the parents.

chromosomes and DNA ploidy level in F6-G3 is shown in **Fig. 6**; Pearson's correlation coefficient was 0.83 (P < 0.01), indicating a strong correlation between them.

The f ratio of the three F5 parents was 27.41–28.39%, with a mean of 27.93%, and that of the 29 F6-G3 individuals was 21.42–34.33%, with a mean of 27.64%, a difference that was not statistically significant (*t* test, P > 0.8). The distribution pattern of the f ratio in F6-G3 individuals is shown in **Fig. 7**, which resembles a normal distribution. The

relationship between the f ratio and the number of chromosomes in F6-G3 is shown in **Fig. 8**; Pearson's correlation coefficient was 0.03 (P > 0.8), indicating a poor correlation.

Discussion

Hexaploid festulolium hybrids of Lolium multiflorum (2x) × Festuca arundinacea (6x) converged to tetraploid in this breeding program

Webster and Buckner (1971) aimed to produce octoploid festulolium (2n = 8x = 56) from hybrids of *L. multiflorum* (2n = 2x = 14) × *F. arundinacea* (2n = 6x = 42) by colchicine treatment, and found a decline toward lower chromosome numbers in later generations. However, they doubted their results because of the small number of plants investigated. Similarly, Kleijer (1987) produced amphiploids having 56 chromosomes by colchicine treatment. Across three successive generations, Kleijer (1987) screened amphiploids having 56 chromosomes in each generation and crossed them, finding that most progeny in each generation had a lower number of chromosomes than the 56 chromosomes of the previous generation. He concluded that ensuring genetic stability at the octoploid level and direct use of genomically unstable plants as breeding material were both difficult. Since earlier breeders failed to obtain stable octoploids following colchicine treatment, we attempted to obtain genetically stable breeding materials at the hexaploid level (2n = 6x = 42 chromosomes) from plants not treated with colchicine. Because F1 hybrids of *L. multiflorum* × *F. arundinacea* are male sterile (Kopecky *et al.* 2008, Webster and Buckner 1971), we open-pollinated the F1 hybrids in a field where diploid and tetraploid *L. multiflorum* were cultivated. Frakes and Matheson (1973) suggested open-pollination or polycross method can produce various genetic progenies in less time and at a low cost. We obtained F2 individuals at various DNA ploidy levels. Regarding the diploid F2 individuals (2n = 2x = 14) obtained in this breeding program, we reported the absence of *Festuca* chromatin but remarkable chromosomal rearrangements (Akiyama *et al.* 2012a).

Three F2 putative hexaploids having sufficient seed

fertility were selected and the F3 generation was produced by open pollination. Low-ploidy plants such as diploid and tetraploid *L. multiflorum* might have crossed with the F2 parents; so not surprisingly, we obtained many individuals of lower DNA ploidy level than the F2 parents. On the other hand, although the F4 generation was produced by polycrossing among 8 F3 individuals with a mean DNA ploidy of 5.5, the mean DNA ploidy level of the F4 generation was reduced to 4.8 and showed a significant difference from the F3 parents. This result clearly indicates the occurrence of genome reduction at the hexaploid level, as with the octoploid.

Even if we selected individuals at the hexaploid level from the F4 generation as parents, our breeding program would result in consequences similar to those for the octoploids of Kleijer (1987). We expected that individuals with the most frequent DNA ploidy level, 4.6–5.0, might have a stable chromosome composition in the F4 generation. Thus, to obtain genomic stability in later generations, we produced an F5 generation by polycrossing among 14 F4 individuals at DNA ploidy levels from 4.6 to 5.0, with a mean of 4.8 (corresponding to 4.8 to 5.3, with a mean of 5.1, based on PI staining). Thus, essential genome reduction measured using PI occurred in the F5 generation.

We assigned the F5 individuals to three groups based on their DNA ploidy level and polycrossed them within each group. The parents of F6-G1, -G2 and -G3 consisted of five F5 individuals at a DNA ploidy level similar to tetraploid festulolium, seven F5 individuals at a DNA ploidy similar to the most frequent DNA ploidy level in the F5 generation, and three F5 individuals at a DNA ploidy similar to the most frequent DNA ploidy level in the F4 generation. F6-G2 and G3 showed genome reduction in the F6 generation, but F6-G1 did not. F6-G3 showed a distribution of DNA ploidy level similar to the F5 individuals. Successive generations might result in genomic stability; however, these results imply that genomic stability in the hybrid depends more on ploidy level than a shift between generations. Although the mean DNA ploidy of F6-G2 was higher than F6-G1, the mode of the distribution was close to the tetraploid level. These results suggest that the DNA ploidy level of our hybrids of *L. multiflorum* and *F. arundinacea* converge to tetraploid.

The observation that the F3 individuals with higher DNA ploidy had lower seed fertility raises the possibility of negative selection pressure on gametes with high DNA ploidy. Lu and Kato (2001) produced interspecific hybrids between *Brassica oleracea* or *B. napus* and *B. rapa*, and examined the number of chromosomes at meiotic metaphase II of pollen mother cells and the number of somatic chromosomes of their progeny. Their study demonstrated different survival rates of gametes with different numbers of chromosomes. Similar selection pressure may exist in progeny of high-ploidy festulolium; that is, gametes with high DNA ploidy may have been eliminated. As a result, gametes with low DNA ploidy survived and a reduction in DNA ploidy occurred in successive generations.

Genome reduction could be related to a move toward genome stabilization. Kato and Tokumasu (1983) examined genomic stability of *Brassicoraphanus* (amphiploids between *Brassica japonica* and *Raphanus sativus*) by counting somatic chromosome numbers of progeny of parents with different chromosome numbers from the 2nd to the 11th generations. Plants with hypoploidy tended to produce progeny with higher ploidy than themselves, while plants with hyperploidy tended to produce progeny with lower ploidy. This phenomenon suggested that chromosome numbers of progeny from aneuploids approach euploidy. In this study, although DNA ploidy variation was biased toward reduction, tetraploids reached stability in DNA ploidy, supporting this hypothesis.

Is the Lolium genome transmitted preferentially in festulolium?

One hypothesis for genome reduction could be preferential transmission of the *Lolium* genome in festulolium hybrids; that is, elimination of the *Festuca* genome (Pašakinskienė *et al.* 1997). Zwierzykowski *et al.* (2011) reported that *Festuca* chromatin was reduced in a festulolium hybrid of *F. pratensis* (4x) × *L. perenne* (4x) across the F2 to F8 generations. We developed the f ratio method for evaluating the amount of the *Festuca* genome in festulolium by GISH and image analysis (Akiyama *et al.* 2010). To investigate whether genome constitution is related to genome reduction, we analyzed the f ratio of F6-G3 individuals and their parents, as comparison of their DNA ploidies suggested a large genome reduction between generations. Although the f ratio of F6-G3 individuals was slightly more dispersed than the parents, the mean f ratio of F6-G3 individuals showed no significant difference from the parents, and the distribution pattern showed an unbiased normal distribution. Moreover, there was no relationship between the f ratio and the number of chromosomes in F6-G3 individuals. These results suggest no preferential transmission of the *Lolium* genome in our festulolium hybrids. On the other hand, the number of recombinant chromosomes increased in F6-G3 individuals, implying that a mixed genome status contributes to maintaining the *Festuca* genome. The amount of observed recombinant chromatin in a genome has a tendency to decrease with the progression of mitosis because of chromosome condensation; thus, use of high resolution pachytene chromosomes at meiosis or mitotic prometaphase chromosomes is considered preferable for acquisition of reliable data (Akiyama *et al.* 2010). We analyzed characteristics of metaphase chromosomes in this study rather than analyzing the amount of recombinant chromatin. However, it is possible that the amount of recombinant chromatin in the F6 generation is higher than the F5 generation.

There are 2 cultivars, 'Lofa' and 'Becva', derived from backcrosses of *L. multiflorum* (2x) × *F. arundinacea* (6x) hybrids with *L. multiflorum* (4x). According to Kopecky (2006), they do not have *Festuca* chromosomes and have a few recombinant chromosomes. As they are introgression

cultivars and selected based on ryegrass type plants showing high forage quality and fast growth, these results are not surprising. Zwierzykowski *et al.* (2011) reported dominance for not only the amount of chromatin from *Lolium* but also the number of chromosomes in their breeding materials that have been selected for agronomic traits. The selection might influence their genomic constitution. Recently, we reported that the f ratio in two festulolium cultivars (*F. pratensis* × *Lolium* species, 'Icarus' and 'Nakei 1') did not decrease across generations in maternally yielded progenies (Kubota *et al.* 2015). The materials without any selection are essential to elucidate whether *Lolium* genome has dominancy in festulolium.

Utilization of FCM for festulolium breeding

The Partec PA ploidy analyzer identified the DNA ploidy level in *F. arundinacea* based on DAPI intensity as 5.6 relative to the diploid *L. multiflorum*. On the other hand, according to genome size estimated more exactly by PI intensity, the DNA ploidy level in *F. arundinacea* was 6.4. This difference in the values obtained by DAPI and PI arose from the difference in GC content between *L. multiflorum* and *F. arundinacea*. Šmarda *et al.* (2008) reported that *F. arundinacea* has higher GC content than *L. multiflorum*, which corresponds to our results. As DAPI intensity is affected by both genome size and GC content, PI is a more preferable dye to estimate the ploidy level of festulolium hybrids by FCM.

Satellite chromosomes at nucleolar organizing regions have been observed in *L. perenne*, *L. multiflorum*, *F. pratensis*, *F. arundinacea* and festulolium (Akiyama *et al.* 2010, 2012b, Huang *et al.* 2008). In agreement with these previous studies, many satellite chromosomes were observed in this study. Although most satellite chromosomes could be distinguished by their small size, host chromosomes having a similar size to satellite chromosomes were observed. Pronounced chromosomal rearrangement accompanied by a change in size has been reported in festulolium (Akiyama *et al.* 2012a). If small host chromosomes were mistaken for satellite chromosomes and ignored, the number of chromosomes would be underestimated. Therefore, accurate chromosome counting in festulolium is difficult without FISH using an rDNA probe detecting nucleolar organizing regions, the centromeres, or telomeres. In this study, we selected a telomere probe that provides clear strong signals and can be produced easily by PCR labeling because its sequence information is public. However, using FISH to count the number of chromosomes is extremely labor intensive and time consuming.

Aneuploidy, which causes genomic instability and sterility, is an undesirable characteristic for a plant breeding program; therefore, information on the number of chromosomes is important. FCM can estimate the amount of DNA in a genome, but not the number of chromosomes. Suda *et al.* (2006) made a distinction between the terms "DNA ploidy level" and "DNA aneuploidy" as referenced in FCM

and "ploidy level" and "aneuploidy" in chromosome counting. We showed a strong correlation between the number of chromosomes and the DNA ploidy level. As counting the exact number of chromosome in festulolium is difficult due to satellite chromosomes, FCM is a tremendous tool to determine its ploidy level, in both meanings of the term, at an early stage of our breeding program. However, some individuals showed small discrepancies between the number of chromosomes and the DNA ploidy level, which might be due not only to combinations of different sizes of chromosomes in the genome but also to changes in chromosome size, indicating that counting the exact number of chromosomes by FISH would be required at a late stage of the breeding program, especially in the case of a seed fertility problem.

In this study, we showed that tetraploid hybrids of *L. multiflorum* (2x) × *F. arundinacea* (6x) could achieve genomic stability at the DNA ploidy level, and FCM valuable tool to screen festulolium at the target ploidy level. In our breeding program, we are targeting tetraploid hybrids with FCM to produce festulolium cultivars suited for the environment of the Tohoku district of Japan.

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