Implementation of GISH (genomic *in situ* hybridization) and DNA marker techniques for *Festulolium* breeding

（GISH および DNA マーカー技術のフェストロリウム育種への利用）

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Table of contents

Chapter 1: General introduction

1.1 Background

Lolium and *Festuca* species are agronomically important grasses that are found in temperate regions worldwide. Italian ryegrass (*Lolium multiflorum* Lam., which is called "Nezumimugi" in Japanese) and perennial ryegrass (*Lolium perenne* L., known as "Hosomugi" in Japanese) have high digestibility, they establish themselves quickly, and grow rapidly. Compared to *Lolium* species, tall fescue (*Festuca arundinacea* Schreb., syn. *Lolium arundinaceum* (Schreb.) Darbysh., syn. *Schedonorus arundinaceus* (Schreb.) Dumort, known as "Oniushinokegusa" in Japanese) and meadow fescue (*Festuca pratensis* Huds., syn. *Lolium pratense* (Huds.) Darbysh, syn. *Schedonorus pratensis* (Huds.) P. Beauv., known as "Hirohaushinokegusa" in Japanese) are robust and well adapted to a wide range of climate. The United States Department of Agriculture (USDA) uses *Schedonorus* instead of *Festuca* for genus name; however, the Organization for Economic Co-operation and Development (OECD) and Ministry of Agriculture, Forestry, and Fisheries (MAFF) in Japan uses *Festuca*. Additionally, "*F. arundinacea*" and "*F. pratensis*" are well established terms in this industry. For the purpose of this work, *Festuca* is used instead of *Schedonorus*.

For efficient livestock production, easily digestible and high yield *Lolium* species are preferable. However, *Lolium* species are not resilient to abiotic stresses (e.g., drought and

cold temperatures). Alternatively, *Festuca* species, especially tall fescue, are resilient to abiotic stresses but are underproductive (per unit of area). *Festulolium*, an intergeneric hybrid of *Lolium* and *Festuca* species, may possess a combination of agronomic benefits from both genera (Yamada *et al*., 2005). Moreover, *Festulolium* is also used as a collective term for several combinations of *Lolium* and *Festuca* species (Ghesquière *et al*., 2010). *Festulolium* is generally classified according to the parental *Festuca* species. If the *Festuca* parent is a tall fescue, F_1 plants are male sterile. Therefore, breeders must adopt the introgression forms. If the *Festuca* parent is a tetraploid meadow fescue, then F_1 plants are partly male fertile. Breeders can choose introgression or amphidiploid forms according to each breeding purpose. In fact, breeders have created a variety of *Festulolium* cultivars. Tall fescue form introgression cultivars have been bred to improve the digestibility of tall fescue. 'Felina', 'Hykor', 'Korina', and 'Lesana' were bred in the Czech Republic. 'Kenhy' and 'Johnstone' were bred in the USA. Mainly in Europe, a number of introgression and amphidiploid form cultivars have been bred from a cross with meadow fescue to improve drought and freezing tolerance (Ghesquière *et al*., 2010).

Over the past four decades, the decrease in rice consumption has brought about a surplus of paddy fields in Japan. Forage cropping is expected in these paddy fields. In the Tohoku region, the orchard grass (*Dactylis glomerata* L., which is called "Kamogaya" in

Japanese) is a major grass. Compared to *Lolium* species, orchard grass is robust and vigorous in summer but is not suitable for growing in fallow paddy fields because it cannot tolerate the excess water conditions (Touno *et al*., 2014; Yonemaru *et al*., 2004a). Since *Festulolium* species can tolerate these conditions, a characteristic inherited from their *Lolium* parents (Yonemaru *et al*., 2004a), they are suitable for growth in fallow paddy fields for harvest. Many researchers have studied *Festulolium* growth characteristics, digestibility, and suitability for grazing in Japan (Kanno *et al*., 1993; Touno *et al*., 2004; Touno *et al*., 2006; Yonemaru *et al*., 2004b). However, there has been no work to study the snow endurance of *Festulolium* cultivars. Considering that the Tohoku region is a snow-covered area, snow endurance is the most important characteristic for cultivars in this region. It is, therefore, essential to compare snow endurance of *Festulolium* to other related species.

Two *Festulolium* cultivars, 'Tohoku 1' (Yonemaru *et al*., 2011) and 'Icarus' (Ueyama *et al*., 2014) were recently bred at the Tohoku Agricultural Research Center, National Agriculture and Food Research Organization (NARO). When 'Tohoku 1' was cultivated in an abandoned paddy field, its yield was over 12 t dry matter ha^{-1} $year^{-1}$ (Touno *et al*., 2014). Touno *et al*. (2012) harvested 'Tohoku 1' for three years in the Tohoku region because *Festulolium* cultivars have better persistence than Italian ryegrass. However,

there are concerns that *Festulolium* cultivation may induce rice bug infestations, as in the case of Italian ryegrass. Italian ryegrass is a major source of two typical shield bugs, namely *Trigonotylus caelestialium* (Kirkaldy) and *Stenotus rubrovittatus* (Matsumura). These rice bugs lay their eggs on the spikes of gramineous plants. Overwintering eggs hatch in spring and become adults in early summer. These adult bugs fly into Italian ryegrass where they lay their eggs. Mass generation adults migrate into rice paddy fields and cause pecky rice seasonally (Hachiya, 1999; Kakizaki, 2004). As a solution to rice bug infestations, Italian ryegrass was successfully artificially inoculated with an endophyte (*Epichloë uncinata* (W. Gams, Petrini & D. Schmidt) Leuchtmann & Schardl) (Kasai *et al*., 2004). The *E. uncinata*-infected Italian ryegrass cultivar, 'Bishamon', was also developed from the progeny of inoculated plants because *E. uncinata* was vertically transmitted. *E. uncinata* produces loline-alkaloids (N-formylloline, N-acetylloline, etc.) that are toxic to insects and not livestock (Kasai *et al*., 2006). In the laboratory, artificially inoculated Italian ryegrass plants efficiently controlled *T. caelestialium* (Shiba *et al*., 2007). 'Bishamon' was also shown to efficiently control *S. rubrovittatus* in the field (Matsukura *et al*., 2014). Endophyte-infected *Festulolium* is expected to also be effective at controlling rice bugs. *E. uncinata* was measured using quantitative real-time PCR using a primer set designed from the nucleotide sequence of the 18S ribosomal RNA gene

(Matsukura *et al*., 2014). A related endophyte (*Neotyphodiumu occultans* C.D. Moon, B. Scott & M.J. Chr.), could be identified using DNA markers (Sugawara *et al*., 2006). However, these DNA markers had not been used in a practical breeding program. Using these DNA marker sets, endophyte infected *Festulolium* cultivars must be bred efficiently.

By the end of the last century, genomic *in situ* hybridization (GISH) techniques, which make it possible to distinguish between the genomes of two species, had been developed. Since then, many *Festulolium* cultivars and strains have been analyzed using GISH (Canter *et al*., 1999; Kopecky *et al*., 2005; Pašakinskienė *et al.,* 1998; Zwierzykowski *et al*., 1998, 2006). This has allowed for the detection of the genome balance drift that results from crosses between *Lolium* species and *F. pratensis*. This genome drift favors *Lolium* in tetraploid amphidiploid *Festulolium* plants. The same tendency was reported in a *Jatropha* interspecific hybrid (Fukuhara *et al*., 2016). Commercial seeds need to be minimally two times of seed multiplication, resulting in advances of two generations. Herein lies another challenge in the cultivation of *Festulolium*. There are concerns that the *Festuca* genome will disappear from allogamous cultivars during commercial seed production. Several hypotheses have been proposed to explain this genome balance drift (Jones *et al*., 2005), including gametic competition, pollination effects, and selection for seedling vigor, fertility, and seed yield. Work by

Zwierzykowski *et al*. (2006) hypothesized that the substitution of *Festuca* chromosomes and chromatin with those from *Lolium* occurs during sexual reproduction, yet the precise mechanisms underlying this genome balance drift remain uncertain. Zwierzykowski *et al*. (1998, 2006) measured the genome lengths of each genus to investigate this phenomenon. Unfortunately, chromatin is not uniformly distributed in chromosomes and euchromatic regions may be overestimated; therefore, the application of this method would likely be challenging. Although Zwierzykowski *et al*. (1998, 2006) tested breeding populations and bulked seeds in each generation, because breeding populations were selected with different agronomic traits, it is not possible to judge whether the genome balance drift was due to selection or chromosome substitutions during sexual reproduction. It is vital to elucidate the cause of genome balance drift using an accurate measurement of genomic constitution in the appropriate population.

1.2 The objectives and composition of this thesis

In chapter 2, to show that *Festulolium* is suitable for growth in the Tohoku region, the author investigated *Festulolium* cultivars and related species in the field, with a specific focus on snow endurance. In chapter 3, to dispel the rice bug concerns, a new set of breeding methods were developed to breed endophyte-infected *Festulolium* simply

using DNA markers. To clarify the cause of genome balance drift, a method to aid the determination of the genomic constitution of *Festulolium* efficiently was developed. The so called “f ratio” uses the ratio of total area of *Festuca*-specific genome regions to the total area of all genome regions (Akiyama *et al*., 2010). In Chapter 4, using the f ratio method, genome balance drifts between maternal plants and their progenies in three populations of *Festulolium* were analyzed. Additionally, the seed yields, ripening rates, and germination rates were monitored in the field over a period of two years. Subsequently, correlations between f ratio values and seed yield-related traits were analyzed. The data generated here show that *Lolium*-favored genome balance drift can be caused solely by phenotypic differences, regardless of any biases, that may occur during meiosis. Chapter 5 is a general discussion of *Festulolium* breeding. Remote crossing (syn. wide cross or distant hybridization) is a standard strategy for crop breeding of which *Festulolium*, the intergeneric hybrid, is a prime example of. There are two problems to overcome in remote crossing; hybrid weakness and hybrid breakdown. The similarities and differences between *Festulolium* and other intergeneric and interspecific hybrids were identified in this study. I have also pointed out problems for cultivating *Festulolium* and suggested ways in which they can be resolved and future work for *Festulolium* breeding methods.

Chapter 2: Potential of *Festulolium* cultivars in Tohoku

2.1 Introduction

The Tohoku region is a major paddy field zone in Japan. Due to changes in Japanese eating habits, rice consumption has declined. Consequently, unused surplus paddy fields are a major economic problem. The government intends to crop forages in these paddy fields. Orchard grass, a major grass found in Tohoku, is more robust and vigorous in summer than *Lolium* species. However, orchard grass is not suitable for growing in fallow paddy fields because it cannot tolerate the excess water conditions. In contrast, *Festulolium*, which have inherited their tolerance to excess water conditions from their *Lolium* parents (Yonemaru *et al*., 2004a), are suitable for growing in fallow paddy fields for harvest. There have been numerous studies into *Festulolium* growth characteristics, digestibility, and suitability for grazing in Japan (Kanno *et al*., 1993; Touno *et al*., 2004; Touno *et al*., 2006; Yonemaru *et al*., 2004b). However, no research have been conducted on the snow endurance (i.e. tolerance to snow molds) of *Festulolium* cultivars. Snow endurance is the most important characteristic for cultivation in the Tohoku region because it is a snow-covered area. The main cause of snow mold in this region is *Typhla incarnata* Lasch:Fries. Pink snow mold (*Microdochium nivale* (Fr.) Samuels & Hallett) is rarely seen (Iida, 1965). In this chapter, the snow endurance of *Festulolium* cultivars

is compared with related species, such as Italian ryegrass, perennial ryegrass, and hybrid ryegrass (*Lolium* × *boucheanum* Kunth, which is called "Nezumihosomugi" in Japanese). Hybrid ryegrass is an interspecific hybrid between Italian ryegrass and perennial ryegrass.

2.2 Materials and methods

2.2.1 Plant materials

Two *Festulolium* cultivars, two Italian ryegrass cultivars, two perennial ryegrass cultivars, and 16 hybrid ryegrass cultivars were tested (Table 1). All cultivars are tetraploid.

2.2.2 Field tests

The experimental field was located in the Tohoku Agricultural Research Center, NARO (Iwate, Japan; 39°44'N, 141°8'E). The average yearly temperature of the experimental site is 10.2°C (Japan Meteorological Agency, 2020). The soil is Andosol. The experimental design utilized a randomized block design with 3 replicates, each spaced 0.75 m apart with a distance of 0.75 m between the rows. Ten plants were tested in each replicate and in total, 30 plants for each cultivar were tested. Seeds from each

Table 1. Characteristics of tested cultivars.

Species †	Cultivar or strain	First heading date (days) ‡	Plant length at early stage (cm)	Culm length (cm)	Vigor in spring (1–9) §	Vigor after third cut (1–9) §	Snow endurance in first year (1–9) §	Snow endurance in second year (1–9) §
FL	Evergreen	21	26	76	6.1	4.5	5.7	4.8
FL	Paulita	23	32	85	6.6	4.2	4.8	3.7
HR	Agata	24	37	85	7.3	5.6	4.6	3.8
HR	Antilope	19	37	84	7.3	5.8	4.9	3.9
HR	Bison	25	37	97	7.8	3.9	5.3	3.8
HR	Delicial	19	36	81	7.5	5.1	5.0	3.9
HR	Dorcas	20	42	85	7.4	6.4	4.5	3.6
HR	Fleurial	24	35	84	7.1	5.5	4.8	4.2
HR	Gazella	16	42	87	7.5	6.6	4.3	3.8
HR	Highflora	20	39	86	7.6	6.8	4.9	4.5
HR	Hymer	24	36	84	7.5	4.4	4.8	3.6
HR	Ibex	23	40	92	7.1	6.8	4.1	3.1
HR	Odra	29	37	81	7.5	4.9	5.2	4.2
HR	Rusa	17	41	83	7.5	6.4	4.5	4.1
HR	Sirene	28	37	84	7.4	4.1	5.2	4.0
HR	Tetrelite	28	39	87	7.6	3.8	4.7	3.6
HR	Tine	19	36	84	7.5	5.2	4.6	3.4
HR	4X–H1	26	35	87	6.9	3.7	5.0	3.8
IR	Ace	22	44	89	6.8	4.6	4.2	3.5
IR	Akiaoba	19	46	99	8.2	5.6	4.2	3.4
PR	Fantoom	24	28	64	6.1	3.9	6.3	4.5
PR	Friend	30	28	67	6.2	4.3	6.4	5.1

† FL, HR, IR, and PR mean Festulolium, hybrid ryegrass, Italian ryegrass, and perennial ryegrass, respectively.

‡ Number of days from May 1st.

§ Relative evaluation. 1: poor – 9: excellent.

cultivar were cultivated in pots containing fertilized granulated soil (Kureha Co., Tokyo, Japan) in a greenhouse. Plants were transplanted on 17th September, 2003 and then mowed on 30th June, 3rd August, and 28th September, 2004. Chemical fertilizer was applied at a rates of 6 g N, 6 g P_2O_5, and 6 g K_2O per m^2 at the time of transplantation, 3 g N, 3 g P_2O_5, and 3 g K_2O per m^2 in early spring, and 2 g N, 2 g P_2O_5, and 2 g K_2O per m^2 after each cutting.

2.2.3 Tested characteristics

The characteristics that were investigated include first heading date, plant length at early stage, culm length, vigor in spring, vigor after third cut, and snow endurance in first year and second year. The dates and details of each investigation are shown in Table 2.

2.3 Results and discussion

During the first winter, snow cover continued from 31st December to 12th March. No plants died but the symptoms of snow mold disease were severe enough to investigate snow endurance. During the second winter, snow cover occurred from 31st December to 22nd March. A few plants died after the second winter because they had been wilted after summer.

Table 2. Plant characteristics investigated and the dates and details of investigation.

Characteristic	Date			Detail
Plant length at early stage	Nov.	18th	2003	
Snow endurance in first year	May.	16th	2004	Observation rating (1: poor – 9: excellent)
First heading date			2004	The day when two stems headed
Vigor in spring	Apr.	9th	2004	Observation rating (1: poor – 9: excellent)
Culm length	Jun.	18th	2004	
Vigor after third cut	Oct.	21st	2004	Observation rating (1: poor – 9: excellent)
Snow endurance in second year	Apr.	1st	2005	Observation rating (1: poor – 9: excellent)

Table 1 showed the tested characteristics in the order of each grass species. Italian ryegrass had higher culm length than perennial ryegrass. *Festulolium* and hybrid ryegrass' were intermediate between Italian ryegrass and perennial ryegrass. In Japan, forage grass is mainly used for cutting (silage or hay). Grazing is not popular. Therefore, higher culm length cultivars are preferable.

Compared to Italian ryegrass, perennial ryegrass had higher snow endurance and lower plant length during the early stages. *Festulolium* and hybrid ryegrass are intermediate between perennial ryegrass and Italian ryegrass in these characteristics. When considering cutting for a multi-year period, snow endurance in the second year is more important than in the first year. Although Italian ryegrass and hybrid ryegrass had higher vigor after the third cut, their snow endurance in the second year was lower than *Festulolium* and perennial ryegrass. Perennial ryegrass is appropriate for grazing, but not for cutting because it has a short plant length. Therefore, *Festulolium* was thought to be the best species to grow in the fallow paddy fields of Tohoku. Recently, two *Festulolium* cultivars, 'Tohoku 1' and 'Icarus' were bred at Tohoku Agricultural Research Center. 'Tohoku 1' was bred from the tetraploid *Festulolium* cultivars, 'Evergreen', 'Paulita', and 'Tandem' by mass selection and maternal-line selection (Yonemaru *et al*., 2011). 'Icarus' was bred from the tetraploid *Festulolium* cultivars, 'Evergreen', 'Paulita', 'Tandem', and

‘Duo’ by mass selection and maternal-line selection (Ueyama *et al*., 2014).

Chapter 3: The development of simple DNA marker-assisted selection of endophyte (*Epichloë uncinata*)-infected *Festulolium*

3.1 Introduction

In Japan, four *Festulolium* cultivars have been bred and registered, namely 'Tohoku 1', 'Icarus', 'Nakei 1', and 'North fest' (Yonemaru *et al*., 2011; Ueyama *et al*., 2014). Since these *Festulolium* cultivars are tolerant to excess water conditions, a trait inherited from their *Lolium* parents, they are suitable for growing in fallow paddy fields for harvest. Moreover, when 'Tohoku 1' was cultivated in an abandoned paddy field, its yield was over 12 t dry matter ha^{-1} $year^{-1}$ (Touno *et al*., 2014). Touno *et al*. (2012) harvested 'Tohoku 1' for three years in northern Japan because *Festulolium* cultivars persist more than Italian ryegrass. However, there are concerns that *Festulolium* cultivation may encourage rice bug infestations, as in the case of Italian ryegrass. Italian ryegrass is a major source of two typical shield bugs, namely *Trigonotylus caelestialium* (Kirkaldy) and *Stenotus rubrovittatus* (Matsumura), which invade rice paddy fields resulting in pecky rice (Hachiya, 1999; Kakizaki, 2004). As a solution to this rice bug infestation, Italian ryegrass was successfully artificially inoculated with an endophyte (*Epichloë uncinata*) (Kasai *et al*., 2004). Subsequently, the *E. uncinata*-infected Italian ryegrass cultivar 'Bishamon' was developed from the progeny of inoculated plants because *E.*

uncinata was vertically transmitted. *E. uncinata* produces loline-alkaloids (such as N-formylloline and N-acetylloline) that are toxic to insects but not livestock (Kasai *et al*., 2006). Artificially inoculated Italian ryegrass plants successfully controlled *T. caelestialium* (Shiba *et al*., 2007) in the laboratory, and 'Bishamon' was also shown to be efficient at controlling *S. rubrovittatus* (Matsukura *et al*., 2014) in the field. Endophyte-infected *Festulolium* is expected to be similarly efficient at controlling rice bugs. *E. uncinata* is an endophyte originating from meadow fescue, as a result, *Festulolium* plants whose maternal parents are meadow fescue may potentially harbor *E. uncinata*. However, some polyploidization procedures, (e.g. sterilization of embryo cultures by bleaching) may eliminate the endophyte from *Festulolium*. Since artificial inoculation of Italian ryegrass with *E. uncinata* has a low success rate (Kasai *et al*., 2004), backcrossing *E. uncinata*-infected Italian ryegrass to *Festulolium* was thought to be a preferable option. Additionally, the *E. uncinata* strain used for the inoculation of Italian ryegrass would also be expected to be compatible with *Festulolium* which contain components of the *Lolium* genome.

In a hybrid fertility test with perennial and Italian ryegrass carried out in an isolated room, Arcioni *et al*. (1983) showed that 7.76% of Italian ryegrass seeds were self-pollinated, using the isozyme phosphoglucoisomerase as a genetic marker. For efficient

backcrossing of *Festulolium*, the self-pollinated progeny need to be excluded from the backcrossed seeds. Hot water emasculation poses the risk of destroying the endophyte, and, because manual emasculation is incomplete, the progeny must still be tested for self-pollination even after the event. Given that backcrossed *Festulolium* parents belonged to the tetraploid amphidiploid cultivar 'Nakei 1', which is the progeny of hybrids between tetraploid meadow fescue and tetraploid Italian, perennial, and hybrid ryegrasses (the author's personal communication), the truly backcrossed seeds of *E. uncinata*-infected Italian ryegrass should have some chromatin elements from the *Festuca* genome. Akiyama *et al*. (2010) developed the "f ratio" method which combines GISH and image analyses, to determine the ratio of the *Festuca*-specific genome region to the whole genome. The f ratio method can be used to determine whether F_1 plants were self-pollinated or truly backcrossed. Using rice genomic information, Tamura *et al*. (2009) developed a polymerase chain reaction (PCR)-based marker system that could distinguish between *Lolium* and *Festuca*. In the first generation of a backcrossing to the *Festulolium* strategy, truly backcrossed hybrids showed some *Festuca*-specific band patterns, whereas the self-pollinated progeny of endophyte-infected Italian ryegrass did not. The objective of this chapter was to investigate whether the f ratio method and the marker system can be successfully used to distinguish self-pollinated progeny. The second aim of this chapter

was to determine the number of backcrossing generations for which these methods could be used for such detection. The presence of *E. uncinata* in our backcrossed progeny was also detected using PCR with a primer set described by Matsukura *et al*. (2014).

3.2 Materials and methods

3.2.1 First backcrossing (F_1 development)

The tetraploid *E. uncinata*-infected Italian ryegrass strain '09MN4X', the progeny of artificially inoculated tetraploid Italian ryegrass, was used as the maternal plant. This was supplied by the Japan Grassland Agriculture and Forage Seed Association. Seedlings were grown in pots containing fertilized granulated soil (Sankensoiru Co., Iwate, Japan) in a greenhouse. Five *E. uncinata*-infected plants were selected by microscopy and named as maternal plant 1, 2, 3, 4, and 5. The paternal parents of the repeated backcrossing were 30 *Festulolium* plants selected for snow endurance from cultivar 'Nakei 1'. 'Nakei 1' had no endophyte-infected seeds (the author's personal communication). The f ratio (ratio of *Festuca*-specific regions to the whole genome) of the 30 paternal plants was 49% on average. The f ratios were calculated using a previously reported method (Akiyama *et al*., 2010). Each Italian ryegrass plant was backcrossed by surrounding it with six *Festulolium* plants in an air-conditioned, isolated greenhouse. F_1 seeds were obtained from the

maternal plants, dried in an airy shaded room, and stored at 4°C. Observation by microscopy showed that the progeny of maternal plant 5 were not endophyte-infected after the preliminary seed infection test. This line was, therefore, excluded from the ensuing tests.

3.2.2 Second backcrossing (BC_1 development)

Twenty-one F_1 seedlings (3—6 from each of four Italian ryegrass parents) were grown in pots in the greenhouse. Each plant was divided into two ramets; one was planted in a field on 30th September 2013, while the other was preserved in the greenhouse for DNA extraction. Six F_1 plants (named F_1–2, F_1–3, F_1–12, F_1–17, F_1–18, and F_1–20) were used for the second backcrossing (BC_1) development the following spring. Each F_1 plant was backcrossed by surrounding it with five *Festulolium* plants in an air-conditioned, isolated greenhouse. BC_1 seeds were obtained from the maternal plants, as with the F_1. Two hundred and forty BC_1 seeds, obtained from six F_1 plants, were grown in pots in the greenhouse and planted in a field on 30th September 2014 for BC_2 development.

3.2.3 DNA extraction

On 29th October 2013, genomic DNA of each F_1 plant was extracted for DNA marker

analysis from leaf blades growing in a greenhouse using the QIAGEN DNeasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany). The leaf blades of each BC_1 plant were sampled in the field on 29th October 2014, and genomic DNA was extracted as with the F_1 plants. The genomic DNA of tetraploid Italian ryegrass clone L503 and tetraploid meadow fescue clone MF07 were extracted for *Lolium* and *Festuca* standards, respectively. Italian ryegrass clone L503 did not contain any endophyte whereas meadow fescue clone MF07 did. For MF07, DNA was extracted from the leaf sheaths because the endophyte is more abundant in the leaf sheaths than in the leaf blades (Matsukura *et al*., 2014).

The genomic DNA of four Italian ryegrass maternal plants (coded as 1, 2, 3, and 4) and six cultivars or strains used to breed 'Nakei 1' were also extracted to study whether the paternal band patterns of truly backcrossed hybrids were *Festuca*-specific. The six cultivars or strains were Italian ryegrass cultivar 'Akiaoba 3', hybrid ryegrass cultivar 'High flora', hybrid ryegrass strain 'Yatsugatake H2', perennial ryegrass cultivar 'YatsukazeII', meadow fescue cultivar 'Tomosakae', and 'First'. For 'Akiaoba 3', 'High flora', 'Yatsugatake H2', and 'YatsukazeII', DNA was extracted from the bulked leaf blades of 10 plants of each cultivar or strain. For 'Tomosakae', DNA was extracted from each of the 10 plants. For 'First', DNA was extracted from bulked seeds because they were old and did not germinate.

3.2.4 DNA marker analyses

Marker analyses for the detection of self-pollinated progeny was carried out according to Tamura *et al*. (2009), with slight modifications. Genomic DNA was extracted using QIAGEN DNeasy Plant Mini Kit (QIAGEN GmbH). PCR amplification was performed using Ampdirect Plus with a BIOTAQ Kit (Shimadzu, Kyoto, Japan). The cycling conditions in Tamura *et al*. (2009) were used with an initial denaturation step of 10 min at 95°C. The banding patterns were visualized using Midori Green DNA Stain (Nippon Genetics Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. For the F_1 progeny, a total of 19 PCR and cleaved amplified polymorphic sequence (CAPS) markers (Table 3) were applied and plants showing paternal band patterns were considered as truly backcrossed hybrids. For the BC_1 progeny, 55 PCR and CAPS markers were applied in total (Table 3) which did not indicate any paternal band in their maternal parents (i.e. F_1 plants). Plants with novel paternal bands in BC_1 were considered to be truly backcrossed hybrids. It was necessary to understand whether the novel paternal bands from the 19 distinguishable markers used in F_1 and BC_1 (Table 3) were from the *Festuca* or *Lolium* genome of 30 *Festulolium* paternal parents. For this, the author used genomic DNA of four Italian ryegrass maternal plants (maternal plant 1, 2, 3, and 4) and six cultivars or strains used to breed 'Nakei 1'. The mean frequencies of *Festuca*-specific

Table 3. List of DNA markers used.

Marker name / corresponding rice locus number in TIGR	Restriction enzyme[†]	Tested in F_1	Tested in BC_1	Specificity	Frequency of *Festuca*-specific bands among BC_1 plants[§] (%)
Distinctive for hybridity					
Os07g01760		○[‡]		*Festuca*-specific band	
Os04g54410		○		*Festuca*-specific band	
Os01g08190		○		*Festuca*-specific band	
Os03g38980		○	○	*Festuca*-specific band	62
Os09g34970		○	○	*Festuca*-specific band	63
Os02g03260		○	○	*Festuca*-specific band	64
Os08g36774		○	○	*Festuca*-specific band	13
Os06g36700		○		*Festuca* and 'Akioaba 3' specific band	
Os08g41830	*Taq*I	○	○	*Festuca*-specific band	81
Os11g47710	*Afa*I		○	*Festuca*-specific band	67
Os06g15420	*Afa*I		○	*Festuca*-specific band	48
Os02g30800	*Afa*I		○	*Festuca*-specific band	81
Os03g36750	*Afa*I		○	*Festuca*-specific band	26
Os01g55540	*Dde*I		○	*Festuca*-specific band	48
Os08g01350	*Dde*I		○	*Festuca*-specific band	55
Os10g02980	*Dde*I		○	'Maternal plant 3' specific band	91
Os12g27830	*Dde*I		○	*Festuca*-specific band	62
Os11g48040	*Taq*I		○	*Festuca*-specific band	57
Os08g33630	*Taq*I		○	*Festuca*-specific band	53
Distinctive but not usable in BC_1					
Os04g43220	*Afa*I		○		
Os05g04190	*Afa*I		○		
Os07g38620	*Afa*I		○		
Os08g03390	*Afa*I		○		
Os11g43900	*Afa*I		○		
Os02g29530	*Dde*I		○		
Os04g27860	*Dde*I		○		
Os08g15080	*Dde*I		○		
Os12g08810	*Dde*I		○		
Os07g30840	*Taq*I		○		
Os08g09940	*Taq*I		○		
Os08g31810	*Taq*I		○		
Not distinctive					
Os07g25430		○			
Os03g06220		○			

Os03g64210		○	
Os11g02580		○	
Os09g03610		○	
Os06g49500		○	
Os06g16350		○	
Os05g43360	*Afa*I	○	
Os10g10244	*Dde*I	○	
Os10g11140	*Taq*I	○	
Os01g02880	*Afa*I		○
Os01g25370	*Afa*I		○
Os01g63270	*Afa*I		○
Os02g47310	*Afa*I		○
Os03g07300	*Afa*I		○
Os03g29950	*Afa*I		○
Os04g37619	*Afa*I		○
Os05g13780	*Afa*I		○
Os07g39630	*Afa*I		○
Os09g20640	*Afa*I		○
Os09g20880	*Afa*I		○
Os12g13320	*Afa*I		○
Os12g40550	*Afa*I		○
Os01g43250	*Dde*I		○
Os01g64720	*Dde*I		○
Os02g57160	*Dde*I		○
Os03g56300	*Dde*I		○
Os05g38330	*Dde*I		○
Os06g04280	*Dde*I		○
Os07g01480	*Dde*I		○
Os08g23320	*Dde*I		○
Os12g23180	*Dde*I		○
Os12g42980	*Dde*I		○
Os05g06330	*Taq*I		○
Os08g27010	*Taq*I		○
Os10g39930	*Taq*I		○
Os11g34130	*Taq*I		○
Os11g38020	*Taq*I		○

† CAPs markers.

‡ The circle means that each marker was tested in F1 or BC1.

§ Mean frequency of Festuca-specific bands among BC1 plants in each maternal line.

bands among BC_1 plants were calculated on each DNA marker set. Firstly, the mean frequencies in each maternal line were calculated and averaged.

PCR detection of *E. uncinata* was performed using the primer set designed for real-time PCR by Matsukura *et al*. (2014). The cycling conditions were as follows: an initial denaturation step of 10 min at 95°C, 30 cycles of 20 s at 95°C, 20 s at 60°C, and 15 s at 72°C, followed by a final extension of 10 min at 72°C. The positive standard for *E. uncinata* was the genomic DNA extracted from the leaf sheaths of MF07, and the negative standard was the genomic DNA from leaves of L503. *E. uncinata*-infected plants show a PCR band while uninfected plants did not.

3.2.5 Microscopy based observations of the endophyte

A common microscopy method (Saha *et al*., 1988), with some modifications, was used to study endophytes. For endophyte detection in F_1 plants, a thin membrane of the leaf sheath was scratched with tweezers, placed on a microscope slide, and a 0.25% (w/v) Rose Bengal aqueous solution was added. For endophyte detection in BC_1 plants, it was checked that whether any of their open-pollinated seeds (n = 15) were infected. Seeds were soaked in a 5% (w/v) NaOH aqueous solution for 24 h, washed, soaked in a 0.25% Rose Bengal aqueous solution, and then squashed on the slide.

3.2.6 Genomic *in situ* hybridization (GISH) analysis

GISH analysis was used to determine whether the F_1 plants were true hybrids of *Festulolium* or self-pollinated Italian ryegrass. All GISH analyses were carried out with a slight modification to the previously described method (Akiyama *et al*., 2010). The f ratios were also analyzed for six selected F_1 plants (F_1–2, F_1–3, F_1–12, F_1–17, F_1–18, and F_1–20). On average, four chromosome spreads (3—5) were tested for each plant. Total genomic DNA from plants L503 (*L. multiflorum*) and MF07 (*F. pratensis*) were extracted using the QIAGEN DNeasy Plant Mini Kit (QIAGEN GmbH), and labeled with Fluorescein-12-dUTP (PerkinElmer, Inc., Waltham, USA) and Texas Red-5-dUTP (PerkinElmer, Inc.), respectively, using a Nick Translation Kit (Roche, Basel, Switzerland). GISH was performed with these probes using a direct–labeling protocol. Next, the chromosome spreads were mounted in a drop of Vectashield mounting medium (Vector Laboratories, Burlingame, USA) containing 1.5 μg mL^{-1} 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). The DAPI images of each chromosome spread were then divided into two areas according to the GISH results. These two areas consisted of either *F. pratensis* or *Lolium*–specific chromatin. The f ratio was then calculated using the integrated DAPI intensity values of *Festuca*–specific chromatin / total chromatin.

3.3 Results and discussion

3.3.1 Hybridity test

Tamura *et al*. (2009) developed a PCR-based marker system using rice genomic information to distinguish between perennial ryegrass and meadow fescue. As suggested by Tamura *et al*. (2009), some of these markers were expected to also distinguish between Italian ryegrass and meadow fescue. Here, the goal was to determine whether these marker sets could also be useful for identifying true hybrids of *Festulolium* backcrossed to Italian ryegrass in F_1 and BC_1 plants. The hybridity and endophyte infection of F_1 plants are shown in Table 4. In this experiment, 19 DNA marker sets were applied to determine hybridity (Table 3), only 9 of which could distinguish between *Lolium* and *Festulolium*. Markers that could not distinguish between *Festulolium* and *Lolium* either showed no bands or no polymorphism between *Lolium* and *Festuca* species. F_1–13 did not present a *Festuca*-specific band pattern, indicating self-pollination of the Italian ryegrass mother plant. Similarly, GISH analysis showed that F_1–13 did not contain a *Festuca*-specific genomic region, unlike the other analyzed F_1 progeny (Figure 1). Thus, both DNA marker and GISH analysis consistently distinguished between cross and self-pollinated progeny plants.

Six F_1 plants (F_1–2, F_1–3, F_1–12, F_1–17, F_1–18, and F_1–20) were selected based on

Table 4. Hybridity and endophyte infection of F_1 plants (*E. uncinata*-infected Italian ryegrass × *Festulolium*) based on DNA marker analysis, GISH, microscopic observations, and PCR detection.

	Maternal plant 1						Maternal plant 2						Maternal plant 3						Maternal plant 4		
F_1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Marker name																					
Os07g01760	F‡	N	F	N	F	N	F	F	F	F	F	F	N	F	F	F	F	F	F	F	N
Os04g54410	N	N	F	N	F	F	F	F	F	F	F	F	N	F	F	F	F	F	F	F	N
Os01g08190	F	N	F	F	?	F	N	F	F	F	F	F	N	F	F	F	F	F	F	N	N
Os03g38980	F	N	N	F	F	F	N	F	F	F	N	N	N	N	F	F	F	F	N	N	F
Os09g34970	F	N	F	N	F	N	F	F	F	F	F	F	N	F	F	F	F	F	N	N	F
Os02g03260	F	N	F	F	?	N	?	N	F	F	F	F	N	F	F	F	F	F	N	N	F
Os08g36774	N	N	N	N	N	N	F	N	F	F	N	N	N	N	N	N	F	N	N	N	N
Os06g36700	F	N	N	N	F	N	N	F	F	F	F	F	N	F	F	F	F	F	F	F	N
Os08g41830§	F	F	N	F	F	?	F	N	F	F	F	N	N	N	F	F	F	F	F	N	F
GISH	F	F	F	F	F	F		F	F	F	F	F	N	F		F	F	F	F	F	F
f ratio¶		11.6	19.1									28.1					29.0	31.1		16.7	
Endophyte infection																					
Microscope	+	+	+		+	+		+	+	+	+	+	+	+		+	+	+	+	+	−
PCR detection	+	+	+	+	+	+	+	−	+	+	+	+	−	+	+	−	+	+	+	+	+

†Marker names correspond to rice locus number in The Institute for Genomic Research (TIGR).

‡F and N indicate that the plant showed and did not show, respectively, *Festuca*-specific bands (or genome).

§CAPS marker.

¶The ratio of *Festuca*-specific genomic regions to the whole genome.
On average, we tested four chromosome spreads (3–5) for each plant.

? indicates that the PCR bands were faint. Blanks mean that the plant was not tested.

+ and − indicate that the plant is endophyte-infected and uninfected, respectively.

Six plants with red letters were selected and developed to BC_1.

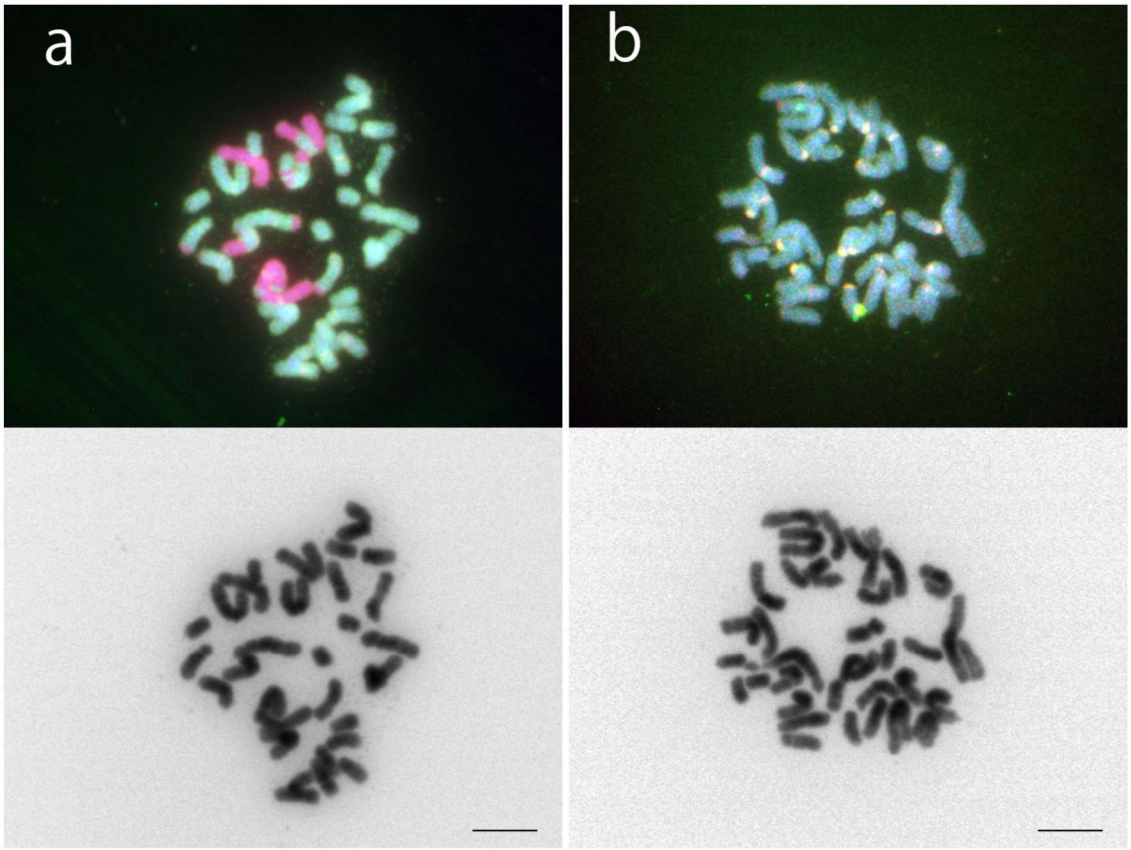

Figure 1 Genomic *in situ* hybridization(GISH) images of F_1 plants.
The upper panels of each image (black background) are GISH images, and the lower panels (white background) are their inverted 4′ ,6-diamidino-2-phenylindole dihydrochloride (DAPI) images.
The red and green regions are derived from *Festuca* and *Lolium* species, respectively.
(a) F_1–3, which was considered truly backcrossed, as inferred from DNA marker analyses.
(b) F_1–13, which was considered self–pollinated.
Scale bars: 10 μm in (a) and (b).

hybridity, endophyte infection (confirmed both by PCR and microscopy), and plant vigor. Using these 6 plants (6 maternal F_1 plants × 40), 240 BC_1 plants were developed. The f ratios of the six F_1 plants were also analyzed. It was shown that the plants with a lower f ratio presented fewer *Festuca*-specific bands, suggesting that it was possible to estimate the f ratios using *Festuca*-specific DNA markers.

The number of true BC_1 hybrids and endophyte-infected plants in each maternal line is shown in Table 5. From 55 PCR and CAPS marker sets, a total of 15 were selected that could distinguish hybridity in at least one maternal line but would not produce *Festuca*-specific bands in its maternal F_1 plant. Two to seven marker sets were tested in each maternal line. The primer information for this is presented in Table 3. It was determined that a plant was a true hybrid when it showed at least one novel *Festuca*-specific band. Then, the percentage of true hybrids was calculated in each maternal line, with the results showing that five maternal lines showed > 97% hybridity (Table 5). One line whose maternal parent was F_1–18 showed only 54% hybridity. However, only two marker sets were suitable to distinguish hybrids in the progeny of F_1–18 because they presented the *Festuca*-specific band for all other marker sets. F_1–18 had the highest f ratio (31.1%) among the six F_1 plants, thereby suggesting that F_1–18 had an almost complete set of *Festuca* genome. It was likely that the remaining 46% of the F_1–18 progeny plants were

Table 5. The percentage of true BC_1 hybrids and endophyte-infected plants in six BC_1 maternal lines using DNA marker analysis, microscopic observations, and PCR detection.

	Maternal plant					
	F_1–2	F_1–3	F_1–12	F_1–17	F_1–18	F_1–20
True BC_1 hybrids (%)	100	97	100	97	54	97
Number of plants	40	39	36	35	35	36
Number of markers used	3	6	7	3	2	7
Endophyte-infected plants (%)						
Microscope†	82	28	81	79	86	91
PCR detection	23	21	11	34	37	42

† Evaluated by the observation of 15 open-pollinated seeds for each plant.

not derived by self-pollination. The chromosomes of *Festulolium* plants were striped with DNA from two genera as a result of recombination (Figure 1a). If exhaustive marker analyses targeting small *Lolium* chromatin regions were to be performed, practically all of the remaining F_1-18 progeny may be found to be true hybrids. Alternatively, if F_1 plants showing *Festuca*-specific band patterns with a low frequency (e.g. F_1–19 or F_1–21) were selected, it would be able to determine the hybridity of their progeny. Although the possibility of false positives owing to recombination in the offspring, or DNA sequence mutations in the *Lolium* genome cannot be ruled out, the likelihood is low as almost all BC_1 plants were shown to be truly backcrossed using more than one DNA marker set.

Here, we showed that the novel paternal bands of 19 distinguishable markers used in F_1 and BC_1 (Table 3) were from the *Festuca* or *Lolium* genomes of 30 *Festulolium* paternal parents, using genomic DNA of four Italian ryegrass maternal plants (maternal plant 1, 2, 3, and 4) and six cultivars or strains used to breed 'Nakei 1'. It was confirmed that 17 out of 19 markers had *Festuca*-specific band patterns in this test, and the novel band patterns in F_1 and BC_1 were from the *Festuca* genome of 30 paternal *Festulolium* plants. The marker "Os06g36700" had band patterns specific to *Festuca* and 'Akiaoba 3'. Although 'Akiaoba 3' is registered as an Italian ryegrass, it is possible that pollen contamination from *Festulolium* in the breeding process may have occurred (the author's

personal communication). Marker “Os10g02980” generally showed two bands, while only ‘maternal plant 3’ and its progeny showed a single band. This marker was not *Festuca*-specific, but distinctive for hybridity. Conveniently, in this thesis all 19 markers are referred to as *Festuca*-specific.

It would be challenging to evaluate BC_2 hybridity using the same DNA marker sets, because there were very few distinguishable DNA markers. The frequency of *Festuca*-specific bands on each DNA marker set in the BC_1 hybridity test varied from 13 – 91% (Table 3), and the mean frequency was 58%. Primer sets such as “Os08g36774”, which showed a *Festuca*-specific band pattern with a lower frequency than other primer sets, are expected to be functional in subsequent generations. However, the potential to determine hybridity would also be low. Following BC_1 generation, Simple Sequence Repeat (SSR) marker analyses would be useful for determining hybridity. In Chapter 4, self-pollinated plants were distinguished from isolated cross progeny in *Festulolium* using Italian ryegrass SSR marker (Hirata *et al*., 2006) analysis. If the polymorphic band patterns of all 30 paternal *Festulolium* plants and all maternal BC_1 plants were analyzed using SSR markers, it would be possible to determine whether each BC_2 plant was a true backcrossed or a self-pollinated plant. However, minor crop breeding stations are rarely equipped with DNA sequencers, a necessary piece of equipment for SSR marker analyses.

In such cases, DNA marker analyses using relatively low-cost equipment is useful. For instance, GISH analyses would only be useful for determination of F_1 hybridity. Given that the mean f ratio of 30 paternal *Festulolium* parents was 49%, the expected f ratio for F_1 plants was 24.5%. In this study, the f ratio of six F_1 plants ranged from 11.6% – 31.1% and, thus, differed from the expected value by more than 50%. Given the variation in the f ratio, it would not be possible to evaluate whether any f ratio increase after the F_1 generation was as a result of pollen from a paternal *Festulolium* parent or variation within the self-pollinated progeny.

In this chapter, only 31 out of 69 PCR and CAPs marker sets allowed for the distinction between *Lolium* and *Festuca* (Table 3). This may be due to the use of a different experimental system compared to Tamura *et al*. (2009). Commercial kits for DNA extraction and PCR amplification, and non-ethidium bromide DNA staining liquid were used. However, the likely reason may be that different populations from those of Tamura *et al*. (2009) were used. In their study on winter hardiness in perennial ryegrass with introgression of meadow fescue, Tamura *et al*. (2017) reported that they could not detect the Quantitative Trait Loci (QTLs) for freezing tolerance as reported by Kosmala *et al*. (2006, 2007). Tamura *et al*. (2017) suggested that the difference in genetic background was the cause of this discrepancy. The lack of versatility is a challenge for

DNA marker-assisted selection in allogamous forage grass species.

3.3.2 Endophyte infection evaluation

Endophyte infection of F_1 plants is shown in Table 4. For four plants, PCR detection of endophytes was inconsistent with microscopy observations. Three plants showed no endophyte-specific band (Figure 2) but the observations made by microscopy showed them to be visibly endophyte-infected. This discrepancy could be because DNA was extracted from leaf blades, while microscopic inspection was performed on leaf sheaths. In a previous study on *E. uncinata*-inoculated Italian ryegrass 'Bishamon', little or no endophyte infection was detected in the leaf blades, while abundant infection was detected in the pseudostem and inflorescences (Matsukura *et al*., 2014). It would be more accurate to detect the endophyte infection with PCR using DNA from leaf sheaths instead of leaf blades. Furthermore, PCR detection could overcome the limitations of analysis by microscopy. Indeed, one of the plants in which it was not identified the endophytic hyphae by microscopy, showed an endophyte-specific band by in the PCR.

The percentages of endophyte-infected plants in each BC_1 maternal line are presented in Table 5. All maternal lines, except for the progeny of F_1–3, had 79% – 91% endophyte infection as judged by microscopic observation of their open-pollinated seeds. PCR

Figure 2 Results of PCR detection of endophyte.
E- and E+ mean negative control (L503) and positive control (MF07) of endophyte, respectively.
Numbers mean each F_1 plants.

analysis showed the percentage of endophyte infection to be 11% – 42% and considerably lower than those detected by microscopy. In the F_1, DNA was extracted from leaf blades growing in the greenhouse; however, in the BC_1, DNA was extracted from leaf blades growing in the field. Environmental stress (e.g. cold temperature) may have suppressed the elongation of hyphae in leaf blades.

PCR detection of endophyte infection is more laborious than observations made by microscopy especially considering the time and effort required for DNA extraction if the DNA is to be used for only one application. However, if DNA is extracted for another purpose, PCR detection of endophyte is very easy. Therefore, if DNA extraction is necessary, it would be better to extract DNA from the leaf sheath. Microscopic observation of open-pollinated seeds showed that only 28% of the progeny of F_1–3 were endophyte-infected. In contrast, microscopic observation of the leaf sheaths of the newly germinated young progeny of F_1–3 indicated that 58% of these plants were endophyte-infected (data not shown). This suggests that environmental stresses in the field suppressed the endophyte in the seeds from the progeny of F_1-3 because these plants had poor compatibility with the endophyte. It may be insufficient to ascertain the presence of endophyte by PCR or microscopy alone because the density of endophyte hyphae should also be taken into consideration.

3.3.3 Conclusion

At least 146 true BC1 and endophyte-infected plants were bred, relatively quickly, using DNA markers. Since Italian ryegrass and *Festulolium* cultivars are allogamous, the phenotypes of plants in a cultivar differed from one another. 'Nakei 1' was bred for silage purposes, and is phenotypically similar to Italian ryegrass. It would, therefore, be difficult to judge whether F_1 and BC_1 progeny are true hybrids of paternal *Festulolium* parents based solely on their phenotypes. As a result, the DNA marker sets from Tamura *et al*. (2009) were useful in the determination of true hybrids in this study. The present study reports the first attempt to utilize these easy-to-use DNA marker sets in a practical *Festulolium* breeding program. However, only a few marker sets were able to distinguish between *Lolium* and *Festuca* because different populations from those of Tamura *et al*. (2009) were used. Moreover, this further exemplifies the difficulty associated with using DNA marker-assisted selection in allogamous forage grass species. GISH analyses would only be useful for determination of F_1 hybridity. Given the variation in the f ratio, it would not be possible to evaluate whether any f ratio increase after the F_1 generation was as a result of pollen from a paternal *Festulolium* parent or variation within the self-pollinated progeny.

Chapter 4: Highly efficient and accurate quantification of the genomic constitution of *Festulolium* using the f ratio method, and analyses of genomic instability in *Festulolium*

4.1 Introduction

It may be possible to cultivate *Festulolium* in fallow paddy fields in the Tohoku region, as discussed in Chapter 2. However, the risk of rice bug infestations can be avoided by infecting *Festulolium* with an endophyte, as discussed in Chapter 3. Even so, another challenge in cultivating *Festulolium* is its genomic instability. Although *Festulolium* is an intergeneric hybrid between *Festuca* and *Lolium* genus, in an amphidiploid forms of *Festulolium*, the genome balance has a tendency to lean towards *Lolium* across the generations (Pašakinskienė *et al*., 1998; Canter *et al*., 1999; Zwierzykowski *et al*., 1998; Zwierzykowski *et al*., 2006).

GISH techniques, developed at the end of the last century, have made it possible to distinguish between the genomes of two species. Many *Festulolium* cultivars and strains have been analyzed using GISH (Pašakinskienė *et al*., 1998; Canter *et al*., 1999; Zwierzykowski *et al*., 1998; Zwierzykowski *et al*., 2006), which has allowed for the study of the genome balance drift in favor of *Lolium* in tetraploid amphidiploid *Festulolium* plants originating from crosses between *Lolium* species and *F. pratensis*. Several hypotheses have been proposed to explain the genome balance drift (Jones *et al*., 2005),

including gametic competition, pollination effects, and selection for seedling vigor, fertility, and seed yield; however, the reason behind this drift remains unclear. Zwierzykowski *et al*. (1998, 2006) support the hypothesis that the substitution of *Festuca* chromosomes and chromatin with those from *Lolium* occurs during sexual reproduction. In Japan, four *Festulolium* cultivars, 'Tohoku1', 'Icarus', 'Nakei 1', and 'North fest' have been bred and registered (Yonemaru *et al*., 2011; Ueyama *et al*., 2014), and some breeders worry that the *Festuca* genome will disappear from their allogamous cultivars during seed multiplication for selling. Thus, it is imperative to uncover the cause of the genome balance drift.

Zwierzykowski *et al*. (1998, 2006) found that the proportion of the total *Festulolium* genome length occupied by *F. pratensis* ranged from 33.3% – 50.8% (mean 40.8%) in four F_8 generations, while it had a mean value of 40.5% in the F_6 generation. These values were much lower than 50%, the theoretical value in amphidiploids. Unfortunately, chromatin is not uniformly distributed in chromosomes and euchromatic regions may be overestimated. The application of this method may, therefore, be challenging. In a recent report, Akiyama *et al*. (2010) developed the "f ratio," a method for determining the genomic constitution of *Festulolium* using the ratio of *Festuca*–specific genome regions to the whole genome. The f ratio quantitatively shows the chromatin rate from the two

genera. Since the f ratio is calculated using integrated intensity values of fluorescence images, it is not affected by the chromatin type (euchromatin or heterochromatin). The f ratio method is more appropriate than the method proposed by Zwierzykowski *et al*. (1998, 2006) for evaluating genome balance drift in favor of *Lolium*. However, it is not clear how many plants are required to estimate the f ratio of a cultivar because *Festulolium* is allogamous and the plants of each cultivar are genetically diverse. Firstly, the variability of the f ratio within and among *Festulolium* cultivars were investigated.

Although Zwierzykowski *et al*. (1998, 2006) described the genome balance drift in favor of *Lolium* across generations, they were unable to determine the cause of the drift because they tested breeding populations and bulked seeds in each generation. Considering that breeding populations were selected with different agronomic traits, it is not possible to judge whether a genome balance drift in favor of *Lolium* was due to selection or chromosome substitutions during sexual reproduction. In this chapter, maternal plants were selected based only on the f ratio without selection for any agronomic traits, crossed separately, and tested each seed to prevent the seed yield of maternal plants from affecting the genome balance. Usually, *Lolium* species produce more than 1000 kg/ha seed yield (sometimes more than 2000 kg/ha), even though *F. pratensis* rarely produces 1000 kg/ha (Alberta, 2004; Havstad, 2016; Hart *et al*., 2003; Mäkelä *et*

al., 2009). *Lolium* species produce more seeds than *Festuca* species which means that bulking of seeds could result in genome balance drift in favor of *Lolium*. In order to avoid this, as plants with low f ratios likely produce more seeds, progenies were maternally-derived and their f ratios were also calculated for the same number of plants in each progeny. The f ratio drift was checked between maternal plants and their progenies in three populations. One population was the progeny of a single cross between parents of the backcrossed cultivar 'Icarus', and the other two were isolated cross progenies from the amphidiploid cultivar 'Nakei 1'. Isolated crossing of a few plants in a greenhouse could result in the inclusion of self–pollinated progeny. Thus, the hybridity of progenies was checked using SSR marker analyses to exclude self–pollinated progeny plants from our genome balance drift tests, because they could disturb the true mean f ratio of the progenies. Second, using these test populations without any breeding selections or biases of seed yields, it was demonstrated that the genome balance drift did not occur.

Using more appropriate plant materials and evaluation methods than previous studies, it was not observed a genome balance drift in favor of *Lolium*. These results suggested that differences in seed yields could cause genome balance drift in *Festulolium*. Next, the seed yields, ripening rates, and germination rates of two populations of 'Nakei 1' were monitored in the field over a period of two years. It was attempted to identify correlations

between f ratio values and seed yield-related traits to show that genome balance drift in favor of *Lolium* could be caused solely by phenotypic differences, regardless of any biases toward *Lolium* occurring during meiosis.

4.2 Materials and methods

4.2.1 Plant materials

4.2.1.1 The variability of the f ratio within and among *Festulolium* cultivars

The f ratios of five tetraploid *Festulolium* cultivars—'Icarus', 'Tohoku 1', 'Evergreen', 'Paulita', and 'Tandem'—were calculated with the number of plants tested being 20, 9, 7, 7, and 7, respectively. All of the cultivars were tetraploid hybrids between *Lolium* species and *F. pratensis* (Ghesquière *et al*., 2010; Yonemaru *et al*., 2011; Ueyama *et al*., 2014). 'Icarus' was bred from the tetraploid *Festulolium* cultivars, 'Evergreen', 'Paulita', 'Tandem', and 'Duo' by mass selection and maternal–line selection (Ueyama *et al*., 2014). 'Tohoku 1' was bred from the tetraploid *Festulolium* cultivars, 'Evergreen', 'Paulita', and 'Tandem' by mass selection and maternal–line selection (Yonemaru *et al*., 2011). 'Evergreen' and 'Tandem' are thought to be introgression cultivars from *L. multiflorum* × *F. pratensis* backcrossed by *Lolium* species (Ghesquière *et al*., 2010). 'Paulita' is an amphidiploid cultivar from *F. pratensis* ×*L. multiflorum* (Kopecky *et al*.,

2006; Ghesquière *et al*., 2010) or *L. multiflorum* × *F. pratensis* (Momotaz *et al*., 2004).

4.2.1.2 Single cross progeny of the backcrossed cultivar ‘Icarus’

Two plants were selected with nearly equal f ratios from the tetraploid *Festulolium* cultivar ‘Icarus’. The two plants selected for our studies were named Icarus–10 and Icarus–19, and had f ratios of 16.9 and 13.0, respectively. A single cross between Icarus–10 and Icarus–19 was carried out in an isolated air–conditioned greenhouse and 24 seeds from the Icarus–19 maternal line (named Icarus–19–1~24) were selected. The genomic DNA of each plant and that of their parents was extracted from leaves using the QIAGEN DNeasy Plant Mini Kit (QIAGEN GmbH) for SSR marker analyses.

4.2.1.3 Isolated cross progenies from the amphidiploid cultivar ‘Nakei 1’

Two groups of plants with high (four plants) and low (five plants) f ratios were selected from the tetraploid cultivar ‘Nakei 1’ based on a preliminary f ratio survey. ‘Nakei 1’ was bred from F_1 hybrids between tetraploid *F. pratensis* and tetraploid *Lolium* species, and the F_3 generation was used as the breeders’ seed (personal communication). In this study, multiplied F_4 generation seeds were used. The plants from the high f ratio group were Na1–7, Na1–16, Na1–20, and Na1–22, while the plants from the low f ratio

group were Na1–6, Na1–9, Na1–10, Na1–17, and Na1–24. Isolated crosses for each group were carried out in an isolated air–conditioned greenhouse, and eight seeds from each maternal plant were cultivated in pots containing fertilized granulated soil (Kureha Co.) in a greenhouse. DNA extractions were performed in the same manner as the single cross progeny of 'Icarus'.

4.2.2 Genomic *in situ* hybridization (GISH) analysis

Seeds were cultivated in pots containing fertilized granulated soil (Kureha Co.) in a greenhouse. Root tips were collected and pretreated for 24–48 h by soaking them in cold water on ice before they were placed in a fixative solution (3:1 ethanol:acetic acid), and stored at 4°C until further use. Molecular cytological experiments were carried out as previously described (Akiyama *et al*., 2010), with the following modifications. Total genomic DNA from plants L503 (*L. multiflorum*) and MF07 (*F. pratensis*) were extracted using the cetyl trimethyl ammonium bromide method or the QIAGEN DNeasy Plant Mini Kit (QIAGEN GmbH), and labeled with Fluorescein-12-dUTP (PerkinElmer, Inc.) and Texas Red-5-dUTP (PerkinElmer, Inc.), respectively, using a Nick Translation Kit (Roche, Basel, Switzerland). With these probes, GISH was performed using a direct–labeling protocol. After GISH treatment, the slides were mounted in a drop of Vectashield

mounting medium (Vector Laboratories) containing 1.5 μg mL^{-1} 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and observed under a fluorescence microscope (Olympus BX61; Olympus Optical Co. Ltd., Tokyo, Japan) equipped with a DP72 CCD camera (Olympus Optical Co. Ltd.). The DAPI, fluorescein, and Texas-Red images were then captured in gray and merged into an RGB pseudocolor image using LuminaVision (Mitani Co. Ltd., Fukui, Japan).

4.2.3 F ratio analyses

Image analyses were conducted using Photoshop CS4 (Adobe Systems Inc., San Jose, USA) in Windows 7 (Microsoft Corporation, Redmond, USA) with a Cintiq–12WX interactive pen display (Wacom, Saitama, Japan). The DAPI images of each chromosome spread were then divided into two areas according to GISH results. These areas correspond to *F. pratensis*– and *Lolium*–specific chromatin. The f ratio was then calculated using the integrated DAPI intensity values of *Festuca*–specific chromatin / total chromatin (Figure 3).

4.2.4 Estimation of necessary sample size for calculating the f ratio

The sample size needed to calculate of the f ratio was estimated from the back

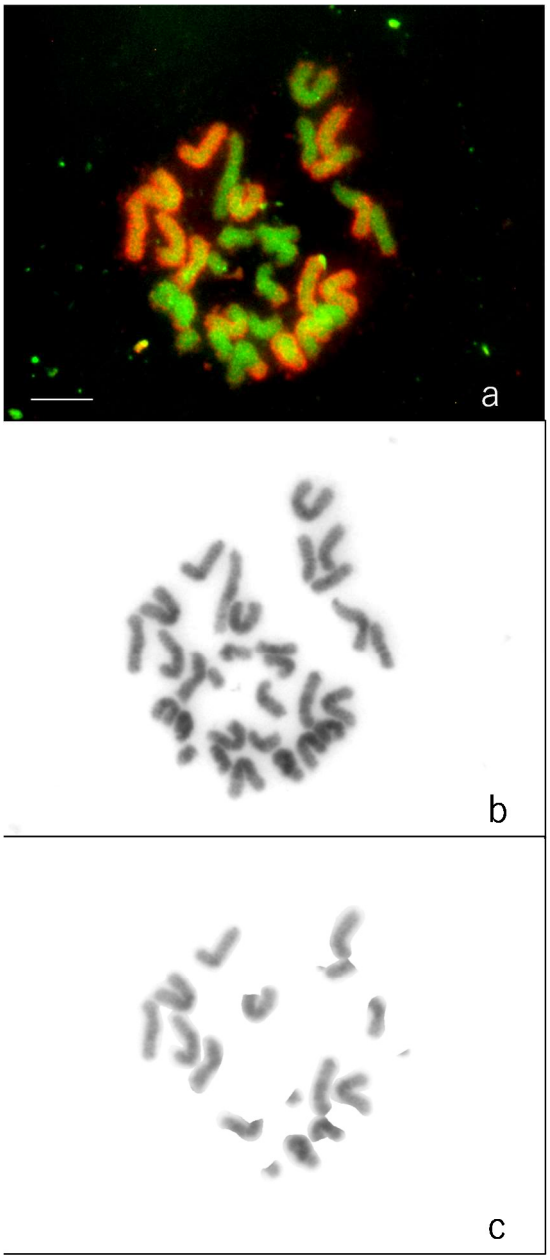

Figure 3 GISH images of a *Festulolium* plant (NA1-17-7) chromosome spread.
(a) Red regions are *Festuca* specific genome.
Green regions are *Lolium* specific genome.
(b) Inverted DAPI image of (a).
(c) Genome of *Festuca* extracted from (b) according to (a).
We calculated 'f ratio' as the ratio of total area of *Festuca* specific genome regions (c) to the total area of all genome regions (b).
Areas were calculated using integrated intensity values.
Scale bar: 10 μ m.

calculation of the statistical interval estimation:

$$\ddot{X} - k\sqrt{s^2/n} \leq \mu \leq \ddot{X} + k\sqrt{s^2/n}$$

$$\rightarrow CI = 2 \times k\sqrt{s^2/n}$$

$$\rightarrow n \geq (2k\, CI^{-1})^2 \times s^2,$$

where $\ddot{X}$, k, s^2, n, μ, and CI refer to the sample mean, critical region of normal distribution, estimate of variance, number of samples, population mean, and confidence interval, respectively.

4.2.5 SSR marker analyses to exclude self–pollinated progeny

First, 25 *L. multiflorum* SSR markers (Hirata *et al*., 2006) were used to select markers that showed polymorphic band patterns between the parents in each population. Marker analysis was carried out according to Hirata *et al*. (2006) with a few changes. SSR primers were fluorescently–labeled with FAM or HEX. PCR conditions using fluorescently–labeled primers were as follows: 95°C for 10 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s; 72°C for 7 min; and holding at 4°C. The PCR products were separated by electrophoresis on 6% (w/v) polyacrylamide gel at 700 V, 26 mA, and 50 min at room temperature. Following electrophoresis, PCR products were detected using a Molecular Imager FX Plus (Bio–Rad, Hercules, USA). Two, six, and nine SSR markers

were selected for the single cross progeny of ‘Icarus’, the isolated cross progeny of the four high f ratio plants of ‘Nakei 1’, and the isolated cross progeny of the five low f ratio plants of ‘Nakei 1’, respectively. Using these SSR markers, it was available to assess whether each progeny plant was a true hybrid or a self–pollinated plant. Self–pollinated plants were excluded from the subsequent analyses.

4.2.6 F ratio calculation in the isolated cross progeny

The f ratios of each maternal progeny were calculated in the two isolated cross progenies assuming random pollination and complete allogamous crossing (i.e. the f ratio of each maternal progeny = (f ratio of the maternal plant + average of the f ratios of the isolated plants except for that of the maternal plant)/2 × 100).

4.2.7 Field tests

Eight plants in the low f ratio group were excluded from the experiment as five of the eight were self-pollinated. Weak, dead, and late-maturing plants were also excluded. In total, 30 plants from the high f ratio group and 28 plants from the low f ratio group were tested. Each plant was propagated into six clones by division from which four were directly transplanted into the field and two remained in a greenhouse for the field test in

the following year.

The experimental field was located in the Tohoku Agricultural Research Center, NARO (Morioka, Iwate, Japan; 39°44'N, 141°8'E). The average yearly temperature of the experimental site is 10.2°C (Japan Meteorological Agency, 2020) and the soil is Andosol. For this experiment, a randomized block design was utilized with four replicates each spaced 0.75 m apart with 0.75 m between each row. Chemical fertilizer was applied at the rates of 8 g N, 8 g P_2O_5, and 8 g K_2O per m^2 at the time of transplantation and 3 g N, 3 g P_2O_5, and 3 g K_2O per m^2 in early spring. Plants were transplanted on 1st October 2013 and 30th September 2014. The seed yield, ripening rate, and germination rate of plants were tested in each seed lot (i.e. four replicates × two years for each plant). Mean values of eight samples were used for later analyses.

4.2.7.1 Seed yield

Spikes were produced by each plant 46 days after their heading date during the first year and 60 days after their heading date during the second year. Spikes were dried in a greenhouse, threshed by hand, winnowed, and weighed.

4.2.7.2 Ripening rate

Every seed, including immature seeds, was threshed by hand. The following equation was used to approximate the ripening rate of each plant:

$$Ripening\ rate\ (\%) = \frac{Seed\ yield\ after\ winnowing}{Seed\ yield\ before\ winnowing} \times 100$$

4.2.7.3 Germination rate

The seeds were exposed to the air from their harvest until the date of the germination testing (July – March of the following year). The temperature ranged from 35°C in summer to –15°C in winter. One hundred seeds were sowed on a petri dish and watered regularly until they sprouted. Seeds were cultivated at 25°C and were exposed to natural daylight. The number of seeds which successfully germinated after 3 weeks were then counted.

4.2.7.4 Seed weight

One hundred seeds were weighed and the number of seeds per gram was calculated.

4.2.8 Simulated f ratio in the next generation

The mean f ratio of plants in the next generation for each group (high or low) was

predicted, thereby assuming that seeds were mixed in equal amounts (i.e., 1 g / progeny) and that pollination occurred randomly. The following equation was used for this calculation:

$$Simulated\ f\ ratio\ (\%) = \frac{\sum 1 \times num \times germ \times F}{\sum 1 \times num \times germ}$$

num = number of seeds / g, *germ* = germination rate, *F* = f ratio of each maternal plant.

4.2.9 Statistical analyses

Statistical analyses were performed using the statistical software package SAS 9.2 or 9.4 (SAS Institute Inc., Cary, USA). One-way analysis of variance (ANOVA) and Tukey's honest significant difference (HSD) test ($P < 0.05$) were applied to the f ratios of five *Festulolium* cultivars. A mean value of 10 chromosome spreads was used as a statistical variable for each plant.

Student's *t*–tests were used to assess whether the f ratio differed between maternal plants and their progeny in each group (high or low). On average, 4 chromosome spreads (minimum 2 and maximum 10) for each progeny plant and 8 chromosome spreads (minimum 5 and maximum 12) for each maternal plant were tested.

To reveal the relationship between the f ratio and seed yield-related traits, a total of

58 plants from both high and low f ratio groups were reallocated to two groups using the threshold value of f ratio = 50%. Plants with over 50% f ratio were named “over 50” and plants with less than 50% f ratio were named “under 50”. Student’s *t*-tests were used to assess whether seed yield-related traits differed between the two groups. Pearson’s correlation coefficient was also used to gauge whether f ratio was correlated with seed yield-related traits. Pearson’s correlation coefficient was used to determine whether seed yield-related traits, except germination rate, were linked within the two study years. For germination rate, Spearman’s correlation coefficient was used.

4.3 Results and discussion

4.3.1 The variability of the f ratio within and among *Festulolium* cultivars.

Figure 4a – f show GISH images on the left (black background) and their inverted DAPI images on the right (white background). The DAPI images show that these chromosome spreads are sufficient for GISH and subsequent analyses of the f ratio. Figure 4a – b show the GISH images for the ‘Icarus’ plants. The red and green regions are derived from *Festuca* and *Lolium*, respectively. Eleven of 20 plants had several lengths of *Festuca* chromatin (Figure 4a), whereas the remainder of the plants did not have any *Festuca*–specific chromatin (Figure 4b).

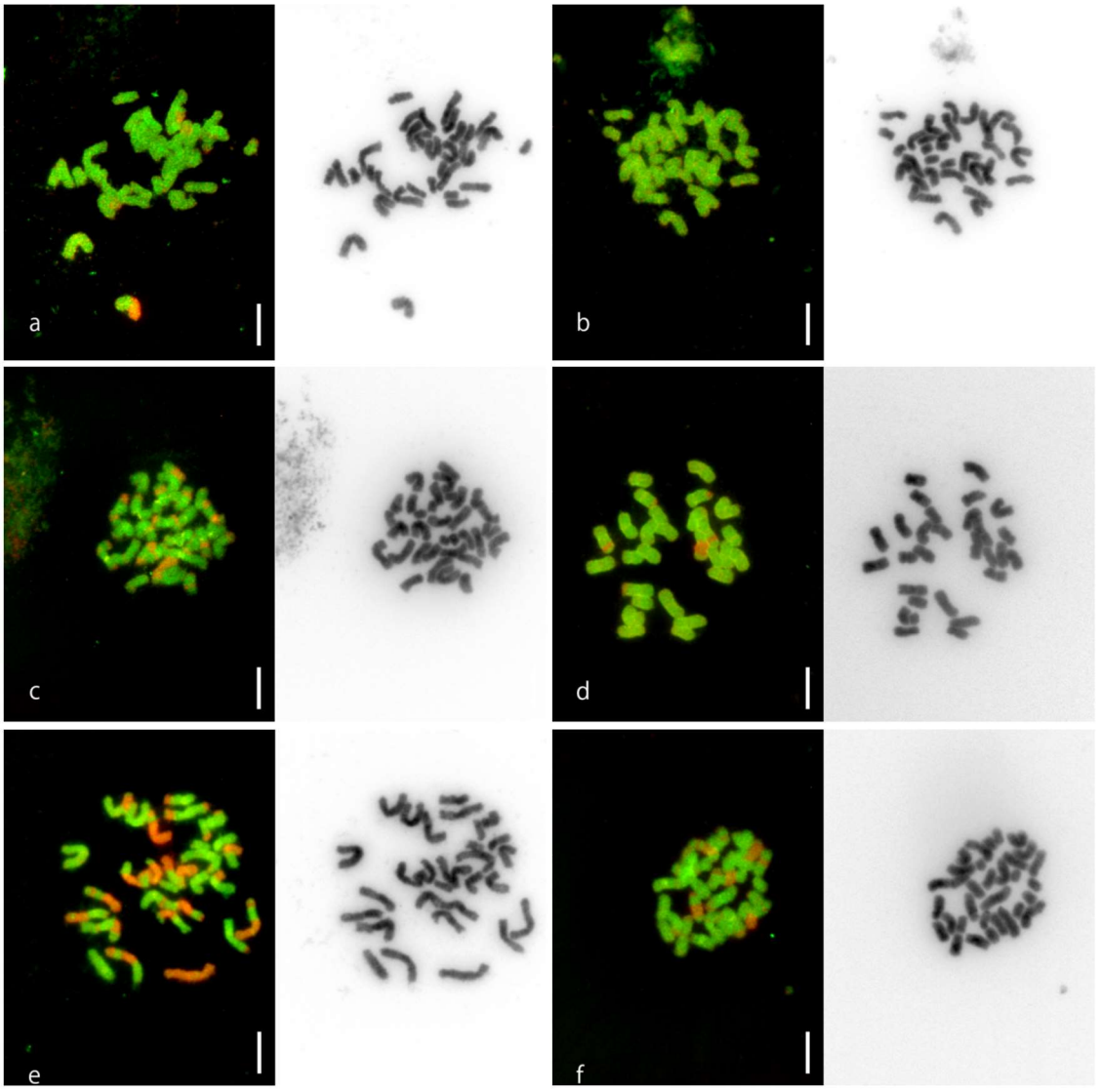

Figure 4 Left sides (black background) of each image are GISH images of plants of tested cultivars (a, c–f) and a plant of Icarus which had an f ratio of 0% (b), and right sides (white background) are their inverted DAPI images. (a, c–f) Plants of Icarus, Tohoku 1, Evergreen, Paulita, and Tandem, respectively.
Scale bars: 10 μm.

Figure 4c – f show GISH images of the ‘Tohoku 1’, ‘Evergreen’, ‘Paulita’, and ‘Tandem’ plants, respectively. Of these cultivars, ‘Paulita’ had the most *Festuca*–specific chromatin, as well as one complete *Festuca* chromosome. The amount of *Festuca*–specific chromatin in the other plants was the largest in ‘Tandem’, lower in ‘Tohoku 1’, and the lowest in ‘Evergreen’. None of these cultivars had any complete *Festuca* chromosomes. ‘Evergreen’ had very little *Festuca*–specific chromatin, and its chromosomes consisted almost entirely of *Lolium* chromosomes.

Recombination between homoeologous chromosomes rarely occurs in hybrids of species or genera such as triticale (Nkongolo *et al*., 2009), rice (Yan *et al*., 1999), wheat (Anamthawat-Jonsson, 1999), and barley (Noda *et al*., 1990). In these hybrids, most of one parental genome is typically lost, or the parental genomes follow allopolyploid formation. In *Festulolium*, a hybrid of *Lolium* species and *F. arundinacea*, breeders must adopt the introgression forms (Ghesquière *et al*., 2010). In addition, the detection of their introgressed chromatin by GISH reveals that it is either slight or not present (Kopecky *et al*., 2006). The *Festulolium* cultivars used in this study are hybrids between *Lolium* species and *F. pratensis* (Ghesquière *et al*., 2010). To date, most of the combinations that have been examined have introgressed chromatin material and recombined homoeologous chromosomes (Zwierzykowski *et al*., 1998; Kopecky *et al*., 2006;

Zwierzykowski *et al*., 2006). It was also observed extensive recombination between homoeologous chromosomes in this study (Figure 4a, c – f).

Although it is possible to distinguish between the genomic constitutions of plants or cultivars that differ markedly from each other using GISH, visual judgments are subjective and difficult to quantify. A better method is needed to evaluate the genome balance drift in favor of the *Lolium* genome. The f ratios, which are calculated using the integrated DAPI intensity values of *Festuca*–specific chromatin / total chromatin, could be a powerful tool because genomic constitutions are quantified objectively (Akiyama *et al*., 2010).

The f ratios of the cultivars tested, as well as their unbiased variances, are shown in Table 6. Every cultivar showed intracultivar variability, particularly 'Icarus' and 'Evergreen', which included plants that appeared to have f ratios of 0% and were more variable than the other cultivars. The f ratios of the tested cultivars were significantly different; 'Paulita' had the highest f ratio (mean ± SD: 32.9 ± 4.2%), followed by 'Tandem' (18.4 ± 5.3%), 'Tohoku 1' (17.7 ± 3.1%), 'Evergreen' (6.3 ± 7.6%), and 'Icarus' (6.3 ± 6.6%).

The unbiased variance of the f ratio of the most variable plant in 'Icarus', which had the largest number of tested plants, was 5.51. In this study, 10 chromosome spreads were

Table 6. F ratios of tested cultivars

Cultivar	No. of plants tested	F ratio (%)				No. of plants
		Mean	Min.†	Max.†	Unbiased variance	f ratio = 0
Icarus	20	6.3 a‡	0.0	19.1	44.0	9
Evergreen	7	6.3 a	0.0	22.2	57.3	2
Tohoku 1	9	17.7 b	11.5	21.3	9.4	0
Tandem	7	18.4 b	10.9	25.4	27.6	0
Paulita	7	32.9 c	27.1	39.6	18.0	0

† Min. and Max. refer to minimum and maximum, respectively.

‡ Values with the same letter are not significantly different. Tukey's HSD ($P < 0.05$).

tested per plant, and the statistical estimation of the confidence interval (CI) at the 0.05 level was 2.9 points (k = 1.96, and $s^2 = 5.51$), which was too strict when the costs of GISH and calculating the f ratio were considered. Using this unbiased variance ($s^2 = 5.51$), the sample size needed to calculate the f ratio was estimated. Four or five chromosome spreads were sufficient to distinguish plants when the f ratios differed by only five points (here, CI = 5, k = 1.96, and $s^2 = 5.51$) at a significance level of 0.05. In the same way, the number of plants needed to calculate the f ratio for cultivars was estimated. Given the unbiased variance in the f ratios of 'Icarus', which had the largest number of tested plants, seven plants were more than sufficient to distinguish among cultivars that had f ratios differing by 10 points at a significance level of 0.05 (here, CI = 10, k = 1.96, and $s^2 = 44.0$). For 'Evergreen', 'Paulita', and 'Tandem', sample size was restricted to seven plants per cultivar. However, the f ratios were significantly different from one another.

4.3.2 There is no decrease in the f ratio of maternally derived progeny across different generations

Using two *L. multiflorum* SSR markers (7–6H and 8–3B), it was confirmed that 23 out of 24 progeny plants were true hybrids in the cross between the maternal parent Icarus–19 and the paternal parent Icarus–10. These two markers showed completely

different band patterns in both parents and enabled us to determine whether the progeny were true hybrids. These *L. multiflorum* SSR markers have previously been used in *Lolium. temulantum* L (which is called “Dokumugi” in Japanese) hybrids (Kiyoshi *et al*., 2012). In this study, these SSR markers were also useful in *Festulolium*.

Arcioni *et al*. (1983) showed that *L. multiflorum* and *L. perenne* seeds were 7.76% and 7.72% self–pollinated, respectively, in a hybrid fertility test between the two species carried out in an isolated room using the isozyme phosphoglucoisomerase as a genetic marker. The mean f ratio of the *Festulolium* cultivar ‘Icarus’ was 6.3% (Table 6), and its genome constitution was nearly all *Lolium*–like. As the parents Icarus–10 and Icarus–19 in this study were two ‘Icarus’ cultivar plants, the percentage of self-pollinated seeds (4.17% = 1/24 ×100) was considered reasonable. Figure 5 shows the frequency distribution of the f ratios in the 23 hybrid plants. The f ratio had a mean of 15.1 (almost equal to that of their parents, 15.0), ranged from 10.9 to 20.2, and showed a normal distribution.

The SSR markers selected for the two isolated cross progenies of the amphidiploid cultivar ‘Nakei 1’ did not show a completely different band pattern among the parents of each group. Figure 6 shows the results of the two selected SSR markers, 25D and 18H, among the five low f ratio parents (Na1–10, Na1–9, Na1–6, Na1–24, and Na1–17).

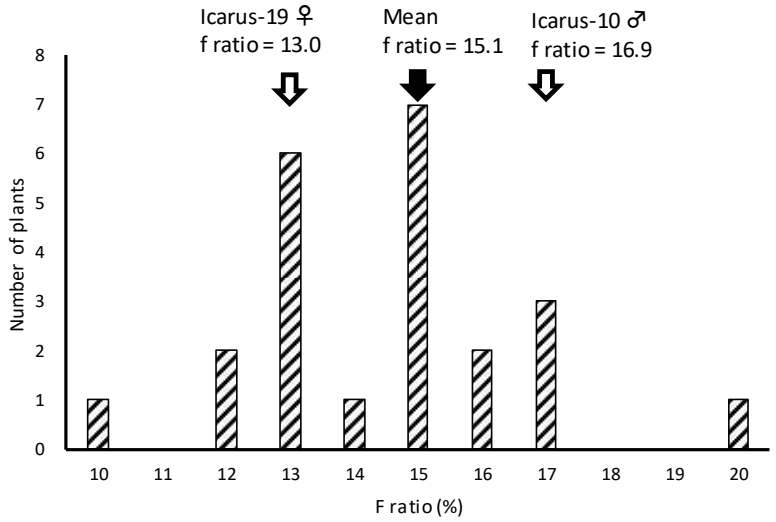

Figure 5 The frequency distribution of the f ratios in the 23 individual hybrids of Icarus–19 and Icarus–10. Open arrows indicate the mean f ratio of each parent and the black arrow indicates the mean f ratio of the progeny.

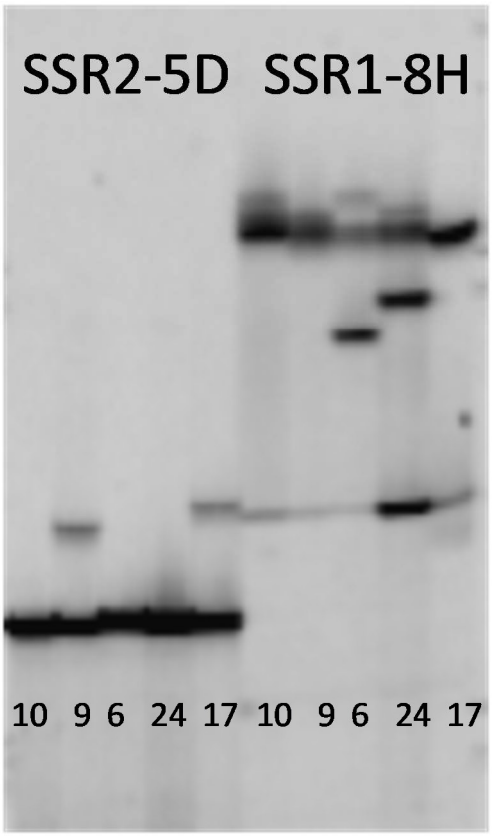

Figure 6 Results for the 2 selected SSR markers, 2–5D and 1–8H, among the 5 low f ratio parents (Na1–10, Na1–9, Na1–6, Na1–24, and Na1–17).
Although we could not distinguish the band patterns among Na1–10, Na1–6, and Na1–24 using 2–5D, the distinction was possible if 2–5D was used in combination with 1–8H. Thus, these SSR markers with partly different band patterns among the parents of the 2 groups of isolated cross progenies were used.

Although it was impossible to distinguish the band patterns between Na1–10, Na1–6, and Na1–24 using 2–5D, distinction was possible if 2–5D was used in combination with 1–8H. Thus, these SSR markers along with partially different band patterns between the parents of the two groups of isolated cross progenies were used.

SSR marker analyses in the isolated cross progeny of four plants of 'Nakei 1' with a high f ratio indicated that all progeny plants showed the band pattern of the paternal parent in at least one SSR marker and were not self–pollinated, except for a progeny plant of Na1–22 (Na1–22–1), which died before extracting genomic DNA. Table 7 shows the f ratios of the maternal plants, their progenies, and the calculated f ratios of the progenies of the two isolated groups. Considering the results of the previous section, an average of eight plants for each progeny and four chromosome spreads (minimum = 2 and maximum = 10) for each progeny plant were tested. Maternal plants were selected from the amphidiploid cultivar 'Nakei 1' based only on their f ratio value. In the high f ratio group, any genome balance drift in favor of *Lolium* was not observed. The mean f ratio of the maternal plants and their progeny plants was 53.8 and 54.3, respectively. The *P* value of the Student's *t*–test comparing the f ratio of maternal plants and their progeny was 0.8117, and therefore, not significant. The investigated f ratios of the four progenies were the same as the calculated f ratios.

Table 7. The f ratios of the maternal plants and their progenies in each groups.

Group	Maternal plant	F ratio (%)	F ratio of progeny (%)		Number of progeny	Caluculation of f ratio (%) †
Low	Na1-6	38.8	43.5	±1.91	8	40.5
Low	Na1-9	35.7	39.6	±1.05	8	39.3
Low	Na1-10	51.2	46.1	±1.63	3	45.1
Low	Na1-17	40.1	45.1	±1.89	8	41.0
Low	Na1-24	41.6	44.3	±1.19	8	41.5
	mean	41.5	43.4	$P = 0.3947$ ‡		
High	Na1-7	54.4	56.0	±1.09	8	54.0
High	Na1-16	55.6	53.8	±0.96	8	54.4
High	Na1-20	46.0	51.2	±1.23	8	51.2
High	Na1-22	59.4	56.2	±0.72	8	55.7
	mean	53.8	54.3	$P = 0.8117$ ‡		

† Values were calculated assuming of random pollination.

‡ P value of the Student's t-test between maternal plants and their progenies.

In the isolated cross progeny of the five low f ratio plants of 'Nakei 1', all progeny plants, except for Na1–10, showed band patterns of the paternal parents and were judged to be true out–crossed plants. Of the eight progeny plants of Na1–10, five did not show the band pattern of the paternal parent and were considered to be self–pollinated. Only three plants were allogamous. The mean f ratio of the low f ratio group did not show a genome balance drift in favor of *Lolium*, instead it showed a drift in favor of *Festuca* (Table 7). The mean f ratios of the maternal plants and their progeny plants were 41.5 and 43.4, respectively. The *P* value of the Student's *t*–test comparing the f ratio of maternal plants and their progenies was 0.3947. The investigated f ratios of the five progenies were the same or a little higher than the calculated f ratios. The mean f ratio of the five self-pollinated plants from Na1–10 was 56.8, which was higher than the Na1–10 f ratio. From the 9 SSR markers tested, at least 3 markers (5 markers on average) detected hybridity between Na1–10 and each paternal plant if they had been crossed. Thus, it was concluded that our self–pollinated categorization of Na1–10 progeny was reliable. When the low f ratio group was crossed in an isolated greenhouse, Na1–10 would flower slightly earlier than other plants. In addition, it was observed that other progenies of the low group had slightly higher f ratios than the calculated f ratios. This might be because Na1–10, which flowered early and had the highest f ratio in the low group, was the paternal parent of

many other progenies. If Na1–10 flowered first in the isolated greenhouse, early flowering spikes of Na1–10 could not be exposed to pollen from other plants, increasing its rate of self–pollination. However, medium to late flowering spikes of Na1–10 could be exposed to pollen from other plants and, additionally, pollinate other plants. It is impossible to explain why the mean f ratio of the five Na1–10 self–pollinated plants was higher than that of Na1–10 because five samples are not enough to interpret the self–pollinated progeny f ratio.

Some researchers have observed genome balance drift of amphidiploid *Festulolium* hybrids over successive generations in favor of the dominant *Lolium* genome (i.e., *Festuca* chromosomes were substituted with those of *Lolium* and recombined at a higher frequency than those of *Lolium*)(Canter *et al*., 1999; Kopecky *et al*., 2006; Zwierzykowski *et al*., 2006). Zwierzykowski *et al*. (2006, 2008) speculated that *Lolium* centromeres may have an advantage, especially during female meiosis, but did not provide any experimental evidences in support of this hypothesis. Another explanation might be that genome balance drift in favor of *Lolium* is due to selection (i.e. *Lolium* phenotypes would be preferentially selected). All *Festulolium* cultivars have been selected for both *Festuca* and *Lolium* phenotypes. Selection pressures cannot be even between the two genera. Therefore, the genome balance drift in favor of *Lolium* might be

due to preferential selection. Several hypotheses have been proposed to explain the drift in favor of *Lolium*, including gametic competition, pollination effects, and selection for seedling vigor (Jones *et al*., 2005). In this chapter, the seed yields of maternal plants were focused on and a drift in favor of *Lolium* could not be detected. Our single cross progeny showed the same f ratio as their parents. Our isolated progenies of the high f ratio group also showed the same f ratio as their parents and the isolated progenies of the low f ratio group showed a drift in favor of *Festuca*. This might be partly explained by the fact that there was no selection for seed yield because our tested progenies were maternally derived. During breeding, seed yield is the most important factor. Generally, *Lolium* species have a greater seed yield than *Festuca* species. Thus, intentional or unintentional selections for seed yield might result in a genome balance drift in favor of *Lolium*. Figure 7 shows the frequency distribution of the f ratios of the isolated cross progenies in the high and low f ratio groups, excluding the five self–pollinated progeny plants of Na1–10. Both groups showed a normal distribution. However, the high f ratio group was slightly biased in favor of *Lolium*. Very few progeny plants had f ratios greater than 58%. The genome balance drift in favor of *Lolium* might be due to the lack of very high f ratio plants. Interestingly, in our study, at least for one generation and in maternally derived progenies, *Festuca* chromosomal fragments did not disappear in either amphidiploid or backcrossed

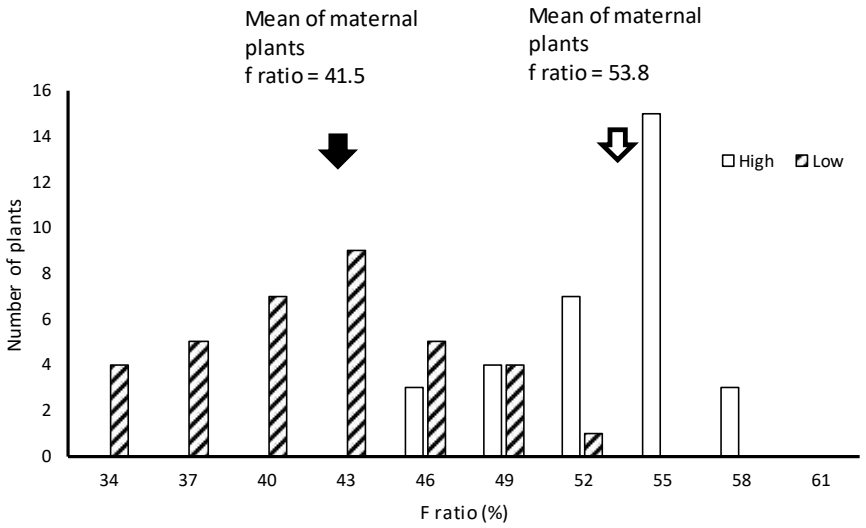

Figure 7 Frequency distribution of the f ratios of the isolated progenies in high and low f ratio groups, excluding the self–pollinated progeny of Na1–10.
The open arrow indicates the mean f ratio of maternal plants in the high f ratio group and the black arrow indicates the mean f ratio of maternal plants in the low f ratio group.

Festulolium cultivars. These results suggest that differences in seed yields can result in a *Lolium*-favored genome balance drift in *Festulolium* cultivars. In the next section, the seed yields, ripening rates, and germination rates of two populations of 'Nakei 1' were monitored in the field over two years. Then, it was attempted to identify associations between f ratio values and seed yield-related traits.

4.3.3 The relationship between f ratio and seed yield-related traits in *Festulolium*

Seed yield, ripening rate, germination rate, and seed weight values from the first and second year were significantly linked to one another ($r > 0.7$, $P < 0.001$, n = 58; Figure 8). Thus, the mean values from both years' data were used in subsequent analyses. Table 8 shows the mean f ratio and seed yield-related trait values for both the under 50 and over 50 f ratio groups. Seed yield, ripening rate, and germination rate in the over 50 f ratio group were significantly lower than in the under 50 f ratio group ($P < 0.05$, 0.01, and 0.001, respectively). In both groups, germination rate was considerably low and the seed weight was equal to each other.

Table 9 shows the Pearson's correlation coefficients and the significance levels between the f ratios and seed yield-related traits in the under and over 50 f ratio group. The f ratio and germination rate were negatively correlated in the over 50 f ratio group

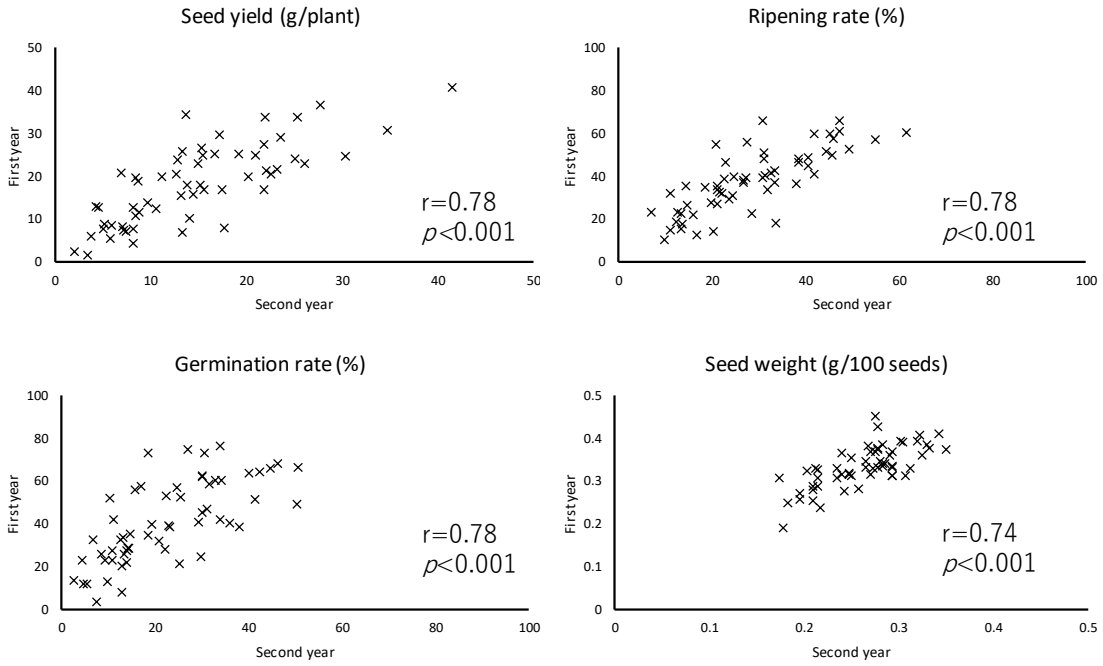

Figure 8 Correlations in seed yield-related traits between first year and second year.
In all parameters other than seed germination rate, “r” indicates Pearson’s correlation coefficient. For germination rate, “r” indicates Spearman’s correlation coefficient. “*p*” indicates the probability levels.

Table 8. Average of f ratio[†] and seed yield related traits.

Group[‡] by f ratio	Number of plants	F ratio %	Seed yield g / plant	Ripening rate %	Seed weight g / 100 grain	Germination rate %
Under 50	30	43.5	18.8	37.4	0.297	40.7
Over 50	28	55.1	13.9	28.6	0.301	22.4
Student's t test		***	*	**	n.s.	***
Mean[§]		49.1	16.4	33.2	0.299	31.8

*, **, *** and n.s. mean that the value is signifficant at $P < 0.05$, 0.01, 0.001 and not signifficant, respectively.

[†] The ratio of *Festuca*-specific genome regions to the whole genome.

[‡] To reveal the relationship between f ratio and seed yield-related traits, 58 plants from Low and High f ratio groups were reallocated to two groups using the threshold value of f ratio = 50%.

[§] Mean of 58 plants.

Table 9. Pearson's correlation coefficints between f ratio† and seed yield related traits in under 50 and over 50 f ratio group.

Group‡		F ratio	Seed yield	Ripening rate	Seed weight	Germination rate
Under 50	F ratio	1				
	Seed yield	-0.00$^{n.s.}$	1			
	Ripening rate	-0.07$^{n.s.}$	0.62***	1		
	Seed weight	0.20$^{n.s.}$	0.50**	0.46*	1	
	Germination rate	-0.24$^{n.s.}$	0.51**	0.43*	0.29$^{n.s.}$	1
Over 50	F ratio	1				
	Seed yield	-0.27$^{n.s.}$	1			
	Ripening rate	-0.27$^{n.s.}$	0.77***	1		
	Seed weight	-0.12$^{n.s.}$	0.64***	0.71***	1	
	Germination rate	-0.47*	0.37*	0.29$^{n.s.}$	0.40*	1

*, **, *** and n.s. mean that the value is signifficant at $P < 0.05$, 0.01, 0.001 and not signifficant, respectively.

† The ratio of *Festuca*-specific genome regions to the whole genome.

‡ To reveal the relationship between f ratio and seed yield-related traits, 58 plants from High and Low f ratio groups were reallocated to two groups using the threshold value of f ratio = 50%.

but not in the under 50 group. In both groups, all seed yield-related traits were positively correlated, except for ripening and germination rate in the over 50 f ratio group, and seed weight and germination rate in the under 50 f ratio group.

Table 10 shows a simulation of the f ratio for the next generation in both f ratio groups. Simulated f ratios for the next generation were 1% lower than the mean maternal f ratio in the high f ratio group and only 0.2% lower in the low f ratio group. In combination, the simulated f ratio for the next generation of both groups was 2% lower than the mean maternal f ratio (n = 58 plants).

Seed yield, but also ripening rate, and germination rate were significantly lower in the over 50 f ratio group than in the under 50 f ratio group. The observed differences in seed yield would likely result in genome balance drift favoring *Lolium* during seed multiplication. The same would be true for the germination rate if maternal lines had been mixed in equal amounts during *Festulolium* breeding. The simulated f ratio for the next generation was 2% lower than the previous generation's mean f ratio. Genome balance drift would, thus, have been avoided by mixing maternal lines in equal numbers in *Festulolium*. However, this is not feasible in industrial seed production. Furthermore, a negative correlation between f ratio and germination rate was observed in the over 50 f ratio group, but not in the under 50 f ratio group. Moreover, the simulated f ratio for the

Table 10. Average of f ratio[†] and simulated f ratio in the next generation.

Group	Number of plants	F ratio	Simulated f ratio[‡]
		%	%
Low	28	43.4	43.2
High	30	54.3	53.3
Mean[§]		49.1	47.1

[†] The ratio of *Festuca*-specific genome regions to the whole genome.

[‡] Simulated f ratio in the next generation assuming that seeds were mixed in equal amount and random pollination.

[§] Mean of 58 plants.

next generation declined by 1% in the high f ratio group, but only 0.2% in the low f ratio group. This suggests that in *Festulolium*, further reductions in the f ratio (of, for example, 40%) could result in f ratio stability.

In this study, germination rate was overall low. The seeds did not undergo dormancy as they were exposed to the air from summer to spring (min. temperature –15°C, max. temperature 35°C) after harvesting. Experimental conditions could also be excluded as interference, as Italian ryegrass plants (L503) showed a germination rate of 77% (Table 11). Since the ripening rate of *Festulolium* plants was also lower than L503, it is possible that hybrid breakdown accounted for germination and ripening rate reductions. For intergeneric hybrids like *Festulolium*, fertility and seed productivity are crucial for breeding (Ghesquière *et al*., 2010). This study is the first to report the differences in not only seed yield and ripening rate, but also germination rate, between the over 50 and under 50 f ratio group. The positive correlations between seed yield-related traits in both f ratio groups agrees with the general principles of crop science (i.e. higher ripening rates result in higher seed yields and seed weights, and higher seed weights ultimately facilitate higher germination rates).

Genome balance drift in favor of *Lolium* has been reported by many studies (Canter *et al*., 1999; Kopecky *et al*., 2005; Zwierzykowski *et al*., 1998, 2006), showing that over

Table11. F ratios and seed yield-related traits of each plants.

Plant name	Group	Reallocated group	F ratio	Seed yield	Ripening rate	100 seeds weight	Germination rate
NA1-6-1	low	under 50	49.2	11.4	37.2	0.264	25.1
NA1-6-2	low	under 50	36.8	9.6	32.1	0.309	45.1
NA1-6-3	low	under 50	43.9	16.5	32.6	0.234	40.9
NA1-6-4	low	under 50	38.5	6.1	17.2	0.226	26.5
NA1-6-5	low	over 50	50.1	20.9	50.7	0.343	37.8
NA1-6-6	low	under 50	37.2	41.2	56.0	0.376	57.1
NA1-6-7	low	under 50	44.6	21.5	43.4	0.363	38.3
NA1-6-8	low	under 50	47.4	26.3	54.2	0.358	46.1
NA1-9-1	low	under 50	41.7	22.4	42.4	0.305	39.0
NA1-9-2	low	under 50	34.9	11.7	26.3	0.184	51.9
NA1-9-4	low	under 50	38.5	29.5	56.5	0.358	52.0
NA1-9-5	low	under 50	37.0	12.8	25.8	0.228	16.6
NA1-9-7	low	under 50	43.3	15.1	34.9	0.245	37.3
NA1-9-8	low	under 50	37.7	21.7	51.8	0.321	58.6
NA1-17-1	low	over 50	50.7	17.0	35.2	0.310	20.9
NA1-17-2	low	over 50	53.0	24.5	50.9	0.365	29.5
NA1-17-5	low	under 50	43.5	19.3	60.9	0.313	55.3
NA1-17-6	low	under 50	42.0	27.5	47.7	0.325	46.4
NA1-17-7	low	under 50	48.9	27.9	44.7	0.270	49.9
NA1-17-8	low	under 50	43.1	7.9	41.5	0.284	38.1
NA1-24-1	low	under 50	37.7	19.9	25.4	0.293	39.0
NA1-24-2	low	under 50	48.0	32.2	38.0	0.278	53.4
NA1-24-3	low	under 50	47.0	16.2	42.7	0.303	46.3
NA1-24-4	low	under 50	43.4	18.8	37.2	0.299	46.8
NA1-24-5	low	under 50	45.0	12.1	48.0	0.314	31.0
NA1-24-6	low	under 50	42.0	15.8	23.6	0.271	50.9
NA1-24-7	low	under 50	44.5	24.6	52.5	0.260	25.0
NA1-24-8	low	under 50	47.0	19.4	34.7	0.349	35.0
NA1-7-1	high	over 50	60.0	10.1	30.7	0.303	11.5
NA1-7-2	high	over 50	56.7	13.7	37.9	0.364	10.4
NA1-7-3	high	over 50	58.8	6.3	18.0	0.281	17.3
NA1-7-4	high	over 50	50.0	10.0	32.2	0.271	21.6
NA1-7-5	high	over 50	55.6	8.5	15.1	0.328	26.8
NA1-7-7	high	over 50	57.5	8.5	21.6	0.319	35.8
NA1-7-8	high	over 50	54.0	21.0	40.9	0.330	38.0
NA1-16-1	high	over 50	55.6	23.0	31.9	0.348	8.8

NA1-16-2	high	over 50	55.6	24.6	35.9	0.311	26.3
NA1-16-3	high	over 50	55.5	20.1	28.2	0.314	21.4
NA1-16-4	high	over 50	52.3	23.4	33.1	0.282	30.9
NA1-16-5	high	over 50	54.7	32.7	39.6	0.313	23.3
NA1-16-6	high	over 50	56.4	7.2	14.1	0.216	5.5
NA1-16-7	high	over 50	52.3	16.5	27.1	0.303	47.3
NA1-16-8	high	under 50	48.3	14.0	24.9	0.353	16.1
NA1-20-1	high	over 50	52.1	4.8	15.6	0.271	17.9
NA1-20-2	high	under 50	49.5	15.5	26.5	0.326	37.8
NA1-20-3	high	under 50	48.6	22.2	27.4	0.323	45.9
NA1-20-4	high	under 50	46.4	13.7	20.4	0.325	55.4
NA1-20-5	high	over 50	56.9	7.2	14.8	0.233	8.4
NA1-20-6	high	over 50	52.0	7.2	10.0	0.283	31.1
NA1-20-7	high	over 50	54.8	14.3	19.0	0.303	22.5
NA1-20-8	high	under 50	49.1	10.3	15.4	0.249	13.9
NA1-22-2	high	over 50	57.0	24.0	48.4	0.355	27.3
NA1-22-3	high	over 50	59.8	5.5	13.0	0.251	19.8
NA1-22-4	high	over 50	55.5	7.6	24.1	0.301	23.4
NA1-22-5	high	over 50	56.2	2.4	27.5	0.240	8.2
NA1-22-6	high	over 50	55.6	6.9	27.1	0.306	19.6
NA1-22-7	high	over 50	57.1	18.3	41.7	0.334	19.3
NA1-22-8	high	over 50	55.9	2.2	17.7	0.261	16.9
Mean			49.1	16.4	33.2	0.299	31.8
Under 50			43.5	18.8	37.4	0.297	40.7
Over 50			55.1	13.9	28.6	0.301	22.4
L503[†]	Italian ryegrass		0.0	26.3	61.1	0.270	77.0
NA1-6	Parent (low)		38.8				
NA1-9	Parent (low)		35.7				
NA1-10	Parent (low)		51.2				
NA1-17	Parent (low)		40.1				
NA1-24	Parent (low)		41.6				
NA1-7	Parent (high)		54.4				
NA1-16	Parent (high)		55.6				
NA1-20	Parent (high)		46.0				
NA1-22	Parent (high)		59.4				

Values are mean of two years tests, except for L503 and "f ratio."

[†]L503 was Italian ryegrass and tested only in second year.

numerous successive cycles of sexual reproduction, chromosomes (or centromeres) and chromatins of *Festuca* in amphidiploid *Festulolium* plants are often substituted by those of *Lolium*. Zwierzykowski *et al*. (2006) suggested that chromosomes or chromatids with *Lolium* centromeres are faster in reaching the poles during female meiosis, thereby suggesting that *Lolium* centromeres or chromatin are dominant to *Festuca*'s. However, they could not provide any evidence upon which this suggestion could be based (Zwierzykowski *et al*., 2008, 2011). Nonetheless, the present study demonstrated that genome balance drift in favor of *Lolium* can be facilitated solely by phenotypic differences, independent of any effects caused by biases during meiosis. In a previous section, it was also demonstrated that genome balance drift did not occur in amphidiploid or backcrossed *Festulolium* cultivars in maternally derived progenies. Comparisons between generations selected for by breeders were likely unreliable and prior works should have considered the effects of selection on the genome balance drift.

Chapter 5 General discussion

5.1 Hybrid weakness and breakdown in *Festulolium*

Festulolium is an intergeneric hybrid between *Lolium* and *Festuca* species. Remote crossing (syn. wide cross or distant hybridization) is also a standard breeding strategy in other crops. Generally, there are two problems to overcome in remote crossing, hybrid weakness being the first. As a result, F_1 plants do not grow normally or F_1 seeds do not mature or germinate. In rice (*Oryza sativa* L.), hybrid weakness occurs between Japanese cultivars and the Peruvian cultivar, 'Jamaica'. The F_1 plants show very weak growth. Two genes (*Hwc1-1* and *Hwc2-1*) complementally bring this hybrid weakness (Amamiya *et al*., 1963). Polyploidization and *in vitro* rescue of hybrid embryos could overcome the hybrid weakness of amphidiploid *Festulolium* between *Lolium* species and *Festuca pratensis*. The F_1 plants grow normally except for low seed and pollen fertility.

The second problem is a hybrid breakdown. Following F_2 generations, there is a reproductive failure caused by incompatibility between interacting genes. Complementary recessive genes (*hbd1* and *hbd2*) controlling hybrid breakdown were mapped in rice (Fukuoka *et al*., 1998). The genome balance drift in favor of *Lolium* in *Festulolium* could be regarded as a hybrid breakdown. The observed differences in seed yields between the over 50 and under 50 f ratio groups could be partly due to species

specific seed yield variations between *Lolium* species and *F. pratensis*. Considering the low germination rate in both groups, the main reason for distinct seed yields is presumably hybrid breakdown. The same tendency (i.e. preferential uniparental chromosome transmission) was reported in a *Jatropha* interspecific hybrid (Fukuhara *et al*., 2016). However, because amphidiploid *Festulolium* could, to some extent, produce seeds, the hybrid breakdown is very slow. *Lolium* species and *F. pratensis* would be comparatively closely related. Recombination between homoeologous chromosomes was normally observed in amphidiploid *Festulolium*. Conversely, recombination rarely occurs in hybrids of species or genera such as triticale (Nkongolo *et al*., 2009), rice (Yan *et al*., 1999), wheat (Anamthawat-Jonsson, 1999), and barley (Noda and Shiraishi, 1990). In these hybrids, most of one parental genome is typically lost, or the parental genomes follow allopolyploid formation. In Chapter 4, it was shown that in an isolated population of high f ratio plants, very few plants had f ratios greater than 58%, which likely contributed to genome balance drift in favor of *Lolium*. This pattern, however, could also be regarded as hybrid breakdown. As F_1 hybrids between *Lolium* species and *F. pratensis* usually require embryo rescue, it might be possible to also use it to grow plants with f ratios greater than 58%. Reproductive barriers between *Lolium* species and *F. pratensis* are more or less present but clearly exist.

5.2 Implementation of GISH and DNA marker techniques for *Festulolium* breeding

In Chapter 2, it was shown that *Festulolium* is useful in fallow paddy fields in the Tohoku region. However, there are two problems with cultivating *Festulolium* in that region. Firstly, there are concerns that *Festulolium* cultivation may induce rice bug infestations, as in the case of Italian ryegrass. In Chapter 3, a new suite of methods to breed endophyte infected *Festulolium* was developed using DNA marker simply. That is backcrossing endophyte-infected Italian ryegrass to *Festulolium*. To remove self-pollinated plants and non-infected plants, DNA marker sets which could distinguish between *Festuca* and *Lolium* and detect *E. uncinata*, were applied, respectively. At least 146 true BC1 and endophyte-infected plants were bred, relatively quickly, using this new suite of methods. Endophyte-infected *Festulolium* would be able to dispel the concerns of pecky rice.

Another problem is the genome balance drift in favor of *Lolium*. There are concerns that the *Festuca* genome will disappear from allogamous cultivars during commercial seed production. In Chapter 4, it was shown that genome balance drift in favor of *Lolium* could occur solely by phenotypic differences (i.e. seed yield-related traits). It was suggested that further reductions in the f ratio (of, for example, 40%) could result in genome stability. However, considering the costs of calculating the f ratio, it is difficult

to combine the f ratio with usual breeding programs. In Chapter 3, the plants with a lower f ratio presented fewer *Festuca*-specific bands, suggesting that it was possible to estimate the f ratios using DNA markers. Developing a dense map of DNA markers for *Festulolium* is difficult because it is allogamous and is mostly polyploid. Some researchers have developed DNA markers for *Festulolium* (Humphreys *et al*., 2005; Tamura *et al*., 2009). In Chapter 3, Tamura's DNA marker sets were used but these DNA marker sets are only useful in introgressed lines, not necessarily in other *Festulolium* populations, especially amphidiploids. Given that Tamura's marker sets can only assess the presence or absence of *F. pratensis*-derived alleles, they cannot judge whether the plant has one or two *F. pratensis*-derived alleles at a certain locus in amphidiploid *Festulolium*. Alternatively, diversity array technology (DArT) markers have been developed for the *Festuca–Lolium* complex (Kopecky *et al*., 2009). DArT markers make it possible to reveal the extensive genetic composition in *Festulolium* cultivars (Kopecky *et al*., 2011). The DArT technique supplies a considerable amount of detailed genomic information and is, therefore, applicable to polyploids and other *Festulolium* populations. Furthermore, it would be possible to estimate the f ratio with the high level of accuracy using DArT, if money is not an issue.

5.3 Challenges and future work

It was demonstrated that genome balance drift in *Festulolium* could be solely induced by phenotypic differences independent of any genetic biases occurring during meiosis. However, even the under 50 f ratio group did not exhibit sufficient germination rates to produce a commercially viable amount of seeds. Commercially sufficient seed yields and germination rates can be reached with introgression cultivars, yet amphidiploid cultivars are more robust and adaptable and thus more suitable for cultivation. In the future, studies should focus on screening amphidiploid plants by seed yield, germination rate, and on estimating the heritability of these traits to better understand their influence on f ratios. This would allow us to evaluate the effect of reproductive barriers on experimental results. Additionally, by comparing high fertility amphidiploids to conventional amphidiploids, evolutionary processes in *Lolium* and *Festuca* species could be better understood.

The outcomes of this thesis can be used to aid efficient breeding of *Festulolium* in Tohoku. The risk of rice bug infestations can be avoided by backcrossing endophyte-infected Italian ryegrass to *Festulolium*. Removing self-pollinated plants and non-infected plants by the DNA marker sets makes it possible to finish backcrossing relatively quickly. The problem of genomic instability can be avoided by further reductions in the f ratio (of, for example, 40%). The breeding strategy is shown in a schematic diagram for the

endophyte-infected, genetically stable, and sufficient seed producing *Festulolium* cultivar (Figure 9).

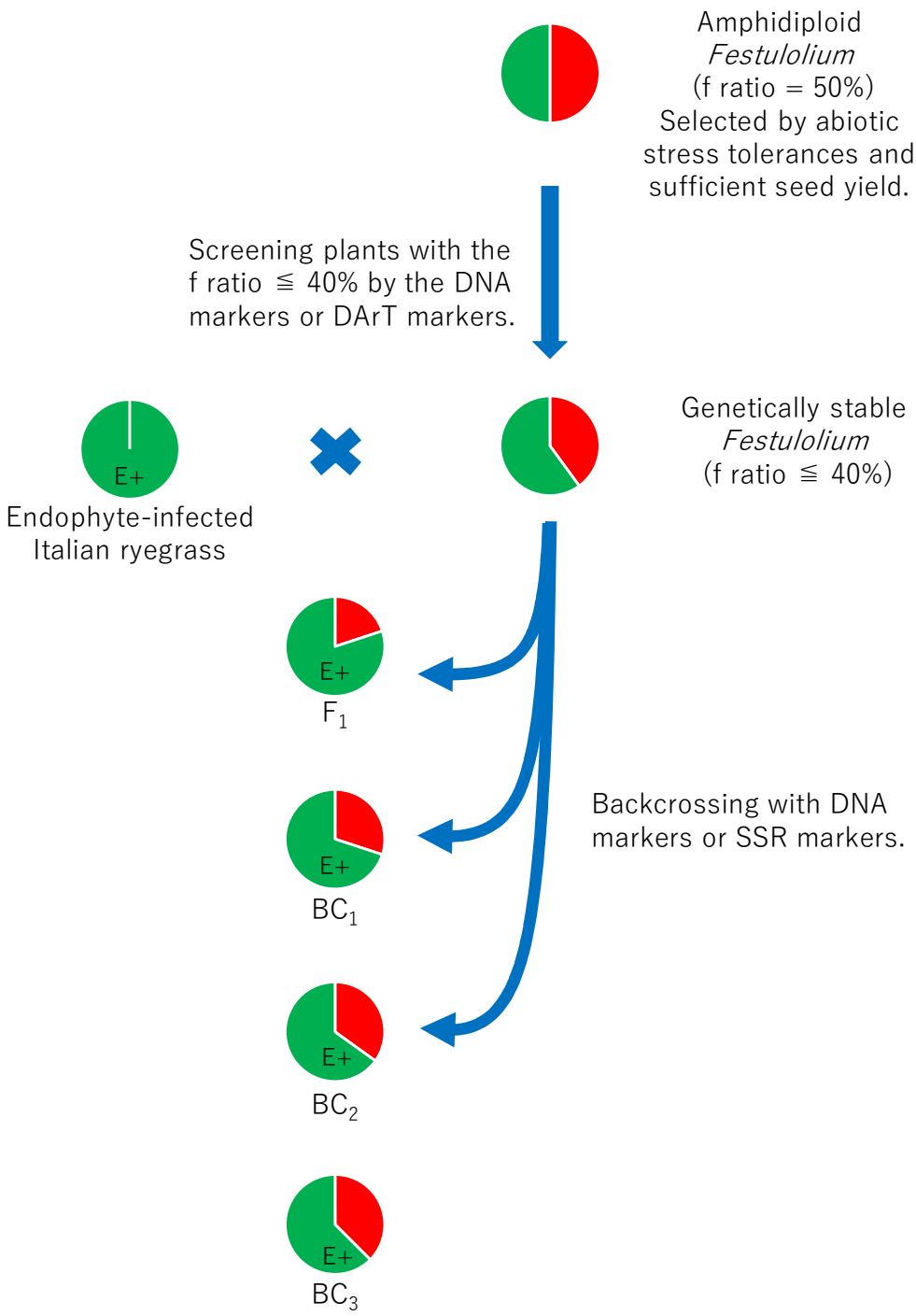

Figure 9 The new breeding strategy of endophyte-infected, genetically stable, and sufficient seed producing *Festulolium*.
Pie charts show genome constitutions in each generation.
Red shows *Festuca*-specific genome.
Green shows *Lolium*-specific genome.
E+ means endophyte-infected.

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Summary

Festulolium is an intergeneric hybrid between *Lolium* and *Festuca* species that may possess a combination of agronomic benefits derived from both genera. *Festulolium* are tolerant to excess water conditions, a trait inherited from their *Lolium* parents, which means that they are suitable for growing in fallow paddy fields for harvest. In Chapter 2, it is shown that *Festulolium* is useful in fallow paddy fields in Tohoku because *Festulolium* has higher snow endurance, especially in the second year of cultivation, compared Italian ryegrass and hybrid ryegrass, and higher culm length than perennial ryegrass, making it suitable for hay or silage.

There are two challenges in the cultivation of *Festulolium* in fallow paddy fields. Firstly, there are concerns that *Festulolium* cultivation encourages rice bug infestations, causing pecky rice, as in the case of Italian ryegrass. To overcome this problem, endophyte-infected *Festulolium* was bred using DNA markers. Endophyte-infected Italian ryegrass was backcrossed to *Festulolium*. To prevent self-pollinated plants being mixed with the progeny, PCR-based DNA marker sets were used to distinguish between *Lolium* and *Festuca* species. In the first backcrossing (F_1 development), 20 of the 21 progeny plants showed *Festuca*-specific band patterns and were endophyte-infected. Six maternal lines were developed (BC_1 development) by backcrossing six selected F_1 plants.

More than 97% of plants from five maternal lines had novel *Festuca*-specific band patterns which their maternal parents lacked. However, only 54% of plants of the maternal line F_1–18 exhibited novel *Festuca*-specific band patterns. F_1–18 showed *Festuca*-specific band patterns with almost all tested DNA marker sets, and its f ratio (the ratio of *Festuca*-specific region to the whole genome) was 31.1%, indicating that F_1–18 had an almost complete set of the *Festuca* genome. Since paternal backcrossing *Festulolium* parents were amphidiploids, an f ratio of approximately 25% corresponds to a complete set of the *Festuca* genome. It was difficult to evaluate BC_2 hybridity using the same DNA marker sets because very few DNA markers were distinctive. In total, at least 146 true BC_1 hybrids and endophyte-infected plants were bred and selected using DNA markers.

Another problem is the genomic instability. Since the GISH technique was developed, many *Festulolium* cultivars had been analyzed (Pašakinskienė *et al*., 1998; Canter *et al*., 1999; Zwierzykowski *et al*., 1998; Zwierzykowski *et al*., 2006), which had allowed for the observation of the genome balance drift in favor of *Lolium* in tetraploid amphidiploid *Festulolium* plants originated from the crosses between *Lolium* species and *F. pratensis*. There are concerns that the *Festuca* genome will disappear from allogamous cultivars during commercial seed production. It is,therefore, imperative to clarify the cause of the drift in favor of *Lolium*. To investigate the genome balance accurately, the f ratio method

was developed by combining GISH and image analyses (Akiyama *et al*., 2010). In Chapter 4, the variabilities of the f ratio within and among cultivars were investigated. Four or five chromosome spreads were sufficient to distinguish plants when the f ratios differed by only five points at a significance level of 0.05. It was also revealed that seven plants were more than sufficient to distinguish among cultivars that had f ratios differing by 10 points at a significance level of 0.05. Then, the genome balance drift was investigated between maternal plants and their progenies in three populations. One population was the progeny of a single cross between parents of the backcrossed cultivar 'Icarus', and the other two were isolated cross progenies from the amphidiploid cultivar 'Nakei 1'. Due to the fact that *Lolium* species have more seeds than *Festuca* species, bulking of seeds could cause the genome balance drift in favor of *Lolium*. In order to avoid this, as plants with low f ratios likely produce relatively more seeds, progenies were maternally-derived and their f ratios were also calculated for the same number of plants in each progeny. Considering the results of variability of the f ratio, eight plants for each progeny and four chromosome spreads (minimum = 2 and maximum = 10) for each progeny plant were tested on average. In maternally derived progenies, *Festuca* chromosomal fragments did not disappear in either amphidiploid or backcrossed *Festulolium* cultivars. These results suggest that differences in seed yields can cause

genome balance drift in favor of *Lolium* in *Festulolium* cultivars. Finally, the seed yields, ripening rates, and germination rates of two populations of ‘Nakei 1’ were monitored in the field. It was shown that genome balance drift in favor of *Lolium* could occur solely by phenotypic differences (i.e. seed yield-related traits). It was suggested that further reductions in the f ratio (of, for example, 40%) could result in genome stability.

Acknowledgements

I express deep gratitude to Professor Toshihiko Yamada from the Field Science Center for Northern Biosphere, Hokkaido University, for his guidance, encouragement, constructive suggestion and excellent supervision. I also extend my sincere gratitude to Associate Professor Yoichiro Hoshino (Field Science Center for Northern Biosphere, Hokkaido University), Professor Hajime Araki (Field Science Center for Northern Biosphere, Hokkaido University), Professor Shiro Tsuyuzaki (Faculty of Environmental Earth Science, Hokkaido University), Professor Yuji Kishima (Research Faculty of Agriculture, Hokkaido University), and Lecturer Yasuyuki Onodera (Research Faculty of Agriculture, Hokkaido University), for their useful comments and careful evaluations of this thesis.

I am indebted to my co-workers at NARO, in particular, I am grateful to Dr. Yukio Akiyama (Hokkaido Agricultural Research Center), for his advice and invaluable mentorship. I am also grateful to Mr. Yasufumi Ueyama (Institute of Livestock and Grassland Science (NILGS)), Dr. Junichi Yonemaru (Institute of Crop Science), Mr. Masahiro Fujimori (Tohoku Agricultural Research Center), Dr. Kenji Tamura (NILGS), Dr. Takako Kiyoshi (NILGS), and Dr. Keiichiro Matsukura (Kyushu Okinawa Agricultural Research Center), for their advice and tremendous support. My gratitude is

also extended to Mses. Setsuko Takahashi, Sayuri Yamagishi, Toshiko Uchitani, Messrs. Toshiyuki Sato, Hisashi Tamura, Nobuyuki Yoshizawa, Keiya Tsunokake, Humitaka Saito, Tadashi Kan, and Daisuke Kato for their technical assistance.

I am also indebted to my co-workers at the Japan Grassland Agriculture and Forage Seed Association, Messrs. Shinichi Sugita, and Tohru Sasaki, and Associate Professor Eri Kasai (present address: Faculty of Home Economics, Gifu Women's University) for their endophyte-infected seed supply.

This work was supported by a grant from the NARO in Tsukuba, Ibaraki, Japan.

Finally, I would like to express my special thanks to my family for their support and encouragement during my studies.