Fescue - locoid introgression as a source of novel germplasm for the pasture industry

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Abstract

Lolium and *Festuca* are two closely related temperate grasses grown worldwide for forage and which are known to hybridise readily. The aim of this research was to characterise F_2 *Festulolium* hybrids so that useful germplasm could be identified.

The DNA content of all F_2 hybrids was estimated using both PI and DAPI stains for flow cytometry. FISH and GISH was performed on selected F_2 hybrids to determine chromosome number and origin. An ISSR specific to *F. arundinacea* (Pašakinskiene *et al.* 2000) was assessed for its ability to identify the percent of parental origins of each F_2 hybrid using qPCR. Growth rates of plants in a randomised complete block, followed by leaf morphology were examined for correlations to estimated ploidy. The fertility of the F_2 hybrids was observed using FDA staining of pollen.

The DNA content of the F_2 hybrids ranged from 5.34 to 26.66 pg of DNA, indicating an estimated ploidy range from diploid to decaploid. Four plants with different chromosome numbers showed recombination between *Lolium* and *F. arundinacea* DNA. Recombination was more frequent in the plants containing more chromosomes. The ISSR marker was unable to be obtained and use of a second ISSR marker was unable to be optimised sufficiently to obtain percent parentage using qPCR, although presence or absence of either parental genome could be detected using PCR. Morphological investigations showed that the *Lolium* plants and some diploid F_2 hybrids had no or few stomata on the underside of their leaves. The number of stomata correlated positively with ploidy. High fertility, judged by pollen viability, of some F_2 hybrids correlated with 2n, 4n, or 6n estimated ploidy; low fertility with aneuploidy.

A diverse range of variation was observed between the F_2 hybrids, ranging from plants that more resembled either parent or were a morphological mixture between the two. The hybrids may be a valuable source of novel variation for future pasture development.

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Chapter 1 - Introduction

1.1 Overview of New Zealand pastures

New Zealand's exports are dominated by the agricultural industry, accounting for $12.8 billion and 6% of GDP in 2016 (Statistics New Zealand 2016). In order to sustain the dairy, beef and sheep industry worldwide, two widely used temperate grass genera, *Lolium* and *Festuca* are planted on farms (Easton *et al.* 1994, Ran *et al.* 2007). *Lolium* is most frequently grown in temperate regions such as New Zealand, Australia and the United Kingdom (Humphreys *et al.* 2010). *Festuca* is commonly grown in the United States of America, especially where drought stress is an important factor affecting plant growth (Rognli *et al.* 2010). *Lolium* and *Festuca* were introduced into New Zealand in the early 19th century from Europe (Lee *et al.* 2012). Since its introduction, *Lolium* rapidly adapted to New Zealand's conditions, and has been planted extensively with about 10 million hectares planted in the North and South Islands combined (Statistics New Zealand 2012). *Festuca* is less commonly used in pastures in New Zealand because it is typically less palatable and takes longer to establish from seed than *Lolium* (Easton *et al.* 1994). However, *Festuca* is better suited to stressful conditions, where it can maintain relatively good growth during drought and cold stresses (Wang and Spangenberg 2007). New Zealand's mild climate has up to now rendered the use of *Festuca* unnecessary (Wang and Spangenberg 2007).

Clovers (*Trifolium* spp.), plantains (*Plantago* spp.), chicory (*Cichorium* spp.) and cocksfoot (*Dactylis glomerata*) are plants sown into New Zealand pastures (DairyNZ 2017). Clover is particularly common because of its association with nitrogen fixing bacteria, supplementing nitrogen to the pastures between fertilising as well as providing excellent forage for animals (Ledgard and Steele 1992). Plantains and chicory are eudicots that have deep roots which allows for utilisation of water deeper in the soil. This gives pastures resilience to drought because some of the pasture is able to grow during drier conditions (Labreveux *et al.* 2004). Cocksfoot is a common grass sown in New Zealand pastures due to its high performance and persistence during the summer and resistance to insect pests (Mills *et al.* 2006). However, due to it poor winter performance it is usually only sown at low rates (Lolicato and Rumball 1994).

Pastures are the backbone of New Zealand's agriculture and, as such, improvement of current cultivars is of great importance to the industry (Lee *et al.* 2012). Within the last decade, droughts have been particularly severe in certain parts of the country, often costing more than $1 billion for each drought event (Carter 2009, NIWA 2017). A drought resistant forage grass is required to help reduce the losses incurred during these periods.

1.2 *Lolium*

Lolium is a genus from the family *Poaceae*, commonly known as grasses, native to Europe, Asia and Northern Africa. Currently 11 species are recognised, but only two species are widely cultivated (*L. perenne* and *L. multiflorum*) (Humphreys *et al.* 2010, The Plant List 2017). All *Lolium* are naturally diploid containing 2n = 2x = 14 chromosomes but can be easily induced to tetraploid 2n = 4x = 28 (Charmet and Balfourier 1994, Humphreys *et al.* 2010). Both *L. perenne* (LpLp) and *L. multiflorum* (LmLm) are commonly used in New Zealand as feed for both cows and sheep and were chosen as a forage grass because of their good agronomic traits such as high yield, nutritional value and palatability (Office of the Gene Technology Regulator 2008). However, *L. perenne* is more widespread due to its perennial nature and its higher tolerance to stresses. On the other hand, *L. multiflorum* is annual or biennial requiring frequent re-sowing. Nevertheless, *L. multiflorum* is often planted in autumn as winter feed because of its higher winter and spring yield (Humphreys *et al.* 2010).

Other *Lolium* species have not been used as a forage grass. One species, *L. temulentum* is a mimic weed, which has evolved alongside the artificial selection pressures of wheat and barley (Thomas *et al.* 2010). This makes *L. temulentum* difficult to identify until inflorescences emerge (Gay and Thomas 1995). Not only does *L. temulentum* compete with wheat and barley for resources, the seed contains a toxic endophyte (Freeman 1904). This was problematic until wheat and barley seeds could be easily sorted for *L. temulentum* seeds. Although *L. temulentum* has not been used for agricultural purposes it has been studied for its flowering responses to long days (Ran *et al.* 2007).

1.3 Festuca

The genus *Festuca*, like *Lolium*, is also a member of *Poaceae* family native to most of the northern hemisphere (Humphreys *et al.* 2010). However, the genus *Festuca* contains more than 400 species, which can be further divided into two groups; broad leaved and tall fescues (*F. pratensis* and *F. arundinacea* respectively), and fine leaved fescues (*F. rubra*, *F. longifolia* and *F. ovina*) (Office of the Gene Technology Regulator 2008). *Festuca*, also like *Lolium* has x = 7 chromosomes. However, the ploidy of the species within the genus *Festuca* ranges from 2n = 2x = 14 to 2n = 12x = 84 chromosomes (Thomas *et al.* 1997, Loureiro *et al.* 2007).

There are two main species of *Festuca* which are commonly used in agriculture; *F. pratensis* (FpFp) and *F. arundinacea* (FpFpFgFgFg'Fg') (Pašakinskienė and Jones 2005). *F. arundinacea* is a hexaploid (2n = 6x = 42 chromosomes) derived from three parental sets of chromosomes, one diploid set from *F. pratensis* (FpFp) and two diploid sets from *F. gigantea* (FgFg and Fg'Fg') (Kopecký *et al.* 2009). Although not widely used in New Zealand, *F. arundinacea* is an alternative to perennial ryegrass and is most widely grown as forage in drought prone areas of the United States of America covering over 15 x 10^6 ha (Rognli *et al.* 2010). The first commercial tall fescue cultivar was released in the early 1940s and brought the species to prominence over the following years. Tall fescue has greater tolerance to abiotic stresses and persistence than ryegrass, although ryegrass is more palatable. However, both tall fescue and both *Lolium* species have high yield and good nutritional quality making them good pastures in their respective adapted environments (Easton *et al.* 1994). *F. pratensis* is a diploid 2n = 2x = 14 and is similarly used as an alternative to *Lolium* in Europe where it is commonly found in hay fields (Kölliker *et al.* 1999). *F. pratensis* naturalised in the United States of America, but *F. arundinacea* is typically favoured for its greater tolerance to drought stress (Rognli *et al.* 2010).

Although not suitable for forage, fine fescues are commonly used as a turf grass. Fine fescues are more tolerant to stresses such as drought and poor soil quality and require relatively low maintenance when compared to other turf grasses (Funk *et al.* 1993). Fescue species commonly used for turf grasses are *F. rubra*, *F. longifolia* and *F. ovina* (Johnson 2003).

1.4 Forage grass improvements

Improving plant cultivars is an ongoing process and depends on whether plants need new traits to produce high quality products in new environments, or simply an improvement of current traits to increase productivity. Breeding programs are dependent on novel variation, which can be selected to introduce new traits or improve current ones (Nguyen and Sleper 1983). The resulting offspring of these crosses can then be selected for desirable traits. This method is often the easiest method for introducing novel variation because it does not reduce the fertility of the breeding individuals (Beddows *et al.* 1962). However, this method is also limited by the variation found within that species (Welch *et al.* 2000).

Another method for introducing novel variation is through the exploitation of polyploids. Polyploidy is also a common occurrence in the plant kingdom where approximately 70% are believed to be of polyploid origin (Masterson 1994) and is known to result in traits not found in at least one of the parents (Notsuka *et al.* 2000, Østrem and Larsen 2008). There are two types of polyploids: autopolyploids, the result of chromosome doubling following fertilisation within the same species; and allopolyploids, the result of fertilisation between two or more different but closely related species (Yousafzai *et al.* 2010).

Allopolyploids are excellent for introducing novel variation which does not exist in the species being improved or traits which would otherwise be difficult to enhance through intraspecific breeding (Humphreys *et al.* 1997, Østrem and Larsen 2008). However, low fertility in allopolyploids may occur, making it difficult to breed for or to introgress traits back into the desired species (Kopecký *et al.* 2009). Consequently, chromosome doubling of infertile allopolyploids, induced through the use of chemicals such as colchicine, can be an important tool for restoring fertility, resulting in a fertile amphiploid (Cao *et al.* 2003). This allows plant breeders to utilise the novel variation in breeding programs.

Doubling of chromosomes, in addition to restoring fertility to allopolyploids, can be used to create autopolyploids that may induce novel variation, such as increasing the expression of some traits including yield (Humphreys *et al.* 2010). Since yield is an important trait, the

induction of chromosome doubling has been done in many plant species (e.g. tetraploid grape vines have larger berries, and tetraploid ryegrass, *Brassica rapa* and *B. oleracea* had greater yield than the diploid counterparts) (Notsuka *et al.* 2000, Abel and Becker 2007, Humphreys *et al.* 2010). However, this may result in founder effects if the initial tetraploid population is too small (Ladizinsky 1985). Trait introductions from either autopolyploidy or allopolyploidy could result in introductions and improvements of a range of desirable traits such as abiotic tolerances, biotic resistances, and yield increases (Ortiz 1997, Notsuka *et al.* 2000, Østrem and Larsen 2008). The ability to develop cultivars that have increased yield under stressful conditions will be particularly important in years to come where increasing demand for food, along with climate change, will require improved plant cultivars.

1.4.1 *Festuca* x *Lolium* hybrids

Until 2004, *Festuca* x *Lolium* hybrids were not formally considered *Festuloliums* unless they were the specific cross between *L. multiflorum* x *F. pratensis*. However, since 2004, the definition of *Festulolium* was extended by the European Commission "to include all hybrids resulting from the crossing of a species from the genus *Festuca* with a species from the genus *Lolium*" (Ghesquière *et al.* 2010). Now, any hybrid *Festuca* x *Lolium* hybrid is considered a *Festulolium*, irrespective of amphiploid or introgression combination.

Broad leaved fescues diverged from fine leaved fescues ca. 9 million years ago (mya), followed by the broad-leaved fescues diverging again into current day broad leaved fescues and *Lolium*, just 1 mya ago (Charmet *et al.* 1997). It is no surprise then that broad-leaved fescues and *Lolium* hybridise readily in nature, considering their relatively recent divergence. However, hybrid offspring typically produce hybrids with reduced fertility. The ability of these two closely related genera to cross, and their offspring to possess desirable traits from both parents, (*Lolium*, high yield and nutritive value; *Festuca*, biotic and abiotic resistances) has led to the investigation and production of artificial crosses (Jenkin 1933). This stimulated *Festulolium* breeding programs leading to the first release of commercial cultivars, 'Prior' and 'Elmet', in the early 1970s (Lewis *et al.* 1973). Since the 1970s several more *Festulolium* cultivars have been made commercially available. These *Festuloliums* are perennials with reasonable persistence (3-4 years) and are at

least as cold tolerant as *F. pratensis*. Furthermore, these hybrids have high yield in both pure cultures and mixture pastures (Kopecký *et al.* 2006).

Akgun *et al.* (2008) examined cultivars Prior (*L. perenne* x *F. pratensis*) (LpFp) and Elmet (*L. multiflorum* x *F. pratensis*) (LmFp) at high elevation in Turkey. Elmet performed the best and, in general, showed greater values for agronomically important traits. *L. perenne* performed the worst as it died during the winter months due to damage caused by the cold weather. In turn, and as expected since Prior contained *L. perenne* genes, Prior generally performed worse than its parent, *F. pratensis*. However, these cultivars did not have chromosome stability with an aneuploidy rate of 41.67% and 73.33% for Prior and Elmet respectively.

To increase the fertility of *Festulolium* hybrids, F_1 hybrids can be backcrossed into either of the parents. Cultivars such as 'Hykor', 'Johnstone' and 'Felina' are examples of introgressed hybrids, backcrossed into *Lolium*, which have greater feeding value than their *Festuca* parent, greater cold tolerance than their *Lolium* parent and higher seed yield than the F1 hybrid. (Kleijer 1987, Kopecký *et al.* 2006). Some *Festuloliums* have also been specifically created to introgress genes from *F. arundinacea* (FpFpFgFgFg'Fg') into *L. multiflorum* (LmLm) (Humphreys 1998, Zwierzykowski *et al.* 1998). One such hybrid is a pentaploid *Festulolium* LmLmFpFgFg' in which crossing over occurs between *Lolium* and *Festuca* genomes. When backcrossed these crossover events between *F. arundinacea* chromosome fragments onto *L. multiflorum* chromosomes can be seen using genomic *in situ* hybridisation (GISH) (Humphreys 1998, Zwierzykowski *et al.* 1998). This method can allow for faster introgression of genes into a diploid *L. multiflorum*, within only two generations (Humphreys 1998).

Another group of *Festulolium* cultivars was produced from the hybridisation of *L. multiflorum* (2n = 2x = 14) x *F. arundinacea* (2n = 6x = 42). F_1 progeny from this cross resulted in a tetraploid (2n = 4x = 28) LmFpFgFg'. These tetraploids were sterile because the differences in chromosome structures of *Lolium* and *Festuca* prevent appropriate pairing and segregation in metaphase during meiosis, resulting in sterile gametes. In order to overcome sterility, gametes must undergo chromosome doubling to produce an octoploid LmLmFpFpFgFgFg'Fg' (2n = 8x =

56) which may restore fertility (Kopecký *et al.* 2009). This is because there are two of each chromosome within a cell which allows for appropriate pairing during metaphase. Chromosome duplication may be done artificially, using chemicals such as colchicine, or naturally through rare chromosome doubling events of the female gametophyte, followed by the development of the egg cell within the embryo without fertilisation from pollen (Kindiger 2012).

Attempts to create a population using 2n = 8x = 56 were initially unsuccessful because successive generations tended to have an unstable chromosome number (Kleijer 1987). However, an apparently stable octoploid was later developed by Pedersen *et al.* (1990), which was reported to have a stable chromosome number of 2n = 56, over successive generations (Pedersen *et al.* 1990). Eizenga *et al.* (1991) suggested that its stability may have been a result of the unintended selection of genes that were involved in chromosome pairing. These genes encourage pairing between two homologous chromosomes (bivalents) and suppress homoeologous pairing. However, upon contacting Eizenga *et al.* (1991) about their germplasm, we found that the meiotic stability of the *Festulolium* had not been maintained in subsequent generations since publication in 1991.

Festuloliums have also been created through protoplast fusion of a metabolically inactivated *F. arundinacea* and *L. multiflorum* (Takamizo *et al.* 1991, Spangenberg *et al.* 1994). Plants regenerated from protoplast fusion had morphologies intermediate between their parents, but also had unstable chromosome numbers, and in the case of Spangenberg *et al.* (1994), *L. multiflorum* chromosomes had been completely eliminated from the hybrid. Spangenberg *et al.* (1994) examined pollen viability of the regenerated and found that 30-40% of the pollen was viable. The presence of viable pollen may allow for desirable traits from these hybrids to be backcrossed into either parent.

1.5 Previous work on this project

All plant material including F_1 and F_2 *Festulolium* hybrids were created at New Zealand Agriseeds Ltd. (-43.45°, 172.18°). F_1 hybrids were created during the summer of 2013-14 by crossing either diploid *L. multiflorum* (LmLm) or *L. perenne* (LpLp) with hexaploid *Festuca*

arundinacea (FpFpFgFgFg'Fg') inside a pollen proof enclosure. Seed from only the *Lolium* parents was collected and sown to minimise the number of progeny that were the result of selfing. Several selection steps ensured that plants being used for subsequent breeding were F_1 hybrids. Firstly, F_1 hybrids were planted in clonal rows and selected for fescue-like traits to ensure that they were a combination of *Lolium* and *F. arundinacea*. Secondly, F_1 hybrids were screened using flow cytometry to determine their ploidy and plants that were determined to be allotetraploid (2n = 4x = 28) were considered hybrids. Finally, one of the allotetraploids was examined using fluorescence *in situ* hybridisation (FISH) and genomic *in situ* hybridisation (GISH) to verify the number and parentage of the chromosomes (Lm/LpFpFgFg') (Figure 1.1, Ansari 2014, unpublished). From this information, a subset of F_1 hybrids was chosen for breeding. During the summer of 2014/15 plants from *L. multiflorum* mothers had 81 F_1 hybrids planted into an isolation block, and given the code name 122 and a modifier (e.g. 122/1), while 51 F_1 hybrids from *L. perenne* mothers were planted in an isolation block, and given the code name with a modifier 123, 125 or 126 (e.g. 123/1).

Seed heads from F_1 plants were collected, dried and planted in compost. Most seed trays did not have seed germinate. However, some seed trays had seeds germinate (Figure 1.2). Seed was also harvested from F_1 hybrids the following year (summer 2015/16) and germinated. The plants produced from the F_1 hybrids are herein referred to as F_2 hybrids and were the plants which were examined in this thesis.

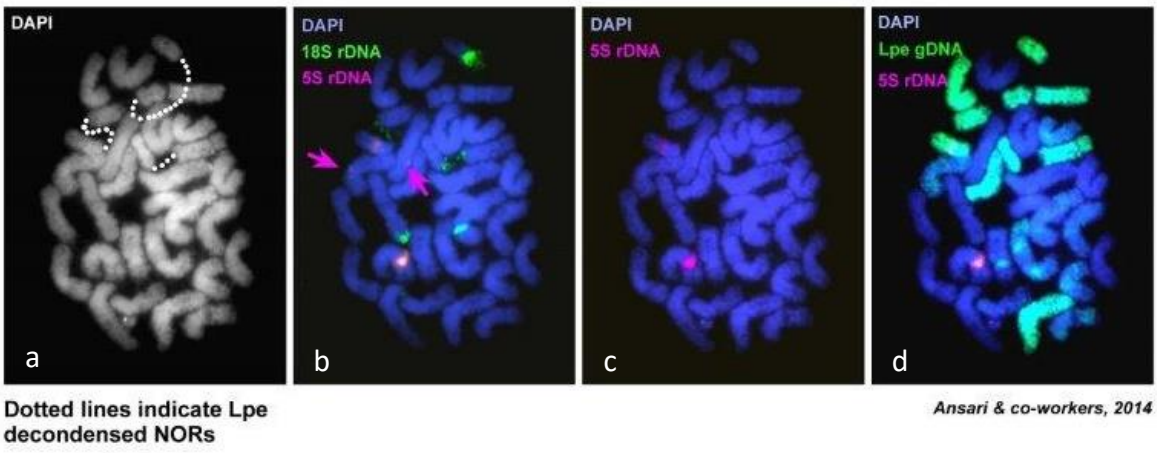

Figure 1.1: Fluorescent in situ *hybridisation (FISH) and sequential genomic* in situ *hybridisation (GISH) of an F_1 hybrid. Grey scale DAPI (a) indicating secondary constriction with the dotted lines. FISH staining of 18S rDNA sites (green) and 5S rDNA sites (red) counterstained with DAPI (blue) (b). Arrows indicate faint 5S rDNA sites. DAPI stained chromosomes (blue) and 5S rDNA sites (red) (c). GISH staining of chromosomes, showing* Lolium *DNA (green) and 5S rDNA (red) counterstained with DAPI (blue) (d) (Ansari et al. 2014, unpublished).*

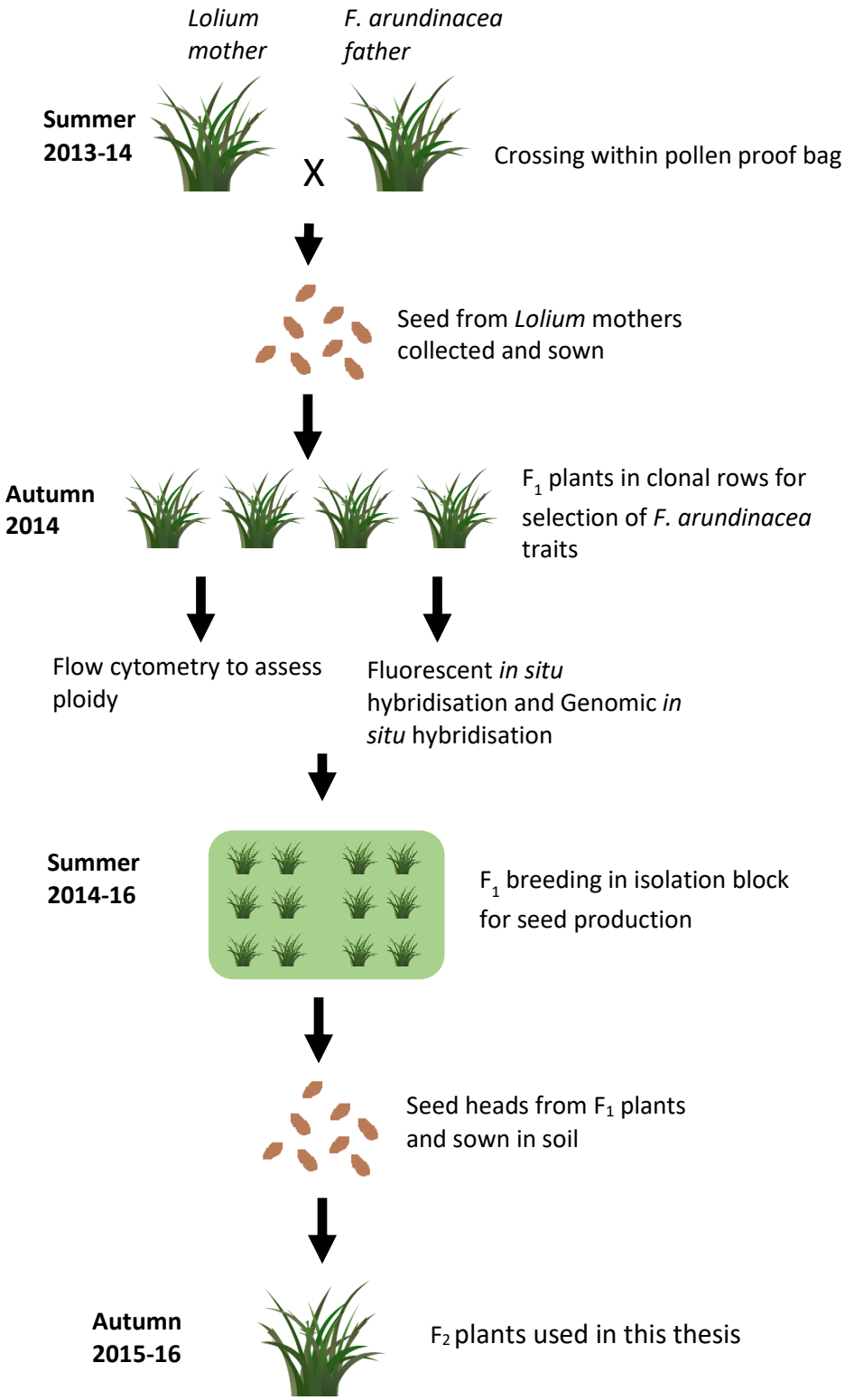

Figure 1.2: Schematic of work completed on Festulolium *breeding before this master's project.*

1.6 Research objectives

The aim of this project was to characterise F_2 *Festulolium* hybrids so that they could be assessed for future breeding.

1. F_2 hybrids were assayed using flow cytometry to identify the ploidy so that a subset of plants could be characterised in more detail in the thesis. It was important to identify F_2 hybrids that had similar amounts of DNA to diploid *Lolium* to test if these plants were doubled haploids using SSR markers developed by Sartie *et al.* (2011)
2. In order to identify individual chromosomes as well as the number of parentage chromosomes, a subset of F_2 hybrids were assayed using fluorescent *in situ* hybridisation (FISH) followed by sequential genomic *in situ* hybridisation (GISH).
3. Due to the time consuming nature of FISH and GISH chromosome staining, a rapid method needs to be developed to estimate the parentage of the F_2 hybrids. *F. arundinacea*-specific sequences were developed by Pašakinskiene *et al.* (2000) using anchored simple-sequence repeats (SSR) as primers. The aim was to replicate this experiment and isolate the DNA fragments for sequencing. Primers specific to these sequences could then be developed and the amount of PCR product quantified using qPCR. Once a standard curve had been created using different ratios of *Lolium* and *F. arundinacea* DNA, it was predicted that a relatively rapid estimation of the amount of *Lolium* and *F. arundinacea* DNA could be obtained.
4. The amount of amplification in the F_2 hybrids from the specific primers could be analysed using qPCR to estimate how much DNA from each parental genome was within each F_2 hybrid.
5. To assess the agronomic characteristics of the hybrids, the morphology and fertility of the F_2 hybrids were characterised by measuring regrowth after defoliation, fresh and dry weight of the forage, leaf morphology, stomata size and density, number of ridges per leaf, crude protein, heading dates and pollen viability.

Chapter 2 – Materials and methods

2.1 Production of plant material used in this thesis

Plant material used in this thesis was generated as described in Figure 1.2.

2.2 Flow cytometry

2.2.1 Plant collection

Leaf samples were collected from F_2 plants growing in a greenhouse at New Zealand Agriseeds Ltd. (-43.45°, 172.18°). Three young leaves were picked from each plant and cut to a length of 3-5 cm. Once all samples were collected, they were stored at 4˚C or on ice until used, but always within 48 h after collection.

2.2.2 Flow cytometry – DAPI

Initially, DAPI (4',6-diamidino-2-phenylindole) flow cytometry was done on the F_2 hybrids without an internal control to get a general idea of what the ploidy of these plants were. The relative fluorescence was manually adjusted regularly to set the *L. perenne* to 100. This allowed for relatively consistent results throughout the day. A second DAPI flow cytometry was done where all F_2 hybrid plants were assayed using DAPI flow cytometry. F_2 samples and diploid *L. perenne* (internal control) were chopped together finely in several drops of Otto I buffer using a sharp razor blade. Leaves were sufficiently chopped when the Otto I buffer had turned a light shade of green and leaf fragments <0.1 mm^2. Once diced, larger leaf material was filtered out using a 20 µm filter. The collected filtered cell suspension had ca. 1 ml of DAPI solution (1 µg/ml DAPI diluted in 0.2 M Na_2HPO_4) added and incubated for 5 min, to stain the DNA. The samples were applied to the flow cytometer (Partec PA II, Germany) as per the manufacturer's instructions. Gated mean values and graphical output were recorded using FloMax (v. 2.70). Each sample was run in the flow cytometer until two clear peaks (sample and control) were obtained or, if the F_2 hybrid was diploid, until it was clear only one peak had formed. The flow cytometry tests were done at Landcare Research, Lincoln, New Zealand. Otto buffer I were

stored at 4˚C, or on ice when in use, and the DAPI solution was stored in a bottle wrapped in foil at 4˚C, to prevent photo-degradation.

2.2.3 Flow cytometry – Propidium iodide

A subset (28 plants) of the total F_2 hybrid population was assayed using PI flow cytometry. This work used the Partec Cyflow Space at Landcare Research in Lincoln. Leaves of the F_2 hybrids were chopped finely in Otto buffer I using a sharp razor blade. Leaf samples were co-chopped with either broad bean (*Vicia faba* ssp. *Faba* var. enquina*,* 'Inovec') or pea (Pisum sativum, 'Ctirrad') as the internal control. Suspended cells were filtered into a 3.5 ml tube using a 20 µm filter to remove large leaf fragments. The cell suspension had ca. 1 ml of PI solution added (10 µg/ml PI diluted in 0.2 M Na_2HPO_4) and was incubated for ca. 5 min. Samples were applied to the flow cytometer (Partec Cyflow Space) where gated mean values and graphical output were recorded using FloMax version 2.70. Each sample was run in the flow cytometer until two clear peaks (sample and control) were obtained.

The calculation used to estimate the amount of DNA per cell (pg) in any F_2 hybrid plant is as follows (Doležel *et al.* 1992, Doležel *et al.* 1998, Šmarda *et al.* 2008):

$$F_2\ pg\ DNA\ per\ \ cell = \frac{mean\ sample\ peak}{mean\ control\ peak} \times pg\ DNA\ per\ cell\ in\ control\ plant$$

Ploidy of the F_2 hybrids could then also be estimated by using the determined pg DNA per *Lolium* cell, and was calculated as follows:

$$F_2\ ploidy = \frac{F_2\ pg\ DNA\ per\ cell}{\text{2n Lolium}\ pg\ DNA\ per\ cell} \times 2$$

2.3 Doubled haploid testing

2.3.1 Plant material collection and testing

Plants that were estimated to be diploid using flow cytometry were tested for homozygosity. Two pseudostems were sampled per plant and cut to lengths of 1.5-2 cm on the afternoon of 8th June 2016. Pseudostems were put into zip lock bags and stored on ice packs, in a polystyrene box until sampling was complete. In total, 12 allodiploid *Festuloliums*, one *L. perenne* and one *L. multiflorum* control plant (cultivars Bronsyn and Tabu respectively) were sampled. Samples were then stored overnight at 4°C. Pseudostems were sent the following morning (9th June 2016) to Grasslands, AgResearch in Palmerston North, New Zealand in a polystyrene box containing an ice pad. Samples were received by AgResearch on the 10th June 2016 and DNA extraction was done that afternoon. The DNA extraction procedure was based on Whitlock *et al.* (2008). Plant DNA isolates were analysed using seven SSR primer pairs, selected from seven ryegrass linkage groups Table 2.1, using procedures described in (Sartie *et al.* 2011).

Table 2.1: Simple sequence repeat (SSR) Lolium *loci used for analysis of F_2 hybrid samples (Sartie et al. 2011).*

SSR marker	Linkage group
pps0002	7
pps0008	1
pps0040	4
pps0164	3
pps0328	2
pps0967	6
pps1068	5

2.4 Fluorescence *in situ* hybridisation and genomic *in situ* hybridisation (FISH and GISH)

Due to the hazardous nature of some of the chemicals used in this experiment, material and safety data sheet recommendations for handling the chemicals was followed.

2.4.1 Root tip collection

Clones of F_2 hybrids were brought from New Zealand Agriseeds in Darfield to Grasslands AgResearch in Palmerston North on the 1st August 2016. These plants were re-potted and grown in a greenhouse at 19-23˚C. Actively growing root tips were harvested and the plants re-potted for later root harvesting when appropriate. Three cm of the actively growing root was harvested and placed in water while the remaining root tips were harvested. Root tips were washed in water using a soft brush to remove all the small dirt particles. The tips were then cut to a length of ca. 2 cm and treated with either 3 mM hydroxyquinoline or 0.05% (w/v) colchicine. Hydroxyquinoline treated roots were incubated for 2 h in the dark at room temperature, followed by 6 h at 4˚C. Colchicine treated root tips were incubated in the dark at room temperature for 2 h, followed by 2 h at 4˚C. Both hydroxyquinoline and colchicine treated root tips were fixed in 3:1 methanol: acetic acid overnight. All fixatives used glacial acetic acid (17.5 M). The fixative was changed the next morning, and root tips were stored at 4˚C for at least one week before being used.

2.4.2 Preparation of F_2 hybrid chromosome slides

The method used for chromosome preparation was called the flame drying technique developed by Ansari *et al.* (1999). Stored root tips were removed from fixative, dried briefly on a tissue and then submerged in citrate buffer, pH 4.8. Citrate buffer was removed and replaced twice to ensure all the fixative had been removed. Under a stereomicroscope the root cap was removed and the region of actively growing cells was sectioned into widths of <1 mm (Figure 2.1). The sections were macerated with an enzyme mix (Table 2.2) for 72 min at 37˚C in a water bath. The enzymatic reaction was halted by rinsing the root tips carefully in citrate buffer three times. The root tip sections were then stored in the third rinse of citrate buffer until used. Two root sections were then placed onto a microscope slide and dissected under a

stereomicroscope, to remove most of the non-relevant outer cells (Figure 2.2). Excess fluid was removed from the slide using a syringe, and the sectioned root cores were crushed, forming a cell suspension. One drop of 55% (9.6 M) acetic acid was added to the cell suspension, and the cells incubated at 22˚C for 150 s. Several drops of cold fixative (3:1 methanol: acetic acid) stored at -20˚C were added to the slide. Excess fluid was drained from the slide and the remaining fixative on the slide lit on fire. The slide was extinguished with a quick flick after 2 s and left to air dry. Once dry, the slide was examined under a microscope in phase contrast. All chromosome observations were done using a Zeiss Axio (Imager.M2) with epifluorescence attachment. All chromosome images were taken using a CoolCube 1 monochrome camera (Metasystems) using ISIS imaging software for FISH (v. 5.8.3) (Metasystems).

Table 2.2: Constitution of enzyme mix for maceration of root tips. All constituents were dissolved in citrate buffer pH 4.8

Constituents	**Amount**
Cellulase (from *Aspergillus niger*) (Calbiochem #21947)	1.2% (w/v)
Cellulase Onozuka R10 (Serva #16419)	0.2% (w/v)
Macerozyme R10 (Serva #28302)	0.1% (w/v)
Pectolyase (Sigma #P3026)	0.1% (w/v)
Pectinase (from *Aspergillus niger*) in 40% glycerol (Sigma #P0690)	9.2% (v/v)

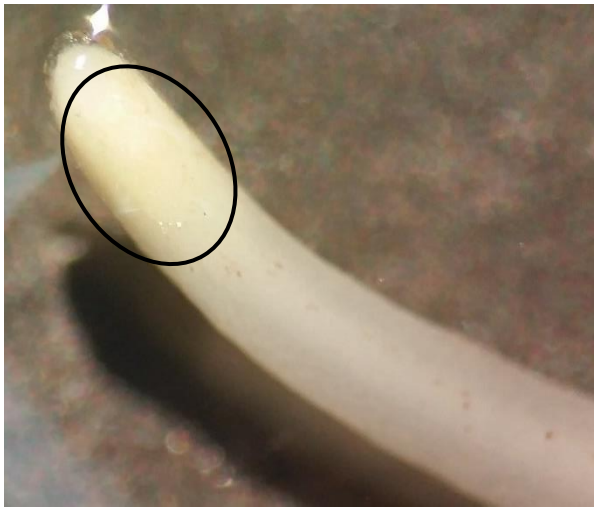

Figure 2.1: Root tip of F_2 Festulolium *hybrid. The whiter region contains the actively growing region where cross sections were made. Non-dividing cells, including the root cap and mature cells (not circled) were discarded. The actively dividing region is the region used for chromosome preparation circled.*

Figure 2.2: Cross section of root tip in F_2 Festulolium *hybrid.* Inner *region of whiter cells (arrow) contains the actively dividing cells, surrounded by non-dividing cells.*

The prepared slide was screened for relevant cells (dividing cells between late prophase to late metaphase stages and with good chromosomal spreading and little cytoplasmic debris). Those slides that had large numbers of these cell types, >15, could be used later for FISH and GISH chromosome staining. Suitable cells had their coordinates recorded so that they could be easily found later. The area of suitable cells was marked by etching the underside of the slide to visualise where the pepsin, FISH and GISH probes were to be applied. Slides were incubated at 37˚C for at least 3 d before starting hybridisation.

2.4.3 Giemsa staining

Giemsa staining was done on some chromosome preparation slides which had several relevant cells, but not enough for FISH or GISH staining. Giemsa staining was done to count the number of chromosomes in the F_2 hybrids before FISH and GISH staining. 1.5 ml of Giemsa stock (Appendix 1) was added to 50 ml of Sorensen buffer (0.067 M, pH 6.8) to make a working Giemsa solution. Chromosome slide preparations was incubated in a Coplin jar of working Giemsa solution (5% w/v) at room temperature for 20 min. Slides were rinsed carefully with water, then air dried. Dried slides were observed under the microscope to check for sufficient staining. Once sufficient staining was confirmed, slides were mounted with immersion oil (Immersol™ Immersion Oil (Carl Zeiss, Inc. 444960)) and a cover slip placed over the top. Immersion oil was also placed on top of the cover slip when viewed and photographed using 630x magnification.

2.4.4 Fluorescence *in* situ hybridisation (FISH) preparation

Slides with many cells in metaphase were chosen for FISH. Relevant sections of slides with high (at least 15 cells in metaphase) mitotic division were treated with 120 µl of 0.1 µg/ml RNase in 2 x SSC (saline-sodium citrate buffer) (Appendix 1), a cover slip added and the slide incubated at 37˚C in a humid chamber for 55 min. The RNase was rinsed off with water and the slides incubated for 5 min in 2 x SSC, followed by 4 min in fresh 2 x SSC. RNase treated slides were immersed in 10 mM HCl for 5 min, excess fluid removed and 5 µg/ml of pepsin solution added to the relevant area. A cover slip was added to the pepsin-treated area and the slide again incubated at 37˚C in a humid chamber for 40 min. Pepsin treated slides were then washed in 2 x

SSC for 1 min, followed by two more washes in fresh 2 x SSC for 5 min each. Washed slides were fixed in 4% (v/v) paraformaldehyde at room temperature, then rinsed in 2 x SSC, and incubated for 5 min in fresh 2 x SSC. Slides were replaced in fresh 2 x SSC for a further 4 min before being dehydrated. Slides were dehydrated using a dehydration series of 70%, 90% and 100% (v/v) ethanol for 2 min each. Air dried, dehydrated chromosomes were then denatured in 70% (v/v) formamide in 2 x SSC at 73˚C for 130 s. Denatured chromosomes were then dehydrated again using a dehydration series at 0˚C of 70%, 90% and 100% ethanol for 2 min each. Slides were air dried.

The hybridisation mixture (Table 2.3) was denatured at 89˚C for 10 min, then immediately transferred to ice for at least 3 min. The denatured hybridisation mixture (Table 2.3) was applied to the relevant area of the slide (0.088 µl/mm^2 and usually the area was ca. 15 mm x 15 mm) covered with a plastic coverslip and incubated at 37˚C overnight in a humid chamber (22 h). DNA was fluorescently labelled using fluorochromes, either Cy3-dCTP (product no. PA5302, GE Healthcare, NZ) or fluorescein-12-dCTP (product no. NEL424001EA, Perkin Elmer). Fluorescent labelling was performed using a nick translation kit (product no. N5500, GE Healthcare NZ) according to the manufacturer's instructions. Briefly two ribosomal DNA (rDNA) probes, pTr5S (GenBank Accession number AF072692) and pTr18S (GenBank Accession number AF071069), which encode for 5S rDNA and 18S-5.8S-26S rDNA (herein referred to as 18S rDNA) respectively, were used as FISH probes. pTr5S was labelled with Cy3-dCTP, while pTr18S was labelled with Fluorescein-12-dCTP.

Table 2.3: FISH hybridisation mixture constituents and final concentration of chemicals.

FISH hybridisation mixture constituents	Volume (µl) of constituents used to make 122 µl	Final concentration
H_2O	16.7	n/a
Formamide (FFA)	61.0	50% (v/v) FFA
20 x Sodium citrate buffer (SSC)	10.7	1.75x SSC
50% (w/v) Dextran sulfate (DS)	24.4	10% (w/v) DS
10% (w/v) Sodium dodecyl sulfate (SDS)	1.2	1% (w/v) SDS
Carrier DNA (Salmon sperm DNA, 1 µg/µl)	0.6	5 ng/µl carrier DNA
DNA probe 1 (pTr5S) 50 ng/µl	3.7	1.5 ng/µl probe 1
DNA probe 2 (pTr18S) 50 ng/µl	3.7	1.5 ng/µl probe 2

Post-hybridisation washing was done to remove excess and non-specific hybridisation. All post-hybridisation washing was done at 42˚C with pre-warmed solutions. Slides were first rinsed in 2 x SSC, then incubated in 2 x SSC for 5 min, followed by a further 4 min in fresh 2 x SSC. Slides were incubated in 50% formamide (FFA) (w/v) dissolved in 2 x SSC for 10 min, then rinsed in 2 x SSC. Slides were incubated again for 5 min in 2 x SSC and for 4 min in 2 x SSC. The slides were transferred to fresh 2 x SSC and allowed to cool to room temperature.

Slides were then counterstained with 1 µg/ml DAPI for 6 min in the dark, then mounted in VECTASHIELD® mounting medium without DAPI (Cat. no. H-1000). Glass cover slips were applied and mounted slides were incubated at room temperature for 30 min, then at 4˚C for 2 h before being viewed under the microscope.

2.4.5 Preparation for sequential hybridisation

Before sequential hybridisation can occur, the slide must be prepared so that new hybridisation can occur. The immersion oil placed on the cover slip must be removed by wiping off as much as possible with a tissue, followed by wiping with xylene to remove any remaining oil. The slides were then incubated in 2 x SSC at room temperature overnight. The cover slips were carefully

removed and slides placed in fresh 2 x SSC for 2 h at 37˚C. Slides were transferred to fresh 2 x SSC for 5 min, then dehydrated in an ethanol series of 70%, 90% and 100% ethanol for 2 min each. The slides were air dried and checked under the microscope in phase contrast to ensure chromosomes had not been damaged. Slides were then kept at 37˚C until use for a second hybridisation.

2.4.6 Genomic *in situ* hybridisation (GISH) preparation

Both fresh slides and slides which had already been used for FISH, underwent GISH. Fresh slides were treated with RNase and pepsin as described for in section 2.4.4. Slides that had previously been used in FISH had to be prepared for sequential hybridisation described above (section 2.4.5).

Fresh slides were fixed in 4% paraformaldehyde and denatured in 50% formamide followed by dehydration as described in FISH (section 2.4.4). The hybridisation mixture used for GISH was slightly different to the FISH hybridisation mixture Table 2.4. The GISH hybridisation mixture contained genomic *L. perenne* DNA fluorescently labelled Fluorescein-12-dCTP using the nick translation kit used for labelling 5S and 18S rDNA probes. *F. arundinacea* genomic DNA (gDNA) was used as blocking DNA and was created by making short fragments of gDNA using sonication. Hybridisation mixture (0.088 µl/mm^2) was added to the relevant area on each slide. Slides were incubated overnight for 22 h before undergoing post-hybridisation washing. Some slides (126, 123/6/A, and 122/80/E) were denatured a second time using a thermocycler (PHC-3) at 72˚C for 150 s followed by gradual cooling to 37˚C dropping 3˚C per min. The thermocycler step was done to maximise success by using a slightly different method to those slides that did not undergo the thermocycler step. Slides remained at 37˚C in the thermocycler for 30 min before being transferred to a humid chamber for incubation for 22 h. Slides underwent post-hybridisation washing as described on FISH followed by DAPI counterstain for 6 min. Slides were mounted in VECTASHIELD and incubated for 2.5 h before viewing.

Table 2.4: GISH hybridisation mixture constituents and final concentration of chemicals.

GISH hybridisation mixture constituents	Volume (µl) of constituents used to make 130 µl	Final concentration
Formamide (FFA)	65.0	50% (v/v) FFA
20 x Sodium citrate buffer (SSC)	11.4	1.75x SSC
45% (w/v) Dextran sulfate (DS)	28.8	10% (w/v) DS
10% (w/v) Sodium dodecyl sulfate (SDS)	1.5	1% (w/v) SDS
Carrier DNA (Salmon sperm DNA 1 µg/µl)	0.7	5 ng/µl carrier DNA
Probe DNA 1 (*L. perenne* gDNA)(50 ng/µl)	7.3	2.8 ng/µl probe 1
Probe DNA 2 (pTr5S, 50 ng/µl)	4.3	1.7 ng/µl probe 2
Blocking DNA (*F. arundinacea* gDNA, 1 µg/µl)	11.0	84 ng/µl blocking DNA

When examining the slides at high magnification, Carl Zeiss Immersol™ Immersion Oil (Carl Zeiss, Inc. 444960) was used. To excite each of the fluorochromes/fluorophores the appropriate DAPI, FiTC and Cy3 filter cubes were used.

Chromosome preparations were made for four F_2 *Festuloliums* (123/33/B, 122/80/E, 126, and 123/6/A). These plants were chosen for study based on the estimated ploidy calculated from flow cytometry. Chromosome preparations were analysed using three different techniques: Giemsa staining, FISH and GISH. The techniques used to analyse the chromosomes of these four plants are summarised in Table 2.5.

Table 2.5: Summary of the chromosome assays performed on F_2 hybrid plants.

Plant name	Estimated ploidy	Giemsa stain (Y/N)	FISH (Y/N)	GISH (Y/N)
123/33/B	Diploid	Y	Y	Y
122/80/E	Tetraploid	N	Y	Y
123/6/A	Octoploid	Y	N	Y
126	Octoploid	Y	Y	Y

2.5 Molecular characterization of F_2 *Festulolium* hybrids

2.5.1 Plant material and DNA extraction

Clones of F_2 *Festulolium* plants were grown at the University of Canterbury 43˚.5' S, 172˚.6' E. Young leaves were harvested from plants and immediately frozen in liquid nitrogen. About 100 mg of frozen leaf sample was ground into a powder, using a mortar and pestle kept cold with liquid nitrogen. Powdered leaves were then either used immediately for DNA extraction, or stored at -80˚C until needed. Genomic DNA from *Lolium* and *F. arundinacea* parents as well as F_2 hybrids was extracted using regenerated silica columns in combination with modified CTAB extraction (Doyle and Doyle 1987, Fu *et al.* 2017). Incubation of plant material with CTAB solution was done at 60˚C for 20 min to ensure long DNA fragments were extracted. DNA quality was tested using a ND-1000 spectrophotometer (NanoDrop Technologies) using NanoDrop 1000 spectrophotometer software v. 3.8.1

2.5.2 PCR of inter-simple sequence repeats (ISSRs)

Two SSR primers (Table 2.6) published in Pašakinskiene *et al.* (2000) were optimised and tested to determine if there were species-specific PCR products being amplified. All optimisation for the molecular characterisation was done using *F. arundinacea*. Each PCR reaction volume was 20 µl consisting of 2 µl 10 x Taq Buffer (Bioline), 2.4 µl of 25 mM $MgCl_2$, 2.5 µl of 2 mM dNTPs, 2 µl of 10 pM primer, 1 µl of 50 ng DNA template, 0.2 µl (5 units/µl) BIOTAQ™ DNA polymerase (Cat. No. BIO-21040) and water to make up to 20 µl. The PCR program was carried out in the following conditions: 1 cycle at 95˚C for 10 min; 35 cycles at 95˚C for 20 s, 50˚C (104H) or 54˚C (78H) for 30 s, 72˚C for 90 s; 1 cycle at 72˚C for 7 min then held at 4˚C. To ensure the PCR was working correctly, an internal control, using primers designed for 18S rDNA (Accession no. U43011). PCR products were separated on 1.5% (w/v) agarose/TAE (Tris-acetate, EDTA, pH 8.3) gels. All PCR product sizes were estimated using HyperLadder™ 1kb (Bioline, Cat. No. BIO-33025). All gels were viewed using G:Box (Syngene) and images analysed using GeneSys (v. 1.0.4.0, Syngene).

Table 2.6: Tested SSR primer nucleotide sequences for species-specific PRC products. 18S rDNA primers (Accession no. U43011) were used as an internal control during primer optimisation.

Primer	Nucleotide Sequence	Abbreviation
104H	5' - GACAGACAGACAGACAGT - 3'	$(GACA)_4GT$
78H	5' - ACGACAGACAGACAGACA - 3'	$AC(GACA)_4$
Ps18SF2	5' – GCTGAAACTTAAAGGAATTGACGGAAG – 3'	
Ps18SR2	5' – TTGAAGACCAACAATTGCAATGATCTATC – 3'	

PCR products of interest (0.6 kb, primer 104H and 1.2 kb, primer 78H) which were estimated to be the same size as those found by Pašakinskiene *et al.* (2000), were purified from the gel using UltraClean® 15 DNA purification Kit, MO BIO (Cat. no. 12100-300). The purified PCR product were either sequenced by sending 10 µl of PCR product with 5 µl of primer to Macrogen, or cloned into One Shot® TOP10 Chemically Competent *E. coli* using TOPO® TA Cloning® Kit for sequencing as per the manufacturer's instructions, then sequenced. Transformed cells (200 µl) were spread onto a LB (Luria broth) agar plate containing 50 µg/ml kanamycin and incubated for 16 h at 37˚C. A subset of the colonies that grew was tested to check that those colonies had been transformed. A cell suspension was created in 20 µl of water for the colonies chosen to confirm that the DNA fragment had been cloned. PCR was done using the cell suspension as the DNA template. The reagents used for the PCR reaction were 3 µl 10x Taq buffer (Roche), 0.3 µl of 20 mM dNTPs, 0.9 µl of 50 mM $MgCl_2$, 0.24 µl of 5 units/µl, (Roche Cat. No. 11 146 165 001), 1 µl M13 primer (Figure 2.3) (0.5 µl forward, 0.5 µl reverse), 1 µl cell suspension and water to make up to 15 µl. The PCR program used was as follows: 1 cycle 95˚C for 2 min; 35 cycles 95˚C for 30 s, 58˚C for 30 s, 72˚C for 60 s; 1 cycle 72˚C for 5 min, then held at 4˚C. PCR product was run on 1% (w/v) agarose/TAE gel. The plasmid was extracted from the *E. coli* using DNA-spin Plasmid DNA purification Kit (Cat. no. 17098) as per the manufacturer's instructions. From each plasmid isolate (15 µl), two tubes had 5 µl of plasmid isolate (>50 ng/µl) added along with 5 µl of either M13 (10 pmol/µl) sense or antisense primer. This allowed for sequencing from both directions and was used as a replicate to verify the sequence.

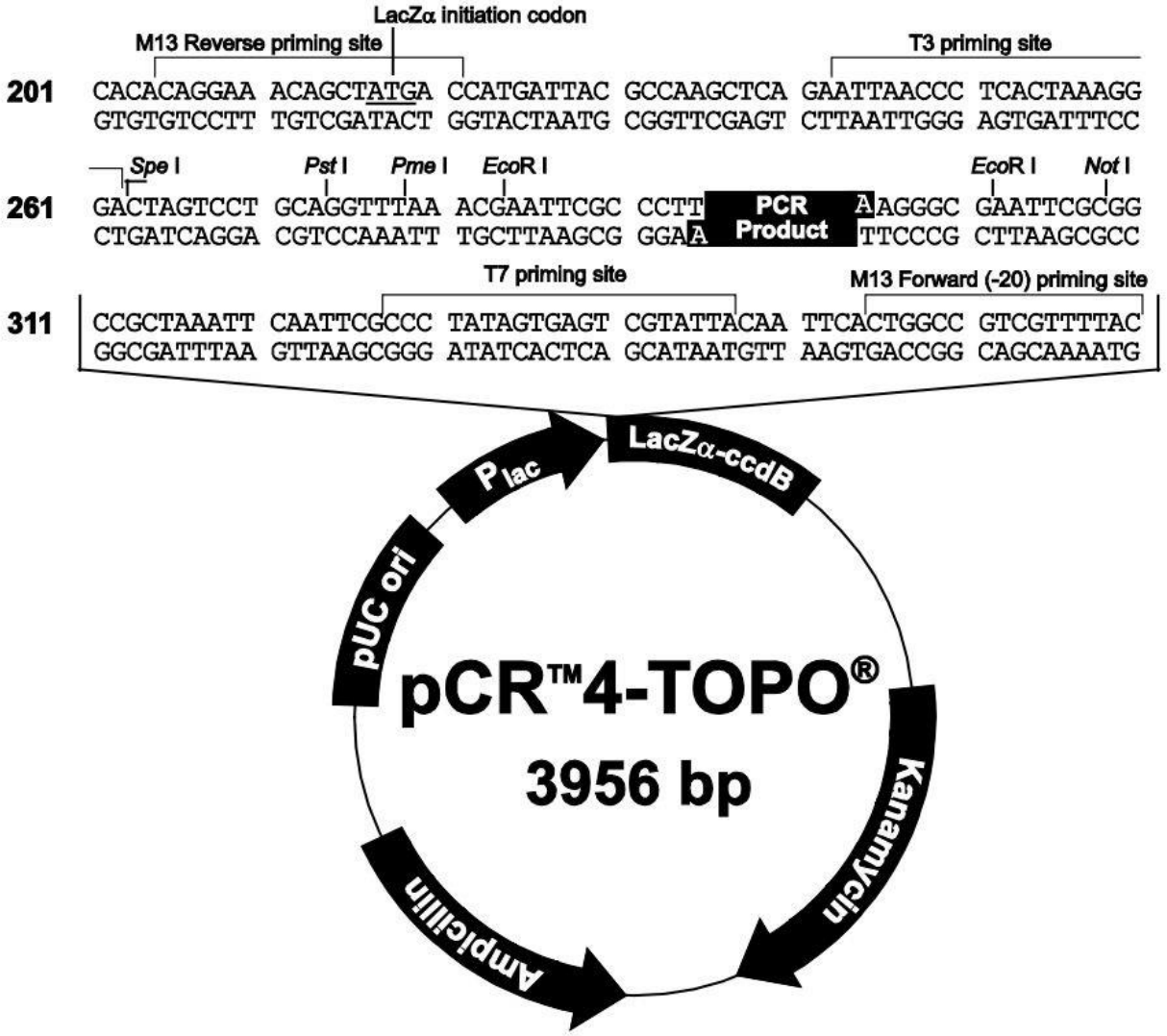

Figure 2.3:Plasmid construct used for cloning the target sequences that were sent for sequencing. The M13 sense and antisense primers were used to identify if the target sequences had been successfully cloned (Topo TA cloning kit, https://tools.thermofisher.com/content/sfs/manuals/topotaseq_man.pdf).

2.5.3 Sequencing of inter-simple sequence repeat

New sense and anti-sense primers inside the GACA repeats were designed to specifically amplify the sequence published in Pašakinskiene *et al.* (2000) and confirm the presence of the sequence in the *F. arundinacea* plant used in the original *Lolium* x *F. arundinacea* cross (Table 2.7). The sequence specific primers (Fest-582seq-F and Fest-582seq-R) were optimised and tested in both *F. arundinacea* and *Lolium* parents. PCR products were visualised using 1% (w/v) agarose/TAE gel. The PCR amplified band was purified from the agarose gel and sent for sequencing to confirm its identity.

2.5.4 Confirmation of parental DNA in F_2 hybrids using PCR

To ensure F_2 hybrid plants contained DNA from both parents, unique markers for *Lolium* and *F. arundinacea* were identified from primer 104H. Three F_2 hybrids 126, 122/80/E, and 123/33/B were assayed. PCR reactions were carried out the same as in section 2.5.2. PCR products were run on 2% (w/v) agarose/TAE gel.

2.5.5 qPCR analysis of *Festulolium* hybrids

To quantify the amount of DNA each F_2 hybrid obtained from its parents, qPCR was used. Primers were designed for qPCR using Primer Premier 6 (v. 6.23). The parameters for primer design were that the primer was between 20-30 bp and the product amplified would be between 150-300 bp. Suggested primer pairs that were highly rated by Primer Premier 6 were chosen for qPCR optimization and analysis and are listed in Table 2.7.

Primers created for qPCR were first optimised for annealing temperature and assayed for species-specificity using PCR. Primers that were found to amplify a product for *F. arundinacea* only were then tested using qPCR.

qPCR reaction volumes were 15 µl and contained 7.5 µl 2 X SYBR Green mix (Appendix 1), 1 µl of sense and anti-sense primer, 1 µl gDNA template and water up to 15 µl. The qPCR machine used was the Rotor-Gene Q, (Qiagen) and the qPCR program used was as follows: 1 cycle 95˚C melt for 10 min; 40 cycles 95˚C for 30 s, 60˚C for 30 s, 72˚C 30 sec; 1 cycle 72˚C for 7 min. A

reference gene primer called elongation factor (5’ – CACCCTGGTCAGATCGGCAAC and 5’ – CACCAACAGCAACAGTCTGCCT) was run with each qPCR as an internal control.

Table 2.7: Sense (F) and antisense (R) primers for qPCR were designed based on the sequence published in Pašakinskiene et al. (2000). Primer sequences tested for species-specific product amplification. Forward primers and reverse primers were tested in all possible combinations to identify species-specific PCR product. Fest-582-F-1 to 3 and Fest-582-R-1 to 6 were designed for qPCR analysis. Fest-582seq-F and Fest-582seq-R were designed to amplify the whole SSR sequence contained between the microsatellite repeats.

Primer Code	Sequence	Code Abbreviation
Fest-582-F-1	5’-TTAACCACAGAGACAACAACAACCA-3’	F1
Fest-582-F-2	5’-CACAGAGACAACAACAACCACTCAA-3’	F2
Fest-582-F-3	5’-GATAATGGCGATGTCAATCAGTGGTT-3’	F3
Fest-582-R-1	5’-ACCACTGATTGACATCGCCATTATC-3’	R1
Fest-582-R-2	5’-CAACCACTGATTGACATCGCCATTA-3’	R2
Fest-582-R-3	5’-TTCGGAAGATATTGTAATTGTTCTGCCTGT-3’	R3
Fest-582-R-4	5’-GATATTGTAATTGTTCTGCCTGTGATGGAG-3’	R4
Fest-582-R-5	5’-CAGACAGACAGACATTCGGAAGATATTG-3’	R5
Fest-582-R-6	5’-TTGTAATTGTTCTGCCTGTGATGGAG-3’	R6
Fest-582seq-F	5’-GAGAGCAACCACACGTGTGAAT-3’	F-seq
Fest-582seq-R	5’-TTCGGAAGATATTGTAATTGTTC-3’	R-seq

2.6 *Festulolium* morphology

2.6.1 *Festulolium* growth

Twenty one F_2 F*estuloliums* were chosen based on their ploidy as a representative sample of F_2 hybrids. The growth experiment took place between 6th January 2016 and 10th February 2017. During this time the average temperature was 16.4˚C (min. = 5.7˚C; max = 30.1˚C). Water was applied evenly to all plants via a sprinkler set on a timer. Estimated ploidy of these plants ranged from 2n = ca. 14 to 2n = ca. 70. From the chosen plants, clones were made by splitting the plants up into one tiller per pot. At least five clones were made from each plant, two of which were grown outside away from the main group to create resilience in case any plants

being used for growth experiments were to die. No *Lolium or F. arundinacea* clones were created as controls for the growth and morphology experiments. However, to get a basic understanding of some morphological characters, external *Lolium or F. arundinacea* plants were used.

All the plants were given time to establish and grow for five months. During these five months, plants were well watered and fertilised fortnightly, using Yates, Thrive® All Purpose Soluble Fertiliser (appendix for nutrient details) at the recommended rate of 40 g/ 4.5 L for 5 square meters to induce maximal growth. Two months before the start of the experiment three clones from the 21 chosen plants were moved from the greenhouse to a sheltered location outside, because the greenhouse was exceeding temperatures of 30˚C almost every day for most of the day. One month before starting the growth experiments, plants were arranged in a randomised block design (Table 2.8). Pots were spaced so that there was 10 cm between each pot. On day zero, plants were cut to 4 cm, and left to re-grow.

Plants were measured five times at 3-4 day intervals, then a final measurement at days 20 days after last measurement. Permanent marker pen was used to mark the leaves at 4 cm during each measurement and the difference between the two most recent marks measured (Figure 2.4). Four random tillers were measured and each tiller given a unique marker so that the tillers could be easily identified later. Growth measurements of the four tillers were averaged to estimate average plant growth. If any tillers on a plant were flowered the plant was excluded from growth, fresh weight, dry weight and crude protein analyses. Once the growth experiment was complete, fresh and dry weight of the plants were recorded. Fresh plant matter was dried at 70˚C for two weeks. Dried material was also ground into a powder (<1 mm) and used to measure the crude protein of the leaves using near infrared reflectance (NIR) spectrometry. Crude protein measurements were done at Lincoln University using NIRSYSTEM 5000 spectrophotometer (Foss). Two measurements per sample were taken per sample and averaged.

Table 2.8: Randomised block design for F_2 hybrid plants used in the growth experiment.

Block 1	North		Block 2			Block 3		
125	123/42	123/12/A	123/12/A	122/8	122/20/2-3	122/8	122/36/A	123/12/B
122/20	122/5B	122/75/A	122/5	126	122/5B	123/33/B	122/75/A	122/10/B
122/10/B	122/1	122/14/E	122/1	122/36/A	122/29	123/34/A	122/20/2-3	123/12/D
122/31	123/33/B	123/12/B	122/10/B	123/12/B	122/20	126	122/29	123/42
122/5	122/8	123/12/D	123/42	123/34/A	125	122/20	122/5	122/80/E
122/29	126	122/80/E	123/33/B	122/14/E	122/80/E	122/5B	122/1	123/12/A
122/36/A	123/34/A	122/20/2-3	122/75/A	122/31	123/12/D	125	122/31	122/14/E

Figure 2.4: Permanent marker markings on the leaf created to show how much the leaf has grown between measurements.

2.6.2 F_2 hybrid Leaf morphology

Three leaves per plant were taken from eight plants after the growth experiment to examine the morphology of the leaves. Stomatal (guard cell) size and density, number of leaf ridges and leaf area estimation were studied. Size of the stomata was estimated by measuring the length and width and calculating the area of the stomata assuming stomata were rectangles. Density of the stomata was also estimated assuming the basal 15 mm of the leaf was rectangular. Stomata size and density were measured by making a leaf imprint with nail polish (Revlon Top Coat 010). The nail polish was 'painted' onto the basal underside of the leaf, allowed to air dry for 30 min, and carefully peeled off using jewellers forceps, ensuring the leaf margins remained intact. A ca. 20 mm long imprint was transferred to a glass microscope slide and a cover slip applied. The basal 15 mm of the imprint was examined for stomata using an Olympus BH-2 compound microscope with bright field illumination at 40x magnification. The numbers of leaf ribs were also counted using a dissecting microscope and values recorded. Leaf area was estimated using the method described in Wilman *et al.* (1996). The formula used to calculate leaf area was:

$$Leaf\ area = length \times width \times 0.905$$

The shape of leaves emerging from the sheath was used as a morphological character to distinguish *L. perenne* (folded leaves) and *L. multiflorum* and *F. arundinacea* (involute or rolled) (Edgar *et al.* 2010). *L. perenne* F_2 hybrids were observed for any deviances from the expected characters.

2.6.3 Heading times and inflorescence morphology of F_2 hybrids

A set of 26 F_2 hybrid clones grown outside were used to ascertain the heading times of vernalised plants. The heading date was defined as the day that the first spikelet of the inflorescence emerged from the sheath. The flower morphology of 11 F_2 hybrids and *L. perenne* were also characterised based on diagnostic characters used to distinguish the difference between both *L. perenne*, *L. multiflorum* and *F. arundinacea* (Edgar *et al.* 2010). Characters by

which the plants were assessed were the length of awns, number of spikelets per inflorescence and the amount of branching per spikelet.

2.6.4 F_2 hybrid pollen viability test

Whole spikes from F_2 hybrid plants were harvested when anthers could be seen from at least several florets. Spikes were cut and base stored in water until used for pollen collection. Under a dissecting microscope, individual mature anthers were chosen for pollen collection. Mature anthers containing mature pollen were defined as anthers which had begun to dehisce but had yet to do so completely so that pollen could still be collected. Pollen was collected on a clean microscope glass slide from at least three different anthers from three different florets on the same spike or panicle. Three replicates per plant were done, each using a different spike/panicle.

Once sufficient pollen was collected on the slide, fresh fluorescein diacetate (FDA) working solution (0.42 mM) was made from FDA stock (2.1 mM). FDA stock solution was diluted using sucrose (1.6 M) and sodium chloride (0.3 M) dissolved in phosphate buffer (0.1 M, pH 7.0) to prevent the pollen from bursting through osmotic stress. Two drops of working FDA solution were added to the pollen and a glass cover slip (24 x 60 mm) was placed on the slide which was incubated at room temperature in the dark for 5 min before viewing. Pollen was viewed using a Nikon Eclipse 80i microscope with bright field illumination, at 100x magnification, to find an appropriate location (>10 pollen grains) to capture an image of the pollen. A second image was captured using a B-2A fluorescent filter cube where the pollen was excited at 493 nm and emitted fluorescence at 520 nm. Pollen was counted and scored using the images, preventing quenching of the pollen fluorescence. To ensure that pollen was not counted more than once, a systematic screening pattern was employed (Figure 2.5). All slides were viewed within 60 min of applying the working FDA solution. Fresh FDA was prepared for every slide to ensure high quality and consistent fluorescence (Pinillos and Cuevas 2008). Images of the pollen were captured at 90 ms exposure time using a Nikon Digital Sight (DS) camera with NIS-Elements software (v. 2.20). Pollen viability was scored from 0 to 5; 0 being no fluorescence, and 5 being the brightest fluorescence. An arbitrary fluorescence scale was constructed to maintain

consistent scoring of the of pollen grain fluorescence (Figure 2.6). Pollen viability was scored in two different ways: 1. Pollen that was scored anywhere on the arbitrary scale was considered viable; 2. Only pollen that scored highly on the arbitrary scale (4-5) was considered viable.

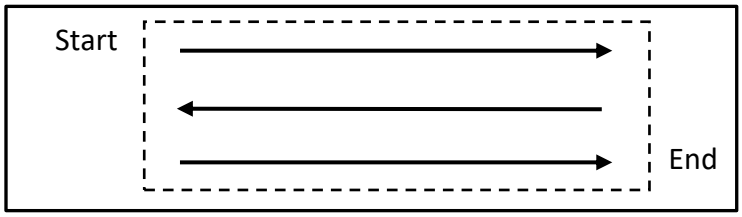

Figure 2.5: Method used to view the whole slide under the microscope. Viewing always started 5 mm in from the top left corner of the cover slip (dashed line) and each subsequent scan across the cover slip was 5 mm down the slide. The X-Y stage micrometre was used as a guide for accurate positioning. No pollen was counted more than once.

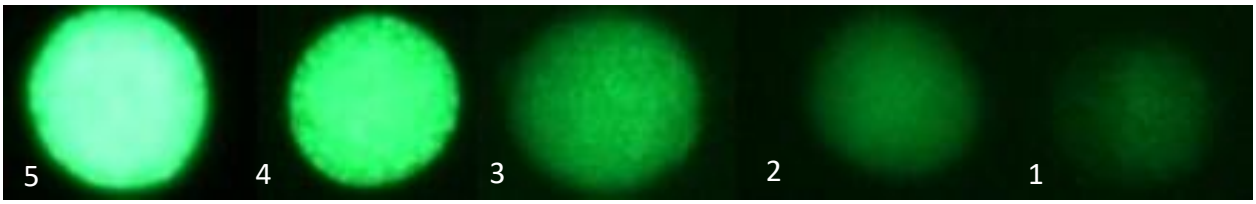

Figure 2.6: Arbitrary pollen scale was used to keep scoring of the pollen consistent. The different levels of fluorescence used in the scale were representative of the range of pollen sampled.

2.7 - Statistical analysis

Linear regression was used to correlate specific morphological characters when estimated ploidy was known. Single factor Analysis of Variance (ANOVA) was used to determine if differences existed between different F_2 hybrid genotypes. Growth data and time between measurements were log transformed for statistical analysis to conform to the assumption of equal variances. Significant p-value threshold was adjusted for multiple ANOVA tests performed by using a Bonferroni correction.

Chapter 3 – Results

3.1 Flow cytometry

3.1.1 DAPI flow cytometry

The 2C nuclear DNA content of 170 F_2 hybrids was measured. Initial flow cytometry results showed a wide range of genome sizes, 5.52 pg to 24.7 pg, in the F_2 hybrid plants, corresponding to allodiploid (2n = 2x = 14) to nearly allododecaploid (2n = 12x = 84) (Figure 3.1). These results were obtained without using an internal control. The gated values for a distinct G_1 peak were obtained and, using the predetermined 2C values for both *L. perenne* and *F. arundinacea* (Šmarda *et al.* 2008), the amount of genomic DNA each F_2 hybrid had per cell was estimated (Figure 3.1). The difference between the *L. perenne* and *F. arundinacea* DNA content estimates were very small and could only be distinguished with higher ploidy plants, where *F. arundinacea* led to estimates of slightly more DNA per cell (Figure 3.1). Clustering of F_2 hybrids around multiples of 2n can be seen. However, the spread was quite broad around those areas. F_2 hybrids retested using DAPI flow cytometry and with diploid L. perenne ('Bronsyn') as an internal control, showed two very clear G_1 peaks (Figure 3.2). Gated G_1 peaks had coefficients of variation (CV) below 5%. Two further peaks were observed, G_2 peaks of *L. perenne* and the F_2 hybrid. F_2 hybrids that were also diploid only showed a single G_1 peak and a much smaller G_2 peak. The similar size of the diploid *L. perenne* and the hybrid combined, obscuring any differences between the two. The estimated genomic DNA and inferred ploidy from gated G_1 peaks range of the F_2 hybrids was from diploid (2n = 2x = 14) to nonaploid (2n = 9x = 63) (Figure 3.3). F_2 hybrids clustered more tightly around multiples of 2n with the internal control than when the internal control was absent (Figure 3.1) and (Figure 3.2). However, very few F2 hybrids have a similar amount of DNA to F. arundinacea and most clustered closely to 2n = 2x, 4x and 8x.

3.1.2 Propidium iodide flow cytometry

A selected group of 21 F_2 hybrids from a range of estimated ploidy levels were chosen for propidium iodide flow cytometry. Gated values of the control and F_2 hybrids had CV below 10%. PI estimated DNA content within the F_2 hybrids was highly correlated with DAPI estimates of

DNA content (p-value <0.0001, R^2 = 0.9888) (Figure 3.4). Although the DNA content was highly correlated, the actual amount of DNA each method estimated was different, by as much 5 pg per cell when more DNA was present. PI flow cytometry estimated DNA content values of F_2 hybrids (Table 3.1). These F_2 hybrids were also investigated for at least one of the following experiments: molecular cytogenetics, molecular characterisation morphological characterisation and/or fertility.

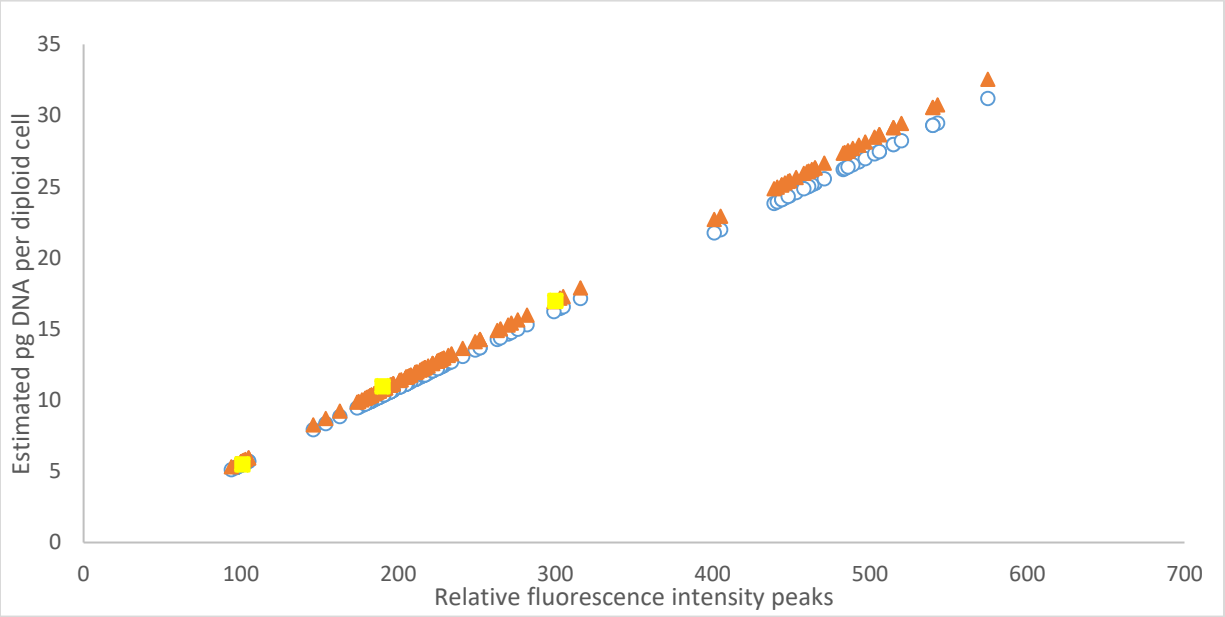

Figure 3.1: Initial DAPI flow cytometry of F_2 hybrid plants with no internal control. Yellow squares represent the diploid Lolium *(100), tetraploid* Lolium *(200) and hexaploid* F. arundinacea *(300). Open circles are the estimated amount of DNA using* Lolium *as the reference to calculate pg DNA. Orange triangles are the estimated amount of DNA using* F. arundinacea *as the reference to calculate pg DNA.*

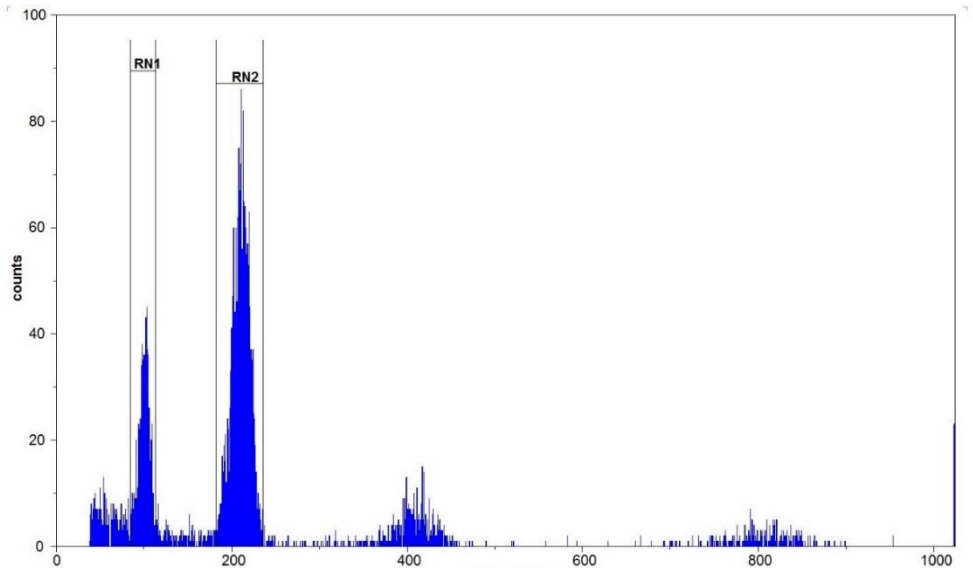

Figure 3.2: DAPI flow cytometry of an F_2 hybrid (122/80/E) co-chopped with L. perenne, 'Bronsyn' as an internal control. Gated mean of L. perenne G_1 peak (RN1) was 100.35 and gated mean of 122/80/E G_1 (RN2) peak was 210.60. G_2 peaks of L. perenne and 122/80/E can be seen at ca. 400 and 800 relative fluorescence respectively.

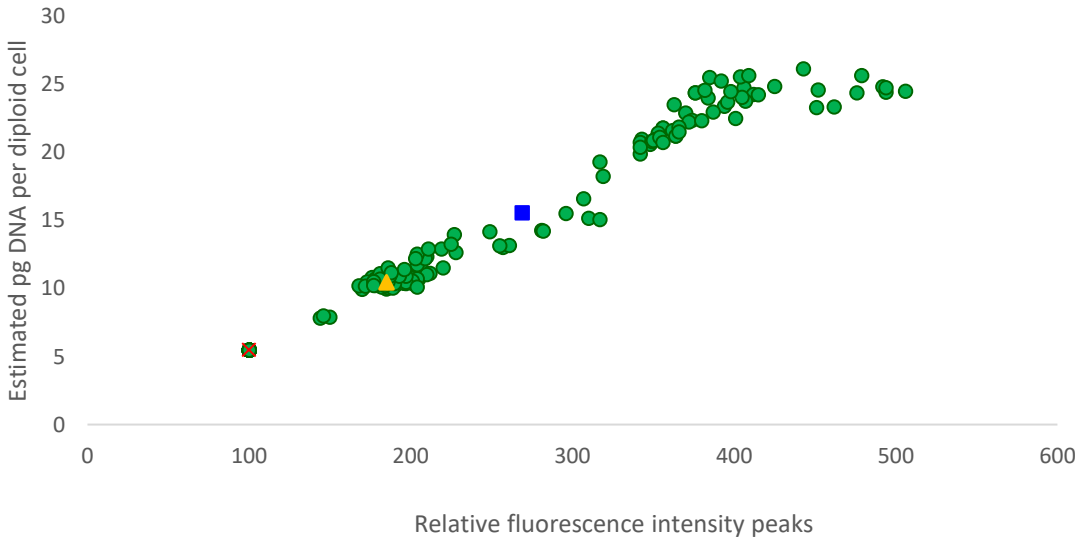

Figure 3.3: DAPI flow cytometry of F_2 hybrid plants using diploid Lolium as an internal control. Putative allodiploid plants had a relative fluorescence peak at 100, putative allotetraploid plants at 200, putative allohexaploid plants at 300 and putative allo- or amphi- octoploid plants at 400. Red cross (×) is diploid L. perenne, orange triangle is tetraploid L. perenne and blue circle is F. arundinacea.

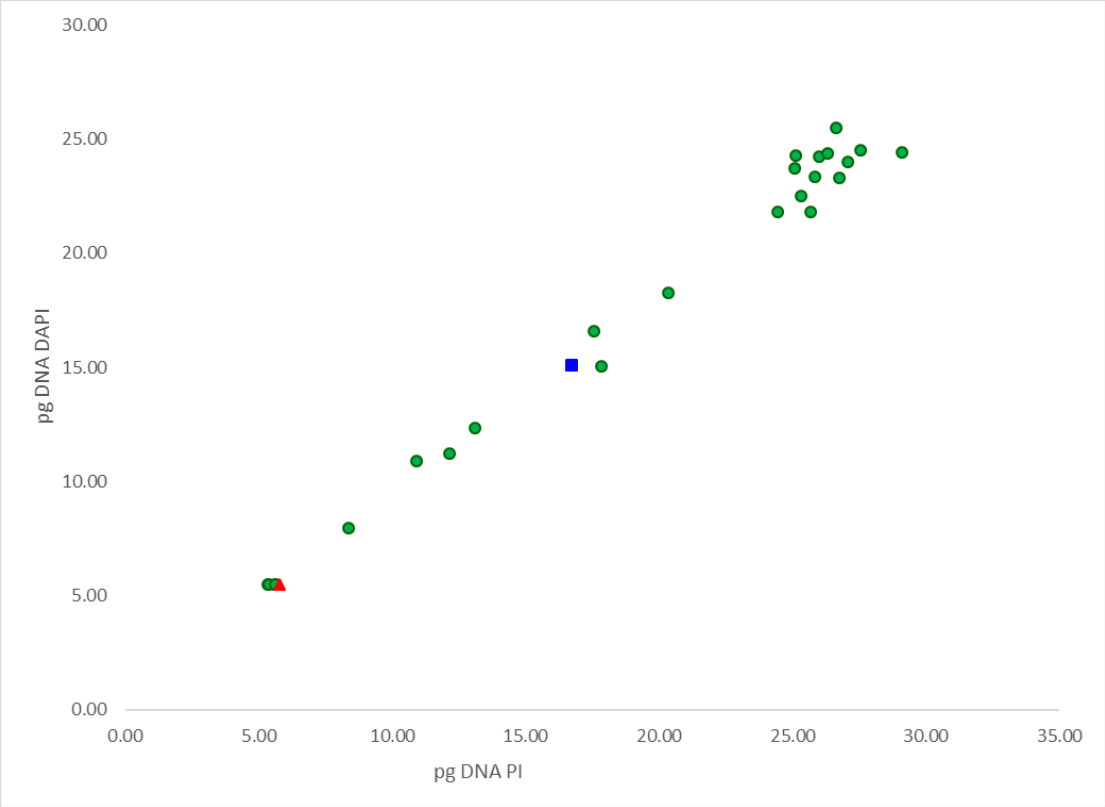

Figure 3.4: Comparison of estimated DNA in F_2 hybrids using DAPI or PI as fluorescent dyes in flow cytometry. Red triangle is diploid L. perenne *and blue square is* F. arundinacea*.*

Table 3.1: Estimated DNA content using PI and DAPI flow cytometry of selected F_2 hybrids used for further characterisation. Not all the plants included in the growth experiments are included here.

	Estimated DNA content (pg DNA)	
Name	PI	DAPI
125	5.34	5.52
122/29	5.38	5.52
123/33/B	5.62	5.52
L. perenne	5.76	5.52
122/75/A	8.35	7.98
122/10/B	10.91	10.90
122/80/E	12.16	11.23
122/1	13.10	12.33
F. arundinacea	16.71	15.11
123/34/A	17.58	16.60
123/12/D	17.85	15.07
126	25.07	23.71
123/6/A	25.34	22.48
122/36/A	25.86	23.35
123/12/B	27.54	24.50

3.2 Doubled haploid testing

Six of the seven loci assayed produced robust data (Table 3.2). The seventh locus was excluded from the results because it generated inconsistent data over the 14 samples. Mean heterozygosity ranged from 0.5 to 0.83 for the 12 potential doubled haploid plants compared with 0.67-0.83 for the four heterozygous control plants. No diploid F_2 hybrids assayed were doubled haploid because mean heterozygosity was not zero. Seven of the F_2 hybrids were as heterozygous as the *L. perenne* controls provided by Agriseeds and AgResearch. The remaining five F_2 hybrids, although more homozygous than the controls, were far from being completely homozygous.

Table 3.2: Allodiploid F_2 hybrids tested for heterozygosity. A mean heterozygosity value of 1, indicates all loci examined were heterozygous, while mean heterozygosity of 0, indicates that all loci examined were homozygous. Control plants were L. perenne *and* L. multiflorum*.*

Sample	Label	Description	Mean heterozygosity
1	123/33/B	putative DH[a]	0.83
2	123/41/A	putative DH	0.50
3	122/20	putative DH	0.83
4	125	putative DH	0.83
5	122/17	putative DH	0.50
6	122/21	putative DH	0.67
7	122/15	putative DH	0.50
8	122/8	putative DH	0.83
9	122/29	putative DH	0.50
10	122/11	putative DH	0.67
11	122/10	putative DH	0.50
12	122/24	putative DH	0.67
13	Bronsyn	het[b] control	0.67
14	Tabu	het control	0.83
	AgResearch 1	control	0.83
	AgResearch 2	control	0.83

[a] DH: doubled haploid; [b] het: heterozygous

3.3 Molecular cytogenetics and chromosome Staining

3.3.1 Giemsa staining

The general karyology of the two putative 2n > 8x plants and one putative 2n = 2x F_2 hybrid were observed in prometaphase to early metaphase cells (Figure 3.5.) 57 chromosome pieces were counted in 123/6/A (Figure 3.5 a), whereas 126 (Figure 3.5 b) had 74 chromosome pieces. The expected number of chromosomes for both F_2 hybrids based on PI flow cytometry was 61 chromosomes. 123/33/B (Figure 3.5 c) had 17 chromosome pieces with an expected chromosome count of 14. Chromosomes are counted as pieces because secondary constriction sites can inflate the true number of chromosomes present.

3.3.2 Fluorescent *in situ* hybridisation (FISH) staining

FISH was performed on three F_2 hybrids two *L. perenne* x *F. arundinacea* and one *L. multiflorum* x *F. arundinacea*. F_2 hybrids were putative 2n = 2x, 2n = 4.2x and 2n = 8.7x (Figure 3.6). The number of chromosomes could be accurately counted in all the chromosome preparations because the NOR regions connecting de-condensed chromosome regions fluoresced green. At least ten cells in prometaphase to metaphase were counted to ascertain a precise chromosome count. The putative allodiploid, 123/33/B, (Figure 3.6 a and b) was confirmed diploid with 14 chromosomes. Six 18S rDNA sites were found and two 5S rDNA sites, one of which shared are chromosome with an 18S rDNA site. The putative allotetraploid, 122/80/E, (Figure 3.6 d and e) contained 31 chromosomes, more than the expected 29 chromosomes estimated from flow cytometry data. There were 13 18S rDNA sites and four 5S rDNA sites, all of which shared chromosomes with 18S rDNA sites. The putative allo-octoploid, 126 (Figure 3.6 j and k), had 68 chromosomes, seven more chromosomes than expected from flow cytometry. Eighteen 18S rDNA sites were observed and seven 5S rDNA sites, only two of which shared chromosomes with 18S rDNA. Some chromosome parentages were identified from FISH images only based on the physical mapping of 18S rDNA and 5S rDNA (Thomas *et al.* 1996, Thomas *et al.* 1997). Chromosomes that only had 5S rDNA sites were of *F. arundinacea* origin, while chromosomes that had both 5S rDNA and 18S rDNA on the same chromosome were of *Lolium* origin.

Chromosomes that only had 18S rDNA sites, or no hybridisation sites, could not be identified as either *F. arundinacea* or *Lolium*.

3.3.3 Sequential genomic *in situ* hybridisation (GISH) staining

Four F_2 hybrid plants underwent chromosome preparation for GISH, three *L. perenne* x *F. arundinacea* (123/33/B, 126 and 123/6/A) and one *L. multiflorum* x *F. arundinacea* (122/80/E) (Figure 3.6). Both 123/33/B and 122/80/E showed some recombined *Lolium-F. arundinacea* chromosomes (arrows), although no pure *F. arundinacea* chromosomes could be seen (Figure 3.6 c and f). Both higher ploidy plants (126 and 123/6/A) had clear chromosome hybridisation. Very clear chromosome recombination could be seen for both 126 and 123/6/A (Figure 3.6 i and l). Sections of recombined chromosomes varied in length, from a short section to half a chromosome. A small amount of green fluorescence can be seen on a chromosome (Figure 3.6 l, star). This is 18S an rDNA site not a *Lolium* chromosome recombination because the same green fluorescence can be seen in the FISH image (Figure 3.6 k and l).

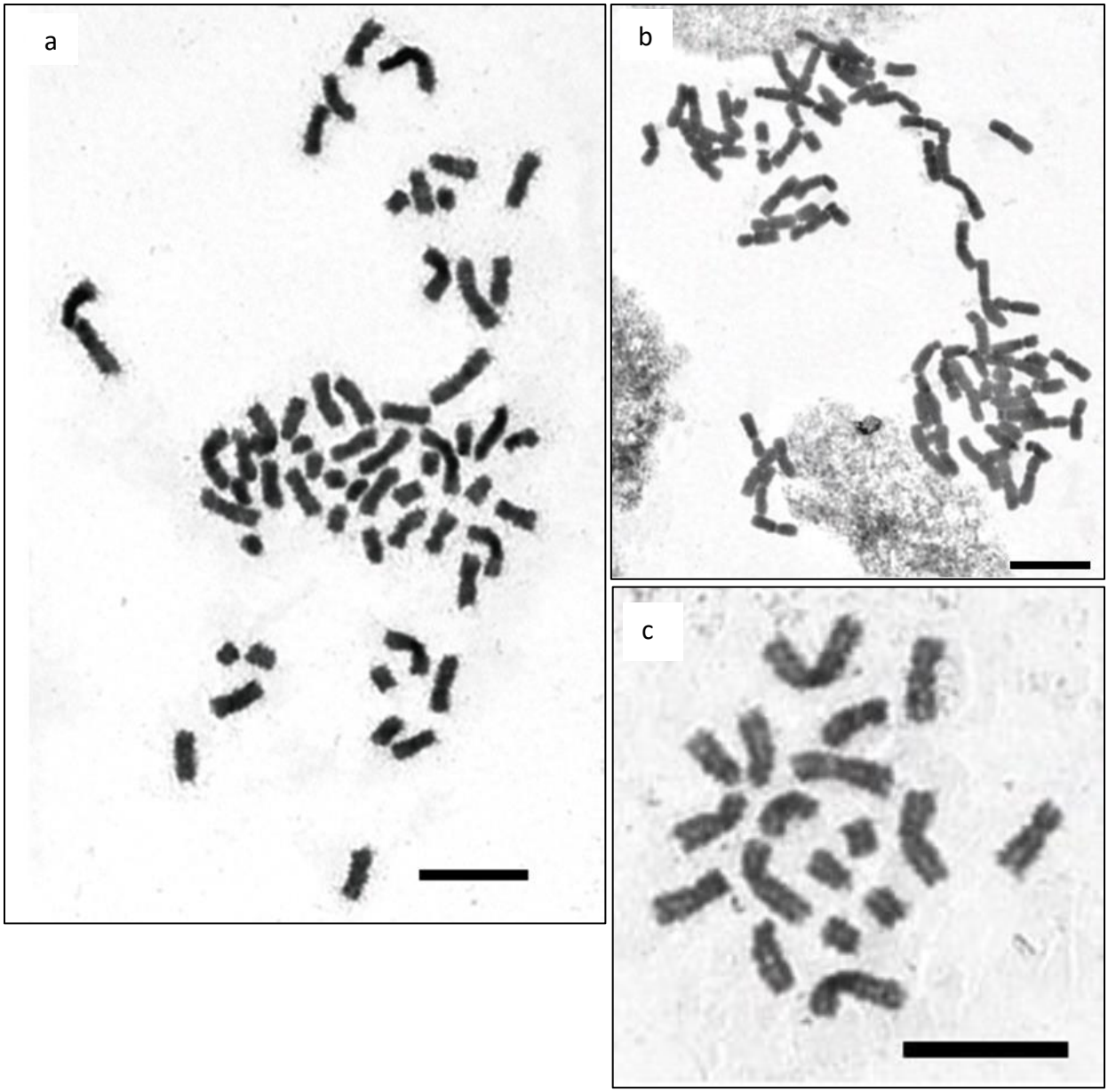

Figure 3.5: F_2 hybrid G*iemsa stained chromosomes. F_2 hybrid 123/6/A has 57 chromosome pieces (a), 126 has 74 chromosome pieces (b) and 123/33/B has 17 chromosome pieces (c). Scale bar = 10 µm.*

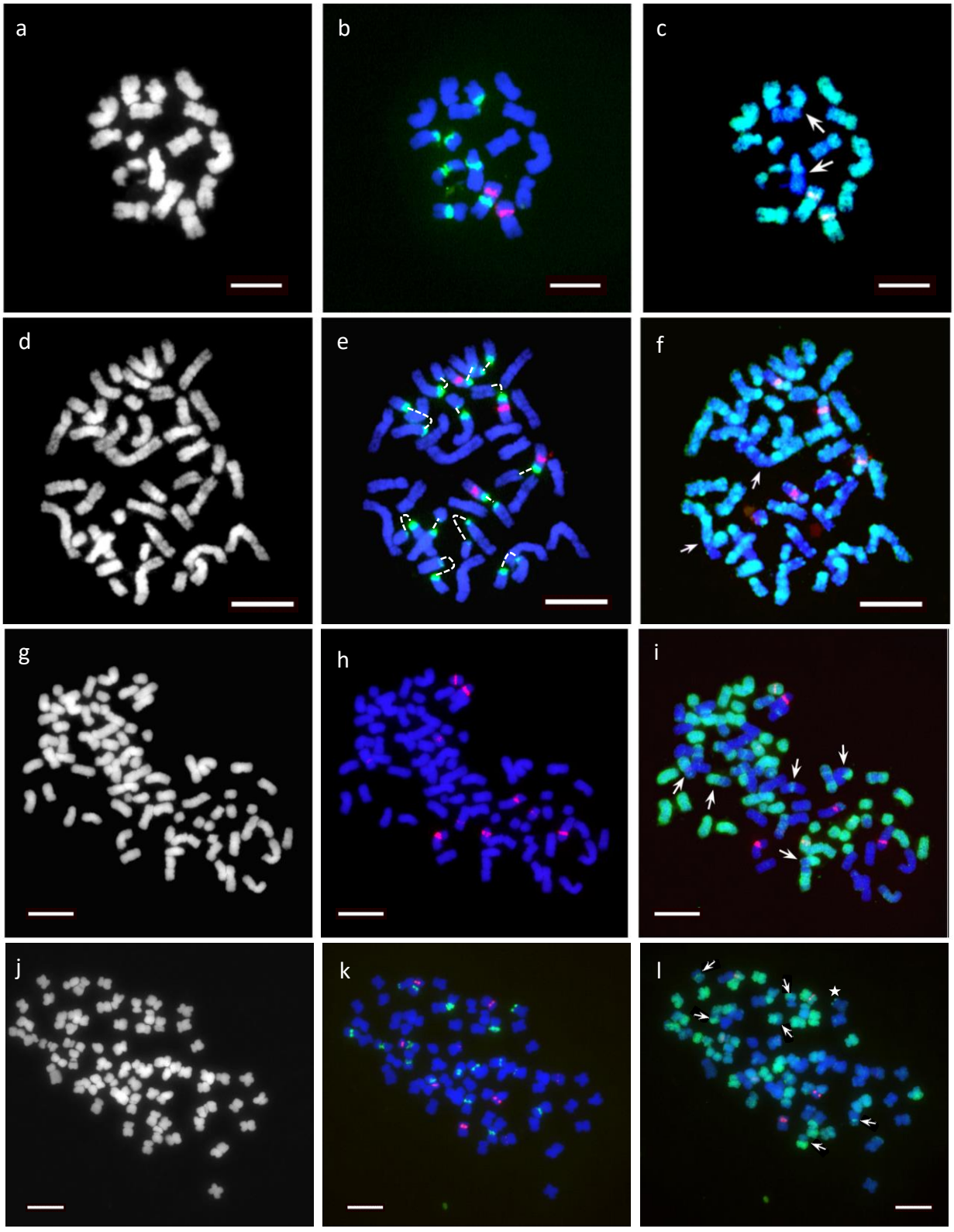
a
b
c
d
e
f
g
h
i
j
k
l

Figure 3.6: Chromosome spreads of four F_2 Festulolium *hybrids. From top to bottom are F_2 hybrids 123/33/B, 122/80/E, 123/6/A, and 126. From left to right are grey scale DAPI, FISH using 5S rDNA probe (red) and 18S rDNA probe (green) counterstained with DAPI, and GISH using* L. perenne *DNA (green) and 5S rDNA FISH probe counter stained with DAPI. Regions of recombination (arrows) can be seen in all four F_2 hybrids. Star shows fluorescently labelled* L. perenne *DNA hybridising with an 18S rDNA site.*

3.4 Molecular characterisation

3.4.1 Identifying species-specific PCR products

DNA extracted from plant material yielded DNA concentrations ranging from 300-800 ng/µl. Younger plant material typically yielded higher concentrations of DNA.

Initial PCR amplification using Bioline Taq, the 78H and 104H primers and the method from Pašakinskiene *et al.* (2000) did not amplify expected band lengths of either 0.6 kb or 1.2 kb for primers 78H or 104H respectively, instead produced products of <0.4 kb (Figure 3.7). However, the control 18S rDNA primer amplified a product of the expected length. The 18S rDNA band was sequenced and had almost 100% homology to sequences in the NCBI database. Optimised temperature and increased extension time resulted in a banding pattern which produced a range of amplification products. The amplification of 104H did not produce a band at the desired band at 1.2 kb (Figure 3.8). The banding pattern produced from 78H did not match the published banding pattern. However, there was a band amplified at ca. 0.6 kb, but did not resemble the published band very well (Pašakinskiene *et al.* 2000) (Figure 3.9). Although the banding patterns of 78H and 104H did not match the published results (Pašakinskiene *et al.* 2000), the regions where these bands were supposed to be were excised and sequenced. Sequence results for both ca. 0.6 kb and ca. 1.2 kb bands did not match any sequences in the NCBI database and did not contain any of the GACA repeats the sequences were expected to contain (Appendix 2.1 and Appendix 2.2).

Ten colonies that had been transformed with either ca. 0.6 kb or ca. 1.2 kb sequences (Figure 3.10) were checked using PCR amplification for the presence of these sequences. Four colonies

were chosen for sequencing from primer 78H which contained products of approximately the correct size (ca. 0.6 kb) (Figure 3.11 a). No colonies transformed with the ca. 1.2 kb band contained a product of ca. 1.2 kb (Figure 3.11 b). Sequenced plasmids did not show sequence homology to the sequenced published by Pašakinskiene *et al.* (2000) nor to any sequences in the NCBI database (Appendix 2.3 a-d). The four sequences did however contain the $(GACA)_4$ repeat at the beginning of the sequence, suggesting they were amplified sequences from the primers, rather than contamination. Furthermore, the sequence from each colony that contained products was confirmed to not only be of a different length, but also a completely different sequence with no sequence homology found between the sequenced products.

The two Taq polymerases (DyNAzyme II DNA polymerase, ThermoFisher Scientific) and GoTaq® DNA polymerase, Promega) that Pašakinskiene *et al.* (2000) used were tried in order to replicate all possible conditions previously used. However, the PCR products amplified did not match the published results. GoTaq® DNA polymerase did not amplify specifically to a sequence, producing a unique banding pattern in each reaction tube even though each reaction tube was aliquoted from the same master solution (Figure 3.12). Amplified PCR products were also very faint indicating poor primer efficiency. DyNAzyme II DNA polymerase only amplified short fragments of DNA <0.6 kb and so was also considered not useful for this experiment (data not shown). DNA from *F. arundinacea*, *Lolium* and three F_2 hybrids (126, 122/80/E and 123/33/B) was amplified using primer 104H (Figure 3.13). Two long species-specific fragments were amplified, a 1.5 kb fragment specific to *F. arundinacea* and a 1.8 kb fragment specific to *L. perenne and L. multiflorum*. Both fragments were amplified in all three F_2 hybrids to varying intensities (Figure 3.13).

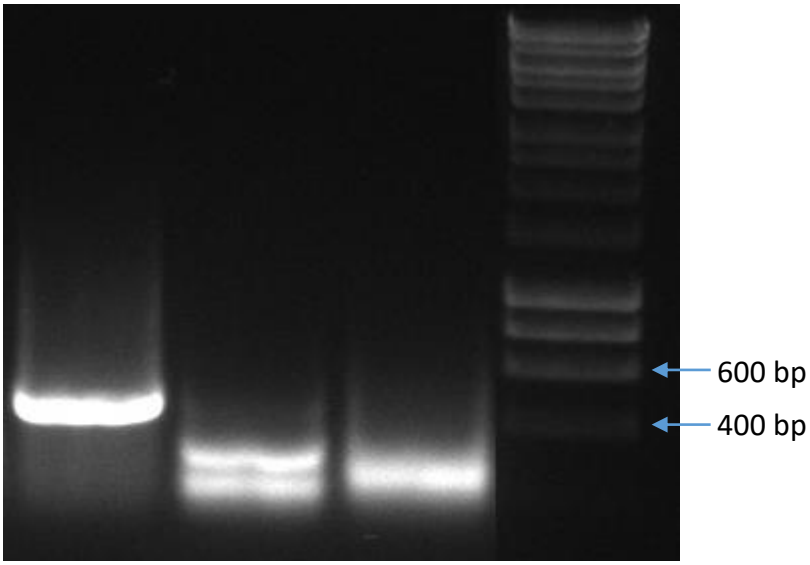

Figure 3.7: PCR products produced using 18S rDNA, 104H and 78H primers (left to right respectively) using BIOTAQ taq polymerase and F. arundinacea DNA. Expected product lengths were ca. 0.6 kb and ca. 1.2 kb. HyperLadder™ I ladder was used in a 1.5% agarose gel.

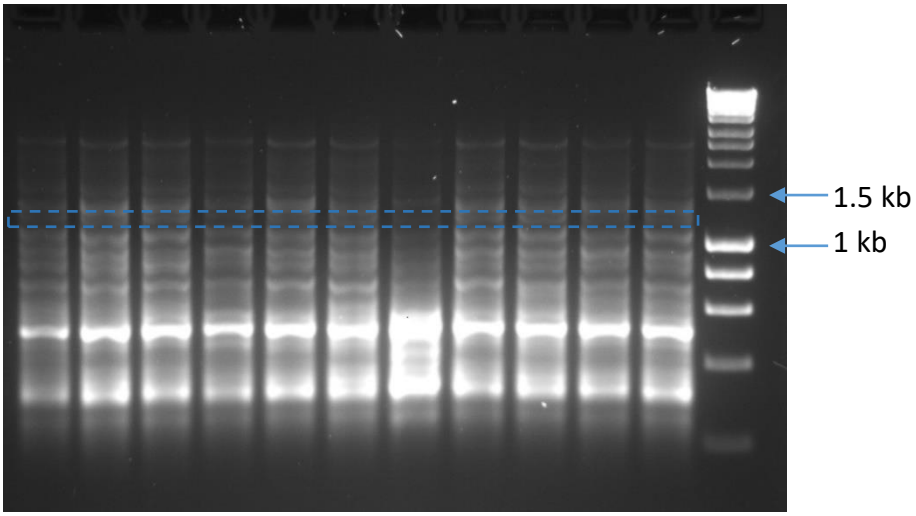

Figure 3.8: Amplified PCR products produced using 104H SSR as a primer and F. arundinacea DNA. A ca. 1.2 kb product cannot be seen of similar size to Pašakinskiene et al. (2000). Furthermore, most of the products show very little resemblance to Pašakinskiene et al. (2000). The region where the ca. 1.2 kb band should have been was excised and purified from the gel in preparation for sequencing (dashed box). HyperLadder™ I ladder was used in a 1.5% agarose gel.

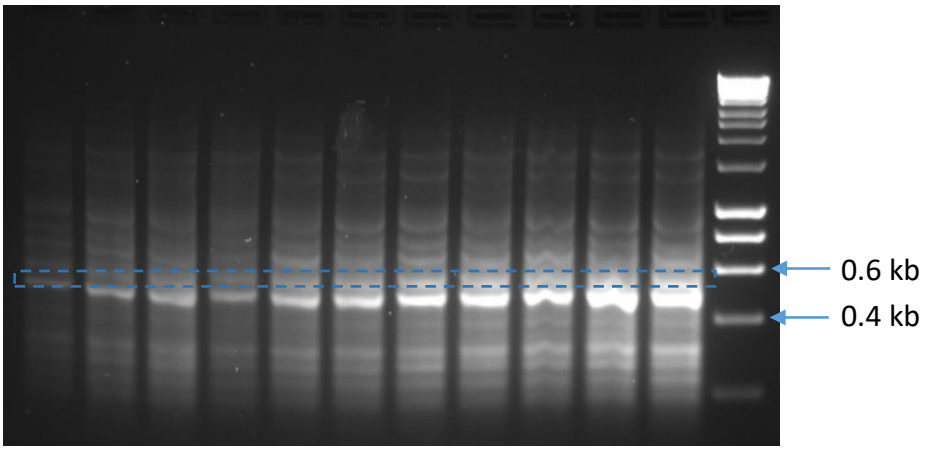

Figure 3.9: PCR products amplified using 78H as the primer and F. arundinacea DNA. The section of gel that contained ca. 0.6 kb fragments were excised and purified from the gel in preparation for sequencing (dashed box). HyperLadder™ I ladder was used in a 1.5% agarose gel.

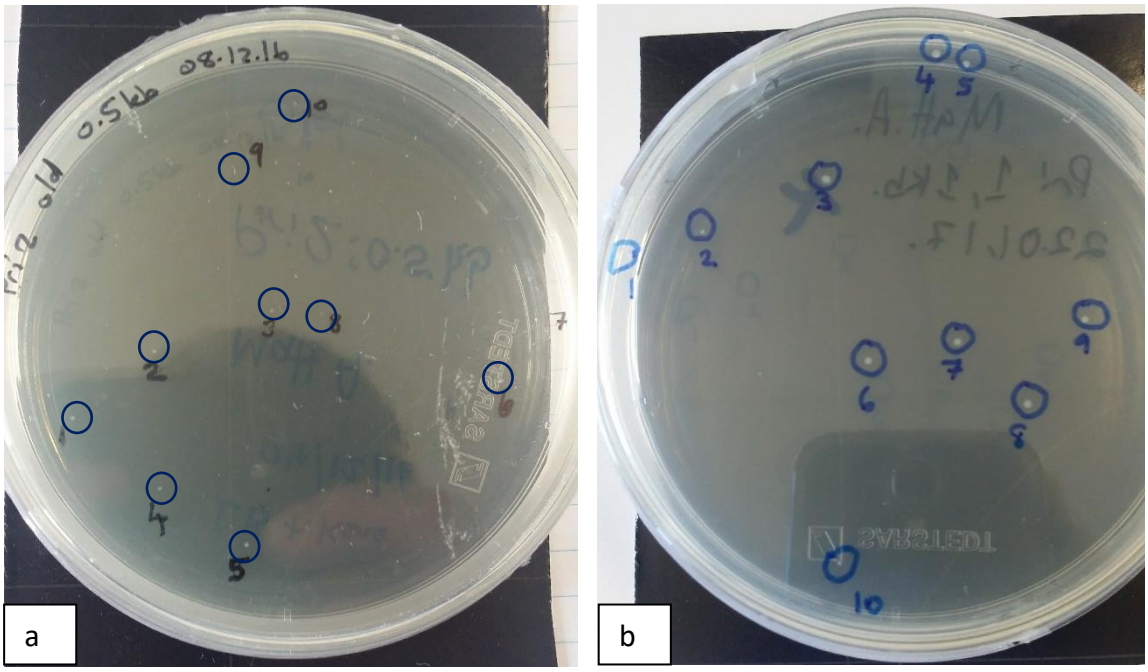

Figure 3.10: Transformed cells growing on a LB 50 µg/ml kanamycin plate. Ten cells from each plate (0.6 kb (a) or 1.2 kb (b)) were labelled.

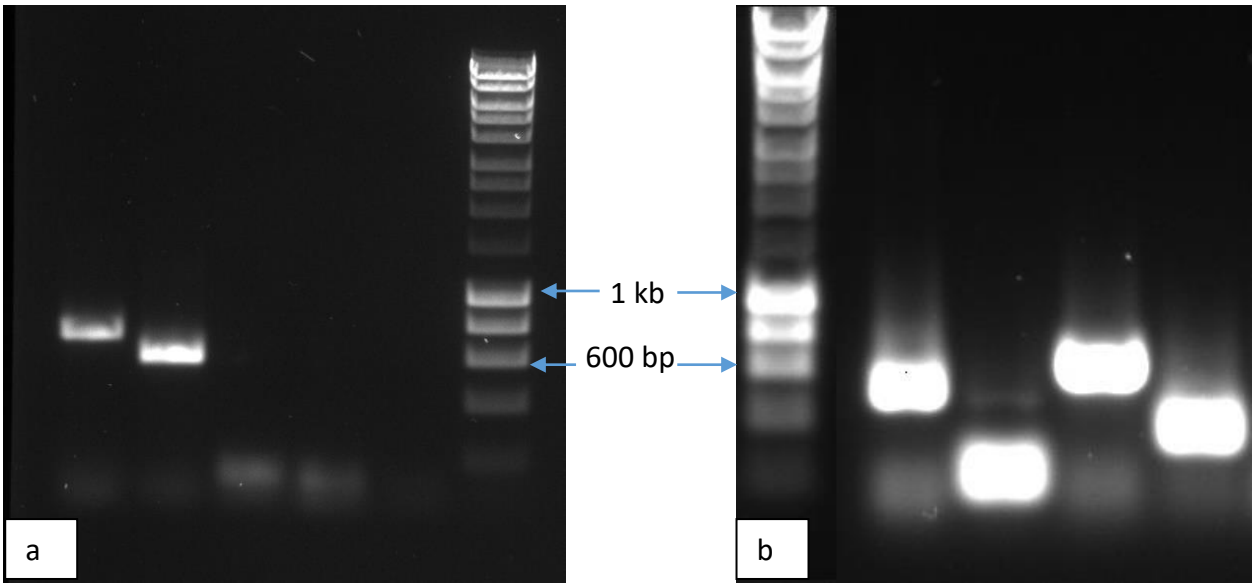

Figure 3.11: PCR of cells transformed with 0.6 kb product (a) and 1.2 kb product (b) using M13 primer (Figure 2.3). Two of the five colonies tested (a.) had been transformed with the 0.6 kb product. Both products also differ in size. All four colonies tested (b.) had varying lengths none of which were ca. 1.2 kb. HyperLadder™ I ladder was used in a 1.5% agarose gel.

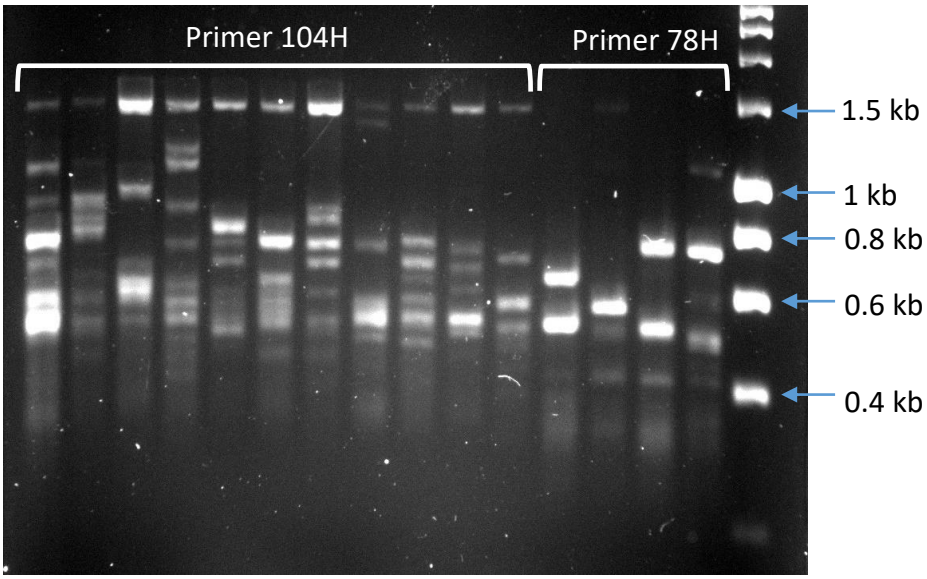

Figure 3.12: Amplification pattern using GoTaq® (Promega) DNA polymerase. The first 10 wells (primer 104H) and the last five wells (primer 78H) amplified F. arundinacea DNA. HyperLadder™ I ladder was used in a 2% agarose gel.

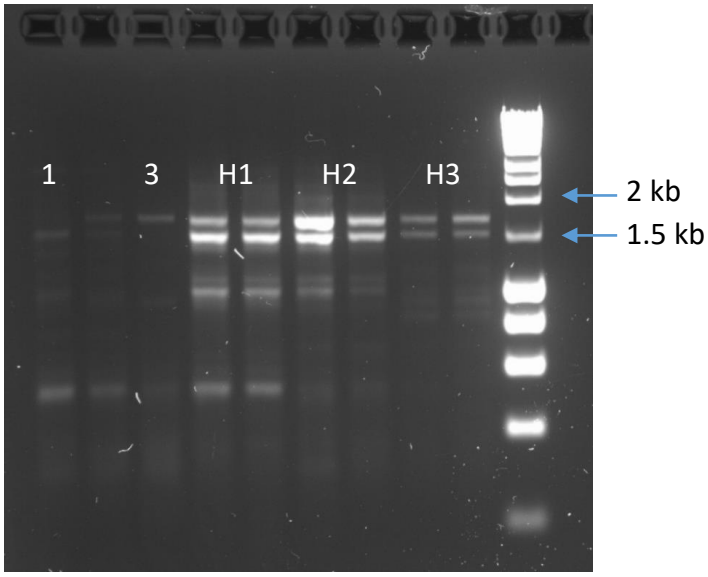

Figure 3.13: Amplification pattern of hybrid parental DNA and F_2 hybrids with primer 104H. F. arundinacea *(1) specific band was amplified at 1.5 kb, while a* Lolium *(3) specific band amplified at 1.8 kb. Lane 2 is the amplification of 50%* F. arundinacea *and 50%* Lolium *DNA. Two lanes per F_2 hybrid H1-3 produced both species-specific bands at varying intensities. H1 = 126, H2 = 122/80/E, H3 = 123/33/B. HyperLadder™ I ladder was used in a 2% agarose gel.*

3.4.2 qPCR analysis of *Festulolium* hybrids

New primers specifically designed for the sequence published in Pašakinskiene *et al.* (2000) (Table 2.7) amplified a product of ca. 0.6 kb (Figure 3.14). The product was a single band showing that the primers designed for this sequence were only annealing and amplifying one sequence in the entire genome. This product was sequenced and showed nearly 100% sequence homology to the published sequence and confirmed its presence in the parental *F. arundinacea* (Pašakinskiene *et al.* 2000) (Figure 3.15). In total four sense primers and seven anti-sense primers were assayed for their specificity towards *F. arundinacea*. Nearly all primers assayed produced a single band as well as some primer dimer following PCR. All but two primer combinations (Fest-582seq-F/Fest-582seq-R and Fest-582seq-F/Fest-582-R-5) also amplified in *L. multiflorum* and *L. perenne* (Figure 3.16). Both primer pairs produced a weak amplification product as well as primer dimer in *F. arundinacea*. These two primer sets were used in qPCR and tested using a DNA concentration gradient (Table 3.3). qPCR results were inconsistent

between qPCR runs. In one run, some PCR amplification of a product can be seen, but in another run, only primer dimer occurred (Figure 3.17).

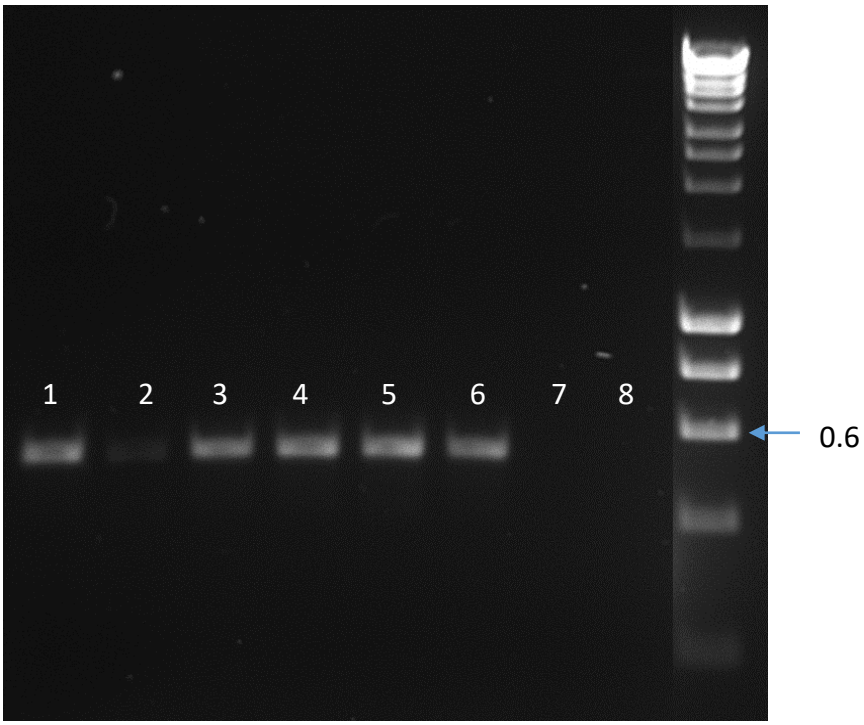

Figure 3.14: PCR product amplified using primer Fest-582seq-F/R using F. arundinacea *DNA. The product is almost 0.6 kb (should be 551 bp), the expected size for this product (lanes 1-6). Primer pair Fest-582seq-F/R were used to amplify* L. multiflorum *and* L. perenne *DNA in lanes 7 and 8 respectively. No amplification was observed in either* Lolium *species.*

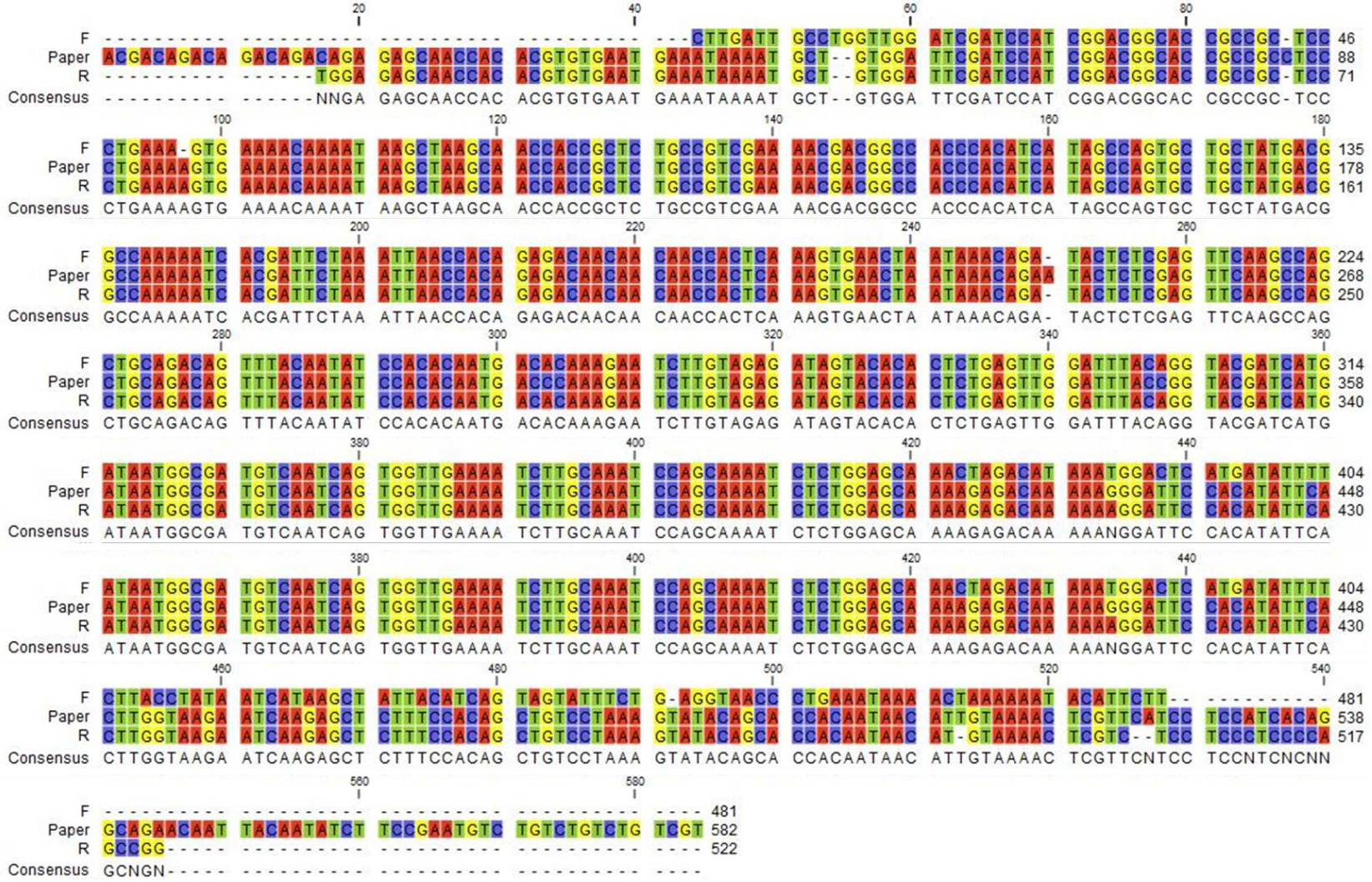

Figure 3.15: Sequence results of the primers specifically designed for the sequence published in Pašakinskiene et al. (2000) (paper). Sequence homology between the sequences is very high (few base pair substitutions or deletions), confirming the presence of the published sequence within the F. arundinacea *line being used in this thesis. Forward (F) sequence was generated using the sense primer, while the reverse (R) sequence was generated using the anti-sense primer.*

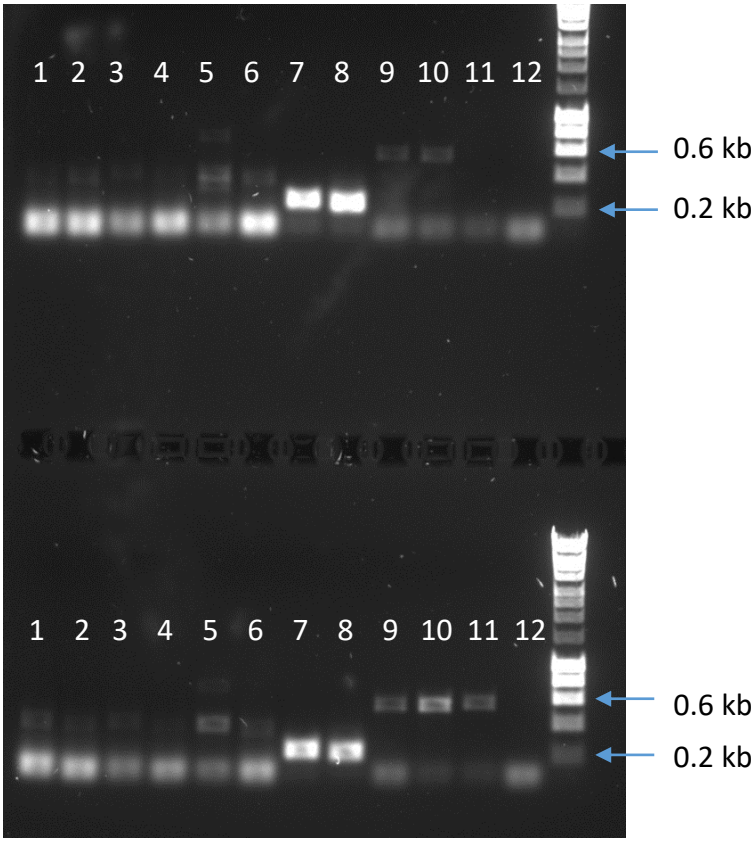

Figure 3.16: PCR amplification of primers being screened for qPCR. Top is the amplification of *Lolium* DNA, bottom is the amplification of *F. arundinacea* DNA. Each well represents a different primer pair. All wells have identical amplification pattern in both species except for well 11 (primer F-seq/R5), which appears specific to *F. arundinacea.* The product that was specific to *F. arundinacea* was ca. 550 bp. The expected length of this sequence was 548 bp based on sequence and primer information. Primer pairs used from wells 1-12 as follows: Fest-582-F-1 with Fest-582-R-3, 4, 5, 6; Fest-582-F-2 with Fest-582-R-3, 4, 5, 6; and Fest-582-F-3 with Fest-582-R-3, 4, 5, 6. Other primer pairs were tested but are not shown here.

Table 3.3: Concentration gradient of F. arundinacea *and* Lolium *DNA used in qPCR to identify whether qPCR would be able to detect different concentrations of* F. arundinacea *or* Lolium *DNA concentrations.*

F. arundinacea	50/50 *L. multiflorum/ L. perenne* mix
100%	0%
50%	50%
0%	100%

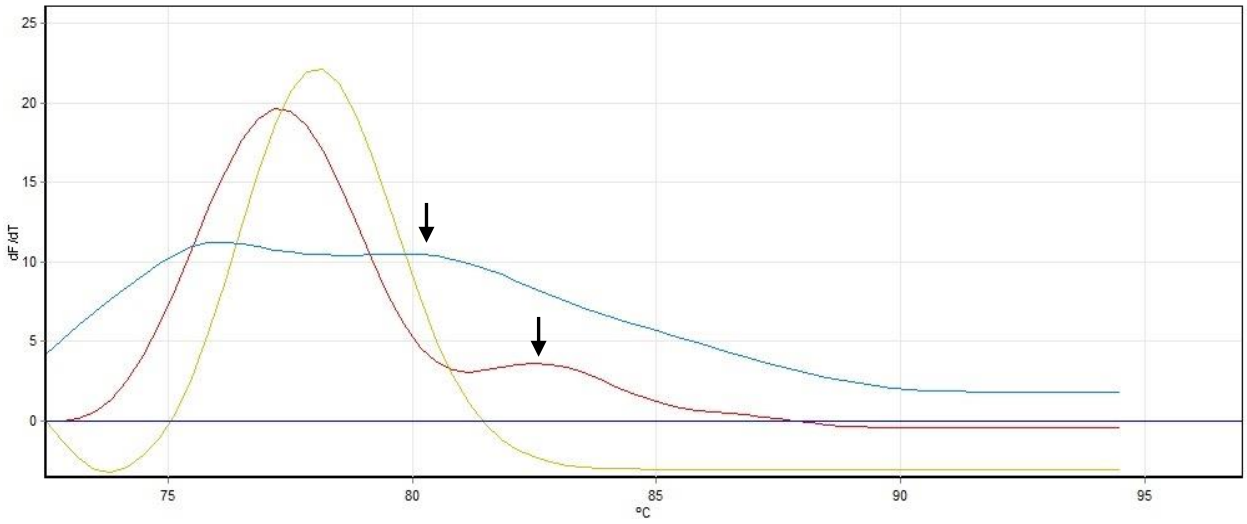

Figure 3.17: Melt curve of qPCR products amplified using the primer pair Fest-582seq-F with Fest-582-R-5 *, where each peak represents a product. No amplification product except for primer dimer was produced for Lolium DNA (light green). F. arundinacea DNA (purple) mostly amplified primer dimer. However, a small amount of amplification product was also observed (arrow). Elongation factor reference gene (dark green) amplified almost equal amounts of primer dimer and expected product (arrow).*

3.5 F_2 *Festulolium* hybrid morphology

3.5.1 Growth of F_2 hybrids

F_2 hybrid growth over the first 17 days after defoliation remained constant with only minor fluctuations when days between measurements varied from 3-4 days. The final measurement at 35 days after defoliation showed significant differences between the F_2 hybrid lines (Figure 3.19). A linear regression showed no correlation between ploidy and total growth (p-value = 0.126, R^2 = 0.0543). The F_2 hybrids which had the greatest mean total growth were 123/34/A (putative allononaploid, 2n = 9x) and 122/10/B (putative allotetraploid, 2n = 4x), which grew a mean total of 651 mm and 601 mm respectively. The least mean total growth was found in 123/12/D (putative allohexaploid, 2n = 6x) and 122/75/A (putative allotriploid, 2n = 3x), where they only grew a mean total of 335 mm and 287 mm respectively, half of the fastest growing plants. One F_2 hybrid (122/80/E) was excluded from the growth analysis because it had bolted.

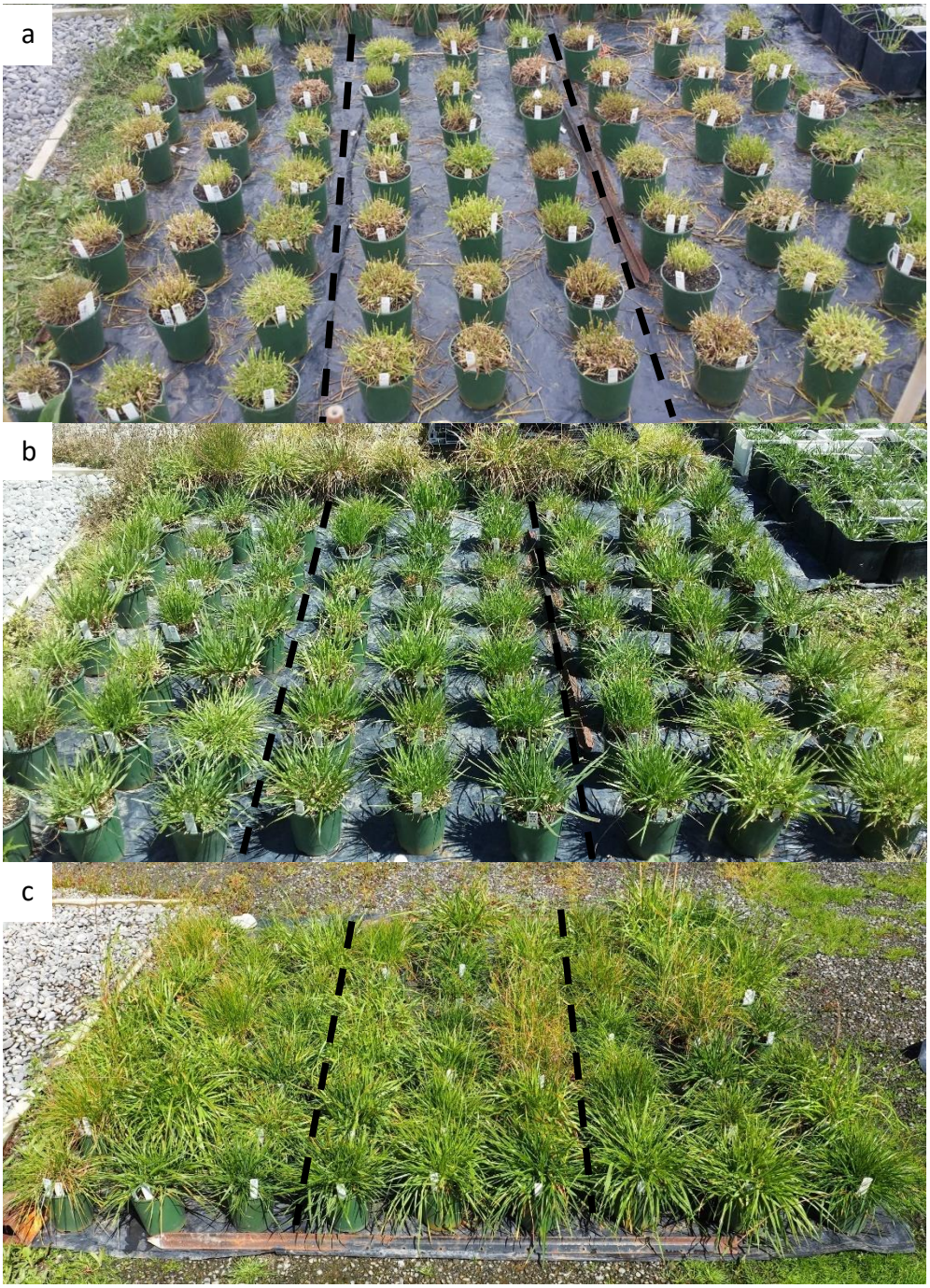

Figure 3.18: Experimental growth set up after seven (a), 17 (b) and 35 (c) days after defoliation. Differences in plant growth habits can be seen after seven days but became more prominent at 17 days after defoliation. Broken lines show the division between blocks.

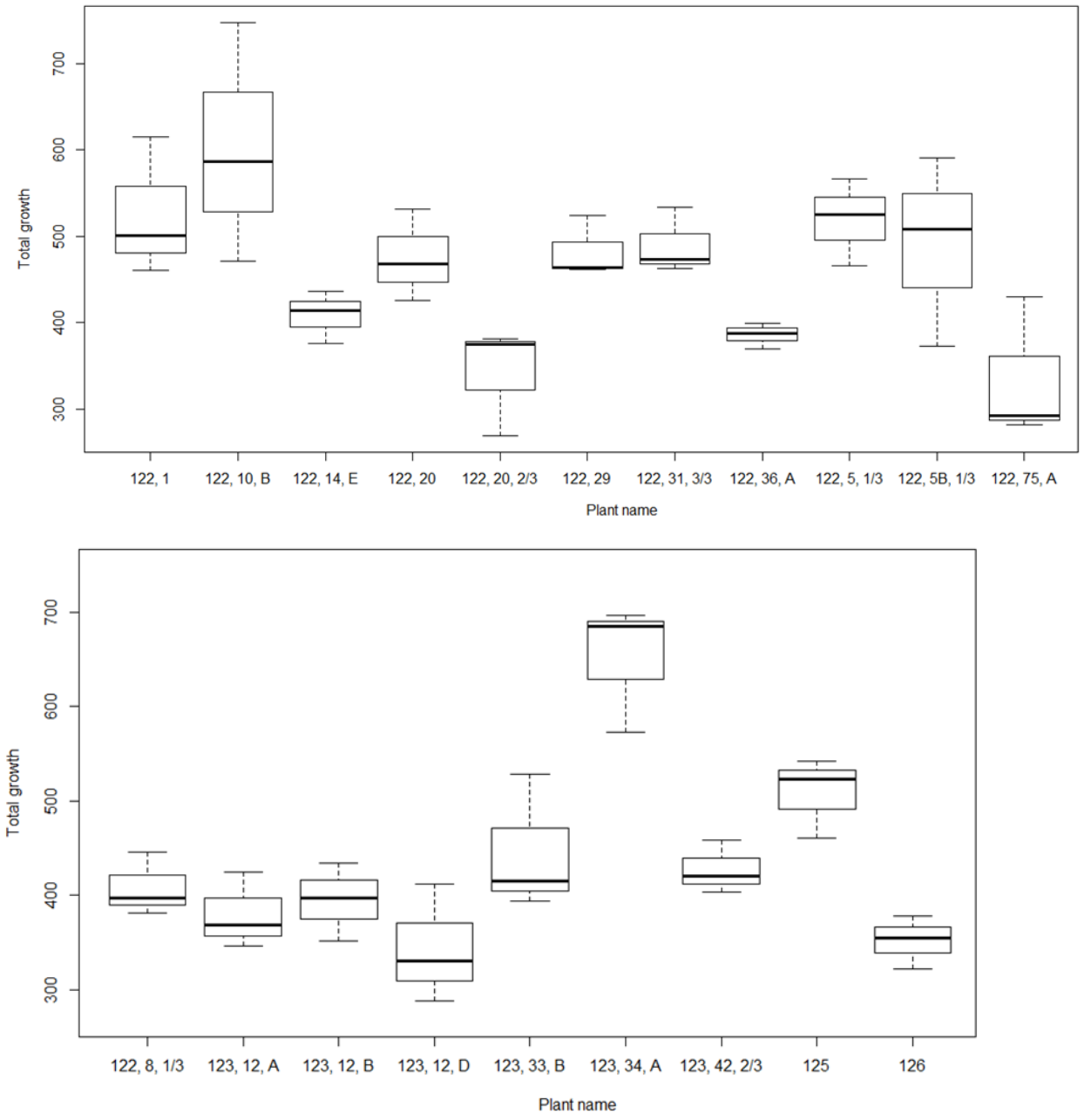

Figure 3.19: Mean total growth of F_2 hybrid plants after 35 days of defoliation. Within the population of F_2 hybrids, a lot of variation can be seen. One of the 21 F_2 hybrids was excluded from this analysis because it had begun to flower.

3.5.2 Leaf morphology and protein analysis

Of 10 leaf characteristics that were studied, only three characters were significantly correlated with ploidy of the F_2 hybrids once statistical significance values were adjusted using a Bonferroni correction (new significant p-value = 0.005). Ploidy was correlated negatively with dry weight (p-value = 0.00245, R^2 = 0.476) and positively correlated with crude protein (p-value = 0.00211, R^2 = 0.416) and stomata size (p-value = <0.001, R^2 = 0.703) (Figure 3.20). There was also a general non-significant trend that the plants from which greater dry weight was harvested tended to have decreased crude protein (p-value = 0.065 R^2 = 0.158). Although most morphological characters did not correlate with other measured characters, there still exists a lot of variation within the F_2 hybrid population. The putative allohexaploid (123/12/D) had the greatest stomata density followed very closely by a putative allotriploid (122/75/A, 2n = 3x) and allononaploid (126, 2n = 9x), while the diploid, 123/33/B, had the lowest stomatal density with no stomata on the leaf underside. Estimated area of the leaves were almost all the same except for two (122/75/A and 123/33/B) plants which had reduced leaf area compared to the other F_2 hybrids, due to significantly narrower and shorter leaves.

F_2 hybrids that were descended from *L. perenne* mothers were assessed for the shape of leaf emerging from the sheath. Only two (123/33/B and 123/34/A) of the eight *L. perenne* F_2 hybrids had folded leaves upon emergence. The remaining six plants had some form of rolled leaves, some more rolled than others, a character of *F. arundinacea*.

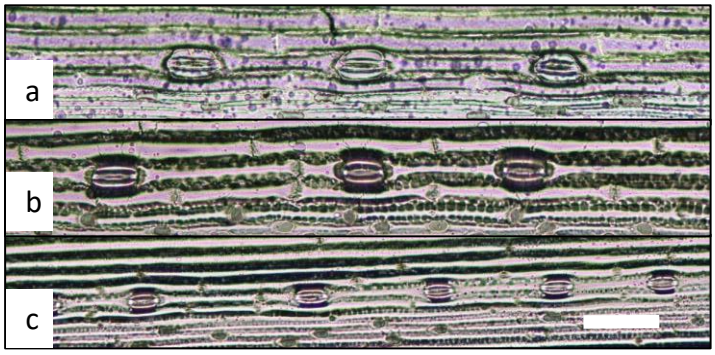

Figure 3.20: Stomata of 2n = 9x (a), 4x (b), and 2x (c) F_2 hybrids respectively. Stomata from the 2n = 2x F_2 hybrid (c) is significantly smaller than the other two plants. Stomata from the 2n = 9x (a) is also larger that the 2n = 4x, but the difference is not as large. Scale bar = 100 µm.

3.5.3 Heading times and inflorescence morphology

Heading times of the F_2 hybrids occurred from 04.11.16 to 15.01.17. In general, F_2 hybrids that were ca. 2n = 4x or less flowered earliest while greater than 2n = 4x typically flowered up to 45 days later (Figure 3.21). One F_2 hybrid (123/33/B) was vegetative for most of the summer and flowered very late compared to other F_2 hybrids. Furthermore, once 123/33/B did flower (15.01.17), only one or two inflorescences appeared at a time and continued flowering for until the middle of March 2017. Both L. perenne and F. arundinacea control plants flowered within the first 10 days of the first hybrid (Figure 3.21).

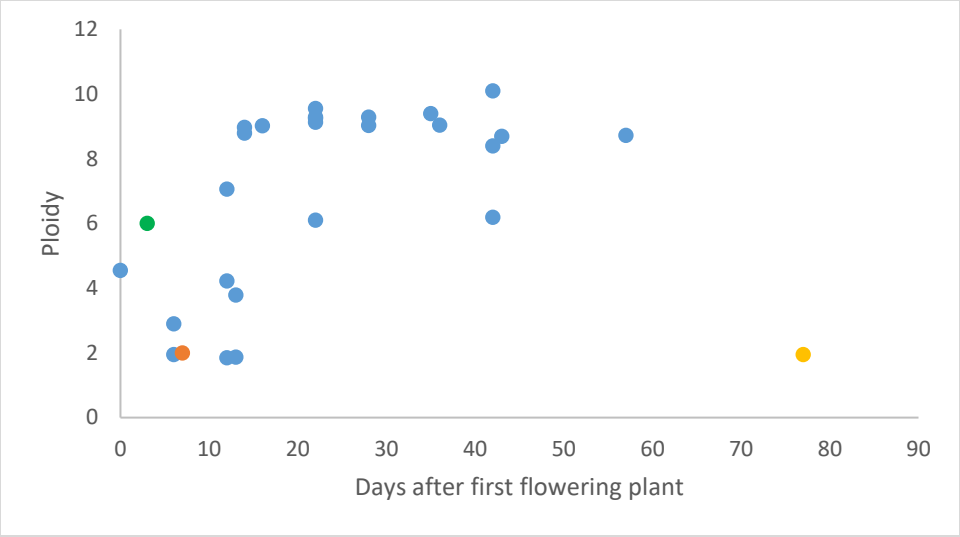

Figure 3.21: Heading times of F_2 hybrids relative to the to the first flower hybrid. The hybrids with lower estimated ploidy (ca. 2n = 4x or less) typically flowered earlier and within a shorter period than higher ploidy hybrids (2n = 6x or higher). Blue, are all F_2 hybrid plants examined. Yellow is one F_2 hybrid (123/33/B) which took exceptionally long to flower relative to other F_2 hybrids. Orange and green are L. perenne *and* F. arundinacea *controls respectively.*

The inflorescence morphologies of the F_2 hybrids were diverse. Both panicle and spike parental inflorescence morphologies were observed as well as intermediates between the two inflorescences types (Figure 3.22). Of the 14 plants examined for inflorescence morphology, 10 spikes (Figure 3.22 a), one compound spike (Figure 3.22 b), one raceme (Figure 3.22 e) and six panicles (Figure 3.22 g and h) were observed. Those F_2 hybrids that had two inflorescence types were counted in each category they belonged to. Two F_2 hybirds shown in Figure 3.22, (123/33/B and 122/75/A) had a spike (Figure 3.22 b, c), but also had a compound spike (Figure 3.22 f) or a compressed panicle (Figure 3.22 g) in appearance, a 'hybrid' of the two parental inflorescences. A raceme, an inflorescence type not normally observed on either *Lolium* or *F. arundinacea* was also observed on one hybrid 123/6/A (Figure 3.22 e). In Figure 3.23 e it can clearly be seen that the spikelet is not sessile. The long awns (>3 mm) normally seen on *L. multiflorum* plants were commonly absent in F_2 hybrids with *L. multiflorum* mothers except for one plant 122/80/E (Figure 3.23 b). However, *L. multiflorum* F_2 hybrids tended to have many more spikelets per inflorescence than *L. perenne* F_2 hybrids, a diagnostic character of their

respective parents. All panicles produced on F_2 hybrids were a compressed form of the parental *F. arundinacea*. Two spikelets were found at each node although, one of the spikelets was sessile while the other resembled a stunted version of its fescue parent (Figure 3.22 g-i and Figure 3.23 d and f). Close-up versions of the main inflorescence types can be seen in Figure 3.23 where a and f are *L. perenne* and *F. arundinacea* respectively and b-e are F_2 hybrid inflorescences.

Figure 3.22: Inflorescence morphology of five F_2 Festulolium *hybrids and parental* L. perenne (a.) *and* F. arundinacea (i.)*. Inflorescences are arranged from most* Lolium *on the left to most* Festuca *on the right. The inflorescence of* L. perenne *was a spike, while* F. arundinacea *was a panicle*. *F_2 hybrids have several different types of inflorescences. A spike on 123/33/B (b.), 122/75/A (c.) and 122/80/E (d.) or a spike containing secondary spikes ('compound spike') on 123/33/B (f.) and 122/75/A (g.). 123/6/A (e.) was found to be a raceme because it does not have sessile spiklets. 122/5B (h.) had a stunted panicle (compared to* F. arundinacea*) narrowing to a spike at the top. Black arrows show a second, shorter or sessile inflorescence from the same node, typical of* F. arundinacea. *Scale bar = 1 cm*

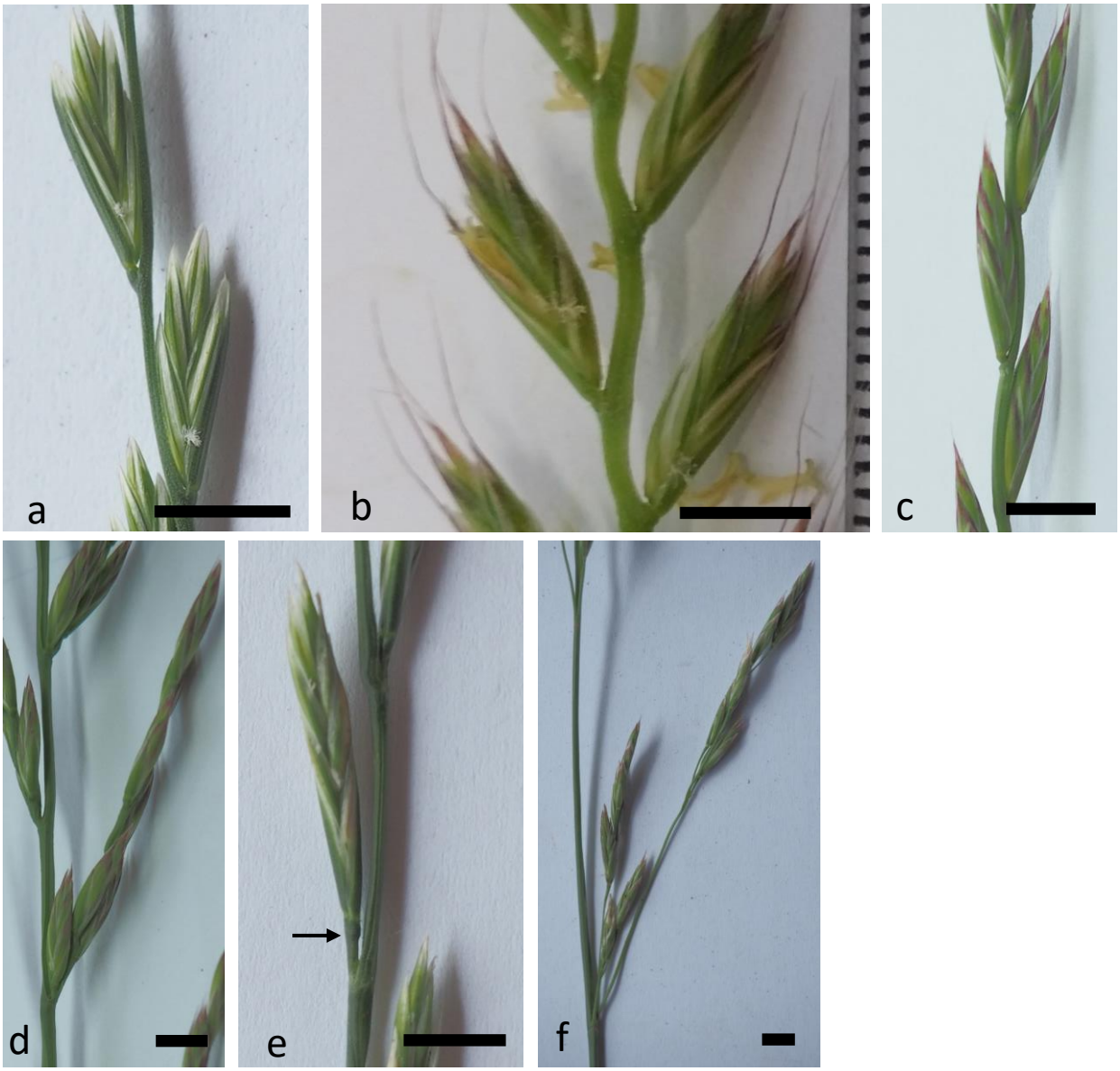

Figure 3.23: Detailed view of F_2 hybrid and parental inflorescences. L. perenne *(a) and* F. arundinacea *(f) were the parental plants to compare hybrid morphology. 122/80/E (b) has spikelets typical of (a) but has very long awns, typical of its* L. multiflorum *ancestry. 122/75/A (c and d) showed different inflorescence morphologies. One inflorescence was a spike (c), while the other a spike, but with a secondary spike where a spikelet should be. The spikelets found one 123/6/A (e) were not sessile (arrow) so the inflorescence was considered a raceme. Scale bars = 5 mm.*

3.5.4 Pollen Viability

Pollen was considered viable if fluorescence was observed and scored anywhere between 1 and 5 (Figure 2.6). When any fluorescence was scored as viable, F_2 hybrids had viable pollen with the highest pollen viability being 95.4% but the lowest 0 % However, if we only consider the pollen which fluoresced very brightly (scored 4 or 5), then pollen viability is much lower with the highest viability at 65 % and the lowest 0 %, although half of the F_2 hybrids examined had <20 % pollen viability. Some F_2 hybrid's anthers abscised before they dehisced. In this case the anthers were carefully opened with a fine tipped needle and any pollen inside carefully scraped out for FCR testing. All plants that had <10% mean pollen viability using the more conservative definition of pollen viability (Table 3.4), shared the common trait of anther abscission before anther dehiscence. The pollen grains obtained from these non-dehisced anthers were often deformed and empty inside when viewed under bright field illumination (Figure 3.25 a). When deformed pollen was observed under the FiTC filter, no fluorescence was observed confirming that they were not viable (Figure 3.25 b). One of the F_2 hybrid's, (123/6/A) anthers remained very small and contained no pollen for FCR testing (Figure 3.25 e). Other F_2 hybrids had much higher pollen viability (Figure 3.25 d). These F_2 hybrids had variable pollen viability, some looked deformed and empty, while other pollen grains that fluoresced very brightly. There was a weak negative correlation of pollen viability with ploidy when using either 1-5 or 4-5 viability (p-value = 0.011 and 0.001, R^2 = 0.15 and 0.25 respectively). However, this R^2 value is very low indicating the data does not fit the regression line very well. Another trend observed was when the plant's estimated ploidy was near a multiple of 2n *Lolium* (e.g. 2n = 2x, 4x or 6x) pollen viability was higher than those F_2 hybrids that have ploidies near 2x = 3n, 5n or ≥8n (Figure 3.24).

Table 3.4: F_2 hybrids investigated for pollen viability using fluorescein diacetate (FDA). Two pollen viability measures were calculated based on the arbitrary pollen scale (Figure 2.6). Mean viability of pollen when any level of fluorescence on the arbitrary scale was observed. Mean viability 4-5 is the mean viability of pollen when only values of 4 and 5 are considered to indicate viable pollen.

Plant Name	Mean (%) viability 1-5	Mean (%) viability 4-5
125	92.5	16.1
122/29	86.4	16.3
123/33/B	81.9	65.0
122/75/A	0.3	0.0
122/10/B	95.4	36.5
122/80/E	94.2	60.5
122/1	8.9	0.9
122/36/A	13.0	3.3
123/12/D	82.1	26.3
123/6/A	0 % (No pollen)	0 % (No pollen)
123/12/B	33.7	0.7
L. perenne	76.8	31.4

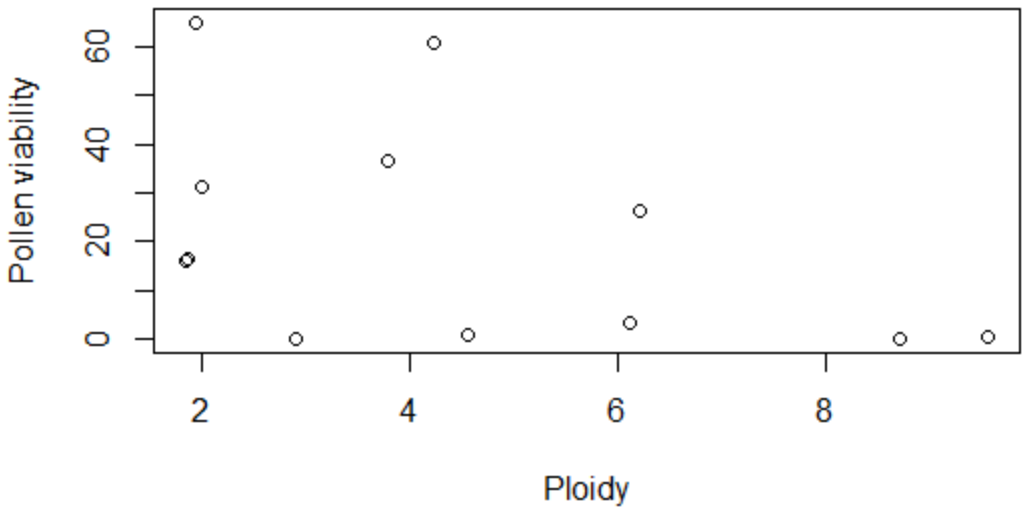

Figure 3.24: Mean pollen viability of F_2 hybrids. F_2 hybrids whose estimated ploidy was approaching multiples of diploid Lolium (2n = 2x, 4x, or 6x) *showed much greater pollen viability than those hybrids whose ploidy was 2x = 3n, 5n or ≥8n. The more conservative 4-5 scored pollen was used, although both scoring methods show a similar pattern.*

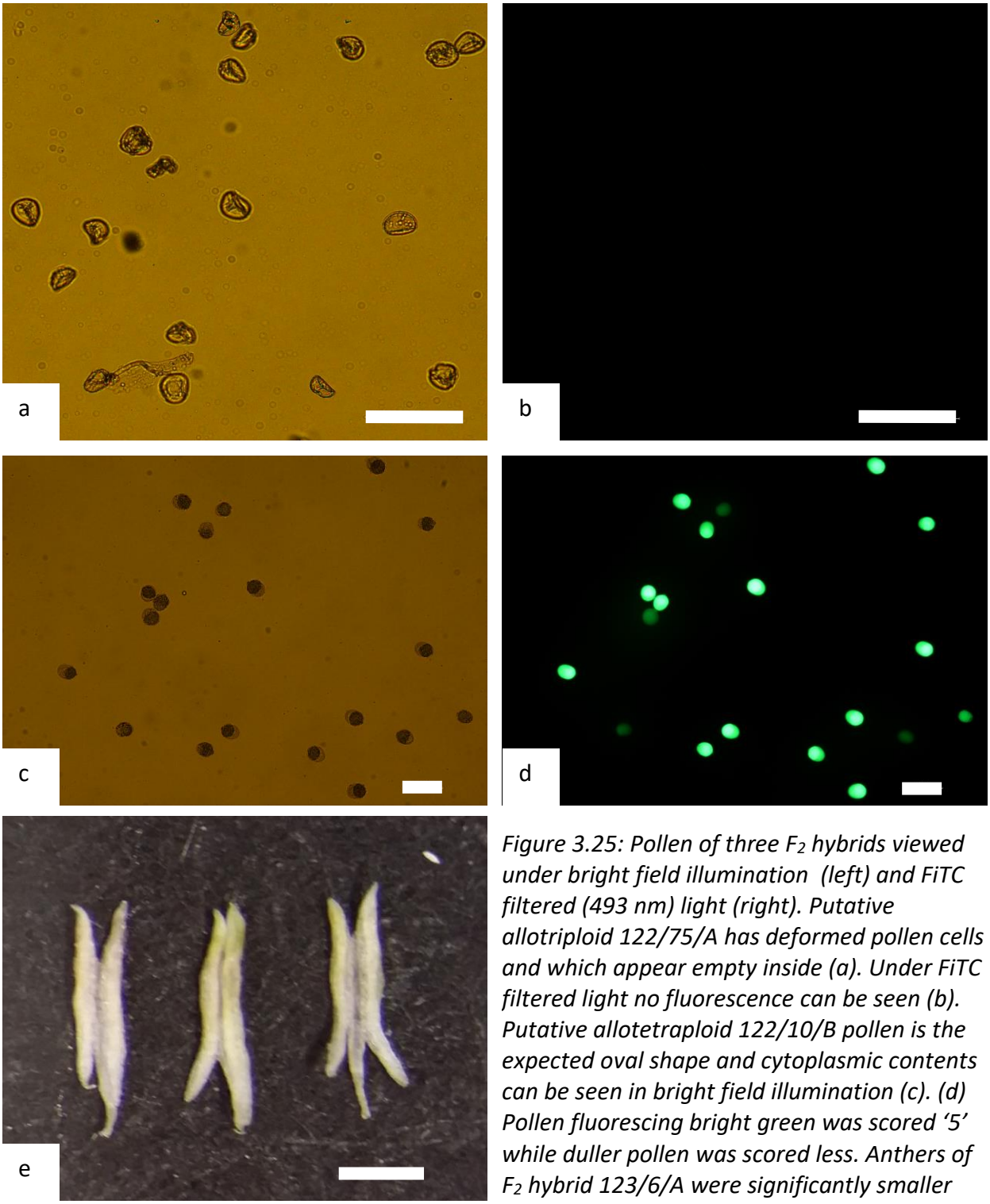

Figure 3.25: Pollen of three F_2 hybrids viewed under bright field illumination (left) and FiTC filtered (493 nm) light (right). Putative allotriploid 122/75/A has deformed pollen cells and which appear empty inside (a). Under FiTC filtered light no fluorescence can be seen (b). Putative allotetraploid 122/10/B pollen is the expected oval shape and cytoplasmic contents can be seen in bright field illumination (c). (d) Pollen fluorescing bright green was scored '5' while duller pollen was scored less. Anthers of F_2 hybrid 123/6/A were significantly smaller than other anthers and did not contain any pollen (e). Scale bars for pollen = 100 µm and scale bar for anthers = 900 µm.

Chapter 4 – Discussion

4.1 *Festulolium* F_2 hybrid ploidy and zygosity of diploids

Flow cytometry has been a useful tool for estimating the amount of DNA contained within a cell for the past 50 years (Kamentsky et al. 1965). There are two fluorescent dyes that are commonly used: 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI), for estimating DNA content (Doležel and Bartoš 2005). DAPI, which fluoresces more brightly when bound to DNA, binds to A-T rich regions of the DNA (Portugal and Waring 1988). As a result, when using DAPI for flow cytometry, care must be taken not to compare species that have different ratios of A-T rich regions within the genome which would give unreliable results (Doležel et al. 1992). However, DAPI flow cytometry is not influenced by chromatin structure, which results in low coefficient of variation (narrow peaks) (Cowden and Curtis 1981). On the other hand, PI binds to the DNA non-specifically, intercalating with all bases (Doležel et al. 1992). This allows for accurate comparisons between any species regardless of the AT:GC ratio, which often makes PI the preferred method for flow cytometry (Doležel et al. 1998, Noirot et al. 2002). However, PI is not without its limitations. PI binds to chromatin and changes in chromatin structure might affect estimation of DNA content (Rayburn et al. 1992).

However, no significant differences were observed when different tissue types were assayed (Blondon et al. 1994, Kamaté et al. 2001). Nevertheless, according to Becker and Mikel (1990) cells that have been fixed in formalin or alcohol have different estimations of DNA content. This is because the different fixatives alter chromatin structure, thus, fixatives can affect the accuracy of flow cytometry results. Furthermore, PI has higher variance (wider peaks) because it does not bind only to DNA. Flow cytometry of F_2 hybrids using both DAPI and PI as fluorescent dyes resulted in similar estimations of DNA per cell when DNA content per cell was below 10 pg DNA. However, when the estimated DNA content was above 10 pg per cell, PI estimates were higher than DAPI estimates (Figure 3.4).

The DNA in the F_2 hybrids with less than 10 pg DNA per cell is likely to contain mostly *Lolium* DNA because plant morphology resembled that of *Lolium*. Furthermore, diploid F_2 *Festulolium*

hybrid plants published in Kindiger (2016) were discovered to be doubled haploid *Lolium*. However, as DNA content increases the amount of *F. arundinacea* DNA present could increase the error of estimated DNA content. Beyond 10 pg DNA per cell DAPI estimated DNA content per cell is from 1-5 pg different to PI and could be due to a different AT:GC ratio in *F. arundinacea*. This error cannot be corrected because, to date, there are no published data on AT:GC ratios for the whole genome in either parental species. A range of pg per cell in the F_2 hybrids could be estimated with DAPI stained cells if *F. arundinacea* was also used as the standard to estimate DNA content. PI would be the best method to estimate absolute DNA content but DAPI was also sensitive enough to roughly identify both polyploids and aneuploids in this study.

Flow cytometry has been used successfully in plant breeding programs for identifying polyploid and aneuploid individuals (Pfosser *et al.* 1995, Faleiro *et al.* 2016). It identifies these different ploidy individuals by estimating absolute DNA content per cell, but may not be an accurate method for estimating the exact number of chromosomes within a cell (Suda *et al.* 2006). Every chromosome within an organism is different in size; hence there will be different amounts of DNA per chromosome. Therefore, if an organism happens to be aneuploid and has lost two or three small chromosomes, it is not possible to tell if it has lost one large chromosome or a couple of smaller chromosomes (Suda *et al.* 2006). This may be particularly problematic with interspecific hybrids, including *Festuloliums,* where aneuploidy is common (Kleijer 1987, Pfosser *et al.* 1995). Therefore, to determine the true ploidy of the F_2 hybrids, direct observation of chromosomes is required.

As mentioned, above Kindiger (2016) found that plants with similar DNA content to diploid *Lolium* may be double haploid. The production of doubled haploid plants is of importance to plant breeders because they are very useful for rapid identification and selection of desirable traits. As a result, doubled haploids have been highly sought after in plant breeding programs. Although doubled haploids have been created in over 250 species, many species remain recalcitrant to the methods commonly employed (Hofinger *et al.* 2013). Previous doubled haploids in *F. arundinacea* and *Lolium* have been created using anther culture, where haploid

plants were regenerated. Chromosome doubling was then induced in the haploid plants to create doubled haploids (Kasperbauer and Eizenga 1985, Begheyn *et al.* 2016). Recently, a new method to produce doubled haploid *F. arundinacea* and *L.* multiflorum has been developed by Kindiger (2016), whereby F_1 hybrids between *F. arundinacea* and inducer line *L. multiflorum* produced offspring, resulting in doubled haploids of their respective parents. Further doubled haploid *F. arundinacea* plants were observed to occur through spontaneous loss of *Lolium* chromosomes in the vegetative plant. This raised the question whether the diploid F_2 hybrids produced in this study were also doubled haploid. However, the six SSRs used to test homozygosity, found that seven diploids were as heterozygous as *Lolium* controls, and the remaining plants were heterozygous at half the loci assayed (Table 3.2).

There may be several reasons why diploid plants in this study were not double haploid. Assuming seed contamination did not occur, the isolation blocks where the F_1 hybrids were grown when flowering occurred may not have been completely isolated from external pollen sources (Griffiths 1950). The F_1 hybrids may have been pollinated by external *Lolium* pollen source resulting in heterozygous diploid offspring (Akiyama *et al.* 2016). This was not originally thought of as an issue because the F_1 hybrids were thought to be sterile, but for future production of doubled haploids, tighter control of external pollen should be considered if doubled haploid production is a priority. Another potential reason for heterozygous F_2 hybrids could be that during meiosis some homoeologous *F. arundinacea* chromosomes underwent recombination with *Lolium* chromosomes (Kopecký *et al.* 2008). Recombination and introgression has been reported in several GISH studies involving *Lolium* x *F. arundinacea* hybrids, although, none of these hybrids were diploid (King *et al.* 2002, Kopecký *et al.* 2006, Kubota *et al.* 2015). However, Pasakinskiene *et al.* (1997) suggest that their diploid plants arose from somatic recombination followed by elimination of the *Festuca* chromosomes. Molecular cytogenetics might be able help elucidate, in part, why the allodiploid F_2 hybrids in this study were not homozygous.

4.2 *Festulolium* molecular cytogenetics

Cytogenetics allows for the direct observation of the chromosomes of an organism. High quality chromosomal preparations must be attained before any detailed observations can be made (Kirov *et al.* 2014). Traditionally, plant chromosome preparations were done by meristematic squashes (root tip squash) to observe cells in anaphase or metaphase (Ahloowalia 1965, Moscone *et al.* 1996, Zwierzykowski *et al.* 1998). This method can produce good quality chromosome preparation, but some cells may become obscured by debris from non-dividing tissues (Schwarzacher and Leitch 1994, Jensen 2014). Another method called the flame drying technique, developed by Ansari *et al.* (1999) removes most of the non-dividing cells, resulting in cleaner preparations. This method also causes the cells to burst resulting in chromosomes that are well spread and cellular debris which have washed away (Figure 3.5). Furthermore, because the chromosomes are well spread and have little cellular debris, hybridisation probes have better access to the chromosomes than the squash method (Ansari *et al.* 2016). Chromosome preparations done using this method may also be viewed months to years later with little or no reduction in quality (pers. com. Ansari, 2016).

In order to observe chromosome details that would otherwise not be visible, chromosomes can be stained using a variety of techniques such as acetocarmine, Giemsa, FISH (fluorescent *in situ* hybridisation) and GISH (genomic *in situ* hybridisation (Evans *et al.* 1973, Dutrillaux and Viegas-Pequignot 1981, Ansari *et al.* 1999, D'Hont 2005). Acetocarmine is typically used when staining chromosomes because it is a simple, well established technique that allows the observer to easily distinguish chromatin from other cellular debris (Lillie and Conn 1969). However, acetocarmine stained chromosomes is limited in use beyond simply observing chromosomes because it binds to heterochromatin and euchromatin equally (Stack 1974). Unlike acetocarmine, Giemsa has different binding affinities to heterochromatin and euchromatin giving rise to chromosomal banding patterns (Dutrillaux and Viegas-Pequignot 1981). These banding patterns (G-banding) can be used for distinguishing chromosomes from one another because the banding patterns are unique to each chromosome (Dutrillaux and Viegas-Pequignot 1981). Furthermore, Giemsa staining results in chromosomes that appear sharper

than acetocarmine when viewed (Mingguang 1981). For this reason, Giemsa was used to initially stain the F_2 hybrid chromosomes. Chromosomes were easily observed and preliminary counting was done. However, several short 'satellite' chromosomes were observed (Figure 3.5), resulting in variable chromosomal counts. These 'satellite' chromosomes are known to be caused by de-condensed 18S rDNA (Ansari *et al.* 1999) and, without 18S rDNA hybridisation, exact chromosome number is difficult to determine (Akiyama *et al.* 2016). There were no suitable chromosome preparations for Giemsa staining of 122/80/E. To accurately and precisely count chromosome numbers FISH was carried out.

Fluorescent in situ hybridisation is an important tool for physical mapping of genes on chromosomes (Jensen 2014). Once specific genes have been mapped to a chromosome, chromosomes can be identified and named for future work (Thomas et al. 1996, Thomas et al. 1997). Both 18S and 5S rDNA probes are often used to identify chromosomes because they are well established markers found in all eukaryotes (Leitch and Heslop-Harrison 1992). They also have two other purposes when used on Festuca or Lolium chromosomes. Firstly, 18S rDNA shows the connection between the 'satellite' chromosome and the main body of the chromosome, allowing for the absolute chromosome number to be determined (Ansari et al. 1999). Secondly, when the 18S rDNA marker is used in combination with the 5S rDNA maker, some chromosomes can be identified and the parentage of some determined (Figure 4.1) (Thomas *et al.* 1996, Thomas *et al.* 1997). The exact number of chromosomes each F_2 hybrid had was obtained from the FISH images (Figure 3.6).

However, some interesting and unexpected observations were made. The allodiploid 123/33/B had 14 chromosomes, which agrees with flow cytometry data (Table 3.1). However, a single chromosome only hybridised with the 5S rDNA, which is indicative of an F. arundinacea chromosome (Figure 4.1) (Thomas *et al.* 1997), while the second chromosome that normally contains both 5S and 18S rDNA was absent. A possible explanation for this is that the *L. perenne* chromosome which contained both 5S and 18S rDNA was eliminated and replaced with the *F. arundinacea* chromosome containing only the 5S rDNA (Akiyama *et al.* 2010). Another explanation is that on rare occasions it has been observed that the 18S rDNA sequence is

absent from the chromosome normally containing both 18S and 5S rDNA (Lideikyte *et al.* 2008). Genomic *in situ* hybridisation should be able to shed some light on the plausibility of these hypotheses as it allows for the differentiation of chromosomes of differing species.

The GISH images obtained for 123/33/B showed that only *L. perenne* DNA had hybridised with the chromosome containing only the 5S rDNA marker (Figure 3.6 c). This confirms that the chromosome was of *Lolium*, suggesting that the personal communication with Dr Helal Ansari (2016) was correct. However, to determine if this is the case, GISH of the *Lolium* parent would be required. Due to the time constraints of a Master's project, another chromosome preparation and GISH experiment for the parental *Lolium* could not be done.

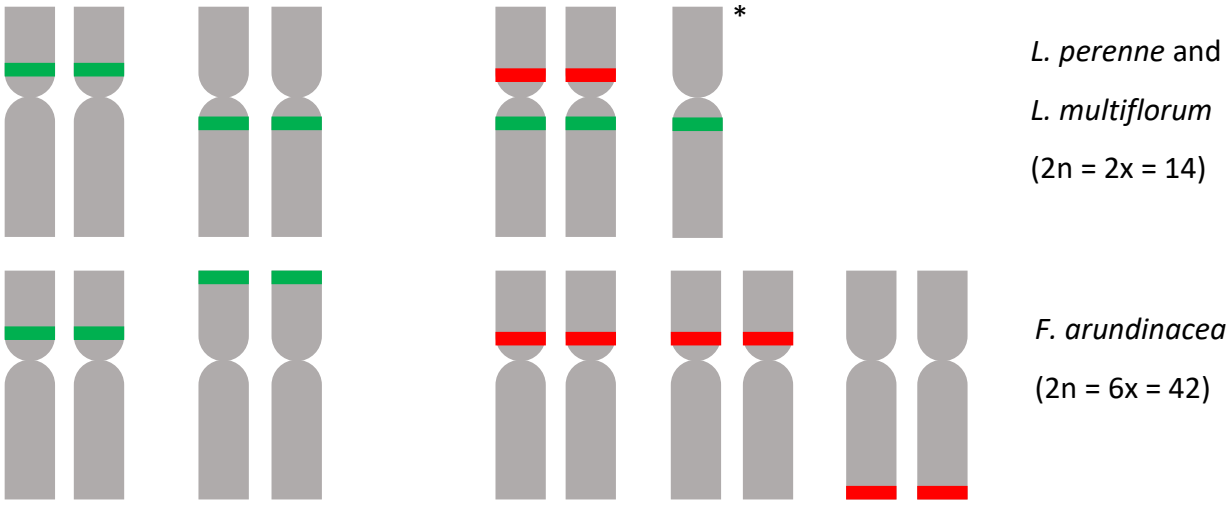

Figure 4.1: Diagrammatic representation of L. perenne*,* L. multiflorum *and* F. arundinacea*. The number and the position of chromosomes containing the 5S rDNA (red) and 18S rDNA (green) sequences are shown (Thomas et al. 1996, Thomas et al. 1997). * A seventh chromosome containing 18S rDNA can be observed in different genotypes of L. perenne (Lideikyte et al. 2008, Książczyk et al. 2010).*

FISH images confirmed the number of chromosomes of 122/80/E as 31, more than the expected number of chromosomes estimated by flow cytometry which was 29.5 (Table 3.1 and Figure 3.6). This could be because of the aforementioned differences in chromosome size (Section 4.1). 122/80/E had very similar chromosome number and constituent 5S and 18S rDNA for a tetraploid *L. multiflorum* (Thomas *et al.* 1996), except for an extra 18S rDNA. This is not unexpected as extra, or missing rDNA sites have been reported in another study where chromosome doubling has occurred (Książczyk *et al.* 2010).

GISH images for 122/80/E revealed that most chromosomes had strongly hybridised with the fluorescently labelled *L. perenne* DNA (Figure 3.6 f). This shows that similar repetitive sequences exist between *L. multiflorum* and *L. perenne (Jones et al. 2001)*, and were sufficient to discriminate between *Lolium* and *F. arundinacea* chromosomes. This is consistent with the finding of King *et al.* (2013) where they found that *L. multiflorum and L. perenne* had high sequence homology of >85%, where the *F. pratensis* homology was significantly lower. Some *L. multiflorum* chromosomes had recombined with *F. arundinacea* chromosomes, resulting in chromosome regions that have not hybridised at all. These chromosome substitutions may also help explain why the flow cytometry data underestimated the number of chromosomes expected to be seen within this plant (Akiyama *et al.* 2016).

Analysis of FISH chromosomes for 126 confirmed the chromosome number was 68 based on chromosome counts, which is seven chromosomes more than flow cytometry estimated. GISH chromosome preparations from 126 and 123/6/A had a similar number of chromosome pieces of 68 and 72 respectively. However, the Giemsa image for 123/6/A only had 57 chromosome fragments, suggesting it is incomplete. Chromosome preparation for 123/6/A was not used for FISH because it was created after the FISH experiments had already been completed. Therefore, exact chromosome number cannot be obtained for 123/6/A until 18S rDNA FISH probe is used but would be fewer than 72 chromosomes.

Clear recombination was visibly evident in both 126 and 123/6/A GISH images (Figure 3.6 i and l). Most chromosomes only had single recombination events. However, a couple of chromosomes had two or three regions where recombination occurred. The high level of detail observed in both chromosome preparations suggests that the stringency attained was nearly optimal (Schwarzacher and Heslop-Harrison 2000). This recombination behaviour has also been observed in other *Festuloliums* and other interspecific hybrids (Humphreys *et al.* 1998, D'Hont 2005, Kopecký *et al.* 2006, Ansari *et al.* 2008, Kopecký *et al.* 2008, Akiyama *et al.* 2012), and inferred by several other studies where increased cold or drought tolerance was observed after several backcrosses into *Lolium* (Humphreys *et al.* 1997, Rapacz *et al.* 2004, Akgun *et al.* 2008, Østrem and Larsen 2008). An *F. arundinacea* chromosomes had fluorescently labelled *L. perenne* DNA hybridised that may be been misidentified as a recombination site without FISH. However, when both the FISH and GISH images are compared it is clear that *L. perenne* DNA hybridised with an 18S rDNA site (Figure 3.6 l). This occurs because 18S rDNA is a repetitive, highly conserved sequence in both *F. arundinacea* and *L. perenne*, hence the 18S rDNA found in *L. perenne* genomic DNA could hybridise with the 18S rDNA site on the *F. arundinacea* chromosome.

The variable number of chromosomes found in all the F_2 hybrids is likely due to an absence of a regular diploid-like pairing system, resulting in unreduced gametes or chromosome elimination (Pasakinskiene *et al.* 1997, Zhao *et al.* 2007, Begheyn *et al.* 2016). Considering about 70% angiosperms are considered to have originated from polyploidy in the past (Masterson 1994), a mechanism must exist to stabilise chromosome number. In a related species to *Festuca* and *Lolium*, hexaploid wheat (*Triticum aestivum*), a single locus called *Ph1* (P*airing Homoeologous 1*) has been found to have a major effect on chromosome pairing and recombination (Griffiths *et al.* 2006). This occurs by suppressing homoeologous chromosome pairing during metaphase I, allowing only homologous chromosomes to pair (Lukaszewski and Kopecký 2010). *Ph1* genes have been documented in *Lolium* (Eizenga *et al.* 1991). Therefore, if higher activity of the *Ph1*-like locus could be found in *Lolium* or *Festuca* and selected for, then stable a *Festulolium* could be achieved.

Several studies attempting to make stable 4n, 6n or 8n *Festuloliums* were unable to achieve this goal (Kleijer 1987, Eizenga *et al.* 1991, Kopecký *et al.* 2006, Zwierzykowski *et al.* 2006, Akiyama *et al.* 2016). Cytogenetic analysis of these hybrid plants showed the formation of monovalents, bivalents, trivalents and sometimes tetravalents. These multivalent pairings result in uneven chromosome numbers in the gametic cells and may cause selective chromosome elimination (Sanei *et al.* 2010). Pedersen *et al.* (1990) registered a 2n = 56 chromosome *Festulolium* hybrid that was allegedly stable and Eizenga *et al.* (1991) suggested it could be a result of unknowingly selecting for *Ph1* activity. However, in a personal communication with the seed bank holding the seed of this hybrid line (2017) (Pedersen *et al.* 1990), it was found in later generations the chromosome stability did eventually break down over subsequent generations resulting in aneuploids. Probably, F_1 hybrids in this thesis did not have an active *Ph1* locus because recombination between homoeologous chromosomes occurred (Figure 3.6 c, f, i, l). Potentially, if enough F_2 hybrids could be created and screened, individuals with more active *Ph1* locus could be found. The production of a 2n = 8x = 56 would have great importance because, if fertility were restored and heterosis fixed in the population, this would allow for the selection of desirable traits (Kleijer 1987).

Another important factor that could affect chromosome pairing and the number of chromosomes in the F_2 hybrids is the presence of a protein called CENH3 (centromere-specific histone H3 variant) (Britt and Kuppu 2016). The formation of the kinetochore is dependent on *Ph1* activity and loss of function and results in the elimination of chromosomes (Allshire and Karpen 2008). The mechanism of CENH3 mediated chromosome elimination has been harnessed to induce haploid production in *Arabidopsis thaliana* (Ravi and Chan 2010). This could have been the underlying mechanism behind the production of doubled haploid *F. arundinacea* described by Kindiger (2016). CENH3 has also been proposed as a mechanism for chromosomal dominance over successive hybrid generations. Although no studies have been published observing this directly in *Festuloliums*, parallels have been drawn to explain the presence of aneuploids and the apparent dominance of the *Lolium* genome found in some studies (Akiyama *et al.* 2010, Sanei *et al.* 2011).

Cytomixis is the movement of some or all the genetic material into an adjacent cell via plasmodesmata (Mursalimov *et al.* 2013) and may also play a role in the variable chromosome numbers observed in F_2 hybrids, resulting in either the loss or gain of chromosomes in gametic mother cells . Cytomixis has been found in many plant species, including both *Lolium* and *Festuca* (Omara 1976, Bellucci *et al.* 2003, Masoud and Bagheri-Shabestarei 2007), which could suggest it may play a role in increasing the ploidy number of these F_2 hybrids beyond 2n = 8x. However, no studies have observed this occurring in *Festuloliums*. Further molecular cytogenetics during meiosis would help elucidate if this does occur and the mechanisms behind the ploidy in F_2 and successive hybrid generations.

4.3 Molecular analysis of F_2 Festulolium hybrids

The use of molecular cytogenetics to analyse interspecific hybrids provides a large amount information. However, this process is very time consuming, thus only very few plants can be screened. A molecular approach is needed to provide a rapid method for detecting the amount of DNA from each parent found within each hybrid instead of the laborious GISH method (Kubota *et al.* 2015).

Reverse transcriptase quantitative real time polymerase chain reaction (RT-qPCR) is a commonly used technique for the analysis of gene expression (Taylor *et al.* 2010). Baseline gene expression is attained with respect to a reference gene (always has the same level of expression) followed by the detection of increased gene expression, often a several hundred-fold increase when it is activated which can be easily detected by the qPCR machine (Schmittgen and Livak 2008). Quantitative PCR (qPCR) is similar to RT-qPCR except the DNA itself is detected, rather than the expression of a gene(s) (Knapp *et al.* 2014). At the detection limits of qPCR, studies have been conducted in transformed cells with low copy number genes (1-3) (Bubner *et al.* 2004, Joshi *et al.* 2008, Jones *et al.* 2014). qPCR was able to detect and predict transgene copy number reliably at as low as two gene copies (Bubner *et al.* 2004). Therefore, qPCR might provide a useful tool for detecting gene copy number in *Festulolium* hybrids. A species-specific DNA sequence that is spread throughout the genome, such as the 1.2 kb sequence, could provide an estimation of the amount of the sequence present in the hybrid.

A dilution series of *F. arundinacea* to *Lolium* DNA can be made to create standard curves to compare hybrids (Joshi *et al.* 2008).

The 1.2 kb sequence discovered by Pašakinskiene et al. (2000) provided a sequenced inter-simple sequence repeat (ISSR) that was distributed throughout the F. arundinacea genome and was almost completely absent in Lolium. The nature of the 1.2 kb sequence being spread throughout the genome suggests that even if a small amount of DNA was present in a hybrid, it should be detectable using PCR (Pašakinskiene et al. 2000). However, neither the amplified product patterns nor sequencing of the bands were similar to Pašakinskiene et al. (2000). However, the cloned sequence results were still of interest because each amplicon sequenced had the GACA primer at the beginning, but also each plasmid had a different length and a different sequence that was completely unique. This suggests that there are many sequences in the Festuca genome with the GACA repeats and the primers must need very specific conditions for anchor specificity, which was not obtained, to amplify the fragment published. In order to completely replicate PCR conditions used by Pašakinskiene et al. (2000) as closely as possible, both Taq polymerases (GoTaq® and DyNAzyme II) were tested, but were unable to produce sufficiently similar amplification products (Figure 3.12). In a personal communication with Dr Andrew Griffiths (2017), a $MgCl_2$ and temperature gradient was suggested to increase the primer 78H. However, due to the time constraints of a Master's thesis, the $MgCl_2$ gradient was unable to be done. Differences in ramp speeds of the PCR machine used in this thesis and the one used by Pašakinskiene *et al.* (2000) may have also contributed to the different banding pattern acquired (Stevens *et al.* 2013). In order to confirm that the 1.2 kb sequence was the one published, southern hybridisation would need to be done because the amplicon was not published (Pašakinskiene *et al.* 2000). If the 1.2 kb product had been sequenced previously it would have allowed for direct primer design for qPCR avoiding initial PCR optimisation.

Since the 1.2 kb *F. arundinacea* species specific sequence could not be obtained in this thesis, the other sequence shown to be species specific (582 bp) was used as proof of concept for qPCR. The ideal sequence size for qPCR is between 150-300 bp, so new primers needed to be developed (Feretzaki and Lingner 2017). However, most of the amplification products of the

primers designed from within the 582 bp sequence were not *F. arundinacea* specific. The one primer pair (Fest-582-F-1 with Fest-582-R-5) that did amplify specifically was too long for good qPCR results. This primer pair was also very similar (only a few base pairs different on one primer) to the primer pair used to specifically amplify the whole sequence. More time was needed to reduce the primer dimer produced in the qPCR. As well as $MgCl_2$ gradients to optimise Mg concentration, adding an extra step, before denaturation of 85˚C for 20 seconds to denature the primer dimer might have allowed for clearer measurements of product amplification (pers. comm. Dr Andrew Griffiths, 2017).

Although qPCR was unsuccessful, qualitative PCR can still be done to detect the presence of a genome in the F_2 hybrids. Using the SSR primer 104H, two long, species specific bands were observed, one for *Lolium* and one for *F. arundinacea* (Figure 3.13). This showed clearly that both *Lolium* and *F. arundinacea* DNA was present in the three F_2 hybrids assayed and confirms the GISH images showing *F. arundinacea* chromosomes. This method could potentially be modified to be semi-quantitative if a reference gene such as 18S rDNA was also amplified as a control to allow for differences in DNA concentrations (Ferre 1992).

Another quantitative method for estimating the percent of parental DNA in hybrid plants is to use many unique DNA markers such as SNPs (single nucleotide polymorphisms) or SSRs (Momotaz *et al.* 2004). Ideally one or more markers per parental chromosome would be used to detect all the chromosomes. Presence or absence of the markers would determine if a chromosome was present (Momotaz *et al.* 2004). However, for SNPs to be a powerful tool, an almost complete genome of *L. perenne or L. multiflorum* and *F. arundinacea* would need to be made public (King *et al.* 2013). However, SSRs have been used with good success. Momotaz *et al.* (2004) used 44 SSR primer pairs to find species specific loci in *Lolium, F. pratensis* and *F. arundinacea*. They found 35 specific *Lolium* markers, four specific to *F. pratensis* and six in *F. arundinacea*. This gave them sufficient data to construct a dendrogram of all three species and the *Festuloliums* produced from these species. Therefore, the information could also be used to estimate the parental DNA found in *Festuloliums*.

4.4 F_2 *Festulolium* morphology

For any plant to be selected for breeding purposes, it must possess some desirable traits. In many crop cultivars, common traits for selection are increased yield, tolerances to biotic and abiotic stresses and palatability (Humphreys *et al.* 1997). Interspecific hybrids are often a good source of novel variation upon which to base selection (Rick and Smith 1953). Interspecific hybrids between *Lolium* and *Festuca* have been created in the past to confer drought and cold resistance in *Lolium* and increased palatability in *Festuca* (Akgun *et al.* 2008). However, by backcrossing these hybrids only a narrow range of traits are selected (Humphreys 2005). Within the small group of F_2 hybrids that were observed for their morphology, a wide range of morphological traits were observed, even though there were no parental controls (*Lolium* or *F. arundinacea*) with which to compare them. The fact that this variation did not correlate with ploidy, suggests that other factors, such as the different combination of chromosomes and acquisition of various quantitative trait loci (QTLs), most likely played a large role (Shinozuka *et al.* 2012). Although the sample size was small, GISH images also showed that the ratio of chromosomes from either parental species was not consistent between hybrids.

Although ploidy was not correlated with growth, it was negatively correlated with dry weight and positively correlated with crude protein. Both results make sense because, although tiller density was not measured, in general, tiller density was much greater in F_2 hybrids of lower ploidy which, therefore, had more biomass. Knowing that more biomass was produced in the lower ploidy plants, crude protein would be 'diluted' by the greater biomass. Crude protein is an important character for pasture because protein is important for muscle growth in livestock (Atti *et al.* 2004).

It was noticed in the early 1900s that with the doubling of chromosomes, there was a corresponding increase in cytoplasmic volume (Wilson 1925). Since then, stomata size was observed and found to also be positively correlated with plant ploidy. This correlation has even been used to estimate relative plant ploidy from fossilised stomata (Sax and Sax 1937, Masterson 1994, Dwivedi et al. 2015). This thesis also confirms that ploidy of interspecific hybrids of Lolium and F. arundinacea can also be estimated by measuring the size of stomata

(Figure 3.20). This is a 'low tech' microscope-based method for determining ploidy that could be used if a flow cytometer is not available. Interestingly, almost no stomata were found on the underside of the *Lolium* leaves and the F_2 hybrids that were diploid also had very few stomata on the underside. Therefore, low stomatal numbers on the underside of the leaf could be a diagnostic character of *Lolium* as it is usual to find stomata on the underside of monocot leaves (Dunn *et al.* 1965). Humphreys *et al.* (1997) found that there was higher stomatal water conductance on the upper surface than the underside of the leaf, which could be because there are no, or very few stomata found on the underside of *Lolium* leaves.

Flowering of the F_2 hybrids resulted in a wide range of inflorescence morphologies, intermediate between the two parents. These intermediate morphologies are common in interspecific hybrids and have been reported in *Festuloliums* as well (Akgun *et al.* 2008, Beatty *et al.* 2016). What was particularly interesting though, was that some F_2 hybrids generated different inflorescence morphologies (spikes and panicles) within the same plant (Figure 3.22). A possible explanation for this is the somatic elimination of chromosomes observed in doubled haploid *F. arundinacea* (Kindiger 2016) or epigenetic changes within the tiller. A flower morphology not previously documented in *Festuloliums* was a raceme because the spikelet was not sessile on the rachis (Figure 3.23 e).

As a result of irregular meiotic division commonly found in interspecific hybrids, the fertility of the hybrid is often compromised (Zhao *et al.* 2007). Viability can be detected in both the pollen and ovule (Stanley and Linskens 1974, Rodriguez-Riano and Dafni 2000, Barrell and Grossniklaus 2005). However, detecting the viability of the ovule is much more technically challenging than in pollen (Barrell and Grossniklaus 2005). Fluorescein diacetate (FDA) staining is a common vital stain used throughout biology, and often used to determine pollen viability (Shivanna and Rangaswamy 1992). Although there are many other pollen viability stains, FDA offers the advantage of having two different signs for viability: one being the test for esterase activity (ubiquitous throughout all of life), the second, cell membrane integrity (Wang *et al.* 2004). When FDA passively moves into the cell, if esterase activity is present, FDA is catabolised to fluorescein and diacetate. However, fluorescein cannot passively pass through the cell

membrane and accumulates within the cell cytoplasm (Pinillos and Cuevas 2008). Thus, if the cell membrane is compromised FDA will leak out into the surrounding medium resulting in high background fluorescence. However, viability can be a difficult thing to define. At what point is there insufficient fluorescence or leaking cytoplasm to say something is not viable? Studies rarely mention a threshold that must be attained for pollen to be viable, merely state that if fluorescence is present it is regarded as viable (Heslop-Harrison and Heslop-Harrison 1970, Heslop-Harrison *et al.* 1984, Shivanna *et al.* 1991, Pinillos and Cuevas 2008). Therefore, two measures of pollen viability were used in this thesis, one that regarded any fluorescence as viable, and a second that considered only the brightest pollen as viable (Figure 2.6). Both scoring methods gave similar trends, although the percent of viable pollen from each plant could be manipulated depending on the stringency of the scoring. While efforts have been made to standardise FDA staining (Pinillos and Cuevas 2008), further standardisation is required to identify what should be considered viable.

Another new method which may provide a more absolute measure of pollen viability, is the use of impedance flow cytometry (Heidmann *et al.* 2015). Impedance flow cytometry measures the capacitance of sampled cells to determine cell properties such as cell membrane permeability and size of cells. Cells with higher membrane permeability (less viable/dead) will have less capacitance. Similarly, smaller cells will have less capacitance than larger cells (Heidmann *et al.* 2016). Using impedance flow cytometry, larger sample sizes can be obtained due to its higher throughput. These larger sample sizes would give higher quality and more precise results. Furthermore, this method would allow for the detection of aneuploid or polyploid pollen grains using cell size as a proxy for ploidy (Heidmann *et al.* 2016). However, an impedance flow cytometer was not available for use, so could not be used in this thesis.

The interesting observation that pollen viability was greatest in plants near 2n, 4n and 6n ploidies suggests that these F_2 hybrids were undergoing more regular meiosis than the F_2 hybrids at 3n, 5n, and >8n (Figure 3.24). Although the pollen from these specify which plants is most likely more viable due to higher chromosome complementation (Kopecký *et al.* 2009), GISH analysis during meiosis would allow for a more in-depth exploration of this observation.

4.5 Conclusion

Increased yield is typically of greatest priority, but in the world's changing climate tolerance to stresses is an increasing focus. Often crop gene pools are limited due to decades or centuries of artificial selection. Crosses with wild progenitors and closely related species are often done to introduce new variation into the gene pool. The use of interspecific hybrids for introducing novel traits into a gene pool may be required to maintain and increase crop production. The interspecific hybrids of *Lolium* x *F. arundinacea* may provide a promising method for introducing drought resistance into a stable amphiploidy or *Lolium* cultivar.

In this project, four different methods for characterising F_2 *Festulolium* were performed. Flow cytometry could potentially be used as a fast and crude method to select F_2 hybrids of similar chromosome number with which to breed. However, hybrids selected for breeding using this method may have as many as many eight chromosomes more than expected with flow cytometry. Use of flow cytometry would be most appropriate on hybrids with small genome sizes where flow cytometry is more accurate.

FISH and GISH will play an important role in the development of *Festulolium* cultivars, especially in determining the ploidy of individuals for breeding. Furthermore, the amount of introgression can be detected using GISH and for more specificity FISH probes could be developed to determine if and how many drought resistance genes present in *F. arundinacea* have been transferred into successive generations of hybrids. Molecular cytogenetics could also be used to understand CENH3 and *Ph1* influences during meiosis.

Although using qPCR to determine the parentage of the F_2 hybrids was not successful, the potential use and plausibility of this method is assessed. If this technique is possible it would also be useful in the breeding of other hybrid cultivars.

Stomata were found to be a diagnostic character for *Lolium* as no or very few stomata were found on the underside of the leaf, in contrast with *F. arundinacea*. This is the first time the lack of stomata on the under surface of the *Lolium* leaf has been documented. While the fertility of

the F_2 hybrids could be determined by staining the pollen with FDA, the need for further standardisation of the technique is required so that a certain brightness of pollen fluorescence can be considered viable.

By identifying novel variation found within the population of F_2 hybrids, plants which have desirable traits can be incorporated into a breeding program. Based on the information obtained in this thesis, one F_2 hybrid, 123/33/B, will be incorporated into the perennial ryegrass breeding program for further investigation. Future research into the meiosis of F_1 hybrids and subsequent generations will be required to identify stable amphiploid F_2 hybrids ($2n = 8x = 56$) and the mechanism behind plants with $>2n = 56$ chromosomes.

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Appendix 1

Chemical Preparations

Otto buffer I

0.1 M citric acid + 0.5% v/v Tween 20

20 x Saline-sodium citrate (SSC) buffer

3 M sodium chloride

300 mM trisodium citrate

To make 1 L dissolve 175.3 g NaCl and 88.2 g $Na_3C_6H_5O_7 \cdot 2\ H_2O$ in 900 ml water. Adjust pH to 7 and NaOH of HCl in necessary, make up to 1 L and sterilise by autoclaving.

Dextran Sulfate

Stock dextran sufate was prepared at 50% (w/v).

To make 50 ml solution add 40 ml water was heated to 65˚C and dextran sulfate added slowly in stages until all was dissolved. Make up volume up to 50 ml and sterilise by autoclaving. Aliquot into smaller volume for storage.

Giemsa Stain

To make 200 ml Giemsa stain

Add 1.5 g of Giemsa powder (Giemsa Azur-eosin-methylene blue) (Merk Cat. no. 9203) to a clean coloured bottle. Add 99 ml of Glycerol Analar reagent and dissolve at 60˚C (shaking in a water bath). Leave dissolving for 4-5 days at 60˚C. Once dissolved, add 99 ml of methanol (acetone free). Leave for a further 5-6 days at room temperature. Filter into a coloured bottle and leave for 1-2 months before use.

Sorensen's Buffer

0.067 M KH_2PO_4

0.067 M Na_2HPO_4

To make 1 L dissolve the 4.5365 g KH_2PO_4 and 5.933 g anhydrous Na_2HPO_4 in separate beakers using water inside a beaker. Once dissolved, each solution was poured into a sterile bottle and stored at 4˚C. To make Sorensen's buffer add the two solutions together to achieve a pH of 6.8. Store at 4˚C in a coloured bottle.

McIlvaine buffer (pH 7.0)

200 mM Na_2HPO_4

100 mM citric acid

To make 100 ml of McIlvaine buffer mix 82 ml 200 mM Na_2HPO_4 with 18 ml 100 mM citric acid.

FDA Stock

5 mg FDA powder (cat. no. F7378, Sigma) dissolved in 1 ml acetone.

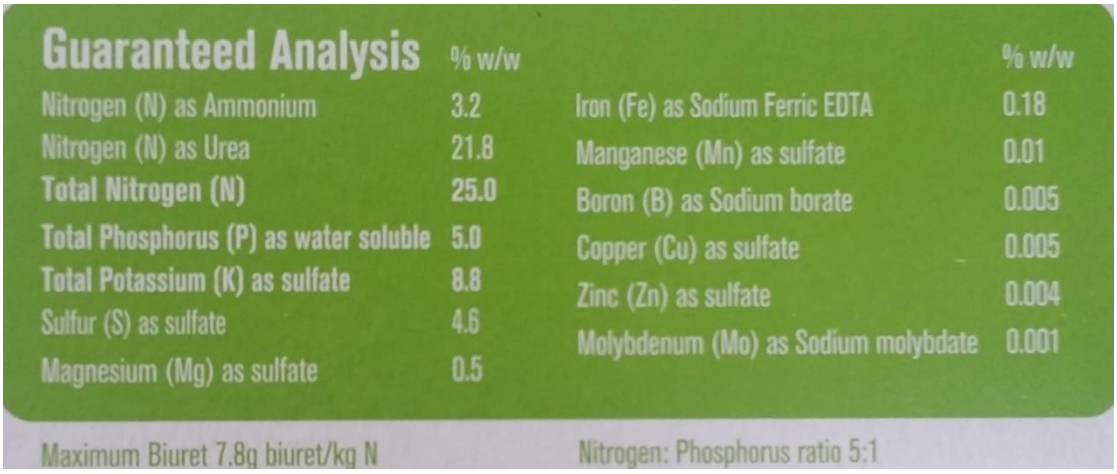
For 10 ml 2 x qPCR buffer

Chemical	Volume/mass
Trehalose	2270 mg
Water	3400 µl
Bioline 10X PCR Buffer (-Mg)	2000 µl
50 mM $MgCl_2$	1600 µl
Dimethyl sulfoxide (DMSO)	800 µl
10% (w/v) Triton-X 100	300 µl

For 1 ml 2 x SYBR Green

Chemical	Volume
qPCR buffer	930 µl
dNTP 20 mM	40 µl
SYBR Green 100X	15 µl
BIOTAQ	15 µl

Yates, Thrive All Purpose Soluble Fertiliser nutrient information



Guaranteed Analysis	% w/w		% w/w
Nitrogen (N) as Ammonium	3.2	Iron (Fe) as Sodium Ferric EDTA	0.18
Nitrogen (N) as Urea	21.8	Manganese (Mn) as sulfate	0.01
Total Nitrogen (N)	**25.0**	Boron (B) as Sodium borate	0.005
Total Phosphorus (P) as water soluble	**5.0**	Copper (Cu) as sulfate	0.005
Total Potassium (K) as sulfate	**8.8**	Zinc (Zn) as sulfate	0.004
Sulfur (S) as sulfate	4.6	Molybdenum (Mo) as Sodium molybdate	0.001
Magnesium (Mg) as sulfate	0.5		

Maximum Biuret 7.8g biuret/kg N

Nitrogen: Phosphorus ratio 5:1

Appendix 2.1

The sequence result for the PCR product from 104H, 1.2 kb. The sequence did not contain the GACA starting GACA repeats and the sequence is ca. 400 bp short.

5’ – AAGCTACGATGGTCTCCTTTGTATTTGTGTATAAATGCTCTAAATTTACCTATATCCTATTTTTTTCAA
ACTTCTTCTTTTACAGCCTTTTATCCCCCCCTCTTTACCAACTACAATAAAAACTTAACTCTCTCTGCAAACT
CCCATCCACCTATAAAAAAAATGGAGGACGATGGGATGCTACAACTGTGAACCAGACCGCAAGTCCCTT
CCTAGCAGAAACGACCGAGGTCTCCTGTTGTGGGGGACGCTTGAGGATGCTGAGTTCGTCTCGCGGAG
GTCGCCGGCGCTGGAATCAAGAGTAAGGACAGCTCAACCAAGTACCAGCTAGGTCACCGATGCTTTGCC
CCGACGATATCATTAACGCAACTCCCTTCCTTCAGACCCCGCCATCAACGCCATGTGTTACCAGAGAAAG
TCAGCCAAAGCAAGAGTCAACATACTTCTAAACTCAGATATAGTTTCATAATCTTTCGAGCGTAACCTCAT
CTCTTCACTAGCAGAAAATGGAACCACTACACCATTGTGACGACGCAACTCTTTTGCTTGATACATCTGT
GACAAAATTCCACCGCGACACTCTATCAGCTTATCCTTAGATCATTAACTAACTGTTGAATCAAACTAAAC
ATACGCATTTTAAAAAGATCGTTTGCCATAGATAGAGTCTTCTTCATTTTCTCACGACCTACACCAGCCAA
CCTCCAGTTCGCACGTGTTTAGTTTCTAATCCCTATGCCTCTTCTCCCTTTCTCTAGTTTCCACGTTCTTTTC
TTTTCCTCCTTATTTAGCTACCATAAAATCATTTACTCGTAGTATATTACTATTTCTCTTACTATTCTTCTTCT
ATTAATAATCAC – 3’

Appendix 2.2

The sequence result for the PCR product from 78H, ca. 0.6 kb. The sequence did not contain the GACA expected repeats at the beginning of the sequence and did not match the sequence published in Pašakinskiene *et al.* (2000). The sequence was ca. 400 bp longer than expected, although the sequence quality was poor.

5’ – ACTTGCGCTCGAGATCAGCACGAGAATGAGGCGTCTTTTTATGTCTGCTTTACGGTGGCCACTTCC
CACGAACCTCACCGGCGCCGCCGTCCACCTCTTAAGATCTAATGGTACCACTCGACGCAGGGTGTCACCA
GCTCCATCACTGTTTTTGAATCAATCAAATCGGTGCATAAACGAAGTCGCCTTCGTCGATCAAAATCGGG
CCAGAAACAGTTTTCGTTTTCAGTTTCGGATCTCGCCCTGTCACGGCCTCAACTTCCGTCGAGCCTCGCCG
TCAAAACGGTACACCTCTTTGAGTCAGACCACCTCCAAAACAATCTCGGCATATCCTTCTTACTCAACCCG
TTGAAACCCACCCGAACCGCTGCTCTGTTTTCTCGCGGCGGGAACTTTAAAATCCGTGCGTAAGAACACG
CATAGTACCACGATCTGTCACGTTTTCATTTCGTTTTCAGTTAACAGAGCTCCCTTCCTTTTTTTGGTCGGT
CGGTCGGGGGTAAACCCGAGGCGAAAGAAGATGTGGAAGACGCGCTGGCGATGGCGCTGGCGCGCG
AGATCGCGCCGAGGAGGTGGAGCGACTACCTCCACATCACCGGCGGGGGCATGGTCACGACGTGAGG
ATCACCGGGCACACTAGCTCGAGCAGCGCGTCGATCTGCACGTGGATAGATTCCGAGTCCCGGTACCGC
TCGCGCCGTTAGGGCCTTGATCCCTGGCTAGTTCCGGGCCTTGCACTGCGATATCTCCGCGAGAACAGAT
ATCCATTGCAGGGGGACCTCGCCGTTGGCGGGAGCATGGAAACTAGACGACGGCGGAGTGGCAAAAA
GCGTGCTTTAGTTCGTTACTTCTTTTACTATTCTGGAAGGTATAAAGATACTCAAAAGTGGTAACCATGAT
GCACCAATGGCCCCTGCCGAGCGAGGATCAAACAGTTTTACCGGCCGGCGACGACATGTTTTCATGTCA
TTGTTTACCGGCGCCGGTCCATTTGGCTTTGACTTGGGTTTTTCCCACGTGTTTTTTGGTCCCGGAGATTT
TTCCCTCTTTCTCT – 3’

Appendix 2.3 a

Sequence results of the plasmid isolated from colony one using the sense primer.

5’ – GAATTACTTCTATAGGGGCGATTGATTTAGCGGCCGCGAATTCGCCCTTCGACAGACAGACAGACA
AGAAAAGGAATGGAGCTCTGTTAACTGAAAATGAATTGAAAACGTGACAGATCGTGGTACTATGCGTG
TTCTTACGCACGGATTTTAAAGTTCCCGCCGCGAGAAAACAGAGCGGCGGTTCGAGTGGGTTTCAACGG
GTTGAGTAAGATGGATATGCCGAGATTGTTTTGGAGGTGGTTTGACTCAAAGAGGTGTACCGTGTCGAC
GGCGAGGCTCGACGGAAGTTGAGGCCGTGACACGGCGAGATCCGAAACTGAAAACGAAAACTGTTTCT
GGCCCGACTTTGATCGACGATGGCAACTTCGTTTCTGCACCGATTTGATTGATTCAAAAACAGTGATGGA
GCTGGTGATGCCATGCGTCGAGTGGTACCATTAGATCGTGCTGAGGTGGACGGTGGCGCCGGTGAGGA
TCGTCGGAAGTGGCCACCGTGAAGCAGACATAAAAAAGACCCCTTTTTCTCGTTTTTGATCTCGATCTCT
CTCTCTCCCTCGTGTCTGTCTGTCTGTCGTAAGGGCGAATTCGTTTAAACCTGCAGGACTAGTCCCTTTAG
TGAGGGTTAATTCTGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCA
CAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAAC
TCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATG
AATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTC
GCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCAC
AGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAA
AAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAA
GTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACAAGGCGTTTCCCCCTGGAAGCTCCCTCGTG
GCGCTCTCCTGGTTCCGACCCTGGCCGCTTAACCGGAATACCTGGTCCGCCTTTTTTCCCTTTCGGGGAA
G – 3’

Appendix 2.3 b

Sequence results of the plasmid isolated from colony two using the sense primer.

5’ – AGAATTAGTCGTATAGGGCGATTGAATTTAGCGGCCGCGAATTCGCCCTTAACGACAGACAGACA
GACATACCCATAGCAGCCCCGGACAACTCATCGCCGGGGGAGGAGCCAACGAGTCCCTCGCATACTGTT
CCCCACTGAATCAAAATGGCGACGCGGATGGACGGAGGGAGATGGAGAAATCTCCAGTGTCATCAGAT
CTCACGGACGAACAGGAAAGCTGGAACGGGGGCCTTCGCCCTTGCAGGTGAAGGAGGTTGTCAGCAAA
CAGGATTTGTCACCGGAATCGCGCCACGGGGAGTTGTCGTCGACTCGTCGAGGCTGCGCTCGCCGGAC
ATGGGGGACTGAAGACTATACACGATTGGGGAATTGCGTGATTAAATGGGCTTCAACTTTGTTTTAACT
GAAAACATCCACCACCTTATCTTGGCCGCTGTCTGTCTGTCTGTCGTAAGGGCGAATTCGTTTAAACCTG
CAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGT
GAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTG
CCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTC
GTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCG
CTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGC
GGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAA
AGGCCAGGAACCGTAAAAAGGCCGCGTTGCCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCAT
CACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCC
CCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCC
TTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAG – 3’

Appendix 2.3 c

Sequence results of the plasmid isolated from colony three using the sense primer.

5’ – CTAGCTTTTGGCCATGTAAGCCCCCTGCAAGCTACCTGCTTTCTCTTTGCGGCTTGCGTTTTCCCTTGT
CCAGATAGCCCAGTAGCTGACATTCATCCGGGGTCAGCACCGTTTCTGCGGACTGGCTTTCTACGTGAAA
AGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGCCTGACATTTATATTCCCCAGAACATCAGGTTAAT
GGCGTTTTTGATGTCATTTTCGCGGTGGCTGAGATCAGCCACTTCTTCCCCGATAACGGAGACCGGCACA
CTGGCCATATCGGTGGTCATCATGCGCCAGCTTTCATCCCCGATATGCACCACCGGGTAAAGTTCACGGG
AGACTTTATCTGACAGCAGACGTGCACTGGCCAGGGGGATCACCATCCGTCGCCCCGGCGTGTCAATAA
TATCACTCTGTACATCCACAAACAGACGATAACGGCTCTCTCTTTTATAGGTGTAAACCTTAAACTGCCGT
ACGTATAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGC
GAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTA
AAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGAATTGAATTTAGCGGCCGCGAATTCGC
CCTTACGACAGACAGACAGACATGCACAATATCTAGCAGATGAGAAATAGCAACAGGAGTTACCAAAA
GTGTGAGAATACCTGAATACCTGATAATAGGGTGTTATTAACTCTAAGTTTGTAGCACTGGACAAGCCTT
CAAGGACCACAGAAAAGCTTTCTTGAGCATACTGCTCACAATCAATAGACTCATTGACGAGGGATCACCT
CGCCAATGGCTACGTATTGTAGACTTGGGTTTTGGGAGAGAGCGACGATGGAGATTCTGGGGACGAGA
TTAGGCACACGACGTACCCAGCTTCGGGTCCCCTCGGTGGAGGATCCCTACGTGCTGCTAGCAATCACA
GTATATGATCATATGTATGTTTACAGGGTGCCGCCATAGGCGGAGCTATATTGTCTATCTGTCTGTCTGT
CTGTCGTAAGGGCGAATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGTAATCTTAGGTTACCCC
– 3’

Appendix 2.3 d

Sequence results of the plasmid isolated from colony three using the sense primer.

5’ – GGATTACTCGTATAGGGCGATTGAATTTAGCGGCCGCGAATTCGCCCTTACGACAGACAGACAGAC
ACGCACACACACACAGACAACTTGTGTTGCGCTCGCAGCTTGATTCCGTTGCGGACTCGATGATATGAG
GCCTCGGAAGTGCCCATTATGTTGCTTGTTAGCGGCCCACAAGGGAGTTGGCGCCACTCTCTTCGGCGC
TCGTTGATGTGTGTCTGTTTTTCATTCAGTTTTGCGCTCACGCCCGATCTTTGCAGTCTCGATAGTGGTGT
TTAGCCAGCACCGATAGCAAGCACCGATTGCAAGCCTCGTCGGCGACAACAACAAACTCGGACTTTGGT
GACGAGCGTCGGCGCCGCGTCACAACGCGCAAGCCGCCCTTCGATCGCCAGAGAACTCGAATCGGGCC
TCAGTCGGTGTGTCAGGCTTGTCGCTTGGGGGCGTGTCCGGCTCGAGAGAGCGTCTTGCCAGATTTGAT
TTGCGCGATTCAACCGAGCAGTGCACTCTAGAGCTAGACCTCTGTCTGTCTGTCTGTCGAAGGGCGAATT
CGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGCTTGGCGTAATCATGGTCATAGC
TGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCCGGAAGCATAAAGTGTA
AAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGGCCGCTTTCCAGTC
CGGAAACCTGTCGTGTCAGCTGCATTAATGAATCGGCCAACGCGCGGGGGAGAGGCGGTTTTGCGTAT
TGTGGCGCTCTTCCGCTTCCGTCGCTCCACTGACTCGCTGCGATTAGGTCGTTCAAGTGCAGGCGAGCG
GATATCAGCTCACTCAGAGGCGGAAAATACGGGTTATCCACAGAAATCAGGGGGATAACGCAAGGAAT
CAACATGTGAGCCAAAAGGCCAGCAAAAATGGCCAGGTAACCGTAAAAAGGGCCGCATTTGCCTGGCC
GTATTTCCATAGGGCTCCAGCCCCCCTTGACCGAGAATAACTAAAAATCGGACGCTTCAAGTCATAAGGT
GGCTGAAACCCTGACAGGGACTATTAAAGCATACCTTGGCCGTTTTCCCGCCTAGGAAAGGTCCCCTTCG
TGCGATCTTCCTGGTTCCGAACCCTTGCCCGCTTTACCGGGAAAACGTGGTCCGGCCTTTTTTCC – 3’