

# VALIDITY OF FLOW CYTOMETRY FOR TESTING PLOIDY LEVEL IN RYEGRASSES

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## Introduction

Of the eight recognized ryegrass (*Lolium*) species, perennial ryegrass (*L. perenne* L.) and annual or Italian ryegrass (*L. multiflorum* Lam.) are the most predominant used commercially worldwide for forage and turf. These two species represent about two thirds of the grass seed acreage in western Oregon. All naturally occurring *Lolium* taxa where chromosomes have been counted are diploid with  $2n=14$  (Terrell, 1968). Starting in the late 1930s, tetraploid ( $2n=4x=28$ ) forms of these two species and their hybrid, intermediate ryegrass (*L. X hybridum* Hausskn.), were developed in attempts to improve forage productivity and nutritive value.

In order to sell cultivars labeled as tetraploids, determining an accurate ploidy level is important to the grass seed industry. Cultivars pure in ploidy level are necessary because crossing among plants with different ploidy levels may result in genomic instability and uneven numbers of chromosomes, often leading to infertility and lower seed yields. Morphological characteristics of grasses with different ploidy levels can be manifested in herbage moisture content, seed size, seedling vigor, forage productivity, establishment ability, milk quality for cows, cold tolerance, competitive ability, and concentration of sugars and digestible organic matter. These different morphological characteristics, however, are not always apparent in detecting ploidy level itself. Furthermore, an accurate ploidy determination, where varieties for USA domestic sale must be at least 98% of a reported ploidy level, is required for certification of cultivar purity.

Ploidy levels in grasses have traditionally been determined via microscopic counting of chromosome numbers. This process involves collecting tissues undergoing cell division, such as root tips or pollen mother cells, and arresting the cells at the metaphase stage. The cells are then stained and squashed on a microscope slide for examination. The process is very time-consuming, and the technique requires experience in preparing and squashing the cells. Accurate counting for rapid testing in seed laboratories is often difficult because chromosomes overlap, are inadequately spread, and polyploid cells have large numbers of very small chromosomes. Such difficulties can lead to observer bias.

In recent years a new technique, flow cytometry, has emerged for ploidy determination. Flow cytometry was first used to examine cell cycles, DNA content, and ploidy

levels in humans and other animals, but by 1982 the technique was also adapted for DNA content determination in plant material. Flow cytometry is now well accepted by turf- and forage-grass researchers.

Flow cytometry involves the separation of intact nuclei from living plant material (usually young, fully extended leaves in the case of grasses) using a lysis buffer. After separating debris by centrifugation or filtration, the nuclear suspension is dyed with a fluorophore. The dyed suspension is then injected into the flow cytometer. Nuclei are taken into a tube via a current of water or buffer solution and passed, one by one, across a tight beam of light filtered to the absorption wavelength of the fluorophore. The light source can be either arc lamp (mercury, mercury-xenon, or xenon) or laser-based. The fluorophores bound to the nuclear material fluoresce, and the emitted wavelength is read by a series of photomultipliers. The larger the amount of DNA in the nuclei, the greater the intensity of the emitted wavelength. Data are fed into a computer, and a peak analysis program produces a histogram that conveys ploidy level and basic statistics from which the amount of DNA may be determined.

Flow cytometry has been recognized as being superior to microscopic chromosome counts (Galbraith et al., 1997) for a number of reasons. With recent technical improvements in modern flow cytometers, it is now a matter of days instead of months for a researcher to become confident with the technique. Leaf material can be collected at any growth stage, leaving the plant alive, and only a small amount of living material is necessary. User bias is virtually eliminated. Samples can be run in bulk, greatly increasing the amount of material that can be examined, and internal standards can be included for use in correcting shifting effects and determining DNA content. Furthermore, tens of thousands of cells can be counted in a single run, producing data with low statistical error.

The present study examined a large number of plants from Italian, perennial, and intermediate ryegrass seed lots using both flow cytometry and root tip chromosome counting. Our objective was to develop methodology for implementing flow cytometry in grass seed testing and verifying that, indeed, flow cytometry is accurate for determining ploidy level in ryegrass.

## Materials and Methods

Three seed lots each of tetraploid Italian, perennial, and intermediate ryegrass cultivars were planted from seed into small conical pots with vermiculite. Each seed lot came from a different cultivar, except two seed lots of intermediate ryegrass (154909 and 170535) came from different production years of the same cultivar. Plants were grown in a greenhouse during the summer and fall of 1998 with a 12-16 hr photoperiod (combination sunlight and full-spectrum, high-pressure sodium lamps), in a temperature of

21°C. Plants were fertilized about every two weeks with a Peters 20-20-20 (N-P-K) combination. Flow cytometric and cytologic analysis of plants began once they had a fully expanded blade and continued through maturity.

Flow cytometry was performed on 200 plants per seed lot, essentially as performed by Galbraith et al. (1983) and Pfosser et al. (1995b). Young leaf tissue was collected by clipping relatively equal portions of blades. Leaf tissue of a tall fescue internal standard, *Festuca arundinacea* Schreb. cv. Arid, determined to have 16.69 pg DNA, was used with all scans to correct for shifting of peaks on the flow cytometer and to calculate relative DNA content of the unknown samples. Plants were examined singly or in bulk samples. Bulk samples were composed of no more than sixteen plants. If a diploid plant was detected, the bulk sample was halved and each half examined separately. In this manner the samples were narrowed down until the diploid plant of interest was isolated.

Tissues were chopped with a double-edged razor blade in 500µl of ice-cold commercial Partec buffer solution to release nuclei and allowed to wash for 1.5 min. The suspension was poured through a 30 µm filter to remove debris. Two ml of ice-cold commercial Partec DAPI solution was added (making a 1:4 buffer:DAPI ratio). The suspension was vortexed and allowed to sit for 5 min before vortexing again. The suspension was analyzed on a Partec PA flow cytometer (Partec GmbH, Otto-Hahn Straße 32, D-48161 Münster, Germany) and allowed to count for at least 20,000 cells. Means and coefficient of variance percentages of peaks were calculated by an internal peak analysis program of the flow cytometer and data values were recorded by hand. Ploidy level was determined based on position of the G1 peaks of unknowns in relation to the internal standard G1 peak.

Mean peak positions of the sample G1 peaks were normalized using the tall fescue internal standard to correct for shift. DNA content was calculated using the normalized peak values and the calculations outlined by Arumuganathan and Earle (1991) and used for relative, intraspecific comparisons.

Root tip squashes and chromosome counts were performed on at least 40 tetraploid plants of each variety and as many diploid and aneuploid plants as possible using the Feulgen technique. Young, translucent root tips were collected around midnight and placed in distilled water for 8-10 hours at 4°C. They were then fixed for 30 minutes in Carnoy's Fluid with chloroform at 4°C, treated with 5N HCl for 2 minutes at 55°C, and stained with Feulgen's solution for 1 hour at room temperature. Root tips were washed with distilled water and frozen for later examination. Once ready for analysis, tips were thawed and counterstained with aceto orcein (1 g orcein to 25 ml glacial acetic acid and 25 ml distilled water) for at least 5 minutes. After

squashing and spreading on a slide, the chromosomes of cells in prometaphase and metaphase were counted at 1600x on a Zeiss Axioplan light microscope with a green filter. At least five cells per plant were counted and averaged. These counts were then compared against the results from the flow cytometer.

## Results and Discussion

Actively dividing cells undergo a series of four temporal phases which are defined by the amount of DNA present, called the cell cycle (Galbraith, 1984). The cell cycle is comprised of a period preceding DNA synthesis (termed G1) where most cells are at any given time; DNA synthesis (S), a period prior to splitting of the cell (G2) which has twice the amount of DNA as G1; and the phase where the cell split (M). For somatic cells, the phase where the cell splits is called mitosis. Flow cytometry measures the amount of DNA present in each cell and a frequency analysis of a large number of cells reveals peaks reflecting that amount. The resulting histogram shows two defined peaks (G1 and G2), of which the G1 peak is larger because more cells are in this phase. The other phases of the cell cycle show up as little more than background noise. The major phases are present regardless of ploidy level, but tetraploid plants, having twice the DNA complement and number of chromosomes, have peaks at double that of their diploid counterparts.

A total of 381 final scans were run using flow cytometry, comprising 1794 individual plants and nine tetraploid ryegrass seed lots in composite samples of no more than sixteen plants each. Diploid contamination in tetraploid seed lots ranged from 0 to 25.5% (Table 1). As expected, DNA content of diploid plants ( $\bar{0} = 5.74$  pg) was about half the DNA content of tetraploid plants ( $\bar{0} = 11.34$  pg). Four seed lots (one Italian ryegrass, 165249, and all three intermediate ryegrasses) failed to meet the two-percent purity level required for seed certification.

Root tip squashes were performed on a total of 397 plants as a means of confirming flow cytometry results. Chromosome counts, representing at least five cells per plant and averaged over all plants in a ploidy level category ranged from 14.2 to 15.0 for diploid plants, 27.8 to 28.6 for tetraploid plants, and 30.9 for aneuploid plants (Table 2). A total of 2495 cells were counted. When testing speed is of the essence, chromosome counts of root tip cells were often inflated by chromosome overlapping and "guessing" demarcation. A careful cytogenetic analysis was not performed on most of the plants studied because techniques and conditions comparable to production testing were desired.

Three aneuploid plants were identified by flow cytometry (Tables 1 and 2). Two of these were confirmed using root tip squashes; the third had died before root tips were collected.

Identification of aneuploids using flow cytometry was determined through the appearance of bimodal peaks (Pfosser et al., 1995 a & b). The difference in DNA between the aneuploid plant and a tetraploid from the same was large enough to form distinctly different peaks, yet similar enough for the peaks to merge, forming the bimodal peak.

Confirmation of aneuploid plants was done using root tip squashes and based on the average chromosome counts. Using rapid chromosome counting techniques, variation in chromosome count averages was about 1.5 chromosomes. Any plant with an average count falling outside that range was an aneuploid suspect and was re-examined.

Flow cytometry is the better method for finding and identifying aneuploid plants because of the speed of the procedure and the ability to examine large numbers of plants. A major drawback to ploidy identification using bulk plant samples is that bulk samples may mask the presence of aneuploid plants, whereupon they would probably be classified as tetraploids. For production seed testing, the industry requires bulk sampling to increase efficiency and speed, running up to sixteen or more samples per scan. In the current study, identifying an aneuploid amongst many euploids was not confidently possible with a bulk sample larger than eight plants. Due to this problem, only one of the three aneuploids in the current study was initially identified using flow cytometry. The other two were initially identified using root tip squashes and later confirmed with flow cytometry.

Though the main goal of the present study was to investigate testing alternatives to determine ploidy level, a determination of relative DNA amounts was also performed (Table 1). While an absolute value for DNA content could not be obtained because of the A-T bias of DAPI dye, for the purposes of ploidy determination in grass seed testing and intraspecific comparisons of relative DNA amounts, DAPI is a suitable choice. With the exception of one of the aneuploids (seed lot 170535), DNA content (and peak position) correlated well with the root tip counts and served well in further distinguishing aneuploidy.

Misclassification errors (i.e. where data indicates that a plant is of a certain ploidy level and is later shown to be of the other) differed depending on the technique. Including the detection of aneuploids, misclassification errors from root tip squashing occurred 1.1% of the time (4 out of 363 plants). Such mistakes may have occurred due to severe overlap of chromosomes, mislabeling, or investigator bias. In contrast, flow cytometry had a misclassification error rate of 0.3% (5 out of 1794 plants) due to erroneously cutting the leaf of a neighboring leaf for the bulk sample and large bulk samples obscuring aneuploids. The difference of 0.8% seems small, but it is highly important when attempting to make the 2% purity level for certification purposes.

Overall, flow cytometry was shown to be the superior technique for ploidy determination. Though the initial cost of a flow cytometer and accessories may seem daunting, the speed, accuracy, and efficiency of the method should more than pay for it in the long run. It can take months for a microscopist to become confident in performing root tip squashes and counts. Even an experienced researcher, counting the chromosomes of five cells per plant, can examine no more than 20 plants in a good day. Yet with less than a week of training, a previously untrained researcher, working alone, can confidently determine the ploidy level of over 400 plants in a good day.

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Table 1. DNA content of nine ryegrass seed lots as determined by flow cytometry.

Seed lot number	Diploid		Tetraploid		Other		% Diploids
	No. of plants	DNA content <sup>†</sup> (pg)	No. of plants	DNA content (pg)	No. of plants	DNA content (pg)	
<b>Italian ryegrass</b>							
172338	2	5.62	198	11.03	0	--	1.0
163303	0	--	200	11.13	0	--	0.0
165249	7	5.91	192	11.91	1	14.32	3.5
<b>Intermediate ryegrass</b>							
167773	51	5.51	148	11.18	1	16.69	25.5
154909	10	5.89	190	11.35	0	--	5.0
170535	13	5.87	186	11.26	1	10.11	6.5
<b>Perennial ryegrass</b>							
167258	0	--	200	11.39	0	--	0.0
172003	1	5.65	199	11.48	0	--	0.5
169515	0	--	194	11.35	0	--	0.0

<sup>†</sup>Calculated DNA content per cell estimated by method of Arumuganathan & Earle (1991), averaged over individual or bulk samples within a seed lot.

Table 2. Chromosome counts in root tips from plants in nine ryegrass seed lots.

Seed lot number	Diploid		Tetraploid		Other	
	No. of plants	Chromosome count	No. of plants	Chromosome count	No. of plants	Chromosome count
<b>Italian ryegrass</b>						
172338	2	14.2 ± 0.1	40	28.0 ± 0.1	0	--
163303	0	--	40	27.8 ± 0.1	0	--
165249	6	14.8 ± 0.3	34	28.2 ± 0.2	†	--
<b>Intermediate ryegrass</b>						
167773	15	14.3 ± 0.1	40	28.6 ± 0.2	1	30.9 ± 0.6
154909	7	14.6 ± 0.2	40	28.4 ± 0.2	0	--
170535	10	14.3 ± 0.1	40	28.4 ± 0.1	1	30.9 ± 0.8
<b>Perennial ryegrass</b>						
167258	0	--	40	28.4 ± 0.2	0	--
172003	1	15.0 ± 0.5	40	28.6 ± 0.1	0	--
169515	0	--	40	28.0 ± 0.1	0	--

<sup>†</sup>Individual died before root tip squashes could be performed.