Journal of Berry Research 3 (2013) 135–149
DOI:10.3233/JBR-130057
IOS Press

Limitations of morphological ploidy estimation methods in *Fragaria*

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Received 04 August 2013; accepted 26 September 2013

Abstract.
BACKGROUND: Strawberry (*Fragaria spp.*) is prone to natural polyploidization. Strawberry shoots regenerated through tissue culture callus may exhibit variations in ploidy. Rapid and accurate ploidy estimation is important for basic research as well as cultivar improvement.
OBJECTIVES: Ploidy-measurement methods are well established in strawberry and other plants. The objective of this work was to define the strengths and limitations to various ploidy-estimation tests.
METHODS: Measurements were performed on a set of known diploids and polyploids as well as synthetic colchiploids. Comparisons were made using petiole size, stomatal size, leaf dimensions, and pollen diameter and compared to flow cytometry results.
RESULTS: Simple methods like leaf proportions and stomatal size may vary greatly based on environmental factors. Pollen measurement proves reliable, but only within tetraploids arising from a single genotype. Measurements made with flow cytometry reliably indicated ploidy and revealed within-plant variation such as mixoploidy.
CONCLUSIONS: Methods for measuring ploidy in *Fragaria* vary in complexity, ease, execution time and precision. This work defines the strengths and limitations of several methods, along with the considerations required for accurate comparisons between genotypes and ploidy levels.

Keywords: Strawberry, *Fragaria*, polyploidy, colchicine, flow cytometry

1. Introduction

The genus *Fragaria* includes about 21 species ranging in natural ploidy levels from diploids ($2\times = 2n = 14$) to decaploids ($2\times = 10n = 70$) [1–3]. This natural diversity in ploidy is of interest as it applies to strawberry ecology and evolution, as well as its role in shaping the genome of the cultivated, octoploid strawberry (*F.* × *ananassa*). The range of ploidy levels is due to the production of unreduced gametes, which is surprisingly frequent in strawberry [4]. Changes in ploidy may also occur from somatic doubling or through tissue-culture regeneration from callus tissue, especially when high hormone levels or mutagens are present [5].

The diploid strawberry *F. vesca* has gained favor as a transgenic system to study gene function [6], yet a significant number of callus-regenerated plants from the accession Hawaii 4, 5AF7, or 'Baron Solemacher' are tetraploid [5]. It is important to reliably identify and eliminate polyploids from downstream analyses, as these plants may exhibit considerable differences in size, physiology or gene expression.

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Breeding efforts in the cultivated strawberry have traditionally emphasized improved shipping, postharvest performance, disease resistance and fruit size often at the expense of flavor [3]. Lower ploidy genotypes possess desirable traits, such as superior flavors and aromas, yet they lack commercial attributes like fruit size and firmness. Ploidy differences create a genetic barrier for introducing these beneficial traits directly into commercial germplasm [7]. There has been interest in developing synthetic octoploids that are interfertile with commercial materials [8, 9]. The generation of synthetic polyploids from lower-ploids (as in 10, 11) with enhanced traits may provide a means to introduce genes into commercial strawberry with benefits throughout the supply chain from farmer to consumer.

While the most accurate method to discern ploidy is chromosome counting, strawberry metaphase chromosomes are small, tightly packed and have similar shapes [12]. Although it is possible to produce karyotypes from higher-ploid strawberries, the techniques require special expertise [13], and are not practical for rapid determinations or for use in the field.

Morphological differences between ploidy levels have been reported [14]. These include variations in leaf size, petiole diameter, pollen size, stomata size, flower size, leaf serrations, leaf thickness, green color, and undulation of the leaf surface. Flow cytometry has been frequently used to estimate ploidy in strawberry [15–17]. These methods vary for ease of ploidy estimation, accuracy, and technical requirements.

The purpose of this study is to compare ploidy estimation methods across a sample of natural species and induced polyploids. Petiole size, stomata size, pollen grain size, and leaf proportions were measured in a set of plants exhibiting various levels of ploidy, including newly-generated materials. The relationship between these traits and strawberry ploidy is discussed.

2. Materials and methods

2.1. Plant materials

All plant materials were greenhouse maintained (University of Florida, Gainesville, FL) under natural lighting. The genetic lines used in this study include both natural and induced *Fragaria* polyploids, and are presented in Table 1. *F. vesca* 'Baron Solemacher' (PI 660766) is an autotetraploid obtained from the USDA-ARS National Clonal Germplasm Repository (NCGR) in Corvallis, Oregon. Other polyploid genotypes were generated as described below. The "GA", "Mig", "FdiB" polyploids were obtained from colchicine treatment of *F. vesca* 'Golden Alpine', 'Mignonette' and 'Fragoli di Bosco', respectively. Three Mig, two FdiB and two GA tetraploids were analyzed in this study. The "Mig5×Fest" line is an F1 hybrid between a *4×* colchiploid of *F. vesca* 'Mignonette' called Mig5 (female) and *8x F* x *ananassa* 'Strawberry Festival' (male). "MaraA13" is a morphologically-distinct, putative 16-ploid genotype from *F.* × *ananassa* 'Mara des Bois' obtained via tissue culture. All experiments on polyploids derived directly from diploids were performed on seed-propagated plants. Trials for all other-ploidy-level genotypes were conducted on stolon-borne clonal plants.

2.2. Colchicine doubling

Colchicine doubling was performed as previously described [18] with minor modifications. Briefly, surface-sterilized seeds were germinated in light. Once seedlings reached the cotyledon stage, they were immersed in aqueous 0.2% colchicine, in the dark, with gentle rocking without complete immersion. After 24 h the seedlings were rinsed in sterile, distilled water and transferred to soilless media. After 1–2 months seedlings exhibiting larger sized, or mis-proportioned leaves were selected for further study. The individual lines are noted in Table 1.

2.3. Flow cytometry

Tissue for flow cytometry was either from two leaf discs (using a hole punch) from a mature leaf, or an entire unexpanded trifoliate leaf. The tissue was chopped with a new razor blade as described [16] in 1 ml of the nuclear isolation buffer used by Marie and Brown (50 mM glucose, 15 mM NaCl, 15 mM KCl, 5 mM Na_2EDTA, 50 mM Sodium Citrate, 0.5% Tween 20 (v/v), 50 mM HEPES pH 7.2, 1 ul/ml 2-mercaptoethanol, 0.1 mg/ml RNase, and 0.05 mg/ml

Table 1

A list of genotypes used in this study. Plant Introduction (PI) numbers are provided for all accessions obtained from the USDA-ARS-NCGR in Corvallis, Oregon

Cultivar/Genetic line	Species	Genotype	Ploidy	GRIN accession
Mig	*F. vesca*	'Mignonette'	2×	PI 616935
Mig3	*F. vesca*	'Mignonette' colchiploid	4×	*this study*
Mig4	*F. vesca*	'Mignonette' colchiploid	4×	*this study*
Mig5	*F. vesca*	'Mignonette' colchiploid	4×	*this study*
Baron	*F. vesca*	'Baron Solemacher'	2×	PI 551507
BS4×	*F. vesca*	'Baron Solemacher' autotetraploid	4×	PI 660766
GA	*F. vesca*	'Golden Alpine'	2×	PI 602576
GA2	*F. vesca*	'Golden Alpine' colchiploid	4×	*this study*
GA12	*F. vesca*	'Golden Alpine' colchiploid	4×	*this study*
FdiB	*F. vesca*	'Fragoli di Bosco'	2×	
FdiB2	*F. vesca*	'Fragoli di Bosco' colchiploid	4×	*this study*
FdiB12	*F. vesca*	'Fragoli di Bosco' colchiploid	4×	*this study*
F. orientalis	*F. orientalis*	*F. orientalis*	4×	PI 637945
F. moschata	*F. moschata*	*F. moschata*	6×	PI 551528
Mig5×Fest	*F. vesca × F. × ananassa hybrid*	*F. vesca* Mig5 x *F. × ananassa* 'Strawberry Festival'	6×	*this study*
F. chiloensis	*F. chiloensis*	*F. chiloensis*	8×	PI 616934
F. virginiana	*F. virginiana*	*F. virginiana*	8×	PI 612495
Festival	*F. × ananassa*	'Strawberry Festival'	8×	PI 664337
Mara des Bois	*F. × ananassa*	'Mara des Bois'	8×	
Elyana	*F. × ananassa*	'Elyana'	8×	
LF9	*F. × ananassa*	'LF9'	8×	
ElyxFvir	*F. × ananassa*	'Elyana' × *F. virginiana*	8×	*this study*
ElyxMara	*F. × ananassa*	'Elyana' × 'Mara des Bois'	8×	*this study*
Guelph S01	Synthetic octoploid	*F. moschata* and *F. nubicola*	8×	PI 551517
Escape	*F. × ananassa*	'Escape'	8×	PI 641087
MaraA13	*F. × ananassa*	'Mara des Bois' 16-ploid	16×	*this study*
F. iturupensis	*F. iturupensis*	*F. iturupensis*	10×	PI 641091

propidium iodide) [19]. Side-by-side comparisons showed that this buffer yielded the most easily deciphered and reproducible data (not shown) compared to five others previously described [20]. The finely chopped samples were filtered through 20 μm filters (Partec, Germany) into amber microcentrifuge tubes, centrifuged for 3 min at 1000× *g*, decanted, resuspended in 500 ul buffer, incubated for 10 min at 4°C, and centrifuged again for 3 min at 1000× *g*. The supernatant was decanted and nuclei resusupended in 30 ul of the buffer used by Marie and Brown [19] for analysis. All nuclei preparation steps were performed on ice or at 4°C. Flow cytometry was performed on a BD Accuri C6 Flow Cytometer using BD software. Diploid *F. vesca* 'Baron Solemacher' (an inbred line obtained from Dr. Janet Slovin, USDA-ARS), tetraploid *F. orientalis* (PI637945; GRIN), hexaploid *F. moschata* (PI551528; GRIN), and octoploid *F. × ananassa* 'Elyana' (from the University of Florida strawberry breeding program) were used as qualitative flow cytometry controls for ploidy level determination.

2.4. Pollen and stomata measurements

For pollen experiments, anthers were collected from naturally flowering, healthy plants and dehisced in petri dishes containing dryrite. Pollen was collected in a drop of distilled water and placed on a microscope slide with cover slip. One-hundred plump pollen grains were measured using images captured by a calibrated dissection scope. Calibration was confirmed using a hemocytometer.

Stomata were measured using the leaf peal method. Fully expanded and healthy leaves were used for analysis. Clear nail polish (Wet n Wild® Wild Shine®) was liberally applied to the abaxial leaf surface and allowed to dry for a few hours at room temperature (~22°C). A leaf peel was then created by pulling off the dried nail polish from the leaf surface with fine forceps. The dried nail polish imprint, or leaf peal, was examined microscopically. Stomatal aperture was defined as the maximal longitudinal size of the stomata, as defined by the length of the guard cells. Fifty stomata were measured using the calibrations described above for pollen.

2.5. Petiole diameter and leaf measurements

Petiole diameter and leaf measurements were taken with a digital caliper. Petiole data was collected from all petioles from five plants of a single genotype when possible. Only one plant of the 16-ploid (MaraA13) was available for analysis. Nine leaves from each of five plants were measured for both length and width using a digital caliper again where possible. The width:length ratio of the entire trifoliate leaf was calculated and averaged across all measurements for each genotype.

2.6. Data analysis

Data analysis was carried out using the JMP 8.0 statistical package. Tukey's HSD was used for all multiple comparisons with a 0.05 α level.

3. Results

3.1. Identification of colchiploid seedlings

Juvenile colchiploids were first distinguished from wild type plants by an increased petiole diameter (see Fig. 1). The emerging leaf from these plants was substantially larger than the wild type emerging leaf. The expanded leaves were visually thicker, darker green, and the serrations were deeper and sharper into the leaf. These putative colchiploid plants were propagated by separating branch crowns and/or planting seeds from self-pollination. Ploidy was eventually confirmed using flow cytometry.

3.2. Verification of ploidy by flow cytometry

Flow cytometry has been widely used to measure the ploidy of *Fragaria* accessions [16, 21, 22]. In this study it was important to measure, or in many cases confirm, the nuclear content of materials used for mophological ploidy tests. This critical step ensures that results from other ploidy evaluation techniques relate to the actual ploidy of the materials used. It also ensures against misidentified materials obfuscating results. The preparation of nuclei for the basic flow cytometry assay required some optimization. A variety of buffers have been reported to prepare strawberry nuclei for flow cytometry. In this study the buffer presented in Marie and Brown [19] provided superior results relative to others tested (data not shown).

To calibrate the assay, nuclei from diploid 'Baron Solemacher' (peak 1), tetraploid *F. orientalis* (peak 2) hexaploid *F. moschata* (peak 3) and octoploid *F. × ananassa* 'Elyana' (peak 4), were measured as size standards (Fig. 2A). Other genotypes used in downstream analysis were then tested for their relative nuclear DNA content compared to these standards. One single fluorescence peak was identified in representative octoploids *F. chiloensis*, *F. virginiana* and *F. × ananassa* (Fig. 2B). Two additional *F. vesca* genotypes with known ploidy differences are shown (Fig. 2C), 'Baron Solemacher' (peak 1) and the Baron Solemacher autotetraploid (peak 2). The G2 peak from 'Baron Solemacher' was in the same position as the G1 peak from BS*4*×. Figure 2D shows the octoploid 'Elyana' (peak 1) compared to the decaploid *F. iturupensis* (peak 2). Results from the diploid *F. vesca* 'Mignonette' and the colchicine-induced polyploid Mig5 are presented (Fig. 2E). The results from a cross between the tetraploid Mig5 and *F. × ananassa* 'Strawberry Festival' are presented (Fig. 2F). The peaks represent the tetraploid *F. orientalis* (peak 1), the hexaploid *F. moschata* co-detected with the tetraploid/octoploid cross (peak 2) and the octoploid parent (peak 3). For clarity, the

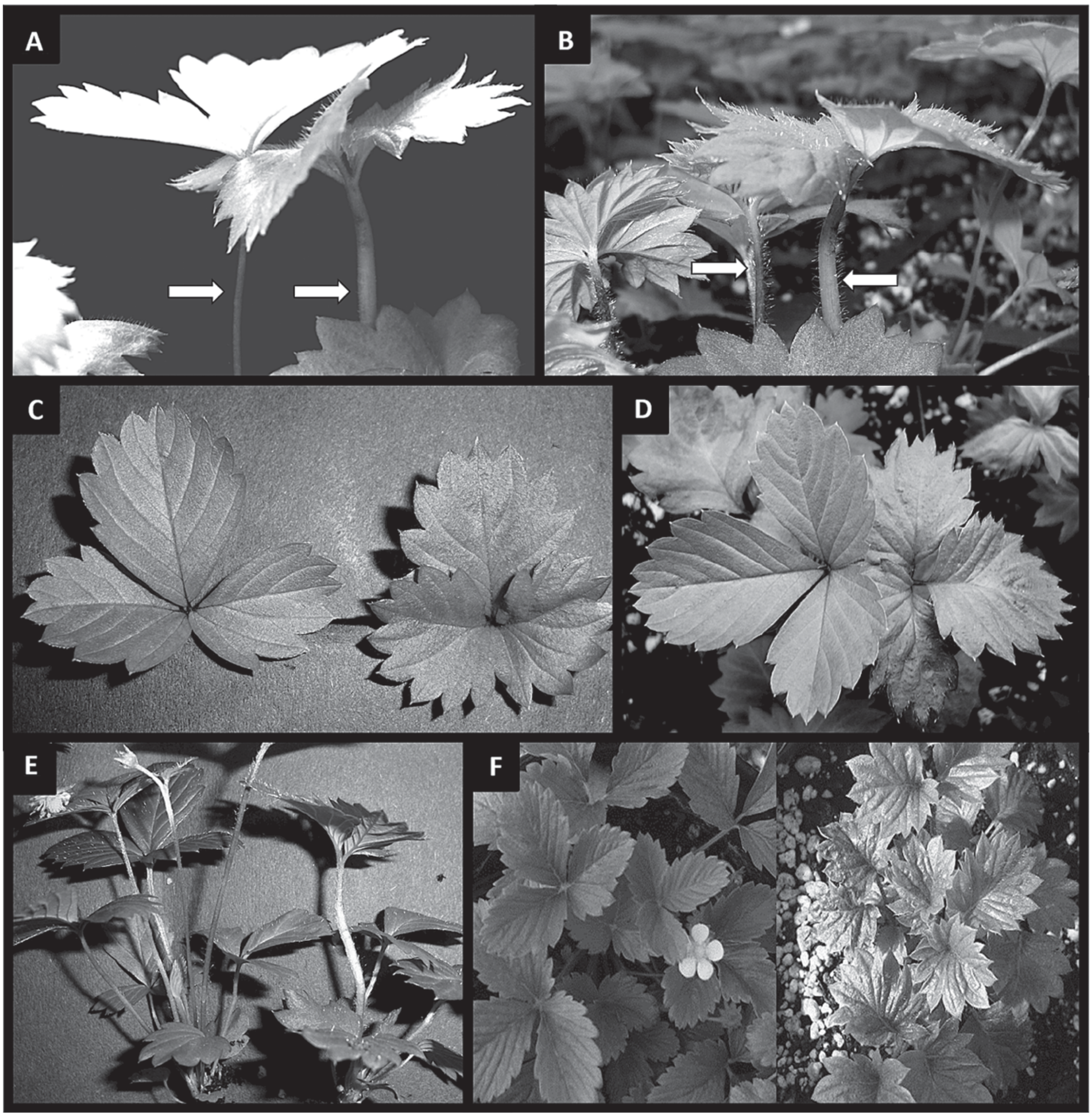


Fig. 1. Variation in *F. vesca* leaflet and petiole morphology between diploid *F. vesca* and induced polyploids. A and B. Representative petiole morphology for water control (left arrow) and colchicine-treated (right arrow) young plants (2-3 leaf stage). C and D. Representative leaf morphology in comparable plants. Petiole and leaflet morphology were developmentally stable over time. Panel E shows water treated (left) and colchicine treated (right) morphology in mature plants. F. *F. vesca* 'Baron' and BS4× canopy and leaf morphology for comparison to colchicine-treated plant morphology. BS4× exhibits typical polyploid leaf morphology and reduced growth rate.

hexaploid *F. moschata* and the hexaploid resulting from a genetic cross are shown (Fig. 2G). The results of another induced colchiploid, in this case the octoploid 'Mara des Bois' (peak 1) and the corresponding 16-ploid (peak 2) are presented in Fig. 2H. Other genotypes relevant to this study were analyzed and produced data consistent with ploidy level as estimated by previously discussed methods. Mig3, Mig4 and Mig5 displayed both diploid and tetraploid nuclei. Nuclei prepared from progeny of the selfed F1 Mig5 were measured (Fig. 2I). A single major G1 peak was

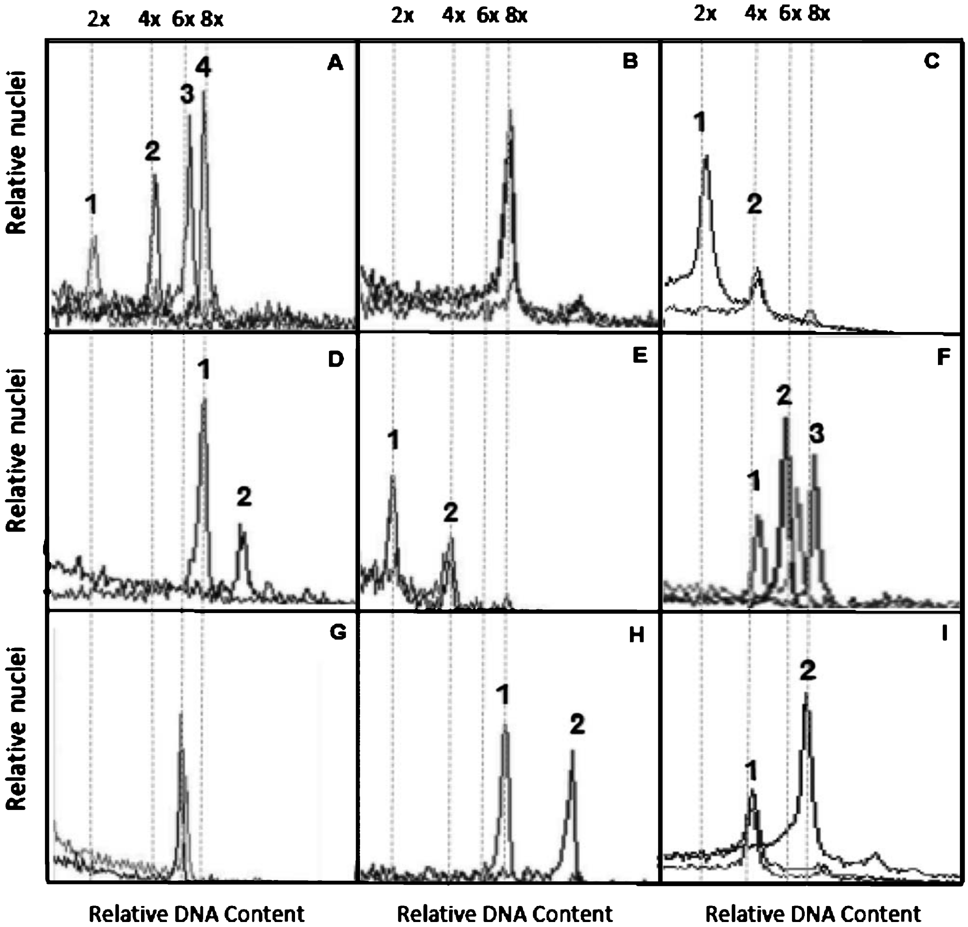
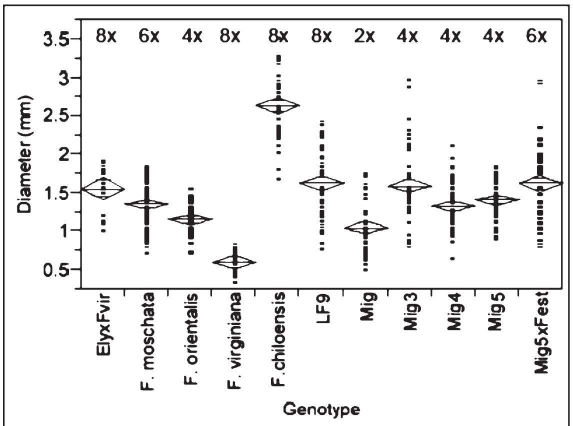


Fig. 2. Comparison of genotypes used in this work, delineated by flow cytometry. The ploidy of all materials used in this study was verified, including new colchiploids. Relative fluorescence intensities are shown for: A) Flow cytometry references of known diploid (1), tetraploid (2), hexaploid (3), and octoploid (4) ploidy levels. B) A single peak representing nuclei from *F.* × *ananassa*, *F. chiloensis* (PI 616934) and *F. virginiana* (PI 612495). C) 'Baron Solemacher' diploid (1) and BS4× tetraploid (2) as ploidy controls showing overlapping G2 peak from 'Baron Solemacher' with G1 of BS4×. D) Octoploid standard (1) and decaploid *F. iturupensis* (PI 641091)(2) E) Mig diploid (1) with Mig3 and Mig5 colchicine tetraploids (2) showing increased nuclei size consistent with polyploidization. F) Evidence for a hexaploid produced from the cross between the tetraploid Mig5 and *F.* × *ananassa* 'Strawberry Festival' (peak 2), compared to tetraploid *F. orientalis* (1), hexaploid *F. moschata* (2) and the octoploid parent (3). G) For clarity, the hexaploids from Panel F with the tetraploid and octoploid controls removed. H) 'Mara des Bois' (1; 8x) and 16-ploid Mara genotype (2) with G2 nuclei from 'Mara des Bois' overlapping G1 nuclei from 16-ploid. I) The self-pollinated F1 plants contain nuclei with equivalent size to *F. orientalis* (1) with *F.* × *ananassa* 'Elyana' shown as control.

detected and overlays the tetraploid *F. orientalis* (both represented in peak 1) precisely. The octoploid *F.* × *ananassa* 'Elyana' (peak 2) is provided for comparison.

3.3. Estimation of ploidy by petiole diameter

Quantitative petiole diameter results (Fig. 3) show that diploid *F. vesca* 'Mignonette' can be separated from its colchiploid progeny Mig3, Mig4 and Mig5. The relative sizes were consistent with phenotypes observed in seedlings. The natural tetraploid, *F. orientalis*, had an average petiole diameter similar to the diploid, *F. vesca* 'Mignonette', and both were statistically smaller than the natural hexaploid, *F. moschata*. The artificial hexaploid Mig5×Fest had larger



Level							Mean
F.chiloensis	A						2.63
LF9		B					1.62
Mig5xFest		B					1.62
Mig3		B	C				1.59
ElyxFvir		B	C	D			1.55
Mig5			C	D			1.41
F. moschata				D			1.35
Mig4				D			1.33
F. orientalis					E		1.16
Mig					E		1.05
F. virginiana						F	0.60

Fig. 3. Oneway ANOVA graph of petiole width measurements in diploids, induced polyploids, and progeny from select crosses. Ploidy, as determined by flow cytometry, is shown at the top of the panel. Box plots (top) depict means diamonds with 95% confidence intervals and includes the range of data points (black dots) for each line tested. Tukey's HSD test for multiple comparisons ($\alpha < 0.05$) is provided in the table (below). Statistically similar means are followed by the same letter.

petioles on average than the natural hexaploid *F. moschata*. Mig*5x*Fest (hexaploid), and the LF9 (a genotype derived from self-pollination of 'Strawberry Festival'; octoploid) had similar average petiole diameters. Wild octoploids were compared, and an *F. chiloensis* accession presented the largest diameters and an *F. virginiana* accession showed the smallest average diameters among all octoploid accessions tested.

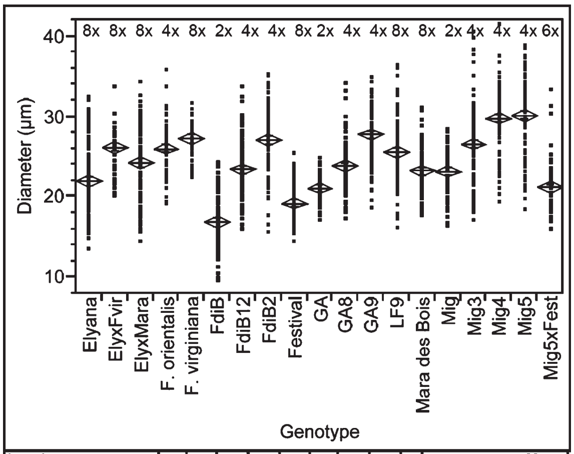
3.4. Ploidy determination by pollen size

The relationships between pollen size and ploidy across natural and induced polyploids were assessed (Fig. 4). The largest pollen diameter was found in the artificial tetraploids Mig4 and Mig5. The diploid *F. vesca* 'Fragoli di Bosco' had the smallest pollen followed by the octoploid *F.* × *ananassa* 'Strawberry Festival'. The tetraploid *F. orientalis* had similar sized pollen to some octoploids and artificial tetraploids but was larger than the artificial hexaploid Mig5×Fest. *F. moschata* was not flowering at the time of data collection and was therefore not included.

Significant differences were observed within any ploidy level. *F.* × *ananassa* 'LF9' is a tissue culture regenerated line of *F.* × *ananassa* 'Strawberry Festival'. Interestingly, the *F.* × *ananassa* line 'LF9' had larger pollen on average than *F.* × *ananassa* 'Strawberry Festival'. *F.* × *ananassa* 'Mara des Bois' had pollen with similar size to the diploid *F. vesca* 'Mignonette'. The largest pollen were found on the wild octoploid *F. virginiana*.

Among diploids 'Migonette' possessed the largest pollen size, and it was significantly different from 'Golden Alpine' and 'Fragoli di Bosco'. Tetraploids also varied greatly with some pollen being among the largest measured.

The colchiploids from each of the three *F. vesca* genotypes tested ('Mignonette', 'Fragoli di Bosco', and 'Golden Alpine') had larger pollen compared to their parental diploids. Pollen size increased to two levels, one larger than the other, and both larger than parental type *F. vesca*.



Level										Mean
Mig5	A									30.13
Mig4	A									29.77
GA9		B								27.76
F. virginiana		B	C							27.25
FdiB2		B	C							27.07
Mig3		B	C							26.55
ElyxFvir		B	C							26.17
F. orientalis		B	C							26.03
LF9			C	D						25.56
ElyxMara				D	E					24.23
GA8				D	E					23.83
FdiB12					E	F				23.43
Mara des Bois					E	F				23.23
Mig					E	F				23.17
Elyana						F	G			21.99
Mig5xFest							G			21.19
GA							G			21.08
Festival								H		19.23
FdiB									I	16.89

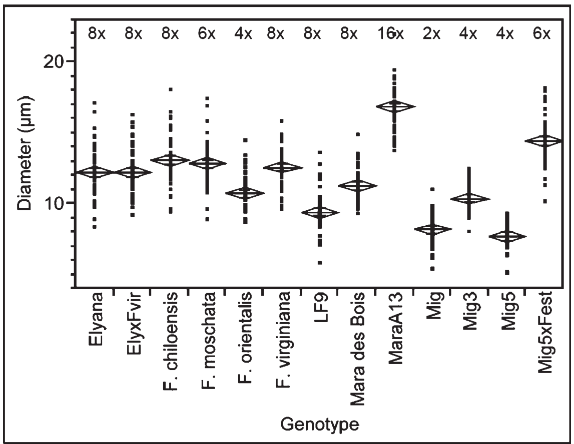
Fig. 4. Oneway ANOVA graph of pollen grain measurements in Fragaria diploids and related colchiploids, along with select parents and genetic crosses between plants representing other levels of ploidy, as noted by the values a the top of the panel. Box plots (top) depict means diamonds with 95% confidence intervals and includes the range of data points (black dots) for each line tested. Tukey's HSD test for multiple comparisons ($\alpha < 0.05$) is provided in the table (below). Statistically similar means are followed by the same letter.

3.5. Use of stomata measurements to detect polyploids

MaraA13 had the largest stomata size of all genotypes tested. The smallest stomata were observed on the 'Mignonette' diploid and the colchiploid Mig5. The artificial hexaploid, Mig5×Fest, had larger stomata than *F. moschata*. The two wild octoploid species, *F. chiloensis* and *F. virginiana*, had similar sized stomata despite large differences in plant morphology including a visibly larger leaf size. *F. orientalis* had similar stomata size comparable to the colchiploid Mig3 and the octoploid 'Mara des Bois'.

3.6. Leaf width: length ratio to detect polyploids

The results from leaf width:length comparisons are shown (Fig. 6). The octoploid *F. virginiana* and diploid Mig had the lowest width:length leaf ratios. The highest ratio genotypes were Mig4, LF9, Mig5 and Mig5×Fest. Mig3 had a similar ratio to the tetraploid *F. orientalis*. *F. moschata* was similar to *F. virginiana* and the 'Elyana' by *F. virginiana* cross. *F. chiloensis* had a similar ratio to *F. orientalis*, Mig3, and the 'Elyana' by *F. virginiana* cross.



Level								Mean
MaraA13	A							16.85
Mig5xFest		B						14.46
F. chiloensis			C					13.07
F. moschata			C					12.83
F. virginiana			C					12.59
Elyana			C	D				12.24
ElyxFvir			C	D				12.21
Mara des Bois				D	E			11.28
F. orientalis					E			10.72
Mig3					E	F		10.38
LF9						F		9.42
Mig							G	8.17
Mig5							G	7.70

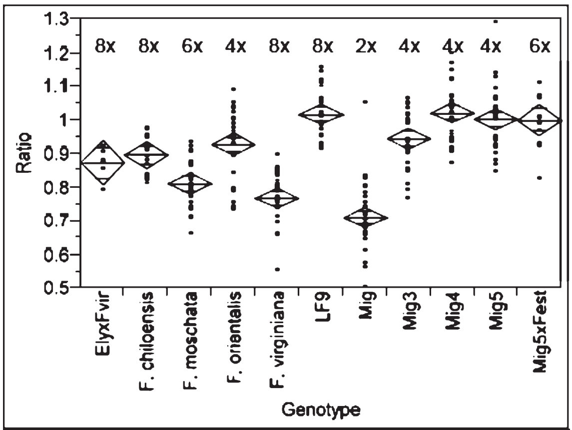
Fig. 5. Oneway ANOVA graph of stomata measurements in diploid strawberries, colchiploids, and parents/offspring from genetic crosses. Ploidy values, determined by flow cytometry, are presented at the top of the panel. Box plots (top) depict means diamonds with 95% confidence intervals and includes the range of data points (black dots) for each line tested. Tukey's HSD test for multiple comparisons ($\alpha < 0.05$) is provided in the table (below). Statistically similar means are followed by the same letter.

4. Discussion

The results of this study show that simple and rapid methods to estimate ploidy in strawberry are not reproducible or reliable between genotypes. Even plants of identical ploidy show great variation in presentation of morphological traits. The exception is that pollen grain size can be used as a more reliable indicator of genome size within a given polyploid series. For instance, pollen of a colchiploid induced from a diploid is reproducibly larger. Flow cytometry confirms these findings.

The variation in strawberry ploidy is important to understand and quantify. For any line of strawberry research it is necessary to remain aware of potential ploidy variation, as such events are relatively frequent and could distort experimental interpretations and breeding decisions. Even as far back as the turn of the 20th century Millardet and Solms-Laubach identified what were referred to as "false hybrids" from what we now know were crosses between plants from different levels of ploidy [23]. In the 1920's Longley counted strawberry chromosomes and confirmed the work from earlier scientists, showing that strawberry exhibited many levels of ploidy [24], findings that could also be reconstructed in the laboratory [18]. Many studies noted relatively high frequency of unreduced gametes in *Fragaria* species [3, 4]. These high ratios lead to fertilization events that occasionally produce auto- and allopolyploid plants, as seen in natural adjacent populations of *F. vesca* and *F. chiloensis* (Bringhurst and Senanayake, 1966).

A contemporary example is seen in what is thought to be the hybridization of *F. vesca* subsp. *bracteata* and *F. virginiana* subsp. *platypetala*, in the formation of the decaploid *F. cascadensis* (Hummer, 2012). Atypical ploidy levels are not always detrimental as strawberry propagates sexually by seed and asexually by stolons and branch



Level							Mean
Mig4	A						1.02
LF9	A						1.02
Mig5	A	B					1.00
Mig5xFest	A	B	C				1.00
Mig3		B	C	D			0.94
F. orientalis			C	D			0.93
F. chiloensis				D			0.90
ElyxFvir			C	D	E		0.87
F. moschata					E		0.81
F. virginiana					E	F	0.77
Mig						F	0.72

Fig. 6. Oneway ANOVA graph of leaf width-to-length measurements in *Fragaria* materials, including wild octoploids, genetic crosses, diploid strawberry and their induced polyploids. Box plots (top) depict means diamonds with 95% confidence intervals and includes the range of data points (black dots) for each line tested. Tukey's HSD test for multiple comparisons ($\alpha < 0.05$) is provided in the table (below). Statistically similar means are followed by the same letter.

crowns, so vegetative expansion of a genotype can occur even in the presence of low fertility. In addition, genomic instability leading to ploidy changes is also common through tissue culture, even when uninduced by mitotic inhibitors [5]. In all cases it is important to identify changes in ploidy.

Flow cytometry has been widely used to determine ploidy in strawberry. However, its implementation is not always practical as an initial test or when screening large numbers of plants. When generating colchiploids or even first-screening tissue culture materials, the ability to rapidly and inexpensively identify polyploids among many individuals can greatly accelerate research efforts. Flow cytometry requires specialized equipment and preparation techniques with a non-trivial learning curve. Today, many researchers report using third-party services to perform these analyses [14, 15, 25]. While convenient and accurate, the time lag and associated costs may outweigh the benefits. In this study, well-characterized materials were measured by flow cytometry to ensure their identity and calibrate the assay for the identification of unknowns (Fig. 2). An extensive comparison of 46 genotypes showed that both chromosome counting and flow cytometry reliably detect ploidy differences across *Fragaria* accessions [22].

The advantage of flow cytometry is that it allows ploidy to be determined across plant morphotypes and it is not subject to environmentally-induced variation. This statement is supported by the data in Fig. 2 when our results match with those previously described for specific accessions. The utility of flow cytometry may be seen in comparisons of *F. chiloensis* and *F. virginiana*, two wild octoploid strawberries. Their morphological characters are extremely variable and not always useful in determining ploidy as shown in Fig. 3–6. However, these two accessions representing separate species have similar nuclear content using flow cytometry. It should be noted that Hummer et al. (2011) have shown slight variations in size between the octoploid *F. chiloensis* and *F. virginiana*, which contrasts with the results here. The differences likely are due to the limited genotypes surveyed here. The trials presented in Fig. 2 demonstrate that it is possible to clearly distinguish each level of ploidy using this method as diploids, tetraploids, hexaploids, octoploids, and hexadecaploids. While these data are nothing new, they demonstrate that they assay works reliably and can be used to measure the putative polyploids produced in this study. The other substantial advantage is that nuclei for this technique may be isolated from small amounts of tissue, meaning a plant may be ploidy-typed long before morphological features are visible.

Polyploids can be identified based on morphological descriptors with some limitations. One instance where a rapid, visual assessment of induced polyploidy is preferred to flow cytometry is when screening hundreds of seedlings after colchicine-induced doubling. In the present study diploid seedlings were treated with colchicine and then grown in soil. Some developing plants displayed a larger emerging apical leaf, and dark-green leaves with deeper serrations relative to their siblings and diploid controls (Fig. 1). These morphological characteristics enabled efficient identification of tetraploid plants among diploids even at high planting densities. The ability to identify variations in ploidy at the seedling stage may be extremely useful to later efforts.

The most conspicuous indicator separating the *F. vesca* diploids from their induced polyploids was a thickened petiole. Their thicker peduncles bore flowers producing fruits and seed that remained tetraploid and gave rise to tetraploid progeny. In general, petiole diameter increased with higher ploidy levels, yet with some significant exceptions (Fig. 3). For example, both the thickest and thinnest petiole diameters recorded in this study were from separate wild octoploid species. Therefore, petiole diameter cannot be used to distinguish ploidy levels across *Fragaria* species. However, when considering induced polyploids petiole diameter effectively discriminated between diploid genotypes and their tetraploid descendants.

Pollen grain diameter has been used to estimate ploidy. Here the method works well in certain circumstances, but not well in others. Like petiole width, pollen grain size does not generally match ploidy level when comparing between genotypes. Figure 4 shows that pollen grain size varies significantly among octoploid accessions. 'Strawberry Festival' and 'Elyana' both arise from the same breeding program, yet clearly their pollen grain sizes have different mean sizes under nearly identical environmental conditions. The octoploid strawberry 'Mara des Bois' has a small pollen grain size, comparable to pollen from the diploid *F. vesca* 'Mignonette' (Fig. 4). Even in this limited survey of octoploid strawberry genotypes pollen grain size is significantly variable. Pollen from genetic crosses was also measured. F1 progeny from an 'Elyana' by *F. virginiana* cross had pollen size matching the *F. virginiana* parent. The hexaploid Mig5 × Fest had pollen distinguishable from both parents, Mig5 and 'Strawberry Festival'. These data indicate that pollen diameter is more influenced by genotype than ploidy level, limiting its application.

However, estimating ploidy by pollen measurement works well within a given genotype and its colchiploid progeny. Figure 4 shows the mean pollen grain sizes from tetraploids arising from colchicine-treated diploids 'Mignonette', 'Golden Alpine', and 'Fragoli di Bosco'. Three size classes are revealed, showing that pollen grain size can be used to distinguish induced tetraploids from diploid foundational materials. Each of the three *F. vesca* genotypes produced colchiploids with distinct and separate pollen size classes. The basis for these distinct size classes is unclear, but because flow cytometry indicates identical size between Mig3 and Mig5, the variation in size must be attributed to ultrastructural changes in the pollen grain itself that vary with ploidy. However, even with this variation within a ploidy level, pollen grain size is a rapid, inexpensive and accurate way to identify polyploid progeny relative to the original genotype. The obvious downside is that the plants must flower before pollen may be collected, requiring mature plants with stamens.

Stomatal size, as defined by guard cell length, has been shown to increase with ploidy in the Rosaceae. In *Polylepis* guard cell length was a strong indicator of ploidy, with discrete classes corresponding to diploid, tetraploid and octoploid materials [26, 27]. Tests in native North American roses also showed faithful estimations of ploidy based on stomatal size [27]. The experiments here in *Fragaria* showed that stomatal aperture diameter was more defined and consistent than guard cell length (not shown). The results in Fig. 5 show that there is substantial variation in stomatal aperture even within a ploidy level.

The results from the stomata aperture data were sometimes inconsistent with pollen-diameter results. Mig5 had larger pollen than the parental, non-colchiploid control, but actually had a similar aperture. Mig3 had pollen smaller than Mig5, yet had larger a larger mean stomatal aperture. The largest stomatal apertures were both from artificial polyploids, MaraA13 (16-ploid) and the hexaploid Mig5 × Fest. Preliminary data was also obtained from the same plants on different days and yielded statistically different results (data not shown). The variation observed is not due to the method, but instead is caused by environmental, morphological, or developmental factors that lead to variation in stomatal size. The data presented in Fig. 5 was all taken from visually healthy, fully expanded leaves from each genotype, from similar parts of the leaf. The stomatal aperture method may prove reliable if extreme attention is given

Table 2
Summary of ploidy estimation methods with strengths and limitations

	Starting material	Precision	Applicable within a genotype	Applicable within a genotypes	Strengths	Limitations
Petiole diameter	Petioles of putative polyploids and wild-type plants	Good	+	–	Rapid and simple. Visual screening possible	Requires a high-precision caliper
Pollen grain size	Anthers from putative polypoids and wild-type plants	Good	+	–	Can quickly measure large numbers	Requires a dissecting microscope with size standards. Cannot perform measurements until plants flower
Stomatal aperture	Epidermal peel	Poor	–	–	Data can be collected before flowering	There is substantial variation due to environmental, developmental or physiological factors. Same genotypes give different results on separate days
Stomatal length	Epidermal peel	Poor	–	–	Data can be collected before flowering	
Leaf measurement	Leaves from putative polyploid and wild type plants	Poor	±	–	Applicable to genotypes examined here	Cannot accurately distinguish between all genotypes of different ploidy levels
Flow cytometery	7 mm hole punch leaf disc	excellent	+	+	Can discern ploidy accurately through a range of ploidy levels	Requires flow cytometter. Many take time to retrieve results. Not good for screening large populations due to time and cost requirements

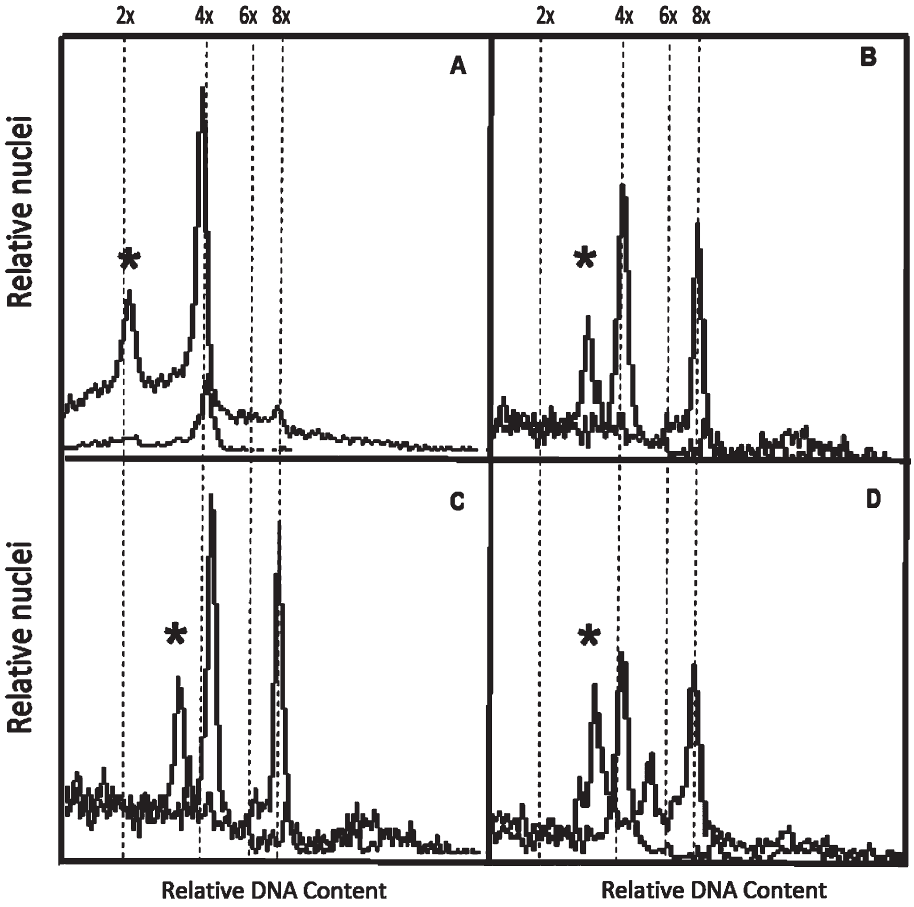


Fig. 7. Detection of nuclei with a one-half expected nuclear content. Analysis of octoploid nuclei occasionally yielded reproducible peaks suggesting variation in ploidy within tissues sampled. In all cases the peak representing a 0.5× complement is noted with an asterisk (*). A) Detection of ploidy variation in the tetraploid, *F. orientalis.* B) The lower-ploid peak detected in *F.* × *ananassa* 'Guelph S01'. C) The lower-ploid peak detected in *F.* × *ananassa* 'Escape', and D) the sub-tetraploid peak in the *F.* × *ananassa* line LF9. In all cases the reference tetraploid peak is *F. orientalis.*

to match leaves of comparable type and grown under the same environment. However, other measures like pollen grain size or petiole diameter are more precise, equally simple, and less affected by acute environmental variables.

The leaf width-to-length ratio can also be used to estimate ploidy, though only between a genotype and its colchiploid derivatives. These changes have recently been reported elsewhere [5]. Diploid *F. vesca* 'Mignonette' had a lower ratio than the colchicine-treated progeny Mig3, Mig4 and Mig5. The method did not successfully differentiate between the tetraploid *F. orientalis* and the octoploid *F. chiloensis*. The octoploid *F. virginiana* was significantly different from the octoploid *F. chiloensis* and both different from the octoploid 'LF9'. Leaf width-to-length ratio can therefore is not recommended as a measure to determine ploidy between different genotypes.

The strengths and limitations of each technique are presented in Table 2. It should be noted that the ultimate test in determining ploidy is counting chromosomes. These cytological techniques have been used reliably to generate karyotypes that confirm ploidy levels [28–30]. However, strawberry chromosomes are exceedingly small and visualization requires special training and methods. The methods described herein, with noted limitations, may serve as a suitable substitute to counting chromosomes.

Some unexpected flow cytometry results are presented in Fig. 7. One sampling of tetraploid *F. orientalis* indicated the presence of both tetraploid (with a corresponding G2 peak) and diploid nuclei sizes (Fig. 7A). This result could not be repeated from the identical source because the entire unexpanded trifolate leaf was processed for nuclei. Similarly, Guelph S01 (Fig. 7B), *F.* × *ananassa* 'Escape' (Fig. 7C) and LF9 (7D) produced a comparable pattern, but unfortunately the first two were only sampled once because of the destructive nature of the assay. Replicated data were obtained from the LF9 genotype and showed haploid-sized nuclei, especially when the edge of the leaf was analyzed (not shown). Mundane explanations, such as contamination with nuclei from pests or pathogens, cannot be ruled out. However, if purely strawberry nuclei these results suggest the potential for within-tissue ploidy variation that is different from polysomaty normally observed in angiosperms [31], as such variations typically reflect endoreduplication in cells. Here a one-half complement is observed. The assay is perhaps detecting variation within a specific cell layer in vascular tissue, or intermittently patterned variation by phyllotaxis. Mixoploid plants have been reported in strawberry plants regenerated from culture [32]. Other reports have identified putative mixoploids in tissue-culture generated plants, but these plants were confirmed chimeras [33, 34].

This study tests and compares several ploidy determination methods in *Fragaria* and defines their strengths and limitations. Implementation of any particular strategy needs to be carefully considered based on experimental need, considering the accuracy desired, time in processing, specialized equipment available, and the ability to measure reliably within and between genotypes.

Acknowledgments

This work was performed with funding from the University of Florida Plant Molecular Breeding Initiative, and the UF Dean for Research and the Florida Strawberry Research and Education Foundation.

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