AN ABSTRACT OF THE THESIS OF

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Nuclear DNA flow cytometry was used to differentiate ploidy level and determine nuclear DNA content in *Rubus*. Nuclei suspensions were prepared from leaf discs of young leaves following published protocols with modifications that included: increasing the stain concentration, adding the stain after the RNase treatment instead of adding it to the chopping buffer, reducing the tissue sample size, and using trout red blood cells (TRBC) as an internal standard. DNA was stained with propidium iodide. Measurement of fluorescence of 40 genotypes, whose ploidy had been determined by chromosome counting, indicated that fluorescence increased concurrently with an increase in chromosome number. Ploidy level accounted for ninety-nine percent of the variation in fluorescence intensity (r^2 = 99%) and variation among the ploidy levels was much higher than within ploidy levels. This protocol was used successfully for genotypes representing eight different *Rubus* subgenera. *Rubus ursinus*, which is widely represented in the USDA-ARS breeding program and has been reported to have 6x, 8x, 9x, 10x, 11x and 12x forms, was extensively tested. Genotypes of *R. ursinus* were predominantly 12x, but

6x, 7x, 8x, 9x and 11x forms were found as well. Nuclear DNA contents of 21 diploid *Rubus* species from five subgenera were determined by flow cytometry. *Idaeobatus*, *Chamaebatus*, and *Anaplobatus* were significantly lower in DNA content than those of *Rubus* and *Cylactis*. In subgenus *Rubus*, *R. hispidus* and *R. canadensis* had the lowest DNA content and *R. sanctus* had the highest DNA content, 0.59 and 0.75 pg, respectively. *Idaeobatus* had greater variation in DNA content among diploid species than the *Rubus* subgenus, with the highest being from *R. ellipticus* (0.69 pg) and lowest from *R. illecebrosus* (0.47pg). Ploidy level of 84 genotypes in the USDA-ARS breeding program was determined by flow cytometry. Flow cytometry confirmed that genotypes from crosses among 7x and 4x parents had chromosome numbers that must be due to the function of non-reduced gametes. Flow cytometry was effective in differentiating chromosome numbers differing by 1x but was not able to differentiate aneuploids.

DETERMINING PLOIDY LEVEL AND NUCLEAR DNA CONTENT IN *RUBUS* BY FLOW CYTOMETRY

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APPROVED:

Major Professor, representing Horticulture

Head of Department of Horticulture

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Rengong Meng, Author

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TABLE OF CONTENTS

TABLE OF CONTENTS, Continued

Page

LIST OF FIGURES

LIST OF TABLES

DETERMINING PLOIDY LEVEL AND NUCLEAR DNA CONTENT IN *RUBUS* BY FLOW CYTOMETRY

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 *RUBUS* POLYPLOIDY AND BLACKBERRY AND RASPBERRY BREEDING

Rubus is a large and important genus that includes an estimated 900 to 1000 species that are widely distributed, from within the Arctic Circle to the tropics, from low to high elevation, from acid to alkaline soils, from very wet to very dry climates, and from fully shaded to open field conditions (Darrow, 1937; 1967; Jennings, 1988; Sherman and Sharpe 1971; Thompson, 1995a). A number of commercial crops are members of this genus including red, black, and purple raspberries, blackberries, cloudberries, and Andean blackberries. *Rubus* is also ecologically important as an important food resource for animals (Thompson, 1997).

The naturally occurring range of chromosome numbers in *Rubus* species is from 2n=2x=14, the diploid state, to 2n=14x=98 or possibly 2n=18x=126, including odd-ploids and aneuploids (Moore, 1984; Thompson, 1995b). *Rubus* has 12 subgenera but the species and cultivars used in blackberry and raspberry breeding have largely been from the *Rubus* or *Idaeobatus* subgenera. The *Rubus* subgenus ranges from primarily diploids in Europe, North and South America, to dodecaploids in the *Ursini*, the native blackberry of the Pacific Coast of North America (Moore, 1984). Presumably, the present day

species and cultivars have arisen primarily from the intercrossing of diploid species and occasionally polyploid species (Jennings, 1988). Genotypes representing many of the different *Rubus* ploidy levels have been used in breeding and released as cultivars. Cultivars have included diploid (e.g. 'Burbank', 'White Pearl', 'Hillquist'), triploid (e.g. 'Philadelphia'), tetraploid (e.g. 'Chester Thornless', 'Navaho', 'Choctaw', 'Cherokee', 'Hull Thornless', 'Shawnee'), hexaploid (e.g. 'Marion', 'Olallie', 'Waldo', 'Lincoln Logan'), septaploid (e.g. 'Kotata', 'Boysen', 'Young'), octoploid (e.g. 'Jenner', 'Douglass', 'Bodega Bay'), and nonaploid (e.g. 'Lincoln Berry', 'Cascade') genotypes as well as aneuploids (e.g. 'Aurora'), with the majority of the cultivars being tetraploid (Hall, 1990).

Heteroploid crosses, crosses having parents of different ploidy levels, are often attempted to facilitate introgression of desirable genes from wild species or from elite material of different species or crop types. Heteroploid crosses generate progenies with varying ploidy levels. Factors such as spontaneous doubling of chromosomes at an early stage of development or the union of a non-reduced gamete (produced by nonreduction of sporocytes during meiosis or reduction of polyploid premeiotic cells that appear non-reduced) with a reduced gamete, both of which are common in *Rubus*, makes the results of heteroploid crosses unpredictable (Thompson, 1997). Fertility is often poor if the hybrid is triploid, pentaploid, or an aneuploid with a chromosome number less than hexaploid. At higher ploidy levels, the odd euploid and aneuploid genotypes may be completely fertile (Lawrence, 1986a; Waldo, 1950).

The USDA-ARS small fruit breeding program in Corvallis, Oregon has two major objectives: 1) to develop new blackberry and raspberry cultivars for the Pacific Northwest

small fruit industry and 2) to collect, evaluate and incorporate new *Rubus* germplasm into breeding material. In a breeding program, if the ploidy level of the potential parents could be determined, problematic progenies could be predicted and crosses could be targeted more effectively. In this effort, *Rubus* genotypes with known ploidy levels ranging from 2x to 12x are used, however, the ploidy levels of most of the breeding material is predicted but not confirmed.

In the Pacific Northwest, the native *Rubus ursinus* Cham.& Schltdl. is widely distributed and a valuable genetic resource for developing new trailing blackberry cultivars. *Rubus ursinus* may be a source of new traits such as increased winter tolerance, disease resistance, and altered fruiting season. *Rubus ursinus* has a known range of ploidy levels from hexaploid to dodecaploid except for septaploid (Brown, 1943). In 1993, *Rubus ursinus* was collected from throughout the Pacific Northwest and established in a common garden in Corvallis (Anderson and Finn, 1996). The populations have been evaluated for horticultural and taxonomic characteristics and superior individuals have been identified. In order to incorporate this material most effectively into erect, semierect and trailing blackberry breeding germplasm, it would be useful to know the ploidy level of the selected genotypes.

Horticultural crop germplasm is being lost at an alarming rate (Moore, 1988) and even though *Rubus* is widely distributed, it is important that germplasm representing this genus is not lost to urbanization, industrialization, or slash and burn agriculture. In addition, some germplasm, such as that in China, has not been accessible in recent history. As new and old collections of *Rubus* are evaluated, ploidy level can serve as a valuable distinguishing taxonomic trait (Thompson, 1995a; 1995b).

For the above reasons, determining ploidy level of *Rubus* genotypes rapidly and inexpensively would be valuable for cultivar improvement and germplasm enhancement.

1.2 DETERMINATION OF PLOIDY LEVEL

Successful chromosome counts have been made on at least 387 *Rubus* species, about 40 percent of the known species in the genus, and 90 cultivars and selections (Thompson, 1997; Thompson, 1995a; 1995b). The determination of ploidy level in *Rubus* genotypes has usually been done by chromosome counts in meristematic tissues, such as root or shoot tips, or pollen mother cells. Thompson (1995a) found that vigorous shoot tips were better than root tips and pollen mother cells for microscopic preparations. Rapidly growing meristems provide abundant mitotic metaphase figures throughout the growing season. In contrast, even when shoots were growing rapidly, root tips from pot-bound plants show few cell divisions. Pollen mother cells provide reliable counts if the appropriate meiotic stages of late diakinesis, metaphase I, or metaphase II can be found. However, due to the varying flower bud sizes in the diverse germplasm, it is difficult to determine the correct stage of meiosis based on bud size. Furthermore, flower buds are only available during certain times of the growing season.

Thompson (1995a) described techniques to count chromosomes using shoot and root tips and pollen mother cells. Detached shoot and root tips, primarily from plants growing in screen houses, were placed immediately in cold water (2 to 4°C) and held overnight. To increase the frequency of meristematic cells in the squash, meristems with only a few leaf primordia were dissected. These were then placed in Carnoy's solution

(three parts 95% ethanol : one part glacial acetic acid) for 4 to 24 hours followed by two changes of 70% ethanol before staining or storing the tissues in a refrigerator. Flower buds were broken open to facilitate the penetration of fluids, placed directly in the killing-fixing solution, and left for 20 to 24 hours followed by two or three changes of 70% ethanol, and refrigerated storage. All tissues were stained in alcoholic hydrochloric acid-carmine (Snow, 1963) at room temperature for 3 to 7 days and then rinsed in two or three changes of 70% ethanol, After the excess stain was rinsed out, tissues are squashed or stored again in 70% ethanol.

Before squashing, the shoot or root tips were hydrolyzed in 45% acetic acid at 60°C for 15 to 30 min to improve cell separation. Then, tissues were pulverized with a scalpel in a drop of 45 % acetic acid on a slide, and the coverslip was mounted with a small drop of Hoyer's medium (Anderson, 1954). Thumb pressure was applied on the coverslip to further separate the cells, to flatten the metaphase plates, and to spread the chromosomes. In some species with very high chromosome numbers, additional pressure applied directly above metaphase plates spread the chromosomes so that as many as 84 chromosomes could be counted accurately.

The procedures just outlined illustrate the challenges of traditional cytological determination of ploidy level. These approaches can be limited by the availability of plant tissues in the appropriate state and by the amount of labor required for every sample. The final limitation of using microscopic techniques is the availability of an expert to conduct the whole procedure. Training, practice and experience are prerequisites for this method.

While this used to be true for flow cytometry analysis, current machines require minimal training and expertise to operate.

These traditional approaches are not practical for the large number of genotypes in a breeding program. Flow cytometry, with its ability to measure nuclear DNA content rapidly, accurately and conveniently, is increasingly the preferred method for determination of nuclear DNA content and ploidy level in plants. Methods for flow cytometric measurement of DNA content and ploidy level have been developed for individual plant cells, protoplasts, and intact plant tissues (Bennett and Leitch, 1995; Galbraith et al., 1983). Arumuganathan and Earle (1991b) established a protocol for nuclear DNA measurement and thus for ploidy level determination for over 100 important plant species.

1.3 *RUBUS* NUCLEAR DNA CONTENT, PLANT GENETICS, AND THE *RUBUS* GENOME

Genome size is a fundamental parameter in many genetic and molecular studies such as: (1) Basic and applied studies involving genome organization, species relationships, gene expression analysis, and germplasm improvement where the knowledge of the haploid uclear DNA content (C value) is important (Baird et al., 1994; Bennett, 1984). For example, enome size estimates are important when constructing and screening genomic or cDNA libraries (Baird et al., 1994; Clark and Carbon, 1976; Friscauf, 1987). (2) Efforts to estimate the recombination length of nuclear genomes and correlate this genetic distance with physical distance where the genome size is necessary for developing linkage maps for genetic analysis and breeding (Baird et al., 1994;

Meagher et al., 1988). (3) Evaluating reproductive and somatic compatibility where the genome size is an important parameter in scion breeding and rootstock selection programs, especially for those using interspecific crosses (Baird et al., 1994).

Genome sizes of only two *Rubus* species, *R. idaeus* L. (Arumuganathan and Earle, 1991b; Bennett and Leitch, 1995) and *R. odoratus* L. (Bennett and Leitch, 1995), have been examined. The genome sizes of more genotypes must be determined if efforts will be undertaken to construct genomic libraries and detect cloned genes.

1.4 MEASUREMENT OF NUCLEAR DNA CONTENT

Several methods have been used to estimate DNA C-values in angiosperms. These are: chemical analysis and microdensitometry (Bennett and Smith, 1976); reassociation kinetics (Britten et al., 1974; Hake and Walbot, 1980) and, in recent years, flow cytometry. Chemical analysis and microdensitometry were the methodology of choice until the 1970's when they were displaced by reassociation kinetics that was in turn displaced by flow cytometry in the 1980's. Chemical analysis and reassociation kinetics, which involve extracting DNA from tissue, were tedious and they contributed only 2.8 and 0.8%, respectively, of the DNA C-values listed by Bennett and Smith (1976; 1991) and by Bennett et al. (1982; 1995). Microdensitometry has been the most widely used method to estimate DNA C-values (Bennett and Leitch, 1995). However, since 1986, there has been a steady decline in the number of species DNA estimates obtained using microdensitometry. On the other hand, there has been a notable increase in the use of flow cytometry for quantifying DNA in plants (Arumuganathan and Earle, 1991a; 1991b;

Costich et al., 1993; Dickson et al., 1992; Dolezel et al., 1992; Dolezel et al., 1994; Figueria et al., 1992; Galbraith et al., 1983; Gomez et al., 1993; Hammatt et al., 1991; Hulgenhof et al., 1988; Keeler et al., 1987; Murray et al., 1992; Rayburn et al., 1989; Sharma et al., 1983; Taylor and Vasil, 1987; Ulrich et al., 1988). The first significant use of flow cytometry in plant DNA studies was for cell cycle analysis (Galbraith et al., 1983). Studies on plant ploidy level and on variation in the amount of plant nuclear DNA followed soon after (Hulgenhof et al., 1988). Today, flow cytometry has largely replaced other methodologies for DNA content determination. Flow cytometry has the advantage that large numbers of nuclei can be examined and the DNA quantified in a relatively short period of time. The large numbers of nuclei scored can produce DNA content estimates with very low sampling error.

While flow cytometry has many advantages over traditional chromosome counting, there are some disadvantages as well. First, even though it is relatively inexpensive to conduct research using a flow cytometer, the initial cost for the equipment is very high. Once the equipment is in place, there are still limitations to the technique and several potential sources of error. DNA content determinations have three primary sources of error including those associated with the stain, the standards, and the isolation and identification of whole 2C sample nuclei (Price and Johnston, 1996). The errors associated with the stain are the most complex, yet the easiest to enumerate. These errors are due to (1) incomplete light saturation of the DNA-dye complex, (2) nonspecific staining of DNA, and (3) sequence-specific bias in staining of the DNA (Price and Johnston, 1996). Light saturation is probably the most ignored aspect of the three. Some

plant nuclei are large compared to the human cells that cytometers were designed to handle. These large genomes may require higher powers to reach saturation.

Flow cytometers produce large amounts of data with no consideration of the source of that data; e.g. it will produce a peak from broken nuclei, just as easily as from 2C or 4C cells. Therefore, it is up to the operator to ensure that the measurement is of intact nuclei. Nuclei can be sorted to permit isolation and direct observation of the counted material, however, this is a time consuming process that is available on only some of the cytometers. Careful nuclei preparation is the better approach. It is important to count only those nuclei that fluoresce brightly in the wavelength appropriate for the dye (called fluorescence activation or fluorescence discrimination) and to frequently observe samples under a fluorescent microscope to ensure that whole nuclei, free of associated cytoplasm, are present. During analysis, improved confidence in the true mean DNA content of a sample (lower coefficient of variation [CV]) is produced by selecting, with appropriate gates, nuclei that are free of associated cytoplasm (Price and Johnston, 1996).

Bias from the binding stoichiometry of the DNA stain is probably the most consistent source of error in DNA studies. Even propidium iodide (PI), which is considered the least preferential fluorochrome, is not free of this kind of bias (Price and Johnston, 1996).

Due to the drawbacks mentioned above, flow cytometry for DNA C- value studies in plants is still not a perfect technique. Nevertheless, several publications have compared the data obtained using flow cytometry and Feulgen microdensitometry, and found that there is a good correlation between measurements made by the two methods over a large

range of DNA amounts in monocotyledons and dicotyledons (Arumuganathan and Earle, 1991a; 1991b; Dickson et al., 1992; Galbraith et al., 1983; Hulgenhof et al., 1988; Michaelson et al., 1991a; 1991b). Price and Johnston (1996) showed that DNA content determined by flow cytometry was very reliable. However, widely different values have been published for the same material, sometimes up to 100% in *Zea mays* spp. *mays* (Bennett and Leitch, 1995).

Using flow cytometry does not obviate the need for cytological work on unknowns and, indeed, this is usually essential for accurate interpretation of the results. Flow cytometry may give a highly accurate DNA value for a taxon but this has limited value if the chromosome number (2n) of the individual plant (or tissue) measured is unknown. The significance of differences among different tissues, plants, populations and species measured using flow cytometry should, therefore, always be assessed in conjunction with cytological analysis (Bennett and Leitch, 1995).

The application of flow cytometry to ploidy differentiation in a single taxon is promising because the errors associated with standards and stains can be avoided. Due to the continual improvement of the flow cytometer and isolation techniques, for those genera with a wide range of ploidy levels, flow cytometry is effective when the chromosome counts have been established in the genus.

1.5 PRINCIPLES OF NUCLEAR DNA FLOW CYTOMETRY

The base pairs in DNA can be stained with propidium iodide (PI), a fluorescent stain. The isolated nuclei stained with PI are passed rapidly through a flow cell that

channels the nuclei into a narrow stream. Then the nuclei are rapidly passed, one at a time, in a stream across a laser beam that is scattered by the passing nuclei while at the same time exciting the dye to produce its characteristic fluorescence. A series of lenses, mirrors and filters are used to collect and direct the scattered light and fluorescence into different detectors, usually photomultiplier tubes. The detectors convert the light into electronic pulses that are amplified, converted into digital signal, and each count is output as a channel number relating to its fluorescence intensity. A histogram of channel numbers from counting of 5,000 nuclei is given to represent the channel position of a sample. In the final output, higher channel numbers represent higher amounts of light output and thus greater light scatter or a greater amount of DNA (Price and Johnston, 1996).

1.6 MEASUREMENT OF DNA CONTENT BY FLOW CYTOMETRY

Generally, the following three steps are necessary to determine ploidy by flow cytometry:

(1) Actively growing, healthy plant tissue (usually leaf tissue) is mechanically chopped to separate intact nuclei. Around 10,000 nuclei are usually required for the analysis and this quantity can usually be obtained from about 50 mg of tissue (Arumuganathan and Earle, 1991b). In our research, we found 40mg leaf tissue was sufficient for producing enough intact nuclei. Larger amounts of tissue not only increased the time spent on chopping, but also increased the amount of debris and decreased the

frequency of intact nuclei. As long as the leaves are fully expanded, the younger the tissue, the better the histograms.

(2) The isolated nuclei are stained with a fluorochrome. The earliest work with flow cytometry used mithromycin, which binds selectively/specifically to GC-rich DNA sequences (Galbraith et al., 1983). However, it is now recognized that mithromycin is not sensitive enough to detect small changes in DNA and has been replaced either by 4' 6-diamidino-2-phenylindole (DAPI), which preferentially binds to the AT-rich regions of the DNA or by the intercalating dyes propidium iodide (PI) or ethidium bromide (EB) that independently bind the DNA sequence (Bennett and Leitch, 1995). There is still considerable disagreement as to which fluorochrome is most sensitive and reliable. While some workers are satisfied that DAPI can be successfully applied to measure DNA and detect intraspecific and intraplant variation (Biradar and Rayburn, 1992; Rayburn et al., 1989), others have suggested that base preference fluorochromes such as DAPI are unreliable (Bennett and Leitch, 1995; Dolezel et al., 1992; Michaelson et al., 1991a;). Dolezel et al. (1992) found that the difference between DNA contents measured with PI and DAPI or mythramycin were statistically highly significant, and concluded that the use of base preference fluorochromes can lead to serious errors. The preferred fluorochrome in animal research is PI because it produces symmetrical DNA fluorescence peaks with low coefficients of variation (CVs). This fluorochrome is now being used more widely for plant DNA estimation because of its sensitivity and base independent binding to DNA (e.g. Arumuganathan and Earl, 1991b; Bennett and Leitch, 1995; Dolezel et al., 1992; 1994; Figueria et al., 1992; Michaelson et al., 1991b).

The accuracy of the determination of DNA content with PI is dependent upon the destruction of the RNAs by RNase (Deitch et al., 1982) or other agent because PI will not only stain DNA, but also double stranded RNA. RNase is relatively inactive in the presence of PI (Price and Johnston, 1996), therefore, it is important to add RNAase to the chopping buffer and to incubate prior to staining with PI.

DNA measurements by flow cytometry are based on the quantitative binding of the fluorochrome to DNA. Thus, for consistent accurate results, it is essential to determine the optimal concentration of fluorochrome needed to stain the DNA. Arumuganathan and Earle (1991b) found that it was important to use a sufficient concentration of PI (at least 50 $\mu g \cdot ml^{-1}$) to saturate DNA binding, as understaining led to decreased fluorescence and to variable results with large CV values. Overstaining with PI or mithromycin was not a problem and there was no evidence of autoquenching of fluorescence (Galbraith et al., 1983). Understaining with mithromycin resulted in lower fluorescence peaks, larger CVs and therefore more variable results (Dolezel, 1991). Similarly, too high or too low concentration of DAPI can result in unreliable DNA estimates (Biradar and Rayburn, 1992; Rayburn et al., 1989). Overstaining with DAPI of nuclei resulted in self-absorption of the stain leading to decreased fluorescence and underestimation of DNA amount, while understaining prevented complete DNA saturation producing variable results. Rayburn et al. (1989) constructed the ratio of stain/nuclei titration curves. They found that the dye concentration per nucleus ($\mu g \cdot ml^{-1} \cdot nucleus^{-1}$) was a more reliable method for obtaining maximum fluorescence than $\mu g \cdot ml^{-1}$ and therefore that accurate determination of nuclei concentration was imperative. Similar experiments were repeated by Biradar and Rayburn (1992) who found that different amounts of DAPI were needed to get maximum DNA

fluorescence when staining nuclei isolated from different tissues. Thus, for accurate measurements of DNA amounts by flow cytometry it seems clear that the optimal stain concentration should be empirically determined for each group of genotypes analyzed.

In our preliminary experiments, when we followed Arumuganathan and Earle protocols (1991a), we found that upon adding more PI to an already stained samples, the DNA fluorescence peak moved to a higher fluorescence channel, which meant that the DNA had not been fully stained with PI. Overstaining with PI created a separate problem where the stain clumped and attached to nuclei and debris and gave inaccurate DNA fluorescence readings. After several trials, we found that 250 $\mu g \cdot ml^{-1}$, compared to the 50 $\mu g \cdot ml^{-1}$ that Arumuganathan and Earle (1991a) used, was the optimum PI concentration for our *Rubus* genotypes.

(3) The sample is passed through a flow cytometer and the relative fluorescence emitted from each nucleus, which is proportional to the DNA content, is measured and analyzed. By including an internal standard the relative fluorescence is converted into absolute amounts.

When selecting an internal standard, the peak position(s) of the standard must not coincide with either the G0+G1 or the G2+M peak. Flow cytometry was first developed using animal systems. Consequently, many DNA measurements for plants have been made using animal standards; the most commonly used being chicken erythrocytes, human leucocytes or rainbow trout erythrocytes. Unfortunately, different workers sometimes assume different values for the same animal standard. For example, 2C values assumed for chicken erythrocytes range from 2.33 pg (Arumuganathan and Earle, 1991a) to 3.00 pg (Berlyn et al. 1986), which differ by 28%. Animal standards are considered

suitable for measuring plant DNA amounts (Arumuganathan and Earle, 1991a) although Price et al. (1980) recommended plant standards.

In our experiments, we originally used tobacco (*Nicotiana tabaccum* L.) as an internal standard. Since tobacco tissue had to be chopped together with the *Rubus* tissue, it took more time to chop the samples and produced more plant debris in the nucleus suspension. We tried chicken red blood cells (CRBC) and rainbow trout blood cells (TRBC) as standards to avoid these problems. We found that with tobacco the peaks had larger CVs and more "noise" (background of debris) than when animal cells were used as standards. CRBC have a DNA content between 2.33 pg and 3.00 pg, so its histogram peak coincides with tetraploid to hexaploid *Rubus* genotypes and interferes with the peaks of *Rubus* genotypes from triploid to octoploid. TRBC DNA content is about 5.05 pg, which is much larger than the *Rubus* with the largest DNA content; the 14x *Rubus* which have a DNA content of about 4.2 pg. Rainbow trout red blood cells were the internal standard that best met our needs.

1.7 FLOW CYTOMETERS

Improvements in cytometers and, in particular, improvements in the acquisition and analysis of data made possible by high-speed personal microcomputers, have greatly increased the utility of these machines. The flow cytometers used for plant DNA analysis are of two basic types depending upon the source of light used to produce fluorescence for the stained DNA in the sample. One type of flow cytometer is based on arc lamps, e.g., mercury, mercury-xenon, and xenon. The arc-based machines are used primarily in

the ultraviolet (UV) spectrum with DNA dyes that are excited at the short UV wavelengths such as DAPI. However, because DAPI preferentially binds with AT-rich sequence of DNA, it is less useful for absolute DNA measurements than PI; and so it is commonly used to compare DNA amounts between tissues in a plant or to compare individuals within a species (Price and Johnston, 1996).

Relatively large, laser-based flow cytometers, as typified by the Coulter "Epics" Elite (Beckman Coulter, Inc., Miami, Fla.) and the Becton Dickinson "FAC-Vantage" (Becton Dickinson and Company, Franklin Lakes, N.J.), use the relatively high-intensity monochromatic visible light output of a laser to excite the fluorescent dye bound to the DNA. Because they produce monochromatic visible light, laser flow cytometers are commonly used to produce an absolute measure of the total genomic DNA. While a number of fluorescent dyes are available and have been used with visible light, PI is the most commonly used stain. Propidium iodide fluoresces relatively brightly, produces a low CV and stains reliably under most conditions. It is an intercalating compound with a slight GC bias (Properi et al., 1991) and with somewhat reduced binding to tightly coiled DNA found in heterochromatin (Properi et al., 1991; Bashir et al., 1993). Because we are concerned with DNA content and ploidy level differentiation, we used a laser beamed flow cytometer (Coulter MCL-XL flow cytometer, Miami, Florida) and chose PI as the stain instead of DAPI.

In recent years, flow cytometers have been improved in many ways primarily related to reliability and ease of use. The greatest improvement, however, have been coincident with improvements in microcomputer technology that permit real-time analysis of DNA content from isolated plant nuclei. The use of real-time analysis

produces faster results and reduces the need to save and rerun data for analysis. Real-time analysis has also permitted more sophisticated use of screens and gates to separate intact nuclei from those that are broken or are tagged with extraneous DNA or RNA.

1.8 COEFFICIENT OF VARIATION

Ideally, the same particle passing repeatedly through the laser beam should produce identical light scatter or fluorescence pulses. Another particle might produce a consistent but different set of pulses. Practically, there is always some variation within the instrument that causes some variation in the pulses even though the particles are the same. Any problems with the sample stream, the laser intensity, the laser alignment, beam focusing and detection would produce too much variation in these pulses and too much variation in the histogram. For instance, it is difficult to determine whether the broadening of a histogram is due to instrument or particle variation or both. Standardizing beads are used to determine the variation due to the machine. Variation due to the sample and the sample preparation can then be addressed. Ideally, the CV for the beads is less than 5%. If samples are run with a CV near or below 5% then both the sample and machine's operation are considered acceptable. If the CV is larger than 5%, the sample must be more carefully prepared or the techniques modified.

The severity of the instrument variation can be determined by calculating a coefficient of variation (CV) on a good uniform test sample, such as Coulter Fluorospheres (Coulter Inc., Miami, Fla.). The basic equation for the CV is:

$$CV = (SD/MEAN) * 100$$

where SD is the standard deviation, and MEAN is the average value for the parameter measured for these particles (for a Gaussian distribution this would be the channel with the highest count).

The instrument actually does all these calculations. However, it is important to know what a normal CV for a cytometer would be so that values that fall outside the normal range can be detected. Assuming the test particles are good, then a high CV for these particles indicates the instrument is at least partially to blame for the broadening of the histogram. A small CV on the test sample would indicate that the broadening is due to the real differences in the particles in that sample. The fluorescence beads can be added to a test sample provided they do not interfere with that sample, or we can calibrate the flow cytometer using beads before testing begins.

1.9 SUMMARY

The primary objective of this study was to develop a rapid, inexpensive and routine methodology to determine ploidy level and nuclear DNA content by flow cytometry. This will allow breeders in blackberry and raspberry breeding programs to efficiently plan crosses and select hybrids and determine the genome size of the diploid *Rubus* species, a fundamental parameter for many genetic and molecular studies.

The secondary objective was to determine the ploidy level of as many genotypes as possible in the USDA-ARS small fruit breeding program in Corvallis. This information will be particularly valuable in planning crosses and, in addition, can help us evaluate germplasm.

CHAPTER 2

MATERIALS AND METHODS

2.1 PROTOCOL FOR BLACKBERRY AND RASPBERRY PLOIDY DIFFERENTIATION BY NUCLEAR DNA FLOW CYTOMETRY

The flow cytometry protocol we used was adapted from that of Arumuganathan and Earle (1991b). The first step was to prepare the nucleus suspension. A $MgSO_4$ buffer solution, which is composed of 0.01M $MgSO_4$, 0.05M KCl, and 0.005M HEPES with a pH of 8.0, and a 10% Triton X-100 (w/v) stock solution were prepared. A chopping buffer was made based on the $MgSO_4$ buffer with the addition of 2% PVP-10, 0.2% dithiothreitol, and 0.275% Triton X-100, to extract and stabilize nuclei. Triton X-100 simultaneously disrupts the plasmalemma, organelles, and particularly chloroplasts (natural fluorescence is thus removed). In addition, it permeabilizes the nuclear membrane and reduces nonspecific fixation. The use of Triton X-100 reduces variation and improves uptake of stain by nuclei.

The addition of PVP-10, a modification of Arumuganathan and Earle procedures (1991b), greatly reduced the debris and increased the number of intact nuclei in suspension. In our preliminary runs, a large amount of debris was attached to the nuclei, preventing the reading of the fluorescence of the stained nuclei and we didn't get any DNA histogram peaks.

About 40 mg of actively-growing *Rubus* leaves were weighed and placed in a 60mm plastic petri dish. Chopping buffer (1 ml) was added and the petri dish was put on

ice. The tissue was chopped with a razor blade to homogenize the tissues and release the nuclei. Leaf tissue was used because it was usually available year round. In preliminary trials, old and young leaf tissues were tried. We found that the younger the leaf tissue, the more intact nuclei we could obtain and the lower the CV. The leaves must be washed to remove soil, chemicals and other organisms that might react with the chemicals and alter the results. While Arumuganathan and Earle (1991b) used 50 mg leaf tissue for each sample, we found 40 mg to be sufficient. Excessive plant tissue increased the amount of debris, gave more background fluorescence and produced peaks with higher CV values. The tissue should be cut into tiny pieces, usually less than 0.5mm in size, to ensure enough intact nuclei are released.

Initially, we used tobacco (*Nicotiana tabacum* L.) as the internal standard and so the tobacco was chopped simultaneously with the *Rubus* tissue. Later, we found that using tobacco produced too much debris and the CVs for the histograms were large. Therefore, we changed our internal standard to trout red blood cells.

The nucleus suspension must be filtered to remove debris that might block the flow cell. The filter system is composed of a 10ml syringe and 30μm nylon mesh. We obtained a small round filter using a number-eight cork borer, whose area is the same as the cross section area of the syringe. The nylon mesh is placed inside of the syringe and held in place at the end of the syringe using the plastic cover provided with the syringe. The nucleus suspension was filtered into a 1.5 ml centrifuge tube.

Because PI can stain RNA as well as DNA, DNAase-free RNase must be used to digest RNA in order to avoid binding of the PI to RNA. DNAase-free RNase (Sigma R-4642, 0.6 μl) was added to the nuclei suspension and the tube was placed in a water bath

at 37 °C for 15 min to digest the RNA. The nuclei in the centrifuge tube were then stained by adding 5mg/ml PI stock solution to a final concentration of 250μg PI / ml. This mixture was held in a water bath at 37 °C for 15 min. Since PI is sensitive to light and heat, the PI stock solution was prepared in advance, covered with aluminum foil and kept in a refrigerator. In the process of preparing nuclei suspension, significant loss of buffer during cutting and filtering should be avoided to keep the PI concentration consistent during staining.

To calculate nuclear DNA content, the standard must be put through a similar procedure. The rainbow trout red blood cells (TRBC) are stained with the same PI concentration as the *Rubus* nuclei and are added to the nuclei suspension to be run in the flow cytometer at a concentration of 10^5 TRBC nuclei /ml.

The samples were run on the flow cytometer within 18 hours of preparation.

2.2 RUNNING SAMPLES ON THE FLOW CYTOMETER

The first step in preparing the equipment for samples was to adjust the laser-emission wavelength to 488 nm which is suitable for PI fluorescence. Then the protocol for PI fluorescence was initiated, which includes acquisition of single-parameter histograms of forward-angle light scatter (FS), a single fluorescence channel (FL3) and logarithmic channel (AUX). A fluorescent bead (Coulter Fluorosphere; Coulter Inc., Miami, Fla.) was used to adjust the optics until the CVs for pulse integral and FS were minimized (typically <2% for fluorescence and 2% to 3% for FS).

The flow cytometer's parameters were set as follows based on preliminary experiments where peaks in linear scale were produced for *Rubus* genotypes and the internal standards:

	Volts	Gain
FL3	608	1.0
Aux	14	7.5

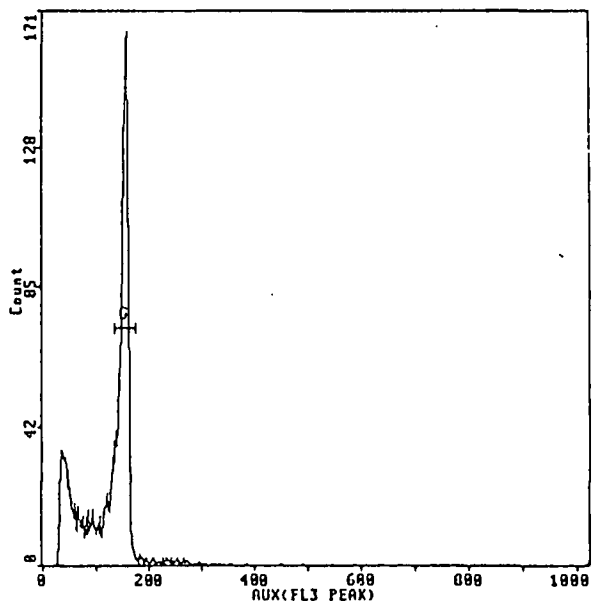
FL3 and Aux represent different photomultiplier tubes (PMTs), serving as detectors and also amplifiers of the weak fluorescence signals, which make normal and log amplifications of the pulses, and in turn, produce a linear scale and a log scale, respectively. A logarithmic scale is used for the detection of all possible peaks that may or may not be showing in the linear scale. The logarithmic amplification makes the small pulses much bigger while amplifying the larger pulses by a lesser amount. The parameters of volts and gain set for the amplifiers directly affect the channel position of sample DNA peaks.

After the flow cytometer was set up, 300 µl of nuclei suspension was transferred to a flow tube for each sample. They were run, including an internal standard if necessary, at a data rate of 50 to 100 nuclei per second. A minimum of 5,000 total events were acquired. The samples were run in a darkened lab to prevent PI degradation. Figure 2-1 and Figure 2-2 are examples of the results generated by the flow cytometer and show histograms from propidium iodide-stained nuclear DNA. Figure 2-1 shows results using only *Rubus* nuclei, but in Figure 2-2, TRBCs were included as an internal standard.

We calculated the nuclear DNA content per 2C nucleus based on the fluorescence of TRBC according to the following equation: Nuclear DNA content of *Rubus* genotypes

Fig. 2-1. An example of a histogram produced by the Coulter Epics XL-MCL flow cytometer (Beckman Coulter, Inc., Miami, Fla.). Propidium iodide-stained nuclear DNA of 'Philadelphia', a triploid cultivar selected from *R. canadensis,* is presented.

7:



Stats: Normalized, Listgating: Disabled

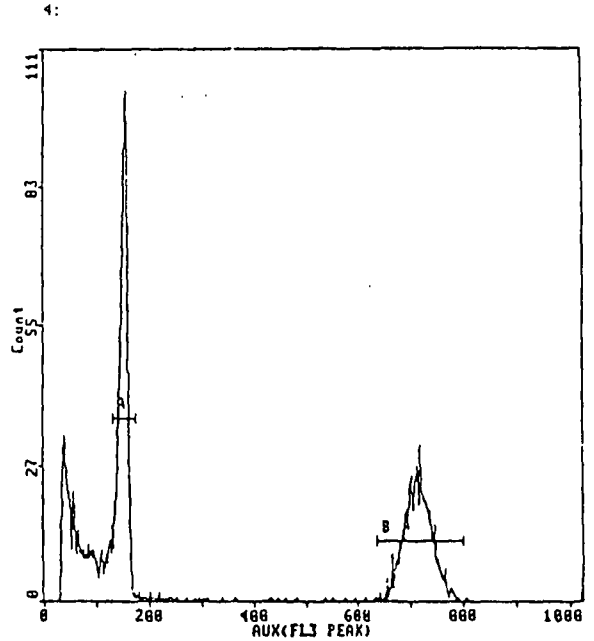
Hist	Region	ID	%	Count	Mn X	Md X	PkPosX	PkCnt	HPCV	Min	Max
7	G	G	55.1	2753	153.1	154.5	159.0	171	3.59	134.0	174.

Sample Info

7:

Run:	G0012754	Specimen:	j6
Date/Time:	22Oct97 15:27:07	Tube:	
Operator:	QQQQ	Panel:	
Cytometer:	XL Z01023	Protocol:	Rengong
Listgating:	Disabled	Protocol File Name:	G0000172.PRO
Total Count:	5,000	Parameters:	AUX(FL3 PEAK)
File name:	G0012754.H03	Gating Parameters:	Not Gated
File Type:	FCS2.0	Facility:	Oregon State University
File Size:	2,816		

Fig. 2-2. An example of a histogram produced by the Coulter Epics XL-MCL flow cytometer (Beckman Coulter, Inc. Miami, Fla.). Propidium iodide-stained nuclear DNA histograms of a *Rubus* genotype (left peak) and trout red blood cell (right peak) are presented.



Stats: Normalized, Listgating: Disabled

Hist	Region	ID	%	Count	Mn X	Md X	PkPosX	PkCnt	HPCV	Min	Max
4	A	A	35.9	1793	151.4	152.9	154.0	111	3.42	130.0	174.
	B	B	33.7	1683	714.8	714.7	719.0	35	1.11	636.0	800.

Sample Info

4:

Run:	G0012762	Specimen:	2-
Date/Time:	22Oct97 15:53:46	Tube:	
Operator:	QQQQ	Panel:	
Cytometer:	XL Z01023	Protocol:	Rengong
Listgating:	Disabled	Protocol File Name:	G0000172.PRO
Total Count:	6,000	Parameters:	AUX(FL3 PEAK)
File name:	G0012762.H03	Gating Parameters:	Not Gated
File Type:	FCS2.0	Facility:	Oregon State University
File Size:	2,816		

(pg DNA) = (*Rubus* sample G_1 peak fluorescence mean) x 5.05 / (mean fluorescence of TRBC G_1 peak), where 5.05 is the nuclear DNA content (pg) of TRBC.

To estimate the putative ploidy level of unknown genotypes, we compared the fluorescence of different *Rubus* genotypes with known ploidy levels and calculated a regression line. Using the prediction interval of the regression line, we estimated the ploidy level of unknown *Rubus* genotypes to the nearest full set of chromosomes.

2.3 PLANT MATERIALS

Five different sets of plant material were analyzed using flow cytometry.

The first group represented the *Rubus* genotypes with known ploidy levels as determined by Thompson (1995a; 1995b). These were available within our breeding program or at the USDA-ARS National Clonal Germplasm Repository (NCGR), Corvallis. Leaf samples of each of 40 genotypes were collected and tested using flow cytometry to determine the relationship between chromosome number and fluorescence intensity (Table 2-1). The results were analyzed with SAS Insight (SAS Institute, Cary, N.C.) to obtain means, variances, and a regression equation (Fig. 2-3). The statistical relationship between ploidy and fluorescence intensity was determined through regression analysis. The amount of total variation in fluorescence intensity explained by ploidy was evaluated through the coefficient of determination (r^2).

The second group was chosen to determine whether this technique would work across the broad range of *Rubus* subgenera. Genotypes were chosen that represented the subgenera, had known ploidy numbers (Thompson, 1995a), and were available to us

Table 2-1. Nuclear DNA flow cytometry measurement of fluorescence intensity of propidium iodide-stained nuclei from *Rubus* cultivars and species of varying ploidy levels.

Genotype[y]	Ancestry[y]	Reported ploidy[z]	Fluorescence intensity (channel no.)
Hillquist	*Rubus argutus* selection	2x	102.6
NC 86-14-02	*R. trivialis* selection	2x	105.7
RUB 817[y]	*R. canadensis* (diploid form)	2x	107.5
White Pearl	*R. allegheniensis* selection	2x	109.5
Burbank Thornless	*R. ulmifolius inermis*	2x	112.4
Flordagrand	(Regal-Ness x *R. trivialis*) x Regal-Ness	2x	113.8
Whitford Thornless	*R. argutus* selection	2x	116.4
Philadelphia	*R. canadensis* selection	3x	154.1
RUB 196[y]	*R. canadensis* (triploid form)	3x	166.0
Choctaw	(Darrow x Brazos) x Rosborough	4x	195.9
Brison	(F_2 of Brainerd x Brazos) x Brazos	4x	196.6
Navaho	ARK 583 x ARK 631	4x	210.0
Cherokee	Brazos x Darrow	4x	214.0
Hull Thornless	(US 1482 x Darrow) x Thornfree	4x	215.4
Shawnee	Cherokee x (Thornfree x Brazos)	4x	219.0
RUB 1151[y]	undetermined species from Pennsylvania	5x	253.3
RUB 1152[y]	undetermined species from Pennsylvania	5x	260.2
Sunberry	*R. ursinus* x Malling Jewel	6x	283.0
Silvan	ORUS 742 (Pacific x Boysen) x Marion	6x	290.7
Waldo	ORUS 1122 x ORUS 1367	6x	293.4
Lincoln Logan	Thornless loganberry	6x	296.2
Bedford Thornless	Thornless mutant of Bedford Giant	6x	297.6
Marion	Chehalem x Olallie	6x	298.6
Olallie	Black Logan x Young	6x	301.2
Tayberry	Aurora x *R. idaeus*	6x	307.1
Boysen 43	clonal selection of Boysen	7x	326.0
Young	Austin Mayes x Phenomenal	7x	328.3
Lucretia	*R. flagellaris*	7x	330.9
Kotata	ORUS 743 x ORUS 877	7x	337.0

Table 2-1. Cont.

Genotype[y]	Ancestry[y]	Reported ploidy[z]	Fluorescence intensity (channel no.)
Douglass[x]	mainly from *R. ursinus*	8x	367.0
Bodega Bay	*R. ursinus* selection	8x	368.0
Jenner	*R. ursinus* selection	8x	370.1
Austin Thornless	Sport or open-pollinated seedling of Austin-Mayes	8x	378.5
Cascade	Zielinski x Logan	9x	408.5
Lincoln Berry	*R. ursinus*, in part	9x	415.6
Tillamook	*R. ursinus*, in part	10x	448.0
Long Black	*R. ursinus*, in part	10x	451.4
Dyke	*R. ursinus* selection	12x	516.0
Zielinski	*R. ursinus* selection	12x	517.0
RUB 197[y]	*R. ursinus* (dodecaploid form)	12x	519.3

[z] As reported by Thompson (1995a; 1995b; 1997)
[y] RUB indicates a USDA-ARS National Clonal Germplasm Repository accession; NC, ARK and ORUS are selections from the North Carolina State University, University of Arkansas and the USDA-ARS/Oregon State University breeding programs, respectively.
[x] Released as 'Black Douglass', patented as 'Douglass'.

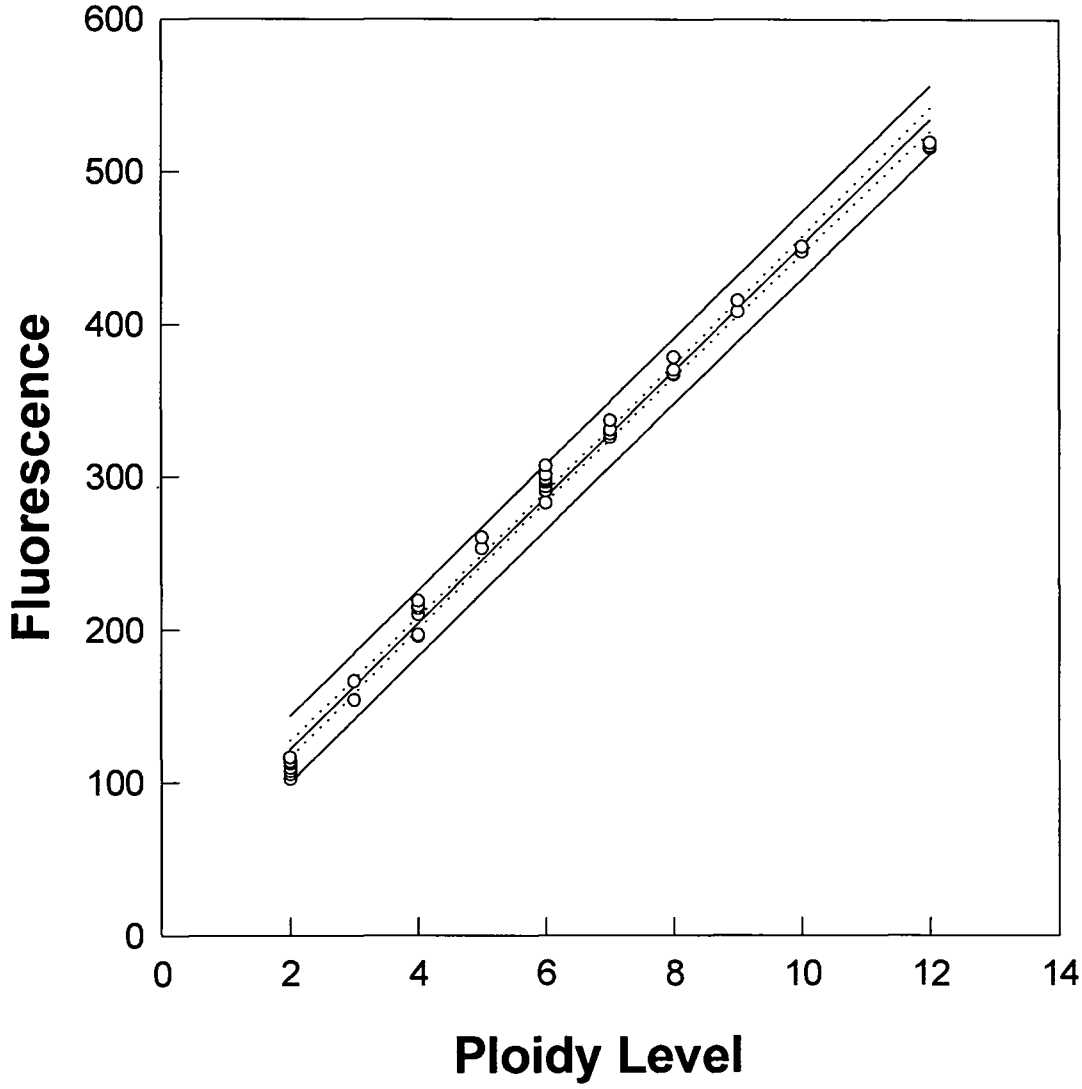


Fig. 2-3. Fluorescence intensity of propidium iodide-stained nuclear DNA of *Rubus* genotypes as a function of ploidy level. The regression line is highly significant (P-value <0.0001). The inner solid line is the estimated mean fluorescence as a function of the ploidy. The dotted lines are the 95% confidence interval for the regression line. The two outer solid lines are the upper and lower endpoints of 95% prediction intervals for the fluorescence at ploidy levels ranging from 2x to 12x.

(Table 2-2). Leaf samples of 30 species representatives from eight subgenera were also evaluated using flow cytometry. The results were analyzed with SAS Insight to calculate means, variances, and a regression equation (Table 2-2 and Fig. 2-4).

In order to better characterize the collection of *Rubus ursinus* from our breeding program, the third group consisted of a broad sampling of this species (Finn and Martin, 1996). While *Rubus ursinus* has been split by some taxonomists into a few different species based on ploidy level and geographic distribution, we used the classification set forth by Jennings (1988) where he considers these to be ecospecies of one polyploid cenospecies. Leaf samples from at least two genotypes in each of 42 *R. ursinus* populations were analyzed with flow cytometry to determine their ploidy level using the 95% prediction interval of fluorescence of the regression line obtained previously from the 40 *Rubus* genotypes with known ploidy levels (Table 2-3 and Fig. 2-5).

The fourth group consisted of 103 genotypes that were of interest to our breeding or germplasm enhancement program. These include species materials, advanced selections and cultivars from the USDA-ARS and other breeding programs (Table 2-4).

A final group of 21 diploid *Rubus* species from five subgenera were analyzed to determine their nuclear DNA content (Table 2-5). Each plant was run three times and the variation in DNA content was evaluated using analysis of variance (SAS, 1990).

Table 2-2. Nuclear DNA flow cytometry measurement of fluorescence intensity of propidium iodide-stained nuclei from *Rubus* species in eight subgenera.

Genotype	Subgenus	Reported ploidy[z]	Fluorescence intensity (channel no.)
Rubus parviflorus Nutt.	*Anaplobatus*	2x	75.8
R. odoratus L.	*Anaplobatus*	2x	93.1
R. nivalis Douglas ex Hook.	*Chamaebatus*	2x	96.5
R. pubescence Raf.	*Cylactis*	2x	77.0
R. lasiococcus A. Gray	*Cylactis*	2x	107.3
R. trifidus Thunb. ex Murray	*Idaeobatus*	2x	79.2
R. microphyllus L. F.	*Idaeobatus*	2x	87.9
R. parvifolius L.	*Idaeobatus*	2x	92.7
R. spectabilis Pursh	*Idaeobatus*	2x	95.5
R. hispidus L.	*Rubus*	2x	96.0
R. sanctus Schreb.	*Rubus*	2x	118.1
R. canescens DC.	*Rubus*	2x	122.3
R. canadensis L.	*Rubus*	3x	166.0
R. sachalinensis Lev.	*Idaeobatus*	4x	175.0
R. parvifolius L.	*Idaeobatus*	4x	196.4
R. tephrodes Hance	*Malachobatus*	4x	188.7
R. lambertianus Ser.	*Malachobatus*	4x	202.6
R. plicatus Weihe & Nees	*Rubus*	4x	189.9
R. hirtus Waldst. & Kit.	*Rubus*	4x	209.3
R. drejeri G. Jensen ex Lange	*Rubus*	4x	224.6
RUB 1151[y]	undetermined species	5x	253.3
RUB 1152[y]	undetermined species	5x	260.2
R. wahlbergii Arrh.	*Rubus*	5x	253.8
R. pectinellus Maxim.	*Chamaebatus*	6x	306.6
R. amphidasys Focke ex Diels	*Dalibardastrum*	6x	288.0

Table 2-2. cont.

Genotype	Subgenus	Reported ploidy[z]	Fluorescence intensity (channel no.)
R. hillii F. Muell.	*Malachobatus*	6x	270.0
R. irenaeus Focke	*Malachobatus*	6x	281.0
R. nubigenus Kunth	*Orobatus*	6x	289.8
R. slesvicensis Lange	*Rubus*	6x	322.8

[z] As reported by Thompson (1995a; 1995b; 1997)
[y] RUB indicates a USDA-ARS National Clonal Germplasm Repository accession.

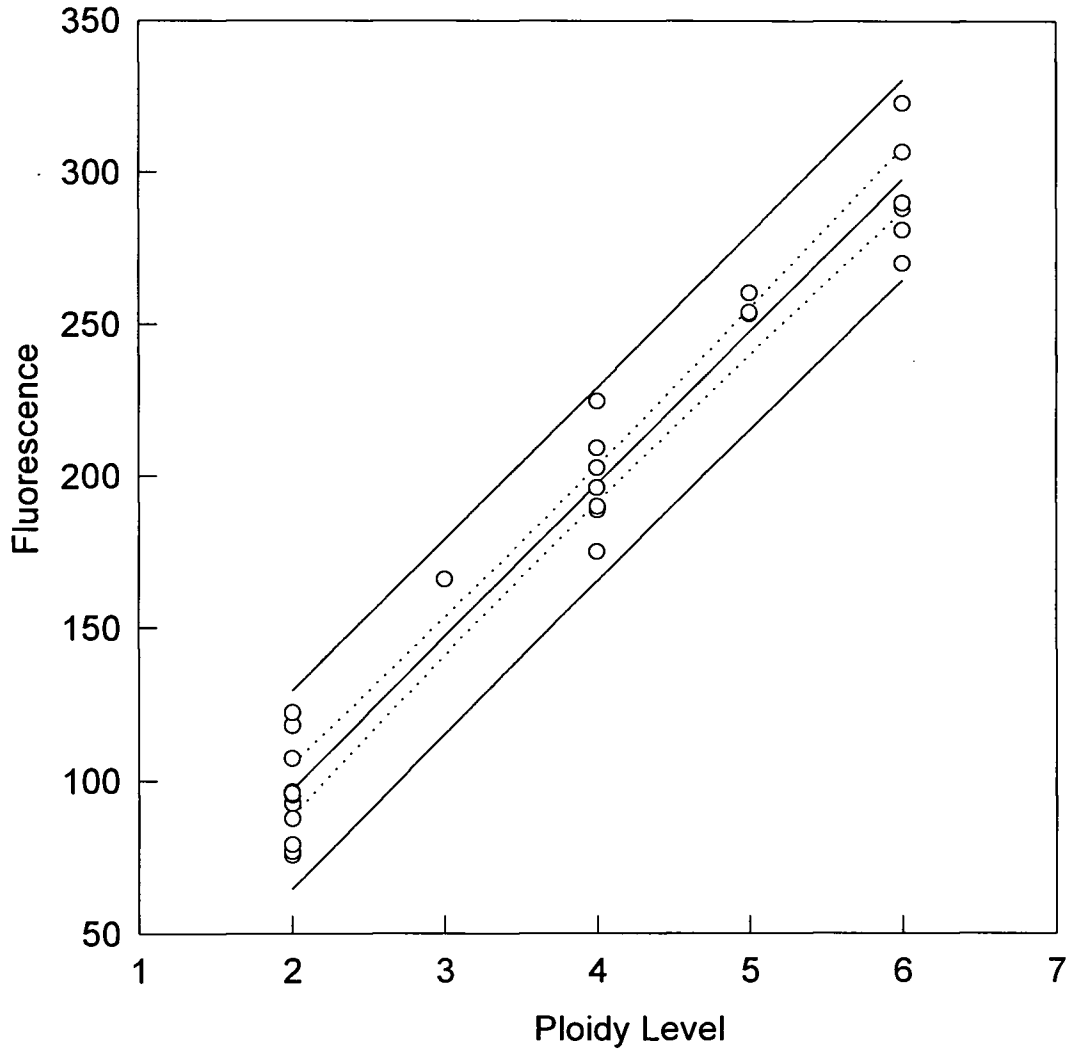


Fig. 2-4. Fluorescence intensity of propidium iodide-stained nuclear DNA of *Rubus* genotypes as a function of ploidy level. The regression line is highly significant (P-value <0.0001). The inner solid line is the estimated mean fluorescence as a function of the ploidy. The dotted lines are the 95% confidence interval for the regression line. The two outer solid lines are the upper and lower endpoints of 95% prediction intervals for the fluorescence at ploidy levels ranging from 2x to 6x.

Table 2-3. Origin, flow cytometry fluorescence and putative ploidy level of 110 *Rubus ursinus* genotypes.

Genotype[z]	Location	Site collected	State	Fluorescence intensity (channel no.)	Estimated ploidy
G2-10	Cascade Mtns.	Huckleberry Mtn.	OR	522.0	12x
G2-6	"	"	"	547.5	12x
G3-12	Coastal Range	S. of Agness	OR	536.5	12x
G3-22	"	"	"	566.9	13x
G4-9	Pacific Coast	Florence	OR	503.1	11x
G4-30	"	"	"	515.4	12x
G4-14	"	"	"	540.4	12x
G4-22	"	"	"	585.0	13x
G5-9R6	Coastal Range	Mary's Peak	OR	494.0	11x
G5-10	"	"	"	529.0	12x
G6-17	Pacific Coast	Aberdeen	WA	509.8	11x
G6-24	"	"	"	538.0	12x
G6-8	"	"	"	541.7	12x
G6-19	"	"	"	561.0	13x
G7-11	Coastal Range	Saddle Mountain	OR	497.7	11x
G7-14	"	"	"	530.8	12x
G7-29	"	"	"	544.0	12x
G7-21	"	"	"	545.3	12x
G7-22	"	"	"	497.4	11x
G8-30	Pacific Coast	Pacific City	OR	520.0	12x
G8-12	"	"	"	553.6	12x
G8-6	"	"	"	574.0	13x
G9-15	Coastal Range	Mt. Hebo	OR	519.4	12x
G9-24	"	"	"	535.0	12x
G10-21	Cascade Mtns.	Iron Mountain	OR	540.4	12x
G10-26	"	"	"	585.7	13x
G11-17	Coastal Range	Triangle Lake	OR	541.8	12x
G11-18	"	"	"	543.0	12x

Table 2-3. Cont.

Genotype[z]	Location	Site collected	State	Fluorescence intensity (channel no.)	Estimated ploidy
G13-7S	Pacific Coast	West Port	WA	525.8	12x
G13-8S	"	"	"	527.3	12x
G14-23	Willamette Valley	Corvallis	OR	536.0	12x
G14-7	"	"	"	544.0	12x
G14-25	"	"	"	561.2	13x
G14-8	"	"	"	569.2	13x
G14-10	"	"	"	598.0	13x
G15-B	Cascade Mtns.	Chilliwack	BC	491.2	11x
G15-A	"	"	"	557.0	13x
G18-15	Cascade Mtns.	Chilliwack Lake	BC	545.0	12x
G18-17	"	"	"	583.6	13x
G19-12	Coastal Range	Horne Lake	BC	532.0	12x
G19-14	"	"	"	546.0	12x
G20-8	Cascade Mtns.	Fraser Valley	BC	542.1	12x
G20-14	"	"	"	584.5	13x
G20-14	"	"	"	544.7	12x
G21-1	Cascade Mtns.	Ryder Lake	BC	585.0	13x
G21-4	"	"	"	554.2	12x
G21-5	"	"	"	544.0	12x
LIG1-A	Coastal Range	SW Quilcene	WA	374.9	8x
LIG1-B	"	"	"	370.3	8x
LIG2-1	Coastal Range	SW Quilcene	WA	524.5	12x
LIG2-6	"	"	"	538.2	12x
LIG2-9	"	"	"	534.0	12x
LIG2-19	"	"	"	539.7	12x
LIG4-9	Coastal Range	NW Quilcene	WA	494.9	11x
LIG4-7	Cascade Mtns.	Lake Wenatchee	WA	515.0	12x
LIG5-B	Pacific Coast	Dungeness	WA	372.0	8x

Table 2-3. Cont.

Genotype[z]	Location	Site collected	State	Fluorescence intensity (channel no.)	Estimated ploidy
LIG5-A	Pacific Coast	Dungeness	WA	510.4	11x
LIG5-8	"	"	"	521.3	12x
LIG5-14	"	"	WA	523.7	12x
LIG5-3	"	"	"	553.8	12x
LIG5-12	"	"	"	584.7	13x
LIG6-6	Pacific Coast	Crescent Bay	WA	520.3	12x
LIG6-3	"	"	"	546.4	12x
LIG6-1	"	"	"	554.6	12x
LIG6-8	"	"	"	560.1	13x
LIG7-B	Coastal Range	SW Crescent Bay	WA	430.6	10x
LIG7-A	"	"	"	498.1	11x
LIG8-B	Coastal Range	SW Crescent Bay	WA	489.0	11x
LIG8-A	"	"	"	493.6	11x
LIG9-1S	Coastal Range	SW Crescent Bay	WA	491.0	11x
LIG9-2S	"	"	"	508.0	11x
LIG17-21	Cascade Mtns.	Mt. Baker	WA	564.2	13x
LIG17-8	"	"	"	578.0	13x
LIG30-8R1	Cascade Mtns.	Lake Wenatchee	WA	499.5	11x
LIG30-7	"	"	"	538.0	12x
LIG30-7R1	"	"	"	543.8	12x
LIG30-4	"	"	"	548.1	12x
LIG33-1	Cascade Mtns.	N. of Packwood	WA	295.1	6x
LIG33-2	"	"	"	525.4	12x
LIG33-3	"	"	"	368.6	8x
LIG33-5	"	"	"	393.7	9x
LIG33-8	"	"	"	298.2	6x
LIG33-4s	"	"	"	337.0	7x
LIG38-30	Cascade Mtns.	Mt.St.Helens	WA	484.1	11x
LIG38-A	"	"	"	498.0	11x
LIG38-B	"	"	"	516.5	12x

Table 2-3. Cont.

Genotype[z]	Location	Site collected	State	Fluorescence intensity (channel no.)	Estimated ploidy
LIG38-6	Cascade Mtns.	Mt.St.Helens	WA	585.0	13x
LIG42-8	Cascade Mtns.	N.of Trout Lake	WA	358.4	8x
LIG42-6	"	"	"	525.9	12x
RUB194-A	Cascade Mtns.	Breitenbush	OR.	491.0	11x
RUB194-B	"	"	"	528.0	12x
RUB395-A	Cascade Mtns.	McKenzie	OR	508.1	11x
RUB395-B	"	"	"	547.0	12x
RUB605-A	Pacific Coast	Port Orchard	WA	433.5	10x
RUB605-B	"	"	"	498.3	11x
RUB649-4	Coastal Range	near Agness	OR	268.9	6x
RUB649-5	"	"	"	333.0	7x
RUB649-7	"	"	"	484.5	11x
RUB649-A	"	"	"	426.0	9x
RUB649-B	"	"	"	540.0	12x
RUB660-B	Cascade Mtns.	N. of Suttle Lake	OR	519.1	12x
RUB660-A	"	"	"	535.3	12x
RUB662-B	Cascade Mtns.	Opal Lake	OR	491.0	11x
RUB662-A	"	"	"	508.0	11x
RUB670-1	Cascade Mtns.	Wind River	WA	522.8	12x
RUB670-2	"	"	"	546.2	12x
RUB686-A	"	"	"	498.0	11x
RUB686-B	Coastal Range	near Quinalt	WA	458.5	10x
RUB708-A	Coastal Range	Deadwood	OR	506.5	11x
RUB708-B	"	"	"	471.6	11x

[z] Genotypes are individuals selected from collected populations. Where the initial number in the accession number is the same, the genotypes are from the same population, e.g.,

LIG 5-14, LIG 5-13 and LIG 5-12 are different genotypes selected from the same population.

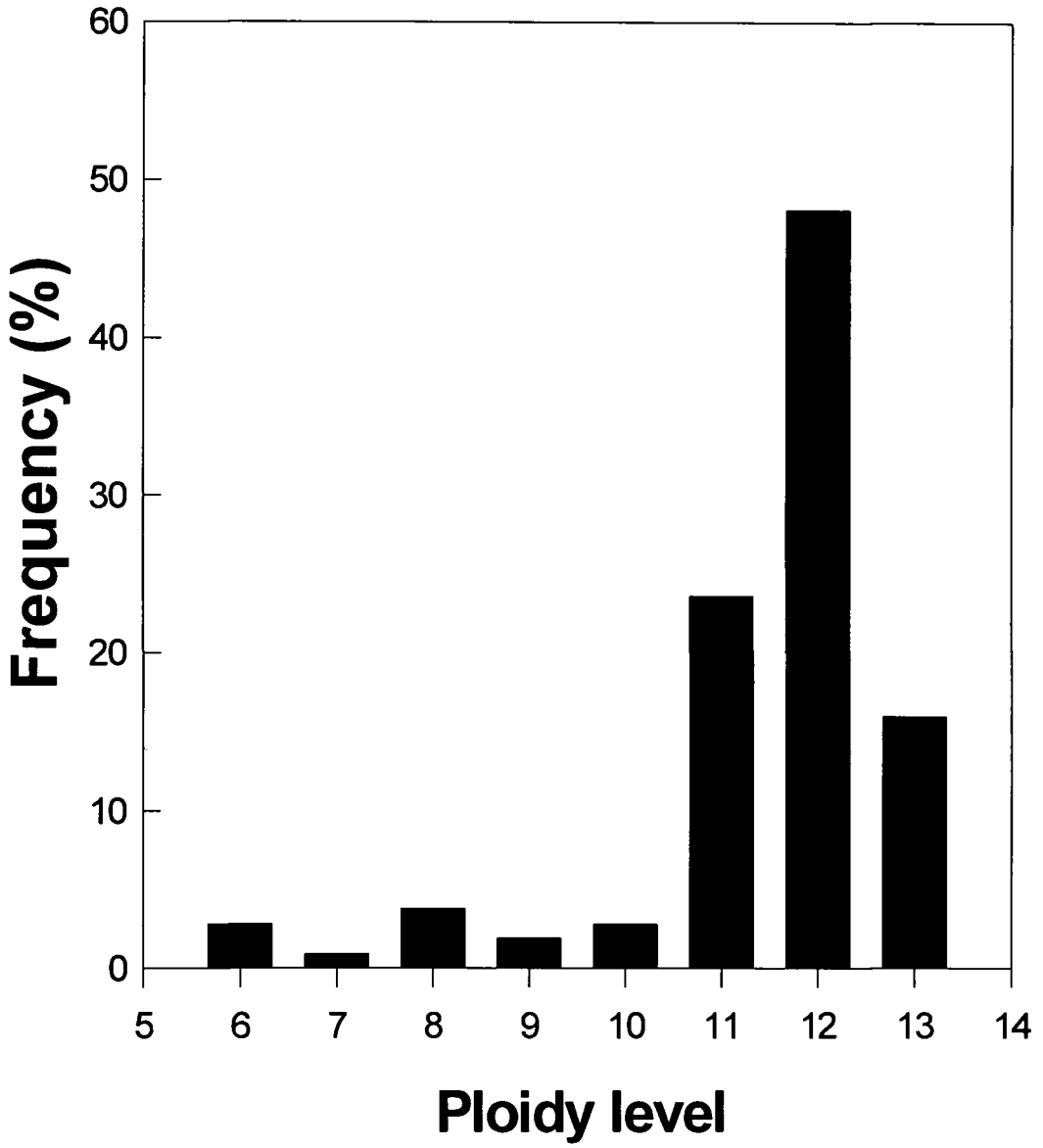


Fig. 2-5. Distribution of ploidy level for 110 *Rubus ursinus* genotypes collected from 42 populations from the Pacific Northwest.

Table 2-4. Nuclear DNA flow cytometry measurement of fluorescence intensity of propidium iodide-stained nuclei, putative ploidy and parents of 84 USDA-ARS selections.

Selection[z]	Fluorescence	Estimated ploidy	Parents[z]
ORUS 742	342.7	7x	Pacific x Boysen
ORUS 965	358.8	8x	ORUS 616 x ORUS 73
ORUS 992	292.1	6x	Chehalem x Olallie
ORUS 993	273.1	6x	Chehalem x Olallie
ORUS 998	268.8	6x	Jenner x Eldorado
ORUS 1063	410.0	9x	ORUS 743 x Chehalem
ORUS 1067	283.8	6x	ORUS 884 x ORUS 743
ORUS 1105	271.0	6x	Olallie x ORUS 878
ORUS 1122	268.8	6x	Marion x ORUS 878
ORUS 1127	301.1	6x	Olallie x ORUS 878
ORUS 1278	308.1	7x	ORUS 1063 x Austin Thnls.
ORUS 1280	320.6	7x	ORUS 1063 x Austin Thnls.
ORUS 1362	270.2	6x	ORUS 1083 x NC 37-35-M2
ORUS 1465	293.6	6x	Olallie x ORUS 998
ORUS 1467	284.3	6x	Olallie x ORUS 998
ORUS 1600	343.0	7x	ORUS 1063 x ORUS 1252
ORUS 1620	370.0	8x	ORUS 917 x ORUS 1282
ORUS 1622	319.3	7x	ORUS 917 x ORUS 1282
ORUS 1683	281.2	6x	Olallie x ORUS 1361
ORUS 1717	398.5	9x	ORUS 1124 x ORUS 1362
ORUS 1826	285.6	6x	ORUS 1122 x Boysen
ORUS 2004	279.0	6x	Marion x ORUS 1683
ORUS 2007	280.0	6x	Marion x ORUS 1683
ORUS 2009	279.9	6x	Marion x ORUS 1683
ORUS 728-3	318.4	7x	ORUS 1717 x ORUS 1826
ORUS 817 R-6	298.6	6x	ORUS 2028 x Kotata
ORUS 826-2	280.6	6x	ORUS 1683 x ORUS 1991
ORUS 828-42	278.1	6x	ORUS 1683 x ORUS 1122
ORUS 887-2	278.3	6x	ORUS 1362 x Himlaya
ORUS 887-3	294.3	6x	ORUS 1362 x Himlaya
ORUS 917-1	291.2	6x	ORUS 1122 x ORUS 2028
ORUS 1052-3	235.0	5x	ORUS 880-5 x ORUS 1826
ORUS 1111-1	282.6	6x	ORUS 728-3 x Siskiyou
ORUS 1112-1	296.1	6x	Siskiyou x ORUS 1717

Table 2-4. Cont.

Selection[z]	Fluorescence	Estimated ploidy	Parents[z]
ORUS 1112-2	329.8	7x	Siskiyou x ORUS 1717
ORUS 1113-1	312.7	7x	Siskiyou x Waldo
ORUS 1113-5	290.0	6x	Siskiyou x Waldo
ORUS 1117-11	291.0	6x	ORUS 1122 x ORUS 2009
ORUS 1120-1	307.0	6x	ORUS 1684 x ORUS 2009
ORUS 1122-1	293.4	6x	Olallie x ORUS 728-3
ORUS 1237-1	331.6	7x	Kotata x ORUS 998
ORUS 1247-1	272.3	6x	ORUS 993 x Kotata
ORUS 1251-2	272.0	6x	ORUS 1112-2 x ORUS 817 R-6
ORUS 1258-1	275.0	6x	ORUS 1127 x Kotata
ORUS 1294-1	272.3	6x	Aurora x Choctaw
ORUS 1295-2	316.0	7x	Aurora x Siskiyou
ORUS 1313-1	274.6	6x	ORUS 1122-1 x Waldo
ORUS 1313-4	280.4	6x	ORUS 1122-1 x Waldo
ORUS 1313-8	276.0	6x	ORUS 1122-1 x Waldo
ORUS 1316-1	298.1	6x	ORUS 817R-6 x ORUS 1122-1
ORUS 1316-7	306.0	6x	ORUS 817R-6 x ORUS 1122-1
ORUS 1332-8	280.6	6x	ORUS 1113-1 x ORUS 817R-6
ORUS 1368-1	276.3	6x	ORUS 828-42 x Black Butte
ORUS 1368-2	277.3	6x	ORUS 828-42 x Black Butte
ORUS 1369-3	291.8	6x	ORUS 828-42 x ORUS 1122-1
ORUS 1378-2	275.1	6x	ORUS 1111-1 x ORUS 1122-1
ORUS 1380-1	400.9	9x	ORUS 1117-11 x ORUS 1122-1
ORUS 1382-1	280.9	6x	ORUS 1117-11 x ORUS 728-3
ORUS 1382-2	343.4	7x	ORUS 1117-11 x ORUS 728-3
ORUS 1392-1 Thornless	204.0	4x	Illini Hardy x Chester
ORUS 1393-1	253.0	5x	Navaho x ORUS 1122-1
ORUS 1393-2	250.0	5x	Navaho x ORUS 1122-1
ORUS 1393-3	261.6	5x	Navaho x ORUS 1122-1
ORUS 1393-4	265.0	5x	Navaho x ORUS 1122-1
ORUS 1394-1	266.3	5x	Navaho x Black Butte
ORUS 1395-1	317.0	7x	Navaho x Kotata
ORUS 1395-2	304.0	6x	Navaho x Kotata
ORUS 1397-1	320.0	7x	Kotata x Navaho
ORUS 1397-2	265.9	5x	Kotata x Navaho
ORUS 1397-3	290.8	6x	Kotata x Navaho
ORUS 1397-4	260.2	5x	Kotata x Navaho
ORUS 1397-5	302.1	6x	Kotata x Navaho

Table 2-4. Cont.

Selection[z]	Fluorescence	Estimated ploidy	Parents[z]
ORUS 1397-6	304.5	6x	Kotata x Navaho
ORUS 1398-1	316.8	7x	Lincoln Logan x Navaho
ORUS 1398-2	260.5	5x	Lincoln Logan x Navaho
ORUS 1410-1	211.7	4x	Chester Thornless x Illini Hardy
ORUS 1413-1	335.7	7x	Marion x Chester Thornless
ORUS 1438-1	328.8	7x	[Douglass x (LB x Mono)] x Walt
ORUS 1438-2	405.9	9x	[Douglass x (LB x Mono)] x Walt
ORUS 1438-5	333.1	7x	[Douglass x (LB x Mono)] x Walt
ORUS 1442-2	425.0	9x	[Douglass x (LB x Mono)] x Rich
ORUS 1442-3	415.4	9x	[Douglass x (LB x Mono)] x Rich
ORUS 1469-1	350.9	8x	Ranui x NW 8729-2
ORUS 1508	287.0	6x	ORUS 913-10 x ORUS 1122-2
ORUS 1532	318.1	7x	ORUS 2024 x Siskiyou
ORUS 1534	323.2	7x	ORUS 2024 x Black Butte
ORUS 1535	349.4	7x	Olallie x Douglass
ORUS 1638-1	278.5	6x	ORUS 1122-1 x NW 9059R-3
N-71	285.0	6x	Aurora x Comanche
NW 90B1-2	288.1	6x	ORUS 817R-6 x Siskiyou
NZ 9368-5	346.6	7x	NZ 8919RDF-7 x NZ 8927RMC.1
NZ 9373-1	336.7	7x	NZ 8927RMC-4 x NZ 8956CC-10
Mac. L.L. San Juan	383.6	8x	B. Douglass selection of *R. ursinus*
Mono x LB	355.3	8x	B. Douglass selection
Black Butte	291.0	6x	Siskiyou x ORUS 728-3
Siskiyou	333.2	7x	ORUS 2027 x ORUS 1826
Triple Crown	222.9	4x	C-47 x ARK 545
96050 (CRUB 1917)[y]	196.0	4x	collected as *R. crataegifolius* in NE China, probably *R. parvifolius*
R. crataegifolius (96064)[y]	76.0	2x	*R. crataegifolius* collected in NE China
R. crataegifolius (96068)[y]	78.7	2x	*R. crataegifolius* collected in NE China
Jokgal	65.3	2x	*R. crataegifolius* cultivar
R. crataegifolius Bunge	87.3	2x	*Unknown*
Jingu Juegal	75.0	2x	*R. crataegifolius* cultivar

[z] Sources of selected genotypes -
ARK = University of Arkansas
Rich, LB, Mono, Walt = Barney Douglass, private breeder, Hillsboro, Ore.
C = USDA-ARS, Carbondale, Ill.
NW = OSU-NWREC, Aurora, Ore.
NZ, N = HortResearch Inc., New Zealand
ORUS =USDA-ARS, Corvallis, Ore.; A new selection numbering system that included a "-" followed by a number was phased in in the late 1970's. Therefore, selection numbers lacking a "-" are from an earlier era in the breeding program and as a result there is the possibility of very similar numbers such as ORUS 1122 and ORUS 1122-1 that are different genotypes.
[y]Thompson et al., 1996

Table 2-5. Nuclear DNA content of 21 diploid *Rubus* species from five subgenera by flow cytometry measurement of fluorescence intensity of propidium iodide-stained nuclei.

			2C Nuclear DNA (pg)	
Genotype	Subgenus	Reported ploidy[z]	Mean	SD
Rubus parviflorus L.	*Anaplobatus*	2x	0.54	0.04
R. odoratus L.	*Anaplobatus*	2x	0.64	0.08
R. nivalis Douglas ex Hook.	*Chamaebatus*	2x	0.56	0.06
R. lasiococcus A. Gray	*Cylactis*	2x	0.69	0.08
R. illecebrosus Focke	*Idaeobatus*	2x	0.47	0.03
R. crataegifolius Bunge	*Idaeobatus*	2x	0.49	0.04
R. leucodermis Doug ex Torrey & Gray	*Idaeobatus*	2x	0.51	0.03
R. simplex Focke	*Idaeobatus*	2x	0.52	0.04
R. parvifolius L.	*Idaeobatus*	2x	0.53.	0.05
R. innominatus S. Moore	*Idaeobatus*	2x	0.54	0.05
R. spectabilis Pursh	*Idaeobatus*	2x	0.54	0.08
R. niveus Thunb.	*Idaeobatus*	2x	0.57	0.05
R. pinfaensis Lev. &Vaniot	*Idaeobatus*	2x	0.59	0.09
R. occidentalis L.	*Idaeobatus*	2x	0.60	0.06
R. lasiostylus Focke	*Idaeobatus*	2x	0.62	0.08
R. ellipticus Sm.	*Idaeobatus*	2x	0.69	0.07
R. hispidus L.	*Rubus*	2x	0.59	0.09
R. canadensis L.	*Rubus*	2x	0.59	0.10
R. trivialis Michx.	*Rubus*	2x	0.71	0.11
R. canescens DC.	*Rubus*	2x	0.73	0.08
R. sanctus Schreb.	*Rubus*	2x	0.75	0.11

[z] As reported by Thompson (1995a; 1995b; 1997)

CHAPTER 3

RESULTS

3.1 RELATIONSHIP BETWEEN CHROMOSOME NUMBER AND FLUORESCENCE INTENSITY

The fluorescence intensity for the group of genotypes with known ploidy levels increased as ploidy level increased (Table 2-1 and Fig. 2-1). There were significant differences in mean fluorescence between ploidy levels (p-value ≤ 0.001; analysis of variance F-test). The fluorescence increased as a result of the increase in ploidy level (one-sided p-value < 0.0001, t-test). Based on the regression analysis, ploidy level accounted for ninety-nine percent of the variation in fluorescence intensity ($r^2 = 99\%$) and variation among the ploidy levels was much higher than within ploidy levels (Table 2-1 and Fig. 2-1).

3.2 RELATIONSHIP BETWEEN CHROMOSOME NUMBER AND FLUORESCENCE INTENSITY ACROSS SEVEN *RUBUS* SUBGENERA.

The fluorescence intensity for the species in all seven subgenera increased as ploidy level increased (one-sided p-value < 0.0001, t-test), although they showed a wider variation in fluorescence intensity than the first group of plant material. Based on the regression analysis, ninety-six percent of variation of fluorescence was explained by the variation among the ploidy levels, which was much higher than within ploidy levels (Table 2-2 and Fig. 2-2).

In order to use the regression equation to determine *Rubus* genotypes of unknown ploidy, the inverse prediction (95%) was made to give the upper and lower limit of fluorescence of all ploidy levels (Table 3-1) (Ramsey and Schafer, 1997). The upper and lower limit as described by Ramsey and Schafer (1997) are as follows:

Upper limit = PF + $t_{38}(0.975)$ x SE [Pred{Fluorescence|ploidy}]

Lower limit = PF – $t_{38}(0.975)$ x SE [Pred{Fluorescence|ploidy}]

PF = Predicted fluorescence = 39.26 + 41.24(ploidy) (This is the regression equation obtained from testing genotypes with known ploidy level, Figure 2-3)

SE [Pred{Fluorescence|ploidy}] = σ {1+1/n + (X_0-Xaverage)/ [(n-1) S_x^2]}

σ = population standard deviation

X_0 = ploidy (i.e. 2,3,4,5,6,7,8,9,10,11,12)

Xaverage = average of ploidy level (independent population)

n = number of samples = 40

S_x^2 = sample variance of ploidy level (independent population)

For example, based on the regression line produced by our data, it was predicted that 95% of the 6x *Rubus* genotypes would produce fluorescence intensity between 265.07 and 308.33. According to this statement, 2.5% of 6x genotypes would have a fluorescence lower than 265.07 and 2.5% would have the fluorescence above 308.33.

3.3 *RUBUS URSINUS* COLLECTION

Using the 95% prediction interval, the ploidy level of 110 *Rubus ursinus* genotypes from 42 populations of *R. ursinus* ranged from 6x to 13x. While 86% of the

Table 3-1. The 90% and 95% prediction intervals for fluorescence intensity produced by all ploidy levels of *Rubus* genotypes.

		Fluorescence intensity			
		95% Prediction interval		90% Prediction interval	
Ploidy	predicted value	Lower limit	Upper limit	Lower limit	Upper limit
2x	121.7	99.6	143.8	103.3	140.2
3x	163.0	140.9	184.9	144.7	181.2
4x	204.2	182.5	226.0	186.1	222.3
5x	245.5	232.2	267.1	227.4	263.5
6x	286.7	265.1	308.3	268.7	304.7
7x	327.9	305.7	349.6	309.9	346.0
8x	369.2	347.0	391.4	351.0	387.3
9x	410.4	392.2	428.7	399.6	421.3
10x	451.7	429.5	473.8	433.2	470.1
11x	492.9	470.5	515.3	474.2	511.6
12x	534.1	511.4	556.9	515.2	553.1

genotypes were 11x, 12x or 13x, there were three 6x, two 7x, five 8x, two 9x and three 10x genotypes (Table 2-3). No 7x, 9x, 10x, and 11x had been previously reported in Oregon, Washington and British Columbia (Brown, 1943) and no 13x has ever been reported. Many samples collected from the same site had different ploidy levels.

3.4 SELECTIONS AND COLLECTIONS IN USDA-ARS BREEDING PROGRAM

Using the 95% prediction interval, the ploidy level of cultivars and selections in our breeding program was estimated (Table 2-4).

3.5 VARIATION OF NUCLEAR DNA CONTENT OF DIPLOID *RUBUS* SPECIES FROM FIVE SUBGENERA

Idaeobatus, *Chamaebatus*, and *Anaplobatus* had significantly lower DNA content than those of *Rubus* and *Cylactis* (Table 3-2). In subgenus *Rubus*, *R. hispidus* L. and *R. canadensis* L. had the lowest DNA content and *R. sanctus* Schreb had the highest DNA content, 0.59 and 0.75 pg, respectively (Table 3-3). *Idaeobatus* had greater variation in DNA content among diploid species than the *Rubus* subgenus, with the highest being from *R. ellipticus* Smith (0.69 pg) and lowest from *R. illecebrosus* Focke (0.47 pg) (Table 3-4).

Table 3-2. Mean nuclear DNA content (picograms) using flow cytometry for 2x *Rubus* species in five subgenera.

Subgenus	Number of samples tested	DNA content (pg)		LSD groupings[z] (*t*-test)
		Mean	SD	
Idaeobatus	36	0.56	0.08	A
Chamaebatus	3	0.56	0.06	A
Anaplobatus	6	0.59	0.08	AB
Rubus	15	0.67	0.11	B
Cylactis	3	0.69	0.08	B

[z] LSD = 0.11. Means with the same letter are not significantly different at 0.05 level.

Table 3-3. Nuclear DNA content (picograms) using flow cytometry for five 2x species of subgenus *Rubus*.

Species	No. samples tested	DNA content (pg)		LSD groupings[z] (*t*-test)
		Mean	SD	
Rubus canadensis L.	3	0.59	0.10	A
R. hispidus L.	3	0.59	0.09	A
R. sanctus Schreb.	3	0.75	0.11	A
R. trivialis Michx.	3	0.71	0.11	A
R. canescense DC.	3	0.73	0.08	A

[z] LSD = 0.18. Means with the same letter are not significantly different at 0.05 value.

Table 3-4. Nuclear DNA content (picograms) using flow cytometry for 12 2x species of subgenus *Idaeobatus*.

Species	No. samples tested	DNA content (pg)		LSD groupings[z] (*t*-test)
		Mean	SD	
Rubus ellipticus Smith	3	0.69	0.07	A
R. lasiostylus Focke	3	0.62	0.08	AB
R. occidentalis L.	3	0.60	0.06	ABC
R. pinfaensis Lev. & Vaniot	3	0.59	0.09	ABCD
R. niveus Thunb.	3	0.57	0.05	BCDE
R. spectabilis Pursh	3	0.54	0.08	BCDE
R. innominatus S. Moore	3	0.54	0.05	BCDE
R. parvifolius L.	3	0.53	0.05	BCDE
R. simplex Focke	3	0.52	0.04	CDE
R. leucodermis Doug ex Torrey & Gray	3	0.51	0.03	CDE
R. crataegifolius Bung	3	0.49	0.04	DE
R. illecebrosus Focke	3	0.47	0.03	E

[z] LSD = 0.10. Means with the same letter are not significantly different at 0.05 level.

CHAPTER 4

DISCUSSION

Polyploidy has played a significant role in plant evolution and many important crops are polyploids. Sexual polyploidization (the fusion of non-reduced gametes) is the principal mode of polyploidization in nature. In fruit breeding, heteroploid crosses have been extensively used in small fruit, especially in blackberry (C. Finn, personal communucation), raspberry (Sanford, 1983), and blueberry (Costich, 1993). Crossability and fertility are always a concern for breeding materials that are polyploid and knowing the exact ploidy level of the genotypes helps in planning crosses and plant identification.

In *Rubus* breeding programs and in nature, 2n gametes are frequently produced, which makes predicting the ploidy level of the progeny difficult. Fertility of the progeny is often correlated with its ploidy level. By using the protocol developed in this research, ploidy level can be determined easily and reliably and problematic progenies can be identified, saving time and resources. In an effort to incorporate valuable traits from materials from outside our breeding program, for example, our blackberries crossed with tetraploid eastern blackberries, we can determine whether what we believe are hybrids are truly hybrids. For example, ORUS 1395-1 and ORUS 1395-2 have the same tetraploid maternal parent and a septaploid paternal parent and are 6x and 7x, respectively, confirming that at least one of our selections was from a successful hybridization.

Most of the genotypes that we tested gave the expected results when the ploidy of the parents were known or suspected. However, because the number of genotypes from

which we produced the regression formula was relatively small compared to the large number of genotypes in a breeding program, it is not surprising that there was some deviation from the regression line. Variation from the flow cytometer and in sample preparation can also contribute to the variation in the results. Theoretically, when the ploidy level is zero, there should be no fluorescence output. However, with our regression equation (Fluorescence = 39.26 + 41.24 * Ploidy), the y-intercept was 39.26 when the ploidy was zero. This is most likely due to other fluorescent materials, such as chloroplasts and mitochondria DNA and ribosomal RNA, which might not have been destroyed completely with the current protocol. Each genotype should be tested at least twice to increase confidence in the results.

Consistent leaf sampling is critical for obtaining consistent results. Surprisingly large variation in nuclear DNA content have been reported to occur in response to factors such as stress (Bassi, 1990; Price 1991), so healthy leaves are required. We also found that the freshness of the leaves directly contributed to the successful isolation of intact nuclei and hence to the accuracy and consistency of the results. New leaves from young canes are ideal for nuclei suspension preparation because older leaves produce fewer intact nuclei and these nuclei fluoresce less.

Rubus ursinus, including *R. ursinus* Cham. & Schlecht *R. macropetalus* Dough., and *R. vitifolius* Cham. & Schlecht., is widely distributed in the Pacific Northwest. *Rubus ursinus* that are 6x, 8x, 9x, 10x, 11x, and 12x are reported throughout California, Oregon, Washington and British Columbia with 12x genotypes most common (Brown, 1943). In this research, we found plants with PI fluorescence that, based on our regression equation and prediction interval, should be 13x. These could be confirmed in the future with

microscopic counting. The occurrence of 8x *R. ursinus* (LIG 1-A and LIG 1-B) in northern Washington (Table 2-3) was surprising as the 8x forms have been hypothesized to only occur in California and southern Oregon (Brown, 1943). Ploidy level, 8x vs. 12 x, and geographical location were used by Brown (1943) to separate *R. ursinus* from *R. macropetalus*. Our results suggest that the 8x types can be found throughout a much broader range than previously suggested. The range of ploidy levels that we determined in *R. ursinus* also suggests that this species exists as several ploidy levels and that, for *R. ursinus*, ploidy level is probably not a valid criteria to use when separating these various forms into different species, i.e. they are not different species.

Somatic chromosome doubling and the sexual functioning of non-reduced gametes can give rise to polyploids. However, spontaneous chromosome doubling, either in the zygote to produce a polyploid plant or in an apical meristem to produce a polyploid chimera, is a rare event. Hence, the common mode of polyploidization is through the formation and sexual functioning of cytologically non-reduced gametes, followed by fertilization with reduced gametes giving, step-by-step, triploids, tetraploids, and higher polyploids. An increase in chromosome numbers can occur in the first or later hybrid generations. To directly produce polyploids by the fertilization of non-reduced gametes from both parents is very rare (deWet, 1980). Therefore, although 13x *R. ursinus* genotypes have not been reported, they are possible. A 13x genotype can be produced during natural crosses with non-reduced gametes, such as 10x x 8x, 12x x 7x, 8x x 9x, 6x x 10x, and even in rare cares, 6x x 7x. Also, genotypes with the same chromosome number but different nuclear DNA content may be produced through somatic doubling or the sexual functioning of cytologically non-reduced gametes in polyploids developed

from diploid species with variable nuclear DNA content. The biggest difference in DNA content in our study was between *R. sanctus* (0.75 pg) and *R. illecebrosus* (0.47 pg), a difference of 0.28 pg. So, in theory the difference between two 12x genotypes could be as large as 1.68 pg (0.28 pg x 6), which equals the DAN from 4.48 to 7.15 sets of chromosomes, if the polyploids are formed by spontaneous somatic doubling. In other words, a 12x genotype could have the same nuclear DNA content of a genotype that is over 5x in ploidy. However, because somatic doubling is extremely rare and the functioning of non-reduced gametes is the major mode in the formation of polyploids, the situation leading to such large differences is not likely. During the formation of polyploids by non-reduced gametes, a diploid with high (or low) DNA content has the same chance to cross with genotypes with high or low DNA content. Therefore, the difference in DNA content within the genotypes with the same ploidy level can't be increased by accumulation. This could explain why we didn't find significant genome differences among the cultivars within the same ploidy levels. However, genome variation can still exist among the genotypes with the same chromosome number, especially in higher polyploids. Actually, the higher the ploidy level, the larger the difference among the genotypes of the same ploidy. Based on the above discussion, the 13x-like genotypes may actually be 12x with a larger nuclear DNA content by the accumulation of DNA content difference during the evolution of 12x from diploid species with a larger genome than those from diploids with a smaller genome. Research needs to be done to study the cytological characters of representatives of populations of *Rubus ursinus*. Greater variation among genotypes at the higher ploidy levels might be another

explanation for genotypes appeared to be 13x might really be 12x. However, when a logarithmic transformation was used to try to reduce variation among the different ploidy levels and to give a more accurate regression equation it widened the prediction band and reduced the accuracy of our inverse prediction. Therefore, genotypes that are predicted to be 13x need to be manually counted to determine if they are truly 13x and not 12x.

Due to abnormal meiosis that leads to non-reduced gametes and uneven chromosome segregation in odd-ploid plants, the phenomenon of different ploidy levels existing in the different selections from the same cross was often evident, eg. ORUS 1112-1 and ORUS 1112-2 (Table 2-4). For selections like ORUS 1398-1 (7x), ORUS 1398-2 (7x) ('Lincoln Logan' x 'Navaho'), and ORUS 1313-1 (7x) ('Marion' x 'Chester Thornless'), they are apparently due to the non-reduced gamete from the 4x parent, 'Navaho' or 'Chester Thornless'.

The usefulness of flow cytometry to aid taxonomic analysis can be illustrated with attempts to determine the species identity of a germplasm accession collected from China. In 1996, a *Rubus* accession (USDA-ARS National Clonal Germplasm Repository accession CRUB 1917) was collected in China as *R. crataegifolius* Bunge (Thompson et al., 1996). The identification was based on fruit morphology in a market sample and the opinion of a local botanist. Seedlings from this and other accessions collected as *R. crataegifolius* were grown and CRUB 1917 appeared very different vegetatively in the first year in the field. Genotypes from these populations were subjected to flow cytometry analysis, along with two *R. crataegifolius* cultivars ('Jokgal' and 'Jingu Juegal') that were known to be 2x Thompson, 1995a). Genotypes from CRUB 1917 tested as 4x, whereas the other *R. crataegifolius* populations (96064, 96068) that appeared morphologically to

be truly *R. crataegifolius* tested as 2x suggesting either a misidentification of CRUB 1917 or a doubling of the chromosome in the species. Subsequently, as the plants have matured and fruited, the population has been identified as a 4x form of *R. parvifolius* L. (Table 2-2; M.M. Thompson, personal communication).

Using laser flow cytometry of isolated nuclei stained with PI provides an opportunity for rapid determination of nuclear DNA content of diploid *Rubus* species. The overall genome size of *Rubus,* as determined here from 21 species in five subgenera, is 0.30 ± 0.05 pg. The genome size can be estimated as 289.5 Mbp/haploid genome by assuming that 1 pg of nuclear DNA has 965 Mbp (Bennette and Smith, 1976), which is about the same as that estimated for the genome of apricot (*Prunus armeniaca* L.), peach (*P. persica* (L.) Batsch) and sweet cherry (*P. avium* (L.) L.), about twice as much as *Arabidopsis*, about 1.5, two and 10 times smaller than prune (*P. domestica* L.), blueberry (*Vaccinium* section *Cyanococcus*) (Costich et al, 1993) and apple (*Malus domestica* Borkh.), respectively, (Arumuganathan and Earle, 1991a)

The results of this research confirm the existence of significant DNA content variation among diploid *Rubus* species and subgenera, from the smallest, 0.47 pg from *R. illecebrosus*, to the largest, 0.75pg from *R. sanctus* (Table 3-3). However, this is not as much variation as in *Helianthus* that has a fourfold variation among diploid species (Sims and Price, 1985). *Anaplobatus* has no significant difference in nuclear DNA content from that of any of the other four *Rubus* subgenera. *Idaeobatus* and *Chamaebatus* have a similar DNA content but both are significantly different from *Rubus* and *Cylactis*. Within the *Rubus* subgenus, there is no significant variation among the five species tested (Table

3-3). Whereas, *Idaeobatus* had significant variation among the 12 species tested (Table 3-4). Several processes could account for this variation. Selection and accumulation of small deletions or duplications may explain the variation in nuclear DNA content, which may result in the interspecific DNA differences distributed throughout the genome (Price, 1976). Part of the variation in nuclear DNA content could result from highly reiterate (redundant) sequences of DNA in the genome. Environmental and genomic stress may activate the amplification and deletion of DNA sequences. The correlation of nuclear DNA variation of diploid *Rubus* with the genetic distance could not be done because of a lack of published information on genetic distance.

Flow cytometry has detected aneuploid variation in mammalian cells, but aneuploid *Rubus* genotypes were not detected in this research. Aneuploids mainly result from the loss or gain of a chromosome(s) during the meiotic process, such as bivalent formation, pairing, crossing over, and segregation, though a small fraction of the aneuploids may have arisen from mitotic malsegregation during embryonic development (Sandhu and Gill, 1987).

To detect aneuploids in *Rubus*, three things need to be done. First, the resolution of the protocol must be improved to detect the DNA difference produced from one single chromosome, which is less than 0.1 pg based on our results. Second, a sufficient number of known aneuploids, differing by one chromosome, would be needed for testing. Finally, nuclear DNA content is not necessarily distributed in each chromosome equally. *Rubus minusculus* Levl. et Vant. and *R. croceacanthus* Levl. were reported to have metaphase chromosomes ranging in size from 0.9 um to 1.4 μm and 1.0 μm to 1.5 μm, respectively

(Yoshikane et al., 1996). Therefore, the number of base pairs of each chromosome would need to be determined as a basis for calculation of differences in DNA content produced from one specific chromosome.

When trying to differentiate aneuploids in our experiments, we had only four aneuploids available with known ploidy. They were 'Tayberry' seedling (RUB 227, 6x +2), 'Carolina' (RUB 102, 7x +4), 'Aurora' (RUB 101 and RUB 134, 8x+2), and 'Santiam' (RUB 79, 6x+5). The fluorescence from 'Aurora' and the 'Tayberry' seedling was not consistently higher than that from other 8x genotypes and 6x, respectively. This could be due to two reasons: 1) the cytological composition is different between 'Aurora' and other 8x genotypes and between 'Tayberry' seedling and other 6x genotypes, and the total number of chromosome base pairs from 'Aurora' (or 'Tayberry' seedling) is not more than that of other 8x (or 6x) genotypes, or 2) 'Aurora's (or Tayberry seedling's) genome is bigger than other 8x (or 6x) genotypes' but our protocol is not sensitive enough to detect the increase of fluorescence produced by two chromosomes. For 'Santiam' and 'Carolina', their fluorescence was higher than other 8x species but it couldn't be separated from the 9x genotypes. There have not been any reports on the cytological or molecular composition of *Rubus* genome that would have been useful for detecting aneuploids.

CHAPTER 5

CONCLUSION

Nuclear DNA flow cytometry can be used to determine ploidy level and nuclear DNA content in *Rubus*. The protocol we developed to differentiate ploidy level in *Rubus* genotypes is effective in differentiating genotypes differing by 1x. It can be used on cultivars and wild species throughout the *Rubus* genera and provides a more efficient technique than microscopic chromosome counting. Flow cytometry provides the opportunity to quickly determine genome size of *Rubus* genotypes, which is an important parameter for many aspects of studies at the molecular level.

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