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Determining Ploidy Level and Nuclear DNA Content in *Rubus* by Flow Cytometry

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Abstract. **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. DNA was stained with propidium iodide. Measurement of fluorescence of 40 genotypes, whose published ploidy ranged from diploid to dodecaploid, indicated that fluorescence increased with an increase in chromosome number. Ploidy level accounted for 99% of the variation in fluorescence intensity ($r^2 = 0.99$) and variation among ploidy levels was much higher than within ploidy levels. This protocol was used successfully for genotypes representing eight different *Rubus* subgenera. *Rubus ursinus* Cham. and Schldl., a native blackberry species in the Pacific Northwest, which 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, 11x, and 13x forms were found as well. Attempts to confirm the 13x estimates with manual counts were unsuccessful. Ploidy level of 103 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 the result of nonreduced gametes. This technique was effective in differentiating chromosome numbers differing by 1x, but was not able to differentiate aneuploids. 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 the *Rubus* subgenus, *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* (0.47 pg).**

Rubus is a large genus that includes an estimated 900 to 1000 species that are widely distributed in various ecosystems (Thompson, 1997). A number of commercial crops are members of this genus including red and black raspberries (*R. idaeus* L. and *R. occidentalis* L.) and blackberries (*Rubus* sp. L.). 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, 1995a). *Rubus* has 12 subgenera but the species and cultivars used in blackberry and raspberry breeding have largely been from the *Rubus* or *Idaeobatus* subgenera, respectively. The *Rubus* subgenera range from diploids to dodecaploids (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 different *Rubus* ploidy levels have been used in blackberry breeding and released as cultivars. Heteroploid crosses are often used to introduce desirable genes from wild species or from elite material of different species or crop types, and can usually generate progenies with varying ploidy levels. Factors such as spontaneous doubling of chromosomes at an early stage of development or the union of a nonreduced gamete (produced by nonreduction of sporocytes during meiosis or reduction of polyploid premeiotic cells that appear nonreduced) 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, 1986; Waldo, 1950).

In blackberry and raspberry breeding programs, knowing the ploidy level of *Rubus* genotypes is essential to predict crossing success and parental combinations that might produce problematic progenies. For germplasm enhancement, ploidy level can also serve as a valuable distinguishing taxonomic trait when collections of *Rubus* are evaluated (Thompson, 1995a, 1995b).

In the Pacific Northwest, the native *R. ursinus* Cham. & Schldl. is widely distributed and has been a valuable genetic resource for developing trailing blackberry cultivars (Finn et al., 1997). *Rubus ursinus* has a known ploidy level range from hexaploid to dodecaploid except for septaploid (Brown, 1943). In 1993, *R. ursinus* was collected from throughout the Pacific Northwest and established in a common garden in Corvallis, Ore. (Anderson and Finn, 1996). These populations were evaluated for horticultural and taxonomic characteristics and superior individuals were identified. 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.

Successful chromosome counts have been made on at least 387 *Rubus* species, ≈40% of the known species in the genus, and 90 cultivars and selections (Thompson, 1995a, 1995b, 1997). Ploidy level in *Rubus* genotypes has usually been determined by chromosome counts in meristematic tissues, such as root or shoot tips, or pollen mother cells. However, these approaches can be

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limited by the availability of plant tissues in the appropriate state and by the amount of labor required for every sample. 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 ploidy level in plants (Bennett and Leitch, 1995; Galbraith et al., 1983). Arumuganathan and Earle (1991) established a protocol for nuclear DNA measurement, and thus for ploidy level determination, for over 100 important plant species. DNA contents of only two *Rubus* species, *R. idaeus* L. (Arumuganathan and Earle, 1991; 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 in these genotypes because an accurate estimation of genome size is helpful to estimate the number of clones needed to create a species gene library (Bennett and Leitch, 1995).

Other than our preliminary work (Meng and Finn, 1999), there has been no published work on the use of flow cytometry in *Rubus*. The primary objective of this study was to develop a rapid, inexpensive and routine methodology to determine nuclear DNA content and ploidy level by flow cytometry. This will allow *Rubus* breeders to plan crosses and select hybrids.

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

Genotype[z]	Ancestry[y]	Reported ploidy[y]	Fluorescence intensity (channel no.)
Hillquist	*R. argutus* selection	2x	102.6
NC 86-14-02	*R. trivialis* selection	2x	105.7
RUB 817	*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	*R. canadensis* (triploid form)	3x	166.0
Choctaw	(Darrow x Brazos) x Rosborough	4x	195.9
Brison	(F2 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	Undetermined species from Pennsylvania	5x	253.3
RUB 1152	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
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	*R. ursinus* (dodecaploid form)	12x	519.3

[z]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.
[y]As reported by Thompson (1995a, 1995b, 1997)
[x]Released as 'Black Douglass', patented as 'Douglass'.

Materials and Methods

Plant material. 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) (Table 1). These were available within our breeding program or at the USDA–ARS National Clonal Germplasm Repository (NCGR), Corvallis. Leaf samples from 40 genotypes were collected and tested using flow cytometry to determine the relationship between chromosome number and fluorescence intensity (Table 1).

The second group was chosen to determine whether this technique would work across the broad range of *Rubus* subgenera. Thirty genotypes were chosen that represented eight subgenera, had known ploidy numbers (Thompson, 1995b), and were available (Table 2).

To characterize the collection of *R. ursinus* from our germplasm development and breeding program, the third group consisted of a broad sampling of this species (Finn and Martin, 1996). 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.

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 3).

A final group of 21 diploid *Rubus* species from five subgenera were analyzed to determine their nuclear DNA content (Table 4). Each genotype was run three times. We would have preferred to analyze more species in the case of subgenera *Chamaebatus* and *Cylactis*, but *R. nivalis* is the only diploid species in subgenus *Chamaebatus*, and *R. lasiococcus* is the only one of the five diploid *Cylactis* species that was available to us.

Nuclei isolation. The protocol for isolating nuclei was adapted from that of Arumuganathan and Earle (1991). A $MgSO_4$ buffer stock solution, which was composed of 10 mM $MgSO_4$, 50 mM KCl, and 5 mM HEPES, pH of 8.0, and a 10% Triton X-100 (w/v) stock solution were prepared. A chopping buffer to extract and stabilize nuclei was made based on the $MgSO_4$ buffer with the addition of 20 $g \cdot L^{-1}$ PVP-10, 1 $g \cdot L^{-1}$ dithiothreitol, and 28 $mL \cdot L^{-1}$ Triton X-100 stock.

About 40 mg of actively-growing *Rubus* leaves, which had been washed to remove soil, chemicals and other organisms that might react with the chemicals and alter the results, was weighed and placed in a 60-mm plastic petri dish. Preliminary experiments had found that young leaves gave much better resolution than old leaves. Chopping buffer (1 mL) was added and the petri dish was put on ice. The tissue was chopped, ≈100 chops per sample, with

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

Genotype[z]	Subgenus	Reported ploidy[y]	Fluorescence intensity (channel no.)
R. parviflorus Nutt.	*Anaplobatus*	2x	75.8
R. odoratus L.	*Anaplobatus*	2x	93.1
R. nivalis Douglas ex Hook.	*Chamaebatus*	2x	96.5
*R. pubescen*ce 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	Undetermined species	5x	253.3
RUB 1152	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
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]RUB indicates a USDA–ARS National Clonal Germplasm Repository accession.
[y]As reported by Thompson (1995a, 1995b, 1997)

Table 3. Nuclear DNA flow cytometry measurement of fluorescence intensity of propidium iodide-stained nuclei, putative ploidy and parents of 88 USDA–ARS selections and 15 other genotypes within the breeding program.

Genotype[z]	Fluorescence	Estimated ploidy	Parents[z]
USDA trailing blackberry selections			
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 Thornless
ORUS 1280	320.6	7x	ORUS 1063 x Austin Thornless
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 Himalaya
ORUS 887-3	294.3	6x	ORUS 1362 x Himalaya
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
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

Genotype[z]	Fluorescence	Estimated ploidy	Parents[z]
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	204.0	4x	Illini Hardy x Chester Thornless
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
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 1508-0	287.0	6x	ORUS 913-10 x ORUS 1122-2
ORUS 1532-0	318.1	7x	ORUS 2024 x Siskiyou
ORUS 1534-0	323.2	7x	ORUS 2024 x Black Butte
ORUS 1535-0	349.4	7x	Olallie x Douglass
ORUS 1638-1	278.5	6x	ORUS 1122-1 x NW 9059R-3
Trailing blackberry cultivars			
Black Butte	291.0	6x	Siskiyou x ORUS 728-3
Siskiyou	333.2	7x	ORUS 2027 x ORUS 1826
Semi-erect blackberry cultivar			
Triple Crown	222.9	4x	C-47 x ARK 545
Miscellaneous trailing selections			
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
Species and cultivars compared to try to ascertain identity of 96050			
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* from NE China
R. crataegifolius (96068)[y]	78.7	2x	*R. crataegifolius* from 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 1970s. Therefore, selection numbers such as ORUS 1122 and ORUS 1122-1 are different genotypes.
[y]Thompson et al., 1996.

Table 4. Nuclear DNA content of 21 diploid *Rubus* species from five subgenera by flow cytometry measurement of fluorescence intensity of propidium iodide-stained nuclei.

Genotype	Subgenus	Reported ploidy[z]	2C Nuclear DNA (pg) Mean[y]	SD
Rubus parviflorus L.	*Anaplobatus*	2x	0.54	0.04
R. odoratus L.	*Anaplobatus*	2x	0.64	0.08
Mean	*Anaplobatus*		0.59 ab	0.08
R. nivalis Douglas ex Hook.	*Chamaebatus*	2x	0.56	0.06
Mean	*Chamaebatus*	2x	0.56 a	0.06
R. lasiococcus A. Gray	*Cylactis*	2x	0.69	0.08
Mean	*Cylactis*	2x	0.69 b	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
Mean	*Idaeobatus*	2x	0.67 b	0.11
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
Mean	*Idaeobatus*	2x	0.56 a	0.08

[z]As reported by Thompson (1995a, 1995b, 1997).
[y]LSD subgenera = 0.11. Means of subgenera with the same letter are not significantly different at $P \leq 0.05$.

a razor blade to <0.5 mm in size to homogenize the tissues and release the nuclei. Leaf tissue was used because it was usually available year round. The nuclei suspension was then filtered to remove debris that might block the flow cell with a filter system that is composed of a 10-mL syringe and 30-μm nylon mesh. The nuclei suspension was filtered into a 1.5-mL centrifuge tube and DNAase-free RNase (Sigma R-4642, 0.06% v/v) was added to avoid binding of the propidium iodide (PI) to RNA. 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 5 $mg \cdot mL^{-1}$ PI stock solution to a final PI concentration of 250 $\mu g \cdot mL^{-1}$. 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.

To calculate nuclear DNA content, rainbow trout (*Oncorhynchus mykiss* Walbaum) red blood cells (TRBC) [source: Oregon Department of Fish and Wildlife-Corvallis Research Laboratory] were used as a standard. The TRBC stained with the same PI concentration as the *Rubus* nuclei and were added to the nuclei suspension to be run in the flow cytometer at a concentration of 10^5 TRBC nuclei/mL.

Flow cytometry. A flow cytometer (EPICS XL-MCL; Coulter Corporation, Miami, Fla.) was used for the nuclei suspension analysis. The laser-emission wavelength was adjusted to 488 nm. Each sample consisted of 300 μL of nuclei suspension, and analysis was conducted at a data rate of 100 to 150 nuclei per second. A minimum of 5,000 total events were acquired. The samples were run in a darkened lab to prevent PI degradation. The nuclear DNA content per 2C (C = minimum DNA content per haploid cell of an organism) nucleus was calculated based on the fluorescence of TRBC (5.05 pg/nuclei) (Arumuganathan and Earle, 1991), using the following formula: sample DNA content = 5.05 × sample fluorescence intensity/TRBC fluorescence intensity.

To estimate the putative ploidy level of unknown genotypes, we compared the fluorescence of different *Rubus* genotypes with known ploidy levels (via chromosome counts) and calculated a regression line with a 95% prediction interval. The inverse prediction (95%) was made to give the upper and lower limit of fluorescence of all ploidy levels (Ramsey and Schafer, 1997).

Results

The fluorescence intensity for the group of genotypes with known ploidy levels increased as ploidy level increased (Table 1, Fig. 1). There were significant differences in mean fluorescence between ploidy levels ($P \leq 0.001$), with fluorescence highly correlated with ploidy level ($r^2 = 0.99$, $P \leq 0.0001$) (Table 1 and Fig. 1). The fluorescence intensity increased as ploidy level increased (one-sided $P \leq 0.0001$, *t* test) for the species in all eight subgenera, although they showed a wider variation in fluorescence intensity than the first group of plant material ($r^2 = 0.96$, $P \leq 0.0001$) (Table 2, Fig. 2).

While *R. ursinus* has been split by some taxonomists into a few different species based on ploidy level and geographic distribu-

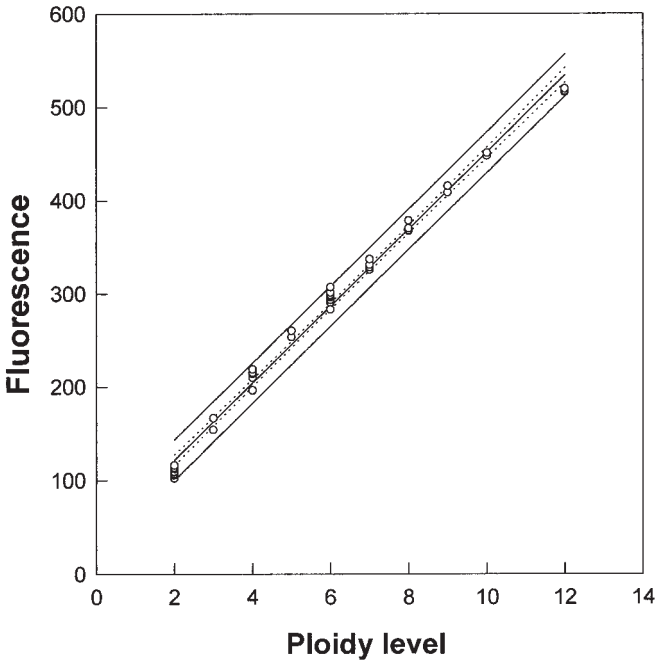

Fig. 1. Fluorescence intensity of propidium iodide-stained nuclear DNA as a function of ploidy level for 40 *Rubus* genotypes with known ploidy levels (Thompson, 1995a, 1995b). The regression line is highly significant ($P \leq 0.0001$). The inner solid line is the estimated mean fluorescence as a function 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.

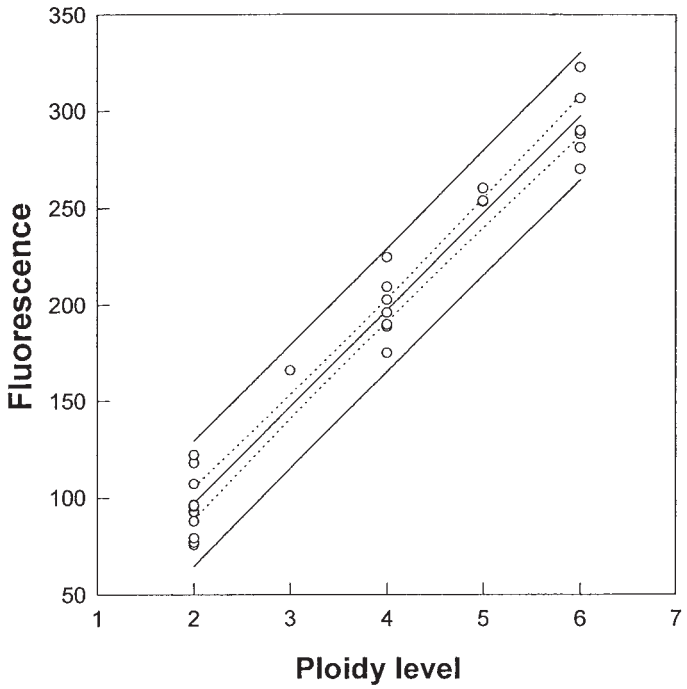

Fig. 2. Fluorescence intensity of propidium iodide-stained nuclear DNA as a function of ploidy level for 30 genotypes representing eight *Rubus* subgenera. The regression line is highly significant ($P \leq 0.0001$). The inner solid line is the estimated mean fluorescence as a function of the ploidy. 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.

tion, we used the classification set forth by Jennings (1988) where he considers these to be ecospecies of one polyploid cenospecies. Using the 95% prediction interval of fluorescence intensity range, the ploidy level of 110 *R. ursinus* genotypes from 42 populations of *R. ursinus* was estimated. Most of the genotypes were 11x, 12x or 13x, but 6x, 7x, 8x, 9x, and 10x genotypes were also represented (Fig. 3). Many samples collected from the same site had different ploidy levels. Our attempts to use microscopy to confirm the 13x genotypes were not successful.

Using the 95% prediction interval, the ploidy levels of cultivars and selections in our breeding program were estimated (Table 3).

Idaeobatus and *Chamaebatus* had significantly lower DNA content than those of *Rubus* and *Cylactis* (Table 4). In subgenus *Rubus*, diploid genotypes, *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 4). *Idaeobatus* had greater variation in DNA content among diploid species than the *Rubus* subgenus, with the highest content being from *R. ellipticus* Smith (0.69 pg) and lowest content from *R. illecebrosus* Focke (0.47 pg) (Table 4).

Discussion

Unreduced gametes are frequently produced in *Rubus*, leading to progeny with varying chromosome numbers, 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 materials from outside our breeding program, high ploid blackberries are crossed with tetraploid eastern blackberries. We can now use flow cytometry to determine if offspring from these crosses true hybrids. For example, ORUS 1395-1 and ORUS 1395-2 have the same 4x maternal and 7x paternal parents, and are 6x and 7x, respectively, confirming that our selections were from a successful hybridization.

Most of the genotypes tested gave the expected results, but there was some deviation from the regression line. Variation from the flow cytometer and in sample preparation can 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 = $39.26 + 41.24 \times$ Ploidy), the y-intercept was 39.26 when the ploidy was zero. This is most likely due to other fluorescent materials, such as residual PI, in solution as well as chloroplasts and mitochondrial DNA and ribosomal RNA, which might not have been destroyed completely with the current protocol.

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 (data not shown). New leaves from young canes are ideal for nuclei suspension preparation, because older leaves can produce fewer intact nuclei and these nuclei fluoresce less.

Rubus ursinus is widely distributed in the Pacific Northwest and 6x, 8x, 9x, 10x, 11x, and 12x have been reported throughout California, Oregon, Washington, and British Columbia, with 12x genotypes most common (Brown, 1943). No 7x, 9x, 10x, and 11x genotypes had been previously reported in Oregon, Washington, and British Columbia (Brown, 1943) and no 13x has ever been

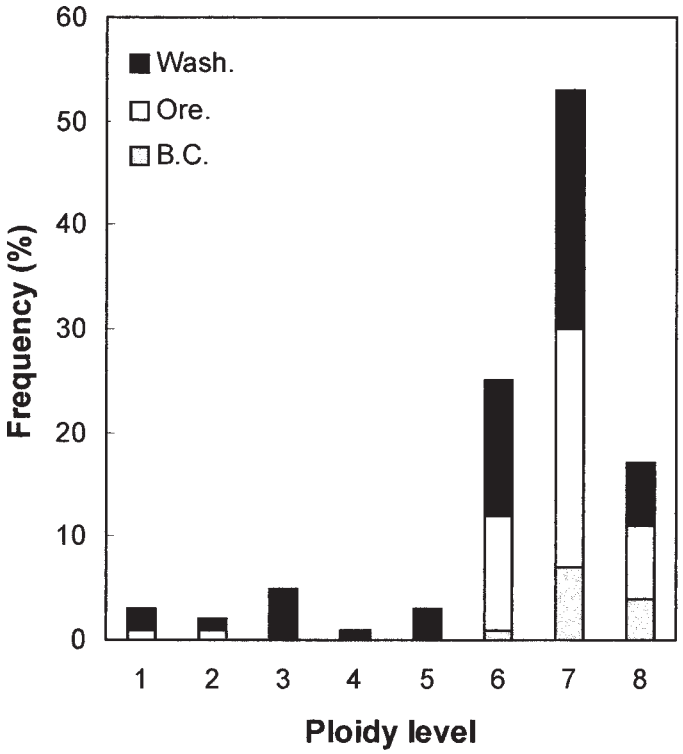

Fig. 3. Distribution of ploidy level as determined by flow cytometry for 110 *Rubus ursinus* genotypes collected from 42 populations from the Pacific Northwest. Actual locations of collections available upon request.

reported. In our material, we found plants with PI fluorescence suggesting individuals with 13x ploidy. While attempted, these could not be confirmed with chromosome counts. The occurrence of 8x *R. ursinus* (2 genotypes) in northern Washington was surprising as the 8x forms have been hypothesized to only occur in California and southern Oregon (Brown, 1943). Ploidy level, 8x vs. 12x, and geographical location were used by Brown (1943) to discriminate *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 at several ploidy levels and thus, for *R. ursinus*, ploidy level is probably not a valid criteria for separating these various forms into different species.

The most common mode of polyploidization is through the formation and sexual functioning of nonreduced gametes, followed by union with reduced gametes. An increase in chromosome numbers can occur in the first or later hybrid generations. To directly produce polyploids by the fertilization of nonreduced gametes from both parents is very rare (deWet, 1980). Therefore, although 13x genotypes had not been reported, they are possible. A 13x can be produced through crosses with nonreduced gametes, such as 10x by 8x, 12x by 7x, 8x by 9x, 6x by 10x, and even in rare cases, 6x by 7x (nonreduced gametes are produced from both parents). Also, genotypes with the same chromosome number but different nuclear DNA content may be produced through somatic doubling or the sexual functioning of nonreduced 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 DNA 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 (fluorescence) of as a genotype that is >8x in ploidy. The situation leading to such large differences is not likely because somatic doubling is extremely rare. However, genome variation can still exist among 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. Therefore, the predicted 13x genotypes may actually be 12x with a larger nuclear DNA content that accumulated during evolution from a 2x species with a larger genome. Obviously, research needs to be done to study the cytological characters of representatives of populations of *R. ursinus*.

Due to abnormal meiosis that leads to nonreduced 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, e.g. ORUS 1112-1 (6x) and ORUS 1112-2 (7x). Selections ORUS 1398-1 ('Lincoln Logan' x 'Navaho') and ORUS 1413-1 ('Marion' x 'Chester Thornless') were 7x instead of the expected 5x and ORUS 1395-1 is 7x. The ploidies of these selections are apparently the result of the nonreduced gamete formation in the 4x parent, 'Navaho' or 'Chester Thornless'.

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, was 0.30 ± 0.05 pg. 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, 0.75 pg from *R. sanctus*. However, this is not as much variation as in *Helianthus*, which has a 4-fold variation among diploid species (Sims and Price, 1985). Analyzing more than one diploid species within the *Chamaebatus* and *Cylactis* would give greater strength to these results. *Chamaebatus* has only one diploid species and there was only one diploid *Cylactis* species available.

Nuclear DNA content of diploid genotypes from five subgenera were compared (Table 4). *Rubus* and *Cylactis* had high DNA content while *Idaeobatus* and *Chamaebatus* had low levels, with *Anaplobatus* as intermediate. Within the *Rubus* subgenus, there was no significant variation among the five species tested (Table 4). Whereas, *Idaeobatus* had significant variation among the 12 species tested (Table 4). Several processes could account for this variation. Selection and accumulation of deletions or duplications may explain the variation in nuclear DNA content, which may result in the interspecific DNA differences (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 have activated the amplification and deletion of DNA sequences (Bennett and Leitch, 1995).

When trying to differentiate aneuploids in our experiments, we had only four aneuploids available with known ploidy. They were a 'Tayberry' seedling (RUB 227, 2n = 6x +2 = 44), 'Carolina' (RUB 102, 2n = 7x + 4 = 53), 'Aurora' (RUB 101 and RUB 134, 2n = 8x + 2 = 58), and 'Santiam' (RUB 79, 2n = 6x + 5 = 47). The fluorescence from 'Aurora' and the 'Tayberry' seedling was not consistently higher than that from other 8x and 6x genotypes, respectively. This could be due to two reasons: 1) the cytological composition is different between 'Aurora' and

other 8x genotypes and between the 'Tayberry' seedling and other 6x genotypes, and the total number of chromosome base pairs from 'Aurora' or the 'Tayberry' seedling is not more than that of other 8x (or 6x) genotypes, or 2) the 'Aurora' or 'Tayberry' seedling's genome is bigger than other 8x (or 6x) genotypes' genomes 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 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.

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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