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Small Genomes in Tetraploid *Rubus* L. (Rosaceae) from New Zealand and Southern South America

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Abstract

The genus *Rubus* contains crop wild relatives of raspberries and blackberries. *Rubus* subgenera *Micranthobatus* and *Comaropsis* are endemic to the Southern Hemisphere in trans-Pacific Ocean environments of Australasia, South America, and the Falkland Islands. The United States Department of Agriculture, National Clonal Germplasm Repository (NCGR) houses a *Rubus* genebank of living plants, including representatives of subgenera *Micranthobatus* and *Comaropsis*. Previously, accessions were determined by chromosome counts to be tetraploid. Our objective was to examine the nuclear DNA content (*C* values) of the tetraploid *R. cissoides, R. parvus, R. schmidelioides, R. squarrosus,* and *R. geoides* in contrast with those of diploid and tetraploid black raspberries (*R. occidentalis*) and diploid red raspberry (*R. idaeus* subsp. *idaeus*). Nuclear DNA content was determined using flow cytometry. Surprisingly, the *C* values of these species were significantly smaller than an autotetraploid clone of *R. occidentalis* or other tetraploid genotypes, and numerically equivalent to about the size of triploid raspberries. The small genomes may provide clues concerning the evolution of these subgenera.

Polyploids, especially allopolyploids, are common in *Rubus* L. (Rosaceae; Rosoideae) and are a major factor confounding its taxonomy and evolutionary history. Reports have recognized divergent ploidy levels of *Rubus* species ranging from diploid to dodecaploid (Thompson, 1997) with tetraploids most abundant. The number of species worldwide ranges from ~400 (Focke, 1894, 1910, 1911, 1914) to 700 (Bailey, 1941; Lu and Boufford, 2003; Alice et al., 2008). Focke, in his publications recognized 12 subgenera (subg.) whereas GRIN-Global database (USDA ARS, 2016) recognizes 15 (including two nothosubgenera). The gametic chromosome number in *Rubus*, like other Rosoideae, is $x = 7$. Nondisjunction, whole genome duplication (WGD), interspecific hybridization and apomixis frequently occur in *Rubus* (Alice et al., 2008). The U.S. Department of Agriculture, National Clonal Germplasm Repository (NCGR) maintains a diverse *Rubus* collection preserved as living plants as well as seed (Hummer, 1996; Hummer et al., 2016). The latest counts for the genebank can be found on the GRIN-Global database (USDA ARS, 2016). Besides preservation, NCGR is responsible for characterization of genetic resources including *Rubus*. Ploidy levels for accessions in the collection were determined through chromosome counts (Thompson, 1995a; 1995b; 1997) and flow cytometry (Meng and Finn, 2002; Hummer et al., 2016).

The New Zealand species of subgenus *Micranthobatus* (Kalkman, 1987) commonly called "bush lawyers" are not well known internationally. These species are sprawling vines with prickles useful for climbing on other plants. Many species have unisexual flowers.

Rubus parvus Buchanan, commonly called "creeping lawyer," is a low growing sub-

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shrub. The long narrow, simple leaves are serrate, with red prickles on the mid-vein. It has solitary, perfect (Webb et al., 1988) or in some reports "unisexual" (Cheeseman, 1925), white flowers about 1.8 cm in diameter that produce red to orange drupelets. A clone at the NCGR genebank has perfect flowers (Fig. 1a). The drupelets form aggregate fruit that ripen red and remain attached to the receptacle when harvested, similar to that of a blackberry (Fig. 1b). Other *Micranthobatus* species, *R. cissoides* A. Cunn. and

Fig. 1a: *Rubus parvus* commonly called "creeping lawyer," has long narrow, simple serrate leaves and solitary, perfect white flowers. Photo by Kim Hummer, USDA.

Fig. 1b: *Rubus parvus* drupelets from aggregate fruit that ripen red and remain attached to the receptacle when harvested, similar to a blackberry fruit. Photo by Kim Hummer, USDA.

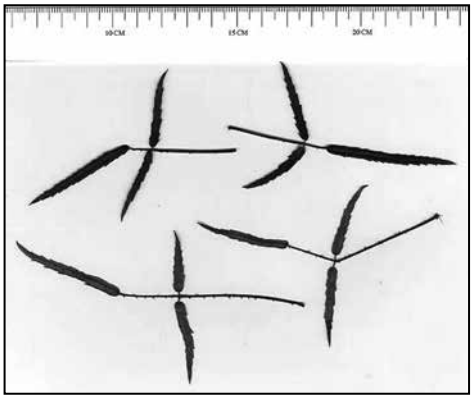

Fig. 2: *Rubus schmideloides* has trifoliate leaves with small lamina. Leaf scan by Adrienne Oda, USDA.

R. schmideloides A. Cunn. are dioecious lianas, with red prickles on stems, petioles, and leaf midrib, small leaves (Fig. 2) relative to others in the subgenus, white to cream-colored petals on a many-flowered panicle-like cyme from 12 to 60 cm long depending on taxon (Webb et al., 1988). *Rubus cissoides* has 10 or more serrations on each simple leaf margin, while *R. schmideloides* has less than 10. The so-called leafless bush lawyer, *R. squarrosus* Fritsch has slender to stout stems, yellow prickles on the petiole and petiolule, and the trifoliate leaves (Fig. 3) lack significant lamina (~1 cm long). It is a climber with intertwining branchlets. This species has not flowered at NCGR.

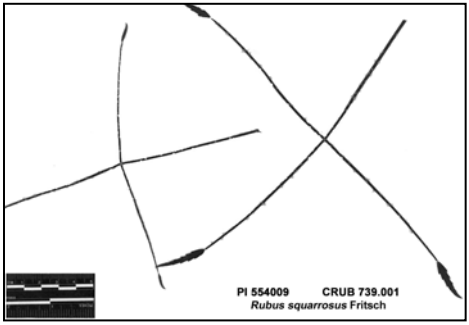

Fig. 3: *Rubus squarrosus* very small trifoliate leaves with prickers on petioles and petiolules. Leaf scan taken by Tyler Young, USDA.

Fig. 4: *Rubus geoides* flower and trifoliate leaves. Photo by Kim Hummer, USDA.

Rubus geoides Sm. (Fig. 4) is a low growing subshrub endemic to southern Argentina, Chile, and the Falkland Islands (Focke, 1910; USDA ARS, 2016). It has trifoliate leaves with small, weak prickles and perfect flowers. It is harvested from the wild for the red raspberry-like fruit. This species was considered for bramble breeding, crossing with species endemic to the northern hemisphere because of hardiness, few prickles, and its ability to produce fruit under windy and extreme environmental conditions; however, crosses between *R. geoides* and northern *Rubus* were unsuccessful and therefore not pursued for commercial development (Haskell and Paterson, 1966). Alice and Campbell (1999) included three members of subg. *Micranthobatus* in their phylogenetic study: Australian *R. moorei* and *R. australis* G. Forst., and *R. parvus* Buchanan from New Zealand. These species form a monophyletic group along with *R. geoides* of subg. *Comaropsis* and Tasmanian *R. gunnianus* Hook. from subg. *Dalibarda*. Hummer et al. (2016) observed that five tetraploid *Rubus* species native to New Zealand and southern South America had relatively small genomes compared to those of other species.

The objective of this study was to determine the amount of nuclear DNA (*C* values) of the tetraploids *R. cissoides, R. parvus, R. schmidelioides, R. squarrosus,* and *R. geoides*. The DNA *C*-value for diploid *R. idaeus* subsp. *idaeus* L. 'Meeker' red raspberry and *R. occidentalis* L. 'Munger' black raspberry, and an autotetraploid 'Munger' produced through tissue culture were determined for comparison.

Materials and Methods

Plant material. Young leaves of *R. cissoides, R. parvus, R. schmidelioides, R. squarrosus, R. geoides*, and diploid and autotetraploid *R. occidentalis* 'Munger' and diploid *R. idaeus* subsp. *idaeus* 'Meeker' growing in greenhouses at the USDA ARS NCGR in Corvallis, Oregon, were collected. Samples were sent overnight to Plant Cytometry Services (Schijndel, The Netherlands) in July 2014. Three leaves (replicates) were analyzed for each accession. Sample leaf material (~1 cm^2/20-50 mg) was combined with leaf material of an internal standard (*Vinca minor* L.). The plant material was chopped with a razor blade in 500 µL of CyStain PI absolute Extraction buffer (Partec GmbH, Münster, Germany) containing RNase, 0.1% DTT (dithiothreitol) and 1% polyvinylpyrolidone (ice-cold), in a plastic Petri dish. After 30-60 s of incubation, 2.0 mL staining buffer containing propidium iodide (PI) as fluorescent dye, RNA-se, 0.1% DTT (dithiothreitol) and 1% polyvinylpyrolidone was added. Remaining cell constituents, large tissue samples, and the internal standard were filtered through a 50 µm mesh nylon filter.

Nuclear DNA determination. After an incubation of at least 30 min at room temperature, the filtered solution with stained nuclei was measured with a CyFlow ML flow cytometer (Partec GmbH, Münster, Germany) with a green diode laser 50 MW 532 nm (for use with PI) and analyzed with Flomax version 2.4 d software. The amount of DNA of the unknown samples was calculated by multiplying the amount of DNA of the internal standard by the DNA ratio of the relative DNA amount of the unknown sample and the internal standard. Flow cy-

tometry determinations were performed by Plant Cytometry Services (AG Schijndel,The Netherlands). The pg/2*C* of nuclear DNA of the *Rubus* samples was calculated based on the value of *Vinca minor* nuclear DNA = 151 pg/2*C* (Bennett and Leitch, 2012). Analysis of variance (ANOVA) was calculated on the pg/2*C*. Least significant difference (LSD) was calculated to separate significantly different means.

Results and Discussion

The amounts of nuclear DNA (pg/2*C*) for the *Rubus* samples are shown (Table 1). The amounts of nuclear DNA of the study group were significantly different as determined by ANOVA ($df = 23$, $F = 850$; $P < 0.01$), therefore LSD was applied for mean separation ($P < 0.01$) and determined three groups (Table 1). The smallest genomes of our samples were diploid 'Meeker' red raspberry, 0.64 pg/2*C* and diploid 'Munger' black raspberry, 0.67 pg/2*C*. These were larger than the genomes reported by Meng and Finn (2002) for *R. illecebrosus, R. crataegifolius*, and *R. nivalis*. The largest genome we sampled was the autotetraploid 'Munger' at 1.39 pg/2*C*, slightly more than twice the amount of diploid 'Munger'. The nuclear DNA amounts for the five tetraploid species from New Zealand and southern South America ranged from 0.89 to 0.93 pg/2*C*, significantly more than the diploids, but significantly less than the autotetraploid 'Munger'.

The amounts of nuclear DNA for the tetraploid species in subgenera *Micranthobatus* and *Comaropsis* were significantly smaller than that of autotetraploid 'Munger', and smaller than that of other tetraploid *Rubus* species, such as *R. alceifolius* Poir. (Amsellem et al., 2001), or cultivated blackberry tetraploids (Hummer et al., 2016). The five *Rubus* species from New Zealand and southern South America had approximately the DNA amount predicted for a triploid, judging from genome size of *Rubus* subg. *Idaeobatus* (raspberry) (Table 1). Gardner (2002) remarked on the small size of bush lawyer chromosomes, and our results were surprisingly low, considering that the species are tetraploid. Whole-genome duplication is widespread in diverse taxa (McGrath and Lynch, 2012) and the combination of genomes through autopolyploidy or allopolyploidy occurs in the plant kingdom at rates comparable to that of point mutations (Lynch and Conery, 2000). When this happens, allopolyploids are expected to have genomes twice as large as their diploid progenitors, and increasing proportionately with ploidy level. The *C* value of the tissue culture-derived autotetraploid 'Munger' was more than

Table 1. Sample identification, mean size (n = 3) of diploid nuclear DNA (pg/2*C*), ± variance, pg/1*C*, and chromosome count. Least significant difference (LSD) was applied to separate means ($P < 0.01$).

Plant Inform. (PI)	Corvallis local identifier	Taxon	Identifier	Mean DNA pg/2*C*	Variance	DNA pg/1*C*	Chromosome Count
553384	989.001	*R. idaeus* L. subsp. *idaeu*	Meeker	0.64a	0.0002	0.32	14
553740	490.001	*R. occidentalis* L.	Munger	0.67a	0.0000	0.34	14
643940	1981.001	*R. geoides* Sm.	Chacao, Chile	0.89b	0.0000	0.45	28
554009	739.001	*R. squarrosus* Fritsch	Hangley Gardens	0.90b	0.0000	0.45	28
553883	741.001	*R. schmideloides* A. Cunn.	SK-NZ-12	0.90b	0.0000	0.45	28
654992	2512.001	*R. parvus* Buch.	rupa576	0.92b	0.0002	0.46	28
654992	772.001	*R. cissoides* A. Cunn.	Lincoln 42	0.93b	0.0002	0.46	28
660944	2573.001	*R. occidentalis* L.	Munger - autotetraploid	1.39c	0.0008	0.69	(28)

twice that of its diploid progenitor consistent with the hypothesis of additivity. In nature *C* values of many polyploid series have DNA amounts less than predicted suggesting that genome reduction can take place immediately following a polyploidization event or can occur over time (Leitch and Bennett, 2004). To get to the tetraploid state, the most recent common ancestor of subg. *Micranthobatus* and subg. *Comaropsis* species must have initially experienced a WGD or allopolyploidization event. The small genomes of these tetraploids may indicate that they were derived from diploid species with small genomes or that genome size has decreased.

Thus, in searching for potential closely related diploids with small genomes, *R. nivalis* Douglas and ancestors of several Asian *Idaeobatus* species, such as *R. illecebrosus* Focke or *R. crataegifolius* Bunge could be considered (Hummer et al., 2016).

The small genomes we observed provide support, in addition to nuclear ITS (Alice and Campbell, 1999) and chloroplast DNA sequences (L. Alice, Western Kentucky University, unpublished data), to the hypothesis that members of the these subgenera likely originated from a single allopoly ploidization event followed by species divergence.

Geographically isolated populations may experience greater speciation rates within polyploid lineages (McGrath and Lynch, 2012). At this time neither the age nor historical biogeography of these taxa is known, therefore dispersal and vicariance, evolution through geographical separation, are viable hypotheses. An alternative is that one or more diploid progenitors with larger genomes were involved in an autopolyploid event followed by genome reduction.

Genome size of polyploids could be expected to be the sum of the genomes inherited from progenitor species.Differences from the expected DNA amounts could be the result of genome size decreases or increases. Increases in genome size following polyploidization are rare (Leitch and Bennett, 2004). Given that our results show smaller DNA amounts than expected for other *Rubus* tetraploids, we can rule out that possibility. Another possibility is the complete additivity of the genomes of diploid progenitors. This is more likely to occur in autopolyploids than allopolyploids.The diploid ancestors of the *Rubus* tetraploids we examined are unknown and may be extinct. Progenitor candidates could include individuals similar to *Rubus nivalis* from northwestern North America which appeared closely related to these *Micranthobatus* and *Comaropsis* taxa (Alice and Campbell, 1999).

Other progenitor candidates might be diploid blackberries which grouped as a sister clade to *R. nivalis* and the Southern hemisphere lineages. Based on flow cytometry data, DNA amounts of subgen. *Rubus* diploids vary from 0.59 to 0.75 (Meng and Finn, 2002). However, doubling the genome size of the blackberry possessing the smallest genome sampled yields a value too large.

Another possibility might be found among the basal members of the *Rubus* phylogeny, such as *R. lasiococcus* Focke or *R. pedatus* Sm. A doubling of the size of those species or *R. crataegifolius* would be close to the size of these New Zealand tetraploids.

The genome size of raspberries in subg. *Idaeobatus* is likely too large to consider as progenitor diploids for *Micranthobatus*, unless significant genome "downsizing" occurred.

We suggest that likely progenitor species for *Micranthobatus* and *Comaropsis* had small genomes initially, such as those for *R. crataegifolius* or *R. lasiococcus*, then moderate downsizing occurred during the development to the modern day species. Molecular phylogeny of *Rubus* species is under investigation and will provide insight to this phylogenic question.

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