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Improved Method of Enzyme Digestion for Root Tip Cytology

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Abstract. **Chromosome numbers are an important botanical character for multiple fields of plant sciences, from plant breeding and genetics to systematics and taxonomy. Accurate chromosome counts in root tips of woody plants are often limited by their small, friable roots with numerous, small chromosomes. Current hydrolysis and enzyme digestion techniques require handling of roots before the root squash. However, optimum chromosome spread occurs when the cell walls have degraded past the point of easy handling. Here, we present a new enzyme digestion protocol that is fast, efficient, and flexible. This protocol reduces handling of the roots allowing for long-duration enzyme digestion. Digestions are performed on a microscope slide, eliminating the need for handling digested cells with forceps or pipettes. To illustrate the flexibility of this method across woody plant taxa, we performed chromosome counts on five angiosperms and one gymnosperm. Ploidy levels included diploids, triploids, and tetraploids with chromosome numbers ranging from $2n = 16$ to $2n = 80$. The range of holoploid 2C genome sizes spanned 1.54–24.71 pg. This protocol will provide a useful technique for plant cytologists working with taxa that exhibit a wide range of genome size and ploidy levels.**

Genome size, chromosome number, and ploidy level are important biological parameters for plant breeding, systematics, and evolution. Since the first published chromosome counts of plants in 1882 (Garbari et al., 2012), ≈25% of angiosperms have been measured for chromosome number (Castiglione and Cremonini, 2012), and 2.1% have measurements of genome size (Garcia et al., 2014). Holoploid 2C genome sizes of plants span about a 2400-fold range, from 0.13 pg (*Genlisea margaretae* Hutch.) to 304.46 pg (*Paris japonica* Franch.) (Bennett and Leitch, 2011; Chen et al., 2014; Fleischmann et al., 2014; Pellicer et al., 2010).

Traditional cytology has many uses in modern studies on woody plants. Combined with flow cytometry, cytology has been used to calibrate genome size with ploidy level and to confirm chromosome and ploidy variation among related taxa and hybrids in both temperate woody species (Contreras et al., 2007, 2009, 2010; Gillooly and Ranney, 2015; Jones et al., 2007; Lattier et al., 2013; Oates et al., 2014; Parris et al., 2010; Ranney et al., 2007; Rothleutner et al., 2016; Rounsaville and Ranney, 2010; Shearer and Ranney, 2013) and tropical woody species (Bationo-Kando et al., 2016; Cai et al., 2013; Dahmer et al., 2009; Schneider et al., 2015). Traditional cytology can be used for identifying aneuploids (additional or missing chromosomes) (Hu et al., 2015) or dysploids (alterations from chromosome fusion or fission) difficult to detect using flow cytometry (Rockinger et al., 2016). To meet the demand for accurate chromosome counts, traditional cytology has proven useful in the development of databases across taxonomic groups and formation of data sets for meta-analysis of chromosome number across plants and animals (Peruzzi et al., 2014).

Although traditional cytology has been an invaluable tool for studies in plant genetics, cytology on woody plants can be challenging compared with their herbaceous counterparts. In general, many woody plants possess small, friable roots with numerous small chromosomes making cytology particularly difficult (Lattier et al., 2013). Chromosome counting techniques can be tedious and require experienced histologists (Ochatt, 2008). In the modern era, cytogenetic studies in hardwood trees have not kept pace with current genomic studies because of small genomes and relatively small chromosomes (Ribeiro et al., 2008). Traditional cytology is also necessary for chromosomal fluorescent labeling techniques, such as fluorescent in situ hybridization and genomic in situ hybridization, and has proven valuable for characterizing hybrids in woody plants with small chromosomes (Van Laere et al., 2010).

Current root tip cytology consists of three broad steps (prefixative, fixative, and root squash) with slight variations for each step. For the prefixative step, roots are treated with one or more spindle fiber inhibitors to allow the cell cycle to continue to metaphase while arresting cytokinesis. In addition, root tip cold treatments have been used to help arrest cells at metaphase and condense chromosomes (Jauhar, 2003). Next, the root tips are fixed in a solution that arrest the cell cycle and are stored in an aqueous ethanol solution until observation. For the root squash step, the roots are hydrolyzed in hydrochloric acid or a combination of hydrochloric acid and ethanol. Alternatively, cell walls may be broken down by enzyme digestion using combinations of cellulase, cytohelicase, and pectolyase. Chromosome stains vary including modified carbol fuchsin, Feulgen, Giemsa, and acetocarmine, as well as fluorochrome stains (Bationo-Kando et al., 2016; Contreras et al., 2010; Jones et al., 2007; Rothleutner et al., 2016; Schneider et al., 2015).

In our observations, to obtain the highest quality chromosome spread, tissue degradation often must proceed beyond the point which the root tip can be easily handled and still remain intact. However, most protocols using enzyme digestion require handling after hydrolysis or digestion. This creates a problem that can be solved by digesting the excised root tip on the same surface used to perform the root squash. Once the cell walls have been fully digested, the weight of the cover slip on to the root tip should be sufficient to initially spread the cells. In the current study, we report a novel root squash protocol from the Ornamental Plant Breeding Laboratory at Oregon State University that has proven to be a fast, effective, and adjustable root tip cytology method applicable across a wide range of woody plant taxa.

Materials and Methods

Plant material. Six taxa were investigated to represent a wide range of genome size and chromosome number, including five angiosperms and one gymnosperm (Table 1). Plants were grown in a temperature-controlled glasshouse at Oregon State University. Although variable growth conditions would likely affect the quality of roots for cytology, all plants were grown under the same standard glasshouse conditions. Plants were container-grown in a 2:1 mixture of Metro-Mix Professional Growing Mix (Sun Gro Horticulture, Agawam, MA) and Perlite (Supreme Perlite Company, Portland, OR). Plants were initially hand-watered using municipal water on an as-needed basis and substrate solution pH and electrical conductivity (EC) were routinely monitored using the pour-through nutrient extraction procedure (Wright, 1986). Once the substrate solution EC was less than EC = 1.0 $\mu S \cdot cm^{-1}$, the plants were fertigated at each irrigation with Peters Professional 20N–4.4P–16.6K plus micronutrients (Everris NA Inc., Dublin, OH), calibrated such that irrigation water EC = 1.0 $\mu S \cdot cm^{-1}$. Plants were grown in a glasshouse with set temperatures of 24 °C day/17 °C night with a 14-h photoperiod.

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Reported chromosome numbers on the taxa investigated span a range of $2n = 16$ to $2n = 80$ and holoploid genome sizes of 1.54–24.7 pg. *Ribes sanguineum* Pursh is reported to be a diploid $2n = 2x = 16$ (Darlington and Wylie, 1956) with a holoploid genome size of 1.94 pg (OPBL, unpublished data). Roots were collected from an open-pollinated seedling of *R. sanguineum* 'Pokey's Pink'. *Quercus robur* L. is reported to be a diploid $2n = 2x = 24$ with a holoploid genome size of 1.85 pg (Favre and Brown, 1996). Roots were collected from an open-pollinated seedling of the columnar *Q. robur* 'Fastigiata'. *Thuja occidentalis* L. is reported to be a diploid $2n = 2x = 22$ with a holoploid genome size of 24.71 pg (Hizume et al., 2001). Roots were sampled from an open-pollinated seedling collected from a plant growing at the Lewis Brown Horticulture Research Farm in Corvallis, OR (field location 09.09). The original seed source for this plant was accessioned as 10-0024 and was received from Lawyer Nursery, Olympia, WA. *Cercidiphyllum japonicum* Siebold & Zucc. is reported to be a diploid $2n = 2x = 38$ with a holoploid genome size of 1.53 pg (Garcia et al., 2010). Roots were collected from an open-pollinated seedling of *C. japonicum* 'Rotfuchs' (Red Fox) at the U.S. National Arboretum (Beltsville, MD). *Acer tataricum* subsp. *ginnala* (Maxim.) Wesm. is reported to be both a diploid $2n = 2x = 26$ (Darlington and Wylie, 1956) with a holoploid genome size of 1.65 pg (Lattier, 2016) and an induced autotetraploid $2n = 4x = 52$ with a holoploid genome size of 3.10 pg (Lattier, 2016). Roots were collected from an open-pollinated seedling (OP2016-04-014) of a tetraploid cytotype (12-0011-010) in an isolation block (field location 75.18) comprised a mixture of tetraploid and diploid cytotypes. *Hibiscus syriacus* L. is reported to be a tetraploid $2n = 4x = 80$ (Darlington and Wylie, 1956) with a holoploid genome size of 4.70 pg (Contreras et al., 2013). Roots were collected from a self-pollinated seedling of *H. syriacus* 'Notwoodtwo' White Chiffon.

Prefixative. Root tips were collected before 1000 HR after two consecutive sunny days and suspended in 1.5 mL microcentrifuge tubes containing a solution of 2 mM 8-hydroxyquinoline and 0.24 mM cycloheximide. The root tips were treated in the dark at room temperature for 2.5 h before a cold period in a refrigerator at 4 °C for 2.5 h.

Fixative. After 5 h in the prefixative solution, the root tips were transferred to a filter paper–lined glass funnel atop a Büchner (vacuum) flask. The root tips were thoroughly rinsed with filter-sterilized water and fixed overnight at room temperature in Carnoy's solution (6 parts 95% ethanol: 3 parts chloroform: 1 part glacial acetic acid; by volume). After fixing the cells, the roots were transferred to a storage solution of 70% ethanol and stored in a refrigerator at 4 °C.

Root squash. The roots were transferred from the storage solution to a small beaker of filter-sterilized water for 5 min, swirling occasionally. This step was repeated twice for a triple rinse of each root over a total of 15 min. Each root was placed on a clean slide under a dissecting microscope (SMZ1500; Nikon, Tokyo, Japan), and the root tip was excised (Fig. 1A). The remaining root was discarded, and excess water was removed with a single-ply, low-lint tissue (VWR International, Radnor, PA) leaving only a small droplet encompassing the root tip to maintain hydration. To localize enzyme digestion to the microscope slide, a circular well was created using an ultraviolet resin pen (Bondic®; Niagra Falls, NY). A circle of ultraviolet resin was drawn around the root tip and water droplet (Fig. 1B). To facilitate removal of the circular well, a "pull tab" of the ultraviolet resin was placed over a small piece of wax paper (Fig. 1B). The slide was then placed in an ultraviolet crosslinker (CL

Table 1. Digestion times for taxa investigated using an improved enzyme digestion protocol for root tip cytology.

Taxa[z]	Digestion time (h)[y]	2C formula[x]	2C genome size (pg)[w]
Cercidphyllum japonicum 'Rotfuchs' Red Fox	0.5	$2n = 2x = 38$	1.53
Ribes sanguineum 'Pokey's Pink'	0.5	$2n = 2x = 16$	1.94
Acer tataricum subsp. *ginnala*	1	$2n = 3x = 39$	2.34[v]
Quercus robur 'Fastigiata'	2	$2n = 2x = 24$	1.85
Hibiscus syriacus 'Notwoodtwo' White Chiffon	2	$2n = 4x = 80$	4.70
Thuja occidentalis	3	$2n = 2x = 22$	24.71

[z]Roots were collected from open-pollinated seedlings from listed taxa.
[y]Optimal digestion times for root tip cytology.
[x]Ploidy and chromosome number of somatic root tip cells.
[w]Reported holoploid 2C genome size.
[v]Estimated triploid 2C genome size based on published diploid and tetraploid genome sizes.

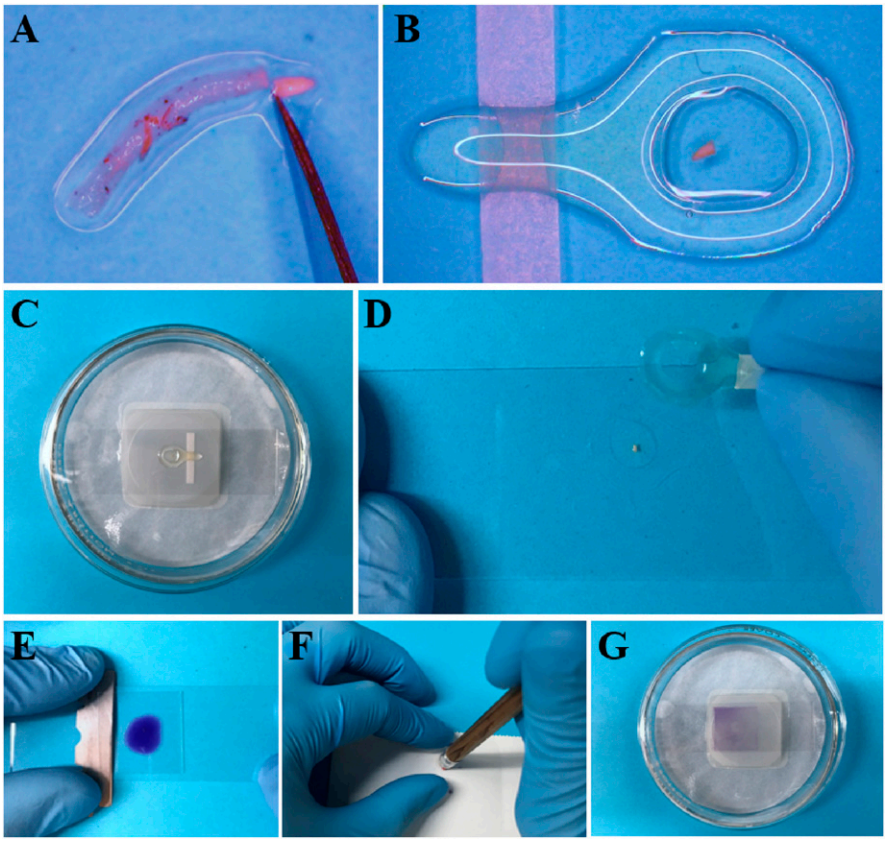

Fig. 1. Steps in an improved enzyme digestion for root tip cytology. (**A**) Rinsed roots are placed on a clean slide under a dissecting microscope and root tip is removed. (**B**) A ring of ultraviolet resin is placed around the root tip and overlapping a waxed paper pull tab. The resin is set in an ultraviolet crosslinker, and enzyme digestion solution is added to the well to encompass the root tip. (**C**) Enzyme digestion takes place in a 37 °C oven with the slide in a petri dish atop a small weighing dish and moist filter paper. (**D**) After digestion, the pull tab is used to remove the enzyme digestion well, and the root tip is rinsed with a droplet of water before being wicked dry. (**E**) A drop of modified carbol fuchsin stain is added, and a glass cover slip is lowered on to the root tip with half of a double-sided razor blade. (**F**) The slide is covered with a bibulous paper, and the root is squashed using gentle pressure from a pencil eraser. (**G**) The prepared slide is stored at room temperature in the petri dish until viewed with a light microscope.

1000 ultraviolet Crosslinker; UVP, LLC, Upland, CA) for 30 s to set the ultraviolet resin. Using a low-lint tissue, the droplet of water containing the root tip was wicked away, and a droplet of enzyme solution was pipetted on the root tip (Fig. 1B). The enzyme solution was composed of 0.5% cellulase (from *Trichoderma reesei*; Sigma, St. Louis, MO), 0.5% cytohelicase (from *Helix pomatia*; Sigma), and 0.5% pectolyase (from *Aspergillus japonicus*; Sigma) in a sodium citrate buffer at pH = 4.5.

Next, the slide was placed in a humid environment to maintain the droplet throughout the digestion period. Humidity was maintained in a glass petri dish with a dampened filter paper; the slide was kept dry by resting it on a small weighing dish (Fig. 1C). The glass petri dish containing the slide was incubated at 37 °C in an oven (Isotemp® Oven 655F; Thermo Fisher Scientific, Waltham, MA). Multiple root tips per taxa were digested at varying durations until an optimal digestion was achieved. Optimal digestion was achieved when the weight of the cover slip provided enough force to break apart the root tip. Less than ten slide preparations per taxa were required to find an optimal digestion time for each taxon.

Once digestion was complete, the glass petri dish and slide were removed from the oven. To prepare the root squash, the paper "pull tab" was used to remove the ring of ultraviolet resin (Fig. 1D). Excess enzyme solution was wicked away before adding a drop or two of filter-sterilized water to the root tip to rinse. The area surrounding the root tip was wiped clean with a low-lint tissue before wicking away excess water on the surface of the root tip. A single drop of modified carbol fuchsin stain (Kao, 1975) was pipetted on the root tip (Fig. 1E). Using half of a double-sided razor blade, a bridge was made to position a 22 × 22 mm cover slip at an angle over the root tip and stain droplet (Fig. 1E). The cover slip was quickly lowered on to the slide to prevent bubbles and covered with a sheet of bibulous paper (Fig. 1F). Pressure was applied to the cover slip using a pencil eraser while the bibulous paper wicked away excess stain (Fig. 1F). Slides mounted with root squashes were placed back into petri dishes and stored at room temperature. This allowed for multiple slide preparations without sealing because the humidity chambers prevented the slides from drying (Fig. 1G).

Chromosome counts. All slides were screened for condensed chromosomes at a magnification of ×200 (×10 adapter and ×20 objective) on a light microscope (Axio imager.A1; Zeiss, Thornnwood, NY). Condensed and spread chromosomes were photographed under oil immersion at ×1000 (×10 adapter and ×100 objective; AxioCam MRm, Zeiss). To maximize resolution of each chromosome and extend depth of field, multiple photos were taken per cell at different focal distances. Bulk focus stacking was performed using the Auto Blend feature in Photoshop CC 2015.5.1 (Adobe Systems, San Jose, CA). Bulk focus stacking of small chromosomes can still leave individual chromosomes out of focus. Therefore, fine editing (selecting sharpest focus for individual chromosomes) was performed when necessary using GIMP 2.8.18 (GNU Image Manipulation Program, https://www.gimp.org/). A minimum of 15 highly resolved cells were observed per taxa.

Results and Discussion

Root squashes were successful across all six taxa. All roots were treated the same in the prefixative and fixative steps. However, we recommend adjusting duration of prefixative treatment to each specific taxon, as recommended in other woody plant cytology protocols (Gamage and Schmidt, 2009). Because the duration of the mitotic cycle is positively correlated with genome size, a longer prefixative process should be used as genome sizes increase (Bennett, 1998; Schneider et al., 2015). Duration of digestion was adjusted for each taxon (Table 1). We observed that the digestion time was influenced by the root tip size and genome size of each taxa. Optimal digestion times were taxon-specific and varied from 30 min for *Ribes sanguineum* to 3 h for *Thuja occidentalis* (Table 1). When calibrating the digestion step, under-digested root tips failed to break apart under the weight of the cover slip whereas over-digested root tips yielded cells with missing and far-spread chromosomes. Modified carbol fuchsin proved a fast and effective stain requiring no extra steps, such as heating the slide (required for acetocarmine), extended incubation (required for Fuelgen), or special light sources (required for fluorochrome stains).

Chromosome counts of metaphase cells revealed diploid, triploid, and tetraploid cytotypes (Fig. 2). *Ribes sanguineum* was confirmed as a diploid $2n = 2x = 16$ (Fig. 2A). *Quercus robur* was confirmed as a diploid $2n = 2x = 24$ (Fig. 2B). *Thuja occidentalis* was confirmed as a diploid $2n = 2x = 22$ (Fig. 2C). *Cercidiphyllum japonicum* was confirmed as a diploid $2n = 2x = 38$ (Fig. 2D). *Acer tataricum* subsp. *ginnala* was confirmed as a triploid ($2n = 3x = 39$) resulting from an interploid cross in our isolation block (Fig. 2E). *Hibiscus syriacus* was confirmed as a tetraploid $2n = 4x = 80$ (Fig. 2F).

With chromosome estimates published for only 25% of angiosperms (Castiglione and Cremonini, 2012), there is a clear need for additional cytological studies. Chromosome counts are an important biological character necessary to multiple fields of plant sciences, from plant breeding and genetics to systematics and taxonomy. Our protocol provides an improved method for root tip cytology that may contribute to the growing number of chromosome surveys. The new enzyme digestion protocol proved an effective tool for producing high resolution metaphase chromosomes across multiple woody plant taxa. Accurate chromosome counting is critical when assessing wide hybrids or interploidy crosses. Confirmation of a triploid cytotype in *Acer tataricum* subsp. *ginnala* illustrates the utility of the protocol in confirming hybrids from interploid crosses.

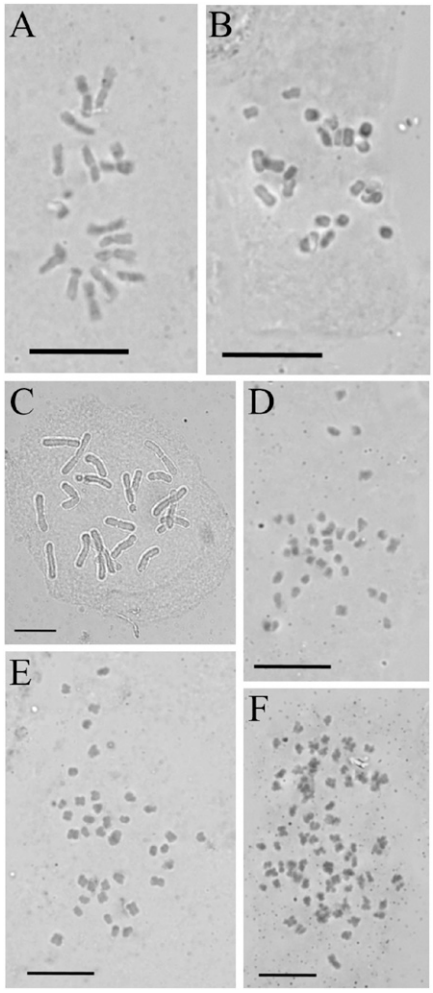

Fig. 2. Photomicrographs from an improved enzyme digestion protocol for root tip cytology. Metaphase chromosomes observed at magnification ×1000 from root apical meristems. Scalebar = 10 µm. (**A**) *Ribes sanguineum*, $2n = 2x = 16$; (**B**) *Quercus robur*, $2n = 2x = 24$; (**C**) *Thuja occidentalis*, $2n = 2x = 22$; (**D**) *Cercidiphyllum japonicum*, $2n = 2x = 38$ (**E**) *Acer tataricum* subsp. *ginnala*, $2n = 3x = 39$; (**F**) *Hibiscus syriacus*, $2n = 4x = 80$.

Minimal handling of root tips combined with long duration digestion makes this protocol a practical method for root tip cytology in woody plants. Its simplicity makes it a fast method for producing quality root squashes. Other hydrophobic barrier pens (PAP pens) have been used for cytology. However, ultraviolet resin pens are less expensive and leave little to no residue, unlike PAP pens which require xylene for residue removal. Although we used a crosslinker, ultraviolet lamps and flashlights are ubiquitous and can be acquired for less than twenty dollars, making the price of this protocol comparable with previous methods. Its flexibility can be combined with other methods with more complicated procedures, such as 4′,6-diamidino-2-phenylindole-staining, to

increase resolution for plants with high ploidy and numerous chromosomes.

Literature Cited

Bationo-Kando, P., J.-D. Zongo, and S. Siljak-Yakovlev. 2016. First genome size assessment, heterochromatin and rDNA chromosome mapping in the genus *Sclerocarya* (Anacardiaceae): Insight into the new basic chromosome number. Bot. Lett. 163(1):11–17.

Bennett, M.D. 1998. Plant genome values: How much do we know? Proc. Natl. Acad. Sci. USA 95:2011–2016.

Bennett, M.D. and I.J. Leitch. 2011. Nuclear DNA amounts in angiosperms: Targets, trends and tomorrow. Ann. Bot. 107:467–590.

Cai, Z.Q., T. Zhang, and H.Y. Jian. 2013. Chromosome number variation in a promising oilseed woody crop, *Plukenetia volubilis* L. (Euphorbiaceae). Caryologia 66(1):54–58.

Castiglione, M.R. and R. Cremonini. 2012. A fascinating island: $2n = 4$. Plant Biosyst. 146(3):711–726.

Chen, S.-C., C.H. Cannon, C.-S. Kua, J.-J. Liu, and D.W. Galbraith. 2014. Genome size variation in the Fagaceae and its implications for trees. Tree Genet. Genomes 10:977–988.

Contreras, R.N., M. Friddle, and J.D. Lattier. 2013. Relative fertility and ploidy levels of selected rose-of-sharon cultivars. Proc. 58th Ann. SNA Res. Conf. 58:232–236.

Contreras, R.N., T.G. Ranney, and S.P. Tallury. 2007. Reproductive behavior of diploid and allotetraploid *Rhododendron* L. 'Fragrant Affinity'. HortScience 42:31–34.

Contreras, R.N., J.M. Ruter, and W.W. Hanna. 2009. An oryzalin-induced autoallooctoploid of *Hibiscus acetosella* 'Panama Red'. J. Amer. Soc. Hort. Sci. 134:553–559.

Contreras, R.N., J.M. Ruter, and B.M. Schwartz. 2010. Oryzalin-induced tetraploidy in *Cryptomeria japonica* (Cupressaceae). HortScience 45:316–319.

Dahmer, N., M.T.S. Wittmann, and P.E. Kaminski. 2009. Chromosome number and karyotype of the endangered Amazonian woody *Centrolobium paraense* Tul. species. Crop Breed. Appl. Biotechnol. 9:382–385.

Darlington, C.D. and A.P. Wylie. 1956. Chromosome atlas of flowering plants. Macmillan, New York, NY.

Favre, J.M. and S. Brown. 1996. A flow cytometric evaluation of the nuclear DNA content and GC percent in genomes of European oak species. Ann. Sci. For. 53:915–917.

Fleischmann, A., T.P. Michael, F. Rivadavia, A. Sousa, W. Wang, E.M. Temsch, J. Greilhuber, K.F. Müller, and G. Heubl. 2014. Evolution of genome size and chromosome number in the carnivorous plant genus *Genlisea* (Lentibulariaceae), with a new estimate of the minimum genome size in angiosperms. Ann. Bot. 114:1651–1663.

Gamage, H.K. and S. Schmidt. 2009. Short communication. A robust method for chromosome quantification and ploidy determination in woody species. Austral. J. Bot. 57:87–93.

Garbari, F., G. Bedini, and L. Peruzzi. 2012. Chromosome numbers of the Italian flora. From the Caryologia foundation to present. Caryologia 65:62–71.

Garcia, S., T. Garnatje, O. Hildalgo, G. Mas de Xaxars, J. Pellicer, I. Sánchez-Jiménez, D. Vitales, and J. Vallès. 2010. First genome size estimations for some eudicot families and genera. Collect. Bot. 29:7–16.

Garcia, S., I.J. Leitch, A. Anadon-Rosell, M.Á. Canela, F. Gálvez, T. Garnatje, A. Gras, O. Hidalgo, E. Johnston, G.M. de Xaxars, J. Pellicer, S. Siljak-Yakovlev, J. Vallès, D. Vitales, and M.D. Bennett. 2014. Recent updates and developments to plant genome size databases. Nucleic Acids Res. 42(D1): D1159–D1166.

Gillooly, D.A. and T.G. Ranney. 2015. Genome size and ploidy Levels in the genus *Kalmia*. HortScience 50:1426–1428.

Hizume, M., T. Kondo, F. Shibata, and R. Ishizuka. 2001. Flow cytometric determination of genome size in the Taxodiaceae, Cupressaceae *sensu stricto* and Sciadopityaceae. Cytologia 66:307–311.

Hu, F.R., H.H. Liu, F. Wang, R.L. Bao, and G.X. Liu. 2015. Root tip chromosome karyotype analysis of hyacinth cultivars. Genet. Mol. Res. 14(3):10863–10876.

Jauhar, P.P. 2003. Haploid and doubled haploid production in durum wheat by wide hybridization, p. 161–166. In: M. Maluszynski, K.J. Kasha, B.P. Forester, and I. Szarejko (eds.). Doubled haploid production in crop plants: A manual. Kluwer Academic Publishers, Boston, MA.

Jones, J.R., T.G. Ranney, and N.P. Lynch. 2007. Ploidy levels and genome sizes of diverse species, hybrids, and cultivars of rhododendron. J. Amer. Rhod. Soc. 61 (4):220–227.

Kao, K.N. 1975. A nuclear staining method for protoplasts, p. 60–64. In: O.L. Gamborg and L.Z. Wetter (eds.). Plant tissue culture methods. L.R. National Research Council of Canada. Prairie Regional Laboratory, Saskatoon, SK, Canada.

Lattier, J.D., T.G. Ranney, and N.P. Lynch. 2013. History and cytological reassessment of *Rhododendron canadense*. J. Amer. Rhod. Soc. 67(2):92–98.

Lattier, K.S. 2016. Inducing and evaluating phenotypic and cytometric variation in landscape plants: Observations from *Acer, Ornithogalum*, and *Penstemon*. Oregon State University, Corvallis, M.S. Diss.

Oates, K.M., T.G. Ranney, and D.H. Touchell. 2014. *Campsis* × *tagliabuana* 'Chastity': A highly infertile triploid trumpet vine. HortScience 49:343–345.

Ochatt, S.J. 2008. Flow cytometry in plant breeding. Cytometry 73A:581–598.

Parris, J.K., T.G. Ranney, and W.V. Baird. 2010. Ploidy levels, relative genome sizes, and base pair composition in magnolia. J. Amer. Soc. Hort. Sci. 135:533–547.

Pellicer, J., M.F. Fay, and I.J. Leitch. 2010. The largest eukaryotic genome of them all? Bot. J. Linn. Soc. 164:10–15.

Peruzzi, L., K.F. Caparelli, and G. Bedini. 2014. A new index for the quantification of chromosome number variation: An application to selected animal and plant groups. J. Theor. Biol. 353:55–60.

Ranney, T.G., N.P. Lynch, P.R. Fantz, and P. Capiello. 2007. Clarifying taxonomy and nomenclature of *Fothergilla* (Hamamelidaceae) cultivars and hybrids. HortScience 42:470–473.

Ribeiro, T., A. Barão, W. Viegas, and L. Morais-Cecílio. 2008. Molecular cytogenetics of forest trees. Cytogenet. Genome Res. 120:220–227.

Rockinger, A., A. Sousa, F.A. Carvalho, and S.S. Renner. 2016. Chromosome number reduction in the sister clad of *Carica papaya* with concomitant genome size doubling. Amer. J. Bot. 103(6):1082–1088.

Rothleutner, J.J., M.W. Friddle, and R.N. Contreras. 2016. Ploidy levels, relative genome sizes, and base pair composition in *Cotoneaster*. J. Amer. Soc. Hort. Sci. 141:457–466.

Rounsaville, T.J. and T.G. Ranney. 2010. Ploidy levels and genome sizes of *Berberis* L. and *Mahonia* Nutt. species, hybrids, and cultivars. HortScience 45:1029–1033.

Schneider, J.V., J. Paule, J. Gitaí, S. Dressler, C.L.S. Gusmão, and A.M. Benko-Iseppon. 2015. Divergent genome sizes reflect infrafamilial subdivision of the Neotropical woody Marcgraviaceae. Bot. J. Linn. Soc. 177:1–14.

Shearer, K. and T.G. Ranney. 2013. Ploidy levels and relative genome sizes of species, hybrids, and cultivars of dogwood (*Cornus* spp.). HortScience 48:825–830.

Van Laere, K., L. Khrustaleva, J.V. Huylenbroeck, and E. Van Bockstaele. 2010. Application of GISH to characterize woody ornamental hybrids with small genomes and chromosomes. Plant Breed. 129:442–447.

Wright, R.D. 1986. The pour-through nutrient extraction procedure. HortScience 21:227–229.