Cytometric and Cytological Analyses of Cultivated Dogwoods (*Cornus* spp.)

Kimberly Shearer and Thomas G. Ranney

North Carolina State University, Department of Horticultural Science, Mountain Crop Improvement Lab, Mountain Horticultural Crops Research and Extension Center, 455 Research Drive, Mills River, NC 28759

kmsheare@ncsu.edu

Index Words: *Cornus*, dogwoods, DNA content, flow cytometry, genome size, ploidy level, interspecific hybridization

Significance to Industry: Dogwoods are an important ornamental nursery crop of the southern region. According to the 2009 USDA Census of Horticultural Specialties, annual sales of dogwoods in southern states exceeded $12 million (1). While breeding, selection and cultivation of dogwoods is widespread, little is known about ploidy and genome size of dogwood cultivars and hybrids. Knowledge of ploidy and genome size is a valuable tool for breeding programs. Ploidy levels can influence fertility, crossability, segregation, and gene expression (2, 3). Relative genome size can be used to determine ploidy among closely related species when calibrated using traditional cytology (4). In this study, flow cytometry and traditional cytology were used to determine relative genome sizes and ploidy levels of 94 accessions of various species, hybrids and cultivars of dogwoods. Although most accessions were found to be diploid, selections of *C. canadensis* were tetraploid and the hybrid cultivar C. 'KN30-8' (Venus™) was triploid. A broad range of interspecific hybrids was also documented based on genome sizes being intermediate between their parents. These results provide new insights into the cytogenetics, reproductive biology, crossability, and systematics of dogwoods.

Nature of Work: The genus *Cornus* is comprised of a wide range of diverse species that includes shrubs, small trees, and herbaceous perennials (7, 8). Many species of dogwoods are used in the landscape providing four seasons of interest with attractive flowers, fruit, foliage, bark, and form (9). While considerable research has been focused on determining systematic relationships among dogwoods, little has been reported relative to genome size and ploidy (7, 8, 10).

In 2006, a species-level phylogeny (8) divided the dogwoods into several major clades including the big-bracted dogwoods (BB), the cornelian cherries (CC), and the dwarf dogwoods (DW). Cytological studies in the past have shown that the base chromosome number for BB is x = 11, for CC is x = 9 or 10, and for DW is x = 11 (8, 11, 12). For DW, the chromosome counts vary and ploidy has been reported as both diploid ($2n$ = $2x$ = 22)(13 – 15) and tetraploid ($2n$ = $4x$ = 44) (11, 16). There are no other known reports of polyploidy for other species of dogwoods. The objectives of this study were

to determine ploidy level and genome size of dogwood species, cultivars and hybrids representing the aforementioned clades.

Flow Cytometry. Flow cytometry was used to determine relative 2C genome size. Over the course of the summer season, expanding leaf tissue, vegetative buds and floral buds were collected from 94 accessions at the Mountain Horticultural Crop Research and Extension Center of North Carolina State University in Mills River, NC. Additionally, Dr. Thomas Molnar at Rutgers University provided leaf tissue and buds from the original *Cornus* 'KN30-8' (Venus ™). Approximately 1 cm^2 or 20 mg of tissue from each accession was co-chopped with a known standard in a petri dish with 400 µL of nuclei extraction buffer (Cystain ultraviolet Precise P Staining Buffer; Partec, Münster, Germany). Chopped tissue and extraction buffer was then filtered through a 50-µm nylon filter and stained using 1.6 mL 4', 6-diamidino-2-phenylindole (DAPI) staining buffer (Cystain ultraviolet Precise P Staining Buffer; Partec). The subsample was then analyzed using a flow cytometer with fluorescence excitation provided by a mercury arc lamp (Partec PA-I; Partec). For most accessions, *Pisum sativum* 'Ctirad' was used (2C = 8.75 pg)(17) as an internal standard. For *Cornus eydeana* (CC), *Magnolia virginiana* 'Jim Wilson' (Moonglow™, 2C = 3.92 pg)(4) was used as an internal standard due to the relatively large genome size of *C. eydeana.* For each replicate, two subsamples were analyzed. 2C DNA contents were calculated as: 2C = DNA content of standard × (mean fluorescence value of sample ÷ mean fluorescence value of standard). Monoploid genome sizes were calculated as: 2C DNA content ÷ ploidy level.

Cytology. Traditional cytology was used to calibrate relative genome size to ploidy and confirm base chromosome counts. Actively growing root tips were collected in the morning prior to 10 am. Root tips were collected from seedlings or rooted stem cuttings and then suspended in a pre-fixative solution of 2mM 8-hydroxyquinolline + 0.24 mM cycloheximide in glass vials. Root tips were incubated in the dark at room temperature for 3 h. Root tips were then refrigerated at 6° C for 3 h. Following pre-fixative treatment, root tips were rinsed four times using refrigerated distilled water and placed into a fixative solution of 3 parts 95% ethanol : one part propionic acid. The following morning, root tips were rinsed four times using 70% ethanol and stored in 70% ethanol until needed. Prior to squashing, cells walls of root tips were hydrolyzed using three parts 95% ethanol: one part 12 M hydrochloric acid for approximately 3 minutes. Root tips were then placed in a staining solution of modified carbol fuschin (18) for approximately 5 minutes. The distal end of the root tip was excised under a dissecting microscope (Leica Stereozoom 6 Photo, Buffalo Grove, IL), placed on a glass slide, and gently squashed with a coverslip using a pencil eraser. Chromosomes were viewed using a light microscope (Nikon Eclipse 80i, Melville, NY). Chromosome counts were determined for *C. florida*, *C. nuttallii, C. capitata, C. hongkongensis, C.* 'KN30-8', *C. canadensis, and C. officinalis*.

Data analysis. Genome size data was subject to analysis of variance by clade, subgenus, and species/grex. Means were separated using Fisher's least significant difference (Proc GLM; SAS Version 9.2; SAS Inst., Cary, NC).

Results and Discussion: Flow cytometry was found to be an effective and efficient tool for determining relative genome sizes and ploidy levels of *Cornus* (Table 1). There was significant variation in genome size among clades, subgenera, and species with a range from 1.07 pg to 5.16 pg (Table 1). There were also differences within clades. In the BB clade, the *Syncarpea* 1Cx values were higher than that of *Cynoxylon;* and hybrids between the two subgenera displayed an intermediate genome size. Differences in 1Cx values were also found when comparing species within subgenera. An example can be found within the subgenus *Cornus. C. eydeana* (5.08 pg) was much larger than *C. mas* (3.31 pg) and *C. officinalis* (3.28 pg). Additionally, within *Cynoxylon, C. florida* (1.58 pg) differed from *C. nuttallii* (1.71 pg). Among the *Syncarpea* species, it was noted that the evergreen species (*C. capitata, elliptica,* and *hongkongensis;* 2.07 to 2.27 pg*)* had a significantly larger 1Cx value than that of *C. kousa* (1.92 pg). The results of this study provide further support for the taxonomic groupings put forth by Xiang et al. (8) with the range of genome sizes for subgenera and clades being distinct and discontinuous.

Bai et al. (17) reported 2C value of one sample of *C. canadensis* to be 4.4 pg using propidium iodide (PI) stain. This result was close to our findings of 4.2 to 4.3 pg using the DAPI stain. In 2005, Zonneveld et al. (19) found the 2C value of *C. mas* to be 6.8 pg using PI stain. This was consistent with our findings of 6.5 to 6.7 pg (DAPI). While different fluorochrome stains will give slightly different estimates of relative genome sizes, both methods have been found to be effective and consistent for use in determining relative genome size and ploidy levels among closely related species (4). However, the DAPI stain is faster, less expensive, less toxic, and generally produces results with a lower CV for mean nuclei fluorescence.

All but one species tested proved to be diploid, confirming the findings of past cytological studies conducted on dogwoods. Only *C. canadensis* was found to be tetraploid with $2n = 4x = 44$. While this is consistent with Dermen and Löve and Löve (11, 16), this conflicts with other reports stating that only *C. unalaschkensis* is a tetraploid species (15). *C. canadensis* is a circumboreal species with a wide-ranging distribution. It is possible that this species displays a ploidy series over its geographic range. Studies of other species with wide ranging geographic distribution have shown that ploidy series is a commonly encountered phenomenon (21, 22). Still others have found that there is ploidy variation within populations (23).

Although there has been speculation that *C. officinalis* 'Spring Glow' might be a triploid due to very low fruit set (personal observations), this study found that its genome size was consistent with other diploids. The only triploid identified in this study was *C.* 'KN30-8' with $2n = 3x = 33$. It has been found that hybrids may produce unreduced gametes (24). This could be the source of the unlikely polyploidy found within the BB clade.

A valuable and practical use of relative genome size in dogwood breeding was found when comparing genome sizes of hybrids to that of parent species. When genome

sizes of parent species varied considerably, hybrid progeny were shown to have intermediate genome sizes. Examples of such hybrids include: *C. capitata* × *C. florida, C. hongkongensis* × *C. florida, C. elliptica* × *C. florida,* and *C.* × *rutgersensis*, *C. capitata* × *C. kousa,* and *C. kousa* × *C. elliptica.*

This study provides new and pertinent information about genome sizes and ploidy levels for widely cultivated dogwoods. It was also found that the use of flow cytometry can be an effective and efficient way to confirm hybridization of BB dogwoods and contributes to the larger census of genome sizes of angiosperms.

Acknowledgements: This work was funded, in part, by the North Carolina Agricultural Research Service (NCARS), Raleigh, N.C., the North Carolina Biotechnology Center, Research Triangle Park, N.C., the Kenan Institute, Chapel Hill, N.C., and the North Carolina Nursery and Landscape Association, Raleigh, N.C.

Literature Cited

1. USDA-NASS. 2010. Census of Horticultural Specialties (2009), Table 20: Deciduous flowering trees. August 2012. <http://www.agcensus.usda.gov/Publications/2007/Online_Highlights/Census_of_Horticulture_Specialties/>
2. Chen, Z.J. and Z. Ni. 2006. Mechanisms of genomic rearrangements and gene expression changes in plant polyploids. BioEssays 28(3):240-252.
3. Soltis, D.E., P.S. Soltis, and J.A. Tate. 2004. Advances in the study of polyploidy since Plant speciation. New Phytologist 161(1):173-191.
4. Parris, J.K., T. G. Ranney, H. T. Knap, and W.V. Baird. 2010. Ploidy levels, relative genome sizes, and base pair composition in Magnolia. J. Amer. Soc. Hort. Sci. 135(6):533-547.
5. Galbraith, D.W., J. Bartos, and J. Doležel. 2005. Flow cytometry and cell sorting in plant biotechnology, p. 291-322. In: L.A. Sklar (ed.). Flow cytometry in biotechnology. Oxford Univ. Press, New York.
6. Keller, E.R.J., L. Schubert, J. Fuchs, and A. Meister. 1996. Interspecific crosses of onion with distant Allium species and characterization of the presumed hybrids by means of flow cytometry, karyotype analysis and genomic in situ hybridization. Theor. Appl. Genet. 92(3-4): 417-424.
7. Fan, C. and J.Q. Xiang. 2001. Phylogenetic relationships within Cornus (Cornaceae). Amer. J. Bot. 88(6):1131-1138.
8. Xiang, J.Q., D.T. Thomas, W.H. Zhang, S.R. Manchester, and Z. Murrell. 2006. Species level phylogeny of the dogwood genus Cornus (Cornaceae) based on molecular and morphological evidence - implication in taxonomy and Tertiary intercontinental migration. Taxon 55(1):9 –30.
9. Cappiello, P. and D. Shadow. 2005. Dogwoods: The genus *Cornus*. Timber Press, Portland, OR.

10. Xiang, J.Q., M. Moody, D.E. Soltis, C.Z. Fan, and P.S. Soltis. 2002. Relationships within Cornales and circumscription of Cornaceae-matK and rbcL sequence data and effects of outgroups and long branches. Mol. Phylogenetics Evolution 24(1):35-57.
11. Dermen, H. 1932. Cytological studies of *Cornus*. J. Arnold Arboretum 13:410-417.
12. Goldblatt, P. 1978. A contribution to cytology in Cornales. Ann. Missouri Bot. Garden 65(2): 650-655.
13. Murrell, Z.E. 1994. Dwarf dogwoods: Intermediacy and the morphological landscape. Systematic Bot. 19(4):539-556.
14. Packer, J.G. 1964. Chromosome numbers and taxonomic notes on western Canadian and Arctic plants. Canadian J. Bot. 42(4):473-494.
15. Zhang, W., J.Q. Xiang, D.T. Thomas, B.M. Wiegmann, M.W. Frohlich, and D.E. Soltis. 2008. Molecular evolution of PISTILLATA-like genes in the dogwood genus *Cornus* (Cornaceae). Mol. Phylogenetics and Evolution 47(1):175-195.
16. Löve, Á. and D. Löve. 1982. IOPB chromosome number reports LXXV. Taxon 31(2):342-386.
17. Bai, C., W.S. Alverson, A. Follansbee, and D.M. Waller. 2012. New reports of nuclear DNA content for 407 vascular plant taxa from the United States. Ann. Bot. 110(8):1623-1629.
18. Kao, K.N. 1975. A chromosomal staining method for cultured cells, p. 63-64. In: O.L. Gambourg and L.R. Wetter (eds.). Plant tissue culture methods. NRC Canada, Saskatoon, Canada.
19. Zonneveld, B.J.M., I.J. Leitch, and M.D. Bennett. 2005. First nuclear DNA amounts in more than 300 angiosperms. Ann. Bot. 96(2):229-244.
20. Wang, X., P.A. Wadl, T.A. Rinehart, B.E. Scheffler, M.T. Windham, J.M. Spiers, D.H. Johnson, and R.N. Trigiano. 2009. A linkage map for flowering dogwood (*Cornus florida* L.) based on microsatellite markers. Euphytica 165(1):165-175.
21. Burton, T.L. and B.C. Husband. 1999. Population cytotype structure in the polyploid *Galax urceolata* (Diapensiaceae). Heredity 82(4):381-390.
22. Whittemore, A.T. and R.T. Olsen. 2011. *Ulmus americana* (Ulmaceae) is a polyploidy complex. Amer. J. Bot. 98(4):1-7.
23. Trávníček, P.B. Kubátová, V. Čurn, J. Rauchová, E. Krajníkova, J. Jersáková, and J. Suda. 2011. Remarkable coexistence of multiple cytotypes of the *Gymnadenia conopsea* aggregate (the fragrant orchid): Evidence from flow cytometry. Ann. Bot. 107(1):77-87.
24. Ramsey, J. and D.W. Schemske. 1998. Pathways, mechanisms, and rates of polyploid formation in flowering plants. Annu. Rev. Ecol. Syst. 29:467-501.

Table 1. Base, monoploid genome sizes (1Cx) for *Cornus* spp. grouped by clade, subgenus, and species/grex.
[1]Values followed by different letters within a column are significantly different, LSD, $P \leq 0.05$.

Clade	1Cx (pg)	Subgenus	1Cx (pg)	Species/grex	1Cx (pg)
Cornelian Cherries (CC)	3.88 A[1]	*Cornus*	3.89 A	*C. eydeana*	5.08 A
				C. mas	3.31 B
				C. officinalis	3.28 B
Big Bracted Dogwoods (BB)	1.89 B	*Syncarpea*	2.00 B	*C. capitata*	2.27 C
				C. elliptica	2.14 D
				C. hongkongensis	2.07 DE
				C. kousa	1.92 HI
				C. capitata × *C. kousa*	2.11 D
				C. hongkongensis × *C. kousa*	2.03 EF
				C. hongkongensis × *C. kousa,* F_2	2.08 DE
				C. kousa × *C. elliptica*	2.01 EFG
		Syncarpea× *Cynoxylon*	1.83 BC	*C. capitata*× *C. florida*	1.98 FGH
				C. hongkongensis× *C. florida*	1.86 I
				C. elliptica× *C. florida*	1.94 GH
				C. ×rutgersensis	1.73 J
		Cynoxylon	1.60 C	*C. florida*	1.58 K
				C. nuttallii	1.71 J
Dwarf Dogwoods (DW)	1.07 C	*Arctocrania*	1.07 D	*C. canadensis*	1.07 L

[1]Values followed by different letters within a column are significantly different, LSD, $P \leq 0.05$

Table 2. List cultivars that were evaluated.

Cornus florida
- 'Appalachian Joy'
- 'Appalachian Spring'
- 'Comco No. 1' (Cherokee Brave ™)
- 'Dixie Colonnade'
- 'Eternal Dogwood'
- 'Little Princess'
- 'Rutnam' (Red Beauty ®)
- 'Spartanburg'
- 'World's Fair'

Cornus kousa
- 'Akabana'
- 'Akatsuki'
- 'Beni Fuji'
- 'Blue Shadow'
- 'Bonfire'
- 'Cherokee'
- 'Dwarf Pink'
- 'Girard's Nana'
- 'Greensleeves'
- 'Little Beauty'
- 'Lustgarten Weeping'
- 'Madison' (Crown Jewel ™)
- 'Milky Way'
- 'Milky Way Select'
- 'National'
- 'Radiant Rose'
- 'Rochester'
- 'Satomi'
- 'Snowbird'
- 'Speciosa'
- 'Spinners'
- 'Square Dance'
- 'Summer Majesty'
- 'Temple Jewel'
- 'Wolf Eyes'

Cornus nuttallii
- 'Barrick'
- 'Colrigo Giant'

Cornus hongkongensis
- 'Gekkou'
- 'Snowcap'

Cornus capitata
- 'Mountain Moon'
- 'Yoko'

(Cornus kousa × C. nuttallii) × C. kousa
- 'KN30-8' (Venus™)

Cornus × rutgersensis (C. kousa × C. florida)
- 'KF1-1' (Saturn ®)
- 'Rutban' (Aurora®)
- 'Rutcan' (Constellation®)
- 'Rutdan' (Celestial®)
- 'Rutfan' (Stardust®)
- 'Rutgan' (Stellar Pink®)
- 'Rutlan' (Ruth Ellen®)

Cornus mas
- 'Aurea'
- 'Elegantissima'
- 'Golden Glory'

Cornus officinalis
- 'Kintoki'
- 'Spring Glow'