HortScience 46(11):1465–1467. 2011.

Differences in Winter Browning among Japanese-cedar Cultivars Are Not Due to Variation in Ploidy Levels

Ryan N. Contreras[1,4]
Department of Horticulture, Oregon State University, 4017 Agricultural and Life Sciences Building, Corvallis, OR 97331-7304

Ron Determann[2]
Atlanta Botanical Garden, 1345 Piedmont Avenue NE, Atlanta, GA 30309

Mara Friddle[3]
Department of Horticulture, Oregon State University, 4017 Agricultural and Life Sciences Building, Corvallis, OR 97331-7304

Additional index words. Cryptomeria japonica, flow cytometry, cytology, photoinhibition, carbol fuchsin, DAPI

Abstract. **There is a great deal of variation among japanese-cedar cultivars with regard to growth form, foliar characteristics, and winter browning. Differences in winter browning have been observed and documented by a number of authors. Previous research has established that there are differences in winter foliage color between cultivars included in the current study; however, no quantitative analysis under standardized conditions was conducted. Because of a previous report that tetraploid forms of japanese-cedar remain green during winter as a result of increased antioxidant enzyme activity, we hypothesized that cultivars that exhibit reduced winter browning were polyploids. We screened 56 accessions of japanese-cedar using flow cytometry analysis of 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei and performed chromosome counts on three cultivars. All accessions were diploid ($2n = 2x = 22$), although there were significant differences in genome sizes among the cultivars. Holoploid genome sizes ranged from 18.9 pg for var. *sinensis* JCRA to 22.3 pg for 'Viridis' with a mean of 20.1 pg. Chromosome counts for cultivars Ogon, Oye Keme, and Viridis supported the flow cytometry results. Although the underlying cause of the variability in morphology and winter browning among cultivars is unclear, our results show that differences in ploidy level are not responsible, because all tested genotypes were diploid. Chemical name: 4′,6-diamidino-2-phenylindole (DAPI).**

Japanese-cedars (*Cryptomeria japonica* D. Don) are large trees in their native range of Japan and China and are often used as timber trees that may reach 36 to 46 m high in the wild (Dallimore and Jackson, 1967). In managed landscapes they are sometimes used as an alternative to leyland cypress [×*Cuprocyparis leylandii* (A.B. Jacks. & Dallim.) Farjon (Farjon et al., 2002)]. They are tolerant of biotic and abiotic stresses (Tripp, 1993; Tripp and Raulston, 1992) and form attractive large screens or specimens in landscapes. However, winter browning caused by photoinhibition (Ida, 1981) is unsightly and likely has reduced the use of japanese-cedars. Winter browning occurs as a result of the accumulation of the pigment rhodoxanthin during photoinhibitory conditions. Rhodoxanthin acts to protect the photosynthetic apparatus by dissipating excess light energy as heat to prevent the accumulation of reactive oxygen species (Han et al., 2003). Plants found in Japanese forestry nurseries that did not exhibit winter browning were identified as tetraploids (Chiba, 1951), and it was found that they did not accumulate rhodoxanthin because they were more resistant to the oxidative damage resulting from increased antioxidant levels (Niwa and Sasaki, 2003). Winter browning is highly variable among ornamental cultivars of japanese-cedar. Rouse et al. (2000) described 45 cultivars of japanese-cedar growing in the eastern United States including variability in foliage color during winter from brown to dark green. Our hypothesis was that a difference in ploidy level among cultivars is related to the variation in winter color and that cultivars that remain greener during winter were tetraploids. The current study was conducted to determine the ploidy level of 56 accessions of japanese-cedar and if variation in ploidy level is related to variation in winter browning.

Received for publication 18 Aug. 2011. Accepted for publication 9 Sept. 2011.
We thank Shawn Mehlenbacher, Jim Myers, and anonymous colleagues for critical review of the manuscript.
[1]Assistant Professor.
[2]Conservatory and Conservation Director.
[3]Faculty Research Assistant.
[4]To whom reprint requests should be addressed; e-mail contrery@hort.oregonstate.edu.

Materials and Methods

Plant material. Fifty-six accessions of *Cryptomeria japonica* were received from the Atlanta Botanical Garden (Atlanta, GA), JC Raulston Arboretum (Raleigh, NC), Hopper Bros. (Woodburn, OR), Bizon Nursery Company (Hubbard, OR), and Youngblood Nursery Inc. (Salem, OR) as unrooted stem cuttings, rooted cuttings, or containerized plants (Table 1). Material received as unrooted stem cuttings was rooted using 2000 ppm indole 3-butyric acid (Dip'N Grow®; Dip'N Grow, Inc., Clackamas, OR), stuck into a 1 peat:1 perlite substrate, and placed under mist at a rate of 10 s every 60 min until root formation. The 56 accessions included in the study were chosen based on availability as well as differences in winter browning. Included in Table 1 are observations of winter color for 33 accessions from the current study from Rouse et al. (2000). These data are included to demonstrate the variability of winter foliage color among japanese-cedar cultivars. Observations by Rouse et al. (2000) were made at multiple locations from Atlanta, GA, to Philadelphia, PA, over a 3-year period. Conditions were not standardized; therefore, observations for winter color cannot be considered as a quantitative tool. However, for the purposes of the current study, these data provide evidence to establish that there is considerable variation in winter foliage color among japanese-cedar cultivars.

Flow cytometry. Approximately 240 mg (1 cm^2) of young leaf tissue from each japanese-cedar clone and 0.5 cm^2 of leaf tissue of *Pisum sativum* L. 'Ctirad' were simultaneously chopped with a razor blade in a 35 × 10-mm petri dish with 400 µL of nuclei extraction buffer (CyStain® ultraviolet Precise P Nuclei Extraction Buffer; Partec, Münster, Germany). The solution was filtered using Partec CellTrics® disposable filters with a pore size of 30 µm to remove leaf tissue. Nuclei were stained with 1.6 mL of DAPI staining buffer (CyStain® ultraviolet Precise P Staining Buffer; Partec) and were incubated for 10 min at 25 °C. The suspension was analyzed using a flow cytometer (CyFlow® Ploidy Analyzer; Partec) to determine mean relative DNA fluorescence [mean relative fluorescence (MRF)]. Ploidy and genome size were determined by comparing the MRF of each sample with the 2C peak of the internal standard [*Pisum sativum* L. 'Ctirad' 2C = 8.76 pg (Greilhuber et al., 2007)]. For all samples, at least 1000 particles were analyzed with the exception of 'Araucarioides', 'Atawhai', 'Gyokomo', 'Koshyi', 'Little Diamond', var. *sinensis* UGA, 'Weed's Dwarf', and 'Yaku', for which fewer than 1000 particles were analyzed. Three samples from each accession were analyzed and data were subjected to analysis of variance and means were separated using Tukey's honestly significant difference test ($\alpha = 0.05$).

Cytology. Plants of selected cultivars were placed in flats containing perlite, and roots were allowed to grow out of pots for collection.

Table 1. Source and relative genome size of *Cryptomeria japonica* taxa based on flow cytometry of 4′,6-diamidino-2-phenylindole-stained nuclei using *Pisum sativum* 'Ctirad' (2C = 8.76 pg) as an internal standard.

Cultivar/selection	Source	Winter foliage color[z]	Relative 2C genome size
Araucarioides	Atlanta Botanical Garden	Brown	19.83 abc[y]
Atawhai	Atlanta Botanical Garden	—	19.32 abc
Beaumont's Dwarf	Atlanta Botanical Garden	—	20.52 abc
Benjamin Franklin	JC Raulston Arboretum	Dark green	20.90 abc
Black Dragon	Atlanta Botanical Garden	Dark green	19.63 abc
Cristata	Hopper Bros. Nursery	Brown	19.43 abc
Dacrydioides	JC Raulston Arboretum	Purplish red	22.24 a
Egmont	Atlanta Botanical Garden	—	19.38 abc
Elegans	Hopper Bros. Nursery	Bronze to purplish red	20.74 abc
Elegans	Youngblood Nursery	Bronze to purplish red	21.18 abc
Elegans Aurea	Youngblood Nursery	Greenish yellow	19.66 abc
Elegans Nana	Atlanta Botanical Garden	Bronze to purplish red	19.82 abc
Gyokumo	Atlanta Botanical Garden	Medium green	19.48 abc
Gracillis	JC Raulston Arboretum	Greenish-yellow	20.08 abc
Gyokuryu (= Gyokumo)	Atlanta Botanical Garden	Medium green	19.51 abc
Jindai-sugi	Bizon Nursery	Bronze	20.79 abc
Kilmacurragh	JC Raulston Arboretum	Bronze	20.53 abc
Knaptonensis	Atlanta Botanical Garden	Bronze	19.44 abc
Koshyi	Atlanta Botanical Garden	—	19.95 abc
Kusari (= Spiraliter Falcata)	JC Raulston Arboretum	Light green	20.35 abc
Little Diamond	Atlanta Botanical Garden	Medium green	19.42 abc
Littleworth Dwarf	Atlanta Botanical Garden	Bronze	21.81 abc
Lobbii	JC Raulston Arboretum	Brown	22.12 ab
Monstrosa	JC Raulston Arboretum	Bronze	20.40 abc
Mushroom	Youngblood Nursery	—	20.16 abc
Nana	JC Raulston Arboretum	Bronze to purplish red	20.43 abc
Ogen	Atlanta Botanical Garden	—	20.00 abc
Ogon (= Aurea)	Atlanta Botanical Garden	Bronze	21.13 abc
Oye Keme	Atlanta Botanical Garden	—	19.66 abc
Pygmea	Youngblood Nursery	—	20.81 abc
Pyramidata	Atlanta Botanical Garden	—	19.30 abc
Radicans	JC Raulston Arboretum	—	20.19 abc
Rasen (Spiralis)	Atlanta Botanical Garden	Medium green	20.65 abc
Rein's Dense Jade (= Bloomers Witches Broom)	Bizon Nursery	Medium green	19.24 abc
Rein's Dense Jade (= Bloomers Witches Broom)	Atlanta Botanical Garden	Medium green	20.11 abc
Ryocogu Coyokyo	Youngblood Nursery	—	19.81 abc
Sekkan	Hopper Bros. Nursery	Light green	19.37 abc
Sekkan	Youngblood Nursery	Light green	20.61 abc
Selaginoides	Atlanta Botanical Garden	—	20.09 abc
Spiralis	JC Raulston Arboretum	Medium green	20.14 abc
Spiraliter Falcata	Atlanta Botanical Garden	Light green	19.73 abc
Taisho-tama	Atlanta Botanical Garden	Brown	19.45 abc
Tansu (revert)	Atlanta Botanical Garden	—	20.11 abc
Tansu (sport)	Atlanta Botanical Garden	—	19.35 abc
Tenzan	Atlanta Botanical Garden	Dark green	19.33 abc
var. *sinensis* (JCRA clone)	Atlanta Botanical Garden	—	18.92 c
var. *sinensis* (weeping form)	Atlanta Botanical Garden	—	20.19 abc
var. *sinensis* (UGA clone)	Atlanta Botanical Garden	—	19.84 abc
Vilmoriniana	Youngblood Nursery	Bronze	21.73 abc
Viridis	Atlanta Botanical Garden	—	22.30 a
Weed's Dwarf	Atlanta Botanical Garden	—	19.02 bc
Wintermint	Atlanta Botanical Garden	—	19.61 abc
Yaku	Atlanta Botanical Garden	—	19.33 abc
Yellow Twig	JC Raulston Arboretum	—	20.44 abc
Yokohama	Atlanta Botanical Garden	—	19.83 abc
Yoshino	Ron Determann Farm	Bronze	19.42 abc

[z]From Rouse et al. (2000).

[y]Means within column followed by the same letters are not significantly different based on Tukey's honestly significant difference ($\alpha = 0.05$).

Cytology was conducted as described by Contreras et al. (2010). Chromosomes were visualized using a Carl Zeiss AxioImager (Carl Zeiss MicroImaging GmbH, Jena, Germany). Images were captured using an AxioCam MRm (Carl Zeiss MicroImaging GmbH) and processed using AxioVision FRET software Version 4.7.2 (Carl Zeiss MicroImaging GmbH). Chromosomes of at least 10 cells of each cultivar were counted and a representative photomicrograph was taken.

Results and Discussion

The 56 accessions included in the current study were found to be diploid based on flow cytometry (Table 1). There were significant differences in genome sizes among taxa; however, we attribute this to variation commonly observed during flow cytometry and not to actual differences in chromosome number or ploidy level. The mean holoploid DNA content for all accessions was 20.1 pg and the CV percentage was 8.5% or less. One would expect to observe values near 30 pg for triploids and 40 pg for tetraploids; however, the observed values ranged from 18.9 pg for var. *sinensis* JCRA to 22.3 pg for 'Viridis'. The three accessions with the largest genome sizes, 'Dacrydioides', 'Lobbii', and 'Viridis', were significantly larger than the smallest, var. *sinensis* JCRA and 'Weed's Dwarf'. There are limitations to calculating genome size when staining with DAPI, which binds preferentially to AT-rich regions of DNA; therefore, our genome size estimates are less exact than when using a DNA intercalator such as propidium iodide. However, DAPI has been shown to be effective and more cost-efficient in analysis of ploidy levels and our results are consistent with Hizume et al. (2001) who reported 22.1 pg/2C for japanese-cedar using propidium iodide.

Chromosome counts were performed on 'Ogon' (2C = 21.1 pg), 'Oye Keme' (2C = 19.7 pg), and 'Viridis' (2C = 22.3 pg). Based on flow cytometry, these cultivars had statistically different genome sizes; however, all were found to be diploids ($2n = 2x = 22$) (Fig. 1). Eckenwalder (2009) reported that some of the variable forms in cultivation have one or two extra sets of chromosomes. However, no cultivars were named and it is unclear if the reference is to the chance triploids and tetraploids found in Japan (Chiba, 1951) or specific cultivars with which the author was familiar. In contrast to reports of a base number of 11 for *Cryptomeria japonica* (Khoshoo, 1961; Sax and Sax, 1933), Dark (1932) reported a base chromosome number of 12, which is common in other genera of conifers. The report by Dark (1932) is the only report of a base chromosome number differing from 11 for japanese-cedar and appears to be an error. Based on flow cytometry, we thought that 'Viridis' might be an aneuploid (e.g., 2n + 2) because it was among the cultivars with a genome size statistically different from the majority of cultivars in the study. However, cytological analysis revealed that it too is diploid (Fig. 1C).

Polyploids of japanese-cedar, tetraploids in particular, are reported to remain green during winter as a result of increases of up to six times the activity of specific antioxidant enzymes such as superoxide dismutase (Niwa and Sasaki, 2003). In an attempt to exploit this phenomenon to develop new ornamental cultivars, induced tetraploids have been developed (Contreras et al., 2010); however, it remains to be seen if the same gains in antioxidant activity and improved winter color will be observed as in previous studies. In addition to Rouse et al. (2000), Dirr (2009) and Krüssman (1985) reported on the variability in winter foliage color among japanese-cedar cultivars, including a number that were included in the current study. There has not been a comprehensive evaluation of cultivars

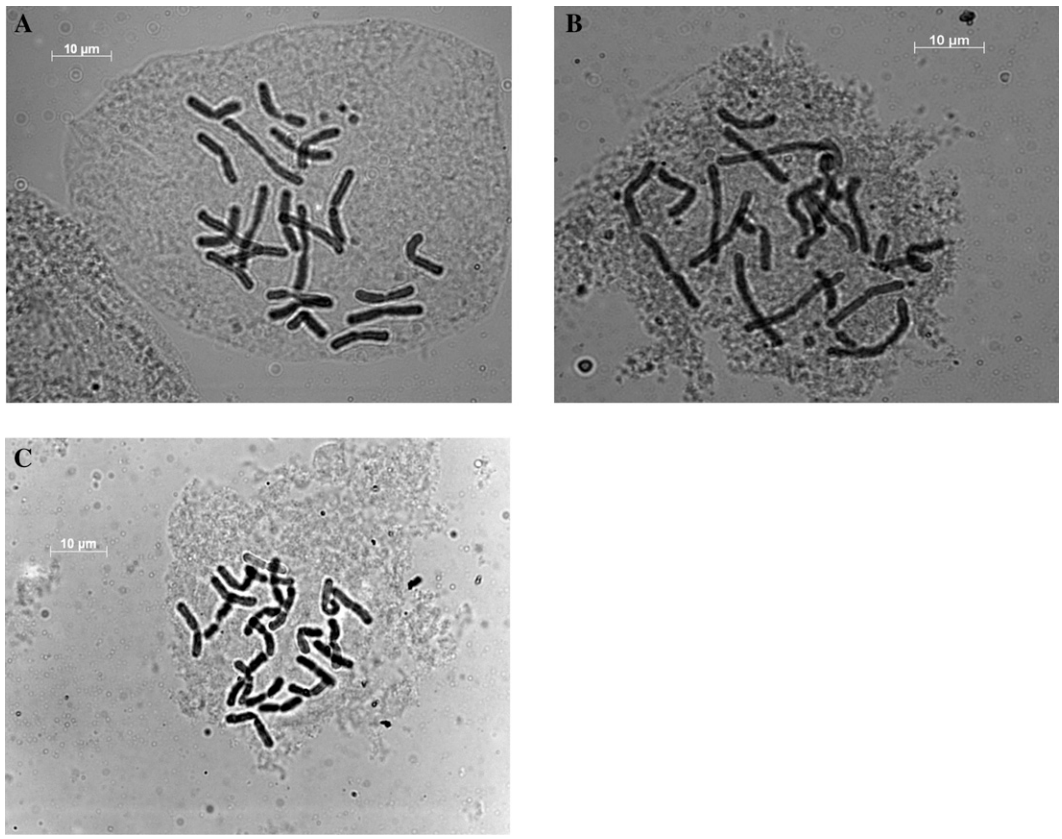

Fig. 1. Photomicrographs of chromosome spreads from root tip squashes of *Cryptomeria japonica* 'Oye Keme' (**A**), 'Ogon' (**B**), and 'Viridis' (**C**) stained with carbol fuchsin. All cultivars were diploid ($2n = 2x = 22$).

of japanese-cedar under standardized conditions to compare winter foliage color between cultivars; however, it is well established that differences exist.

We report the analysis of 56 accessions of japanese-cedar for ploidy level and genome size using flow cytometry. Cytological analysis of selected cultivars supported the findings obtained by flow cytometry and all accessions were found to be diploid ($2n = 2x = 22$). The cultivars and selections included in the current study vary widely in growth form, morphology, and winter browning. Rouse et al. (2000) reported varying winter foliage color (Table 1) for 33 of the accessions included in the current study from brown to dark green. Although not standardized, their report highlights the fact that variability in winter foliage color exists among japanese-cedar cultivars. Some cultivars can be expected to remain greener during winter than others. The underlying cause of this variability is unclear, but it is not related to differences in ploidy level.

Literature Cited

Chiba, S. 1951. Triploids and tetraploids of sugi (*Cryptomeria japonica* D. Don) selected in the forest nursery. Bul. Govt. For. Expt. Sta. (Tokyo) 49:99–109.

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

Dallimore, W. and A.B. Jackson. 1967. A handbook of Coniferae including Ginkgoaceae. 4th Ed. E. Arnold, London, UK.

Dark, S.O.S. 1932. Chromosomes of *Taxus, Sequoia, Cryptomeria* and *Thuya* [sic]. Ann. Bot. (Lond.) 46:965–977.

Dirr, M.A. 2009. Manual of woody landscape plants. 6th Ed. Stipes Publ., Champaign, IL.

Eckenwalder, J.E. 2009. Conifers of the world. Timber Press, Portland, OR.

Farjon, A., N. Tien Hiep, D.K. Harder, P. Ke Loc, and L. Averyanov. 2002. A new genus and species in Cupressaceae (Coniferales) from northern Vietnam, *Xanthocyparis vietnamensis*. Novon 12:179–189.

Greilhuber, J., E.M. Temsch, and J.M. Loureiro. 2007. Nuclear DNA content measurement, p. 67–101. In: Doležěl, J., J. Greilhuber, and J. Suda (eds.). Flow cytometery with plant cells: Analysis of genes, chromosomes and genomes. Wiley-VCH, Weinheim, Germany.

Han, Q., K. Shinohara, Y. Kakubari, and Y. Mukai. 2003. Photoprotective role of rhodoxanthin during cold acclimation in *Cryptomeria japonica*. Plant Cell Environ. 26:715–723.

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

Ida, K. 1981. Eco-physiological studies on the response of taxodiaceous conifers to shading, with special reference to the behaviour of leaf pigments. I. Distribution of carotenoids in green and autumnal reddish brown leaves of Gymnosperms. Bot. Mag. Tokyo 94:41–54.

Khoshoo, T.N. 1961. Chromosome numbers in gymnosperms. Silvae Genet. 10:1–9.

Krüssman, G. 1985. Manual of cultivated conifers. Timber Press, Portland, OR.

Niwa, Y. and Y. Sasaki. 2003. Plant self-defense mechanisms against oxidative injury and protection of the forest by planting trees of triploids and tetraploids. Ecotoxicol. Environ. Saf. 55: 70–81.

Rouse, R.J., P.R. Fantz, and T.E. Bilderback. 2000. Descriptions and a key to cultivars of japanese cedar cultivated in the eastern United States. HortTechnology 10:252–266.

Sax, K. and H.J. Sax. 1933. Chromosome number and morphology in the conifers. J. Arnold Arbor. 14:356–375.

Tripp, K.E. 1993. Sugi: The ancient Japanese cedar finds new life in a profusion of outstanding cultivar forms. Amer. Nurseryman 178: 26–39.

Tripp, K.E. and J.C. Raulston. 1992. *Cryptomeria* evaluations at the NCSU Arboretum. Proc. 37th Ann. Southern Nurs. Assoc. Res. Conf. 37: 336–339.