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Genome Sizes and Ploidy Levels in the Genus *Kalmia*

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Abstract. ***Kalmia*** **is a highly ornamental genus of shrubs native to North America and Cuba and grown as a valuable nursery crop throughout much of the temperate world. Although most species of** ***Kalmia*** **have previously been found to be diploid with $2n = 2x = 24$, one species,** ***Kalmia polifolia*****, has been found to be tetraploid. However, sampling within the genus has been limited, and information on the ploidy levels of specific cultivars is lacking. The objective of this study was to determine the relative genome sizes and ploidy levels of species, hybrids, and cultivars of** ***Kalmia*****. Flow cytometry was used to determine the relative genome sizes of 67 accessions representing species, interspecific hybrids, cultivars, and chemically induced polyploids. Traditional cytology was used to calibrate genome sizes with ploidy levels. Results showed that relative genome sizes were conserved with 1Cx values ranging from 0.57 pg for** ***Kalmia carolina*** **to 0.70 pg for** ***Kalmia latifolia*****. Most species of** ***Kalmia*** **were diploid including** ***K. buxifolia*** **(*Leiophyllum buxifolium*), *K. carolina*, *K. cuneata*, *K. hirsuta*, *K. latifolia*, and *K. microphylla*. Although plants of *K. carolina* (*Kalmia angustifolia* var. *carolina*) were uniformly diploid, the closely related, but more northerly distributed, *K. angustifolia* was primarily tetraploid, providing additional justification for treating these as separate species. An unusual triploid of *K. angustifolia* f. *candida* was also documented. *Kalmia polifolia* included both tetraploid and potentially pentaploid individuals, indicating a ploidy series within this species. *Kalmia latifolia* cultivars also included one triploid, two cytochimeras, and two chemically induced tetraploids. Overall, polyploidy was more prevalent in *Kalmia* than previously reported and varied both within and among species. This broader survey of relative genome sizes and ploidy levels in *Kalmia* provides valuable information for plant breeders and new insights into the systematics and cytogenetics of the genus.**

Kalmia L. is a small but diverse genus containing nine to 11 species (Ebinger, 1974; Southall, 1973; Weakley, 2012) native to North America and Cuba (Jaynes, 1997). Deciduous or evergreen woody shrubs, *Kalmia* spp. have a varied morphology and are each distinct. Some of their diverse characteristics include plants that are "… erect, or ascending, branching shrubs or rarely trees. The leaves are simple, alternate, opposite or whorled, coriaceous, dark green above, light green beneath, entire and often revolute (Southall, 1973)." *Kalmia latifolia*, and to a lesser degree, *K. angustifolia* L. and *K. polifolia* Wangenh. are valuable nursery crops and have been cultivated in North America and Europe since the early 1700s (Jaynes, 1997).

Cytology was performed on *Kalmia* species by Dr. Richard Jaynes of the Connecticut Agricultural Experiment Station in the late 1960s (Jaynes, 1969). His examinations found most species of *Kalmia* to be diploid ($2n = 2x = 24$), with the exception of *K. polifolia* that was tetraploid ($2n = 4x = 48$). However, that study only examined material from one or two plants per species to determine ploidy, and in the case of *K. angustifolia*, only samples from North Carolina were obtained as representatives of that species (Jaynes, 1969). *Kalmia angustifolia* and *K. carolina* Small [*K. angustifolia* var. *carolina* (Small) Fernald] are closely related with North Carolina provenances now generally classified as *K. carolina* (Gillespie and Kron, 2010; Southall and Hardin, 1974; Weakley, 2012). Cytology on Ericaceous plants, including *Kalmia,* is notoriously difficult, due in part to the small chromosomes (Jaynes, 1997). Flow cytometry provides a more efficient approach to broaden sampling within and among species and can provide accurate estimates of ploidy, particularly for Ericaceous plants (Jones et al., 2007).

The objective of this study was to survey genome sizes and ploidy levels of a broad range of species, hybrids, and cultivars of *Kalmia* from throughout North America.

Materials and Methods

A diverse collection of *Kalmia* taxa was obtained from cooperators in the United States and Canada (Table 1). The Cuban species *Kalmia ericoides* C. Wright ex Griseb. was not available for analysis. Relative 2C genome sizes were determined using flow cytometry on recently expanded leaves (Greilhuber et al., 2007). Sample tissue was combined with an internal standard (*Pisum sativum* L. 'Ctirad', 2C DNA content = 8.76 pg, Greilhuber et al., 2007) and diced with a razor blade in a petri dish containing 400 µL of extraction buffer (CyStain Precise P; Partec, Münster, Germany). The nuclei suspension was poured through a 50-µm filter and stained with 1600 µL of a nucleotide staining buffer solution (CyStain ultraviolet Precise P Staining Buffer, Partec) containing 4', 6-diamidino-2-phenylindole . Stained nuclei were analyzed using the PA-II flow cytometer (PA-II; Partec). A minimum of 3000 nuclei counts were analyzed for each sample with three samples for each species. Holoploid, 2C genome sizes for each sample were calculated as: 2C = DNA content of standard × (mean fluorescence value of sample ÷ mean fluorescence value of the standard). The relationship between genome sizes and ploidy levels was based on samples with confirmed chromosome numbers. Mean 1Cx monoploid genome size (i.e., genome size of the nonreplicated base set of chromosomes) was calculated as 2C genome size ÷ ploidy. Taxa were sampled in a completely randomized fashion. Data for 1Cx values were subjected to analysis of variance and means. Different taxa were separated using Tukey's honestly significant difference, $P \leq 0.05$ (SAS version 8.02; SAS Institute, Cary, NC).

Cytology. To confirm ploidy and calibrate with genome size the cultivar *K. latifolia* 'Elf' was selected and traditional cytology was conducted using a root squash technique following procedures outlined in Lattier et al. (2014). Root tips were collected from potted plants and placed in vials of prefixative solution (2 mM 8-hydroxyquinoline + 70 mg·L^{-1} cycloheximide). Vials were stored in the dark for 2 h at ambient room temperature after which they were placed in a dark refrigerator at ≈4 °C for an additional 2 h. Following the prefixative period, roots were removed and rinsed thoroughly in deionized water. The

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Table 1. Relative genome sizes and estimated ploidy levels for 67 accessions of *Kalmia* species, cultivars, and hybrids.

Taxa/cultivar	Source/accession no.	Relative 2C genome size (pg)	Est. ploidy level (*x*)	Mean relative 1C genome size (pg) by taxa
Kalmia angustifolia 'Royal Dwarf'	NCSU 2003-131[z]	2.36 ± 0.02[y]	4	0.58 ± 0.00 DE[x]
Kalmia angustifolia 'Hammonasett'	Highstead	2.31 ± 0.01	4	
Kalmia angustifolia 'Royal Dwarf'	Highstead	2.35 ± 0.00	4	
Kalmia angustifolia 'Poke Logan'	Highstead	2.32 ± 0.01	4	
Kalmia angustifolia 'Wintergreen'	Highstead	2.38 ± 0.02	4	
Kalmia angustifolia f. *candida*	Highstead	1.74 ± 0.00	3	
Kalmia buxifolia	Southern Highlands 1	1.21 ± 0.02	2	0.60 ± 0.00 DE
Kalmia buxifolia	Southern Highlands 2	1.20 ± 0.04	2	
Kalmia buxifolia	Southern Highlands 3	1.21 ± 0.01	2	
Kalmia carolina	NCSU 2003-110	1.17 ± 0.06	2	0.57 ± 0.01 E
Kalmia carolina	NCSU 2012-126	1.15 ± 0.02	2	
Kalmia carolina	BRCC 1	1.12 ± 0.00	2	
Kalmia carolina	BRCC 2	1.10 ± 0.00	2	
Kalmia carolina	BRCC 3	1.12 ± 0.00	2	
Kalmia hirsuta	Miller/NCSU 2013-068	1.35 ± 0.00	2	0.67 ± 0.01 ABC
Kalmia hirsuta	Miller/NCSU 2013-069	1.32 ± 0.01	2	
Kalmia hirsuta	Miller/NCSU 2013-070	1.33 ± 0.02	2	
Kalmia latifolia 'Richard Jaynes'	NCSU 2004-074	1.34 ± 0.02	2	0.70 ± 0.00 A
Kalmia latifolia 'Hearts Desire'	NCSU 2011-011	1.39 ± 0.01	2	
Kalmia latifolia 'Kaleidoscope'	NCSU 2011-153	1.38 ± 0.02	2	
Kalmia latifolia 'Elf'	NCSU 2011-154	1.35 ± 0.10	2	
Kalmia latifolia 'Olympic Fire'	NCSU 2011-156	1.38 ± 0.02	2	
Kalmia latifolia 'Tinkerbelle'	NCSU 2011-157	1.37 ± 0.05	2	
Kalmia latifolia 'Minuet'	NCSU 2011-158	1.39 ± 0.01	2	
Kalmia latifolia 'Sarah'	NCSU 2011-159	1.36 ± 0.04	2	
Kalmia latifolia 'Fire Cracker'	NCSU 2011-160	1.41 ± 0.03	2	
Kalmia latifolia 'Little Linda'	NCSU 2011-161	1.42 ± 0.01	2	
Kalmia latifolia 'Tiddlywinks'	NCSU 2011-162	1.38 ± 0.00	2	
Kalmia latifolia 'Tidewater'	NCSU 2013-053	1.37 ± 0.01	2	
Kalmia latifolia	NCSU H2012-231-008	1.39 ± 0.03	2	
Kalmia latifolia	NCSU H2012-231-009	1.37 ± 0.02	2	
Kalmia latifolia 'Waxy Max'	Jaynes	1.39 ± 0.00	2	
Kalmia latifolia	Jaynes pk-74	1.37 ± 0.01	2	
Kalmia latifolia 'Big Red'	Jaynes	1.42 ± 0.00	2	
Kalmia latifolia 'Emerald Sheen'	Jaynes	1.40 ± 0.01	2	
Kalmia latifolia	Jaynes 2-06-1	1.40 ± 0.01	2	
Kalmia latifolia	Jaynes 2-06-2	1.45 ± 0.01	2	
Kalmia latifolia	Jaynes 2-06-3	1.42 ± 0.01	2	
Kalmia latifolia	Jaynes 2-06-4	1.42 ± 0.01	2	
Kalmia latifolia	Jaynes 2-06-5	1.43 ± 0.01	2	
Kalmia latifolia	Jaynes 2-06-6	1.49 ± 0.03	2	
Kalmia latifolia	Jaynes 2-06-7	1.48 ± 0.01	2	
Kalmia latifolia	Jaynes 2-06-8	1.45 ± 0.01	2	
Kalmia latifolia	Jaynes 2-06-9	1.44 ± 0.01	2	
Kalmia latifolia	Jaynes 2-06-10	1.41 ± 0.01	2	
Kalmia latifolia	Jaynes 2-06-11	1.46 ± 0.01	2	
Kalmia latifolia	Jaynes 2-06-12	1.47 ± 0.02	2	
Kalmia latifolia	Jaynes 2-06-13	1.48 ± 0.04	2	
Kalmia latifolia 'Big Boy'	Woodlanders	2.10 ± 0.00	3	
Kalmia latifolia 'Showtime'[w]	Jaynes	1.40 ± 0.00	2	
		2.75 ± 0.00	4	
Kalmia latifolia 'Silver Dollar'[w]	Jaynes	2.09 ± 0.00	3	
		4.21 ± 0.03	6	
Kalmia latifolia	NCSU H2014-222-002	2.77 ± 0.00	4	
Kalmia latifolia	NCSU H2014-222-004	2.74 ± 0.00	4	
(*K. latifolia* × *K. hirsuta*) × *K. latifolia*	Jaynes 1	1.40 ± 0.01	2	0.70 ± 0.01 AB
(*K. latifolia* × *K. hirsuta*) × *K. latifolia*	Jaynes 2	1.41 ± 0.00	2	
(*K. latifolia* × *K. hirsuta*) × *K. latifolia*	Jaynes 3	1.38 ± 0.01	2	
Kalmia microphylla var. *microphylla*	UBC 1	1.37 ± 0.03	2	0.69 ± 0.01 AB
Kalmia microphylla var. *microphylla*	UBC 2	1.36 ± 0.03	2	
Kalmia microphylla var. *microphylla*	UBC DM/2012-008PT 41559	1.41 ± 0.02	2	
Kalmia polifolia	HVNC	2.59 ± 0.01	4	0.65 ± 0.01 BC
Kalmia polifolia	Thompson 1	3.32 ± 0.03	≈5	
Kalmia polifolia	Thompson 2	3.23 ± 0.02	≈5	
Kalmia polifolia	Thompson 3	3.34 ± 0.00	≈5	
Kalmia polifolia ×*microphylla* var. *microphylla* 'Rocky Top'	Highstead	2.37 ± 0.00	4	0.59 ± 0.00 DE

[z]BRCC = Blue Ridge Community College, Flat Rock, NC; Highstead = Highstead Arboretum, Redding, CT; HVNC = Hidden Valley Nature Center, Jefferson, ME; Jaynes = Richard Jaynes, Broken Arrow Nursery, Hamden, CT; Miller = Ron Miller, Pensacola, FL; NCSU = North Carolina State University, Mountain Crop Improvement Laboratory, Mills River, NC; Southern Highlands = Southern Highlands Reserve, Lake Toxaway, NC; Thompson = Elizabeth Thompson, University of Vermont, Burlington, VT; UBC = University of British Columbia, Vancouver, BC, Canada; Woodlanders = Woodlanders Nursery, Aiken, SC.
[y]Values are means ($n = 2$) ± SE.
[x]Means followed by a common letter are not significantly different, Tukey's honestly significant difference, $P \leq 0.05$.
[w]Mixoploid/cytochimera.

rinsed root tips were then placed in a 3:1 solution of ethanol and propionic acid and left overnight at ambient room temperature to complete the fixation process. The following day, the roots were rinsed with 70% ethanol three times and stored in 70% ethanol at ≈4 °C until root squashes could be performed. Root tips were removed from the ethanol storage solution and placed in a hydrolyzing solution made up of a 3:1 solution of 95% ethanol and hydrochloric acid (12.3 M) for 10 min. After hydrolyzing, the root tips were transferred into a modified carbol-fuchsin staining solution for 10 min. Once stained the terminal end of the root tip was excised under a dissecting stereo microscope and placed on a clean microscope slide in a drop of staining solution. A cover slip was applied, and the root tip tissue was squashed. Slides were observed using a light microscope (Nikon Eclipse 80i; Nikon, Melville, NY). Photographs, taken at multiple focal points, were layered (Photoshop CS6; Adobe, Mountain View, CA) to create an enhanced depth of field.

Results and Discussion

Cytology confirmed that *K. latifolia* 'Elf' is a diploid with $2n = 2x = 24$ (Fig. 1) as has been reported previously for that species (Jaynes, 1969). Flow cytometry was also an efficient and consistent method for determining relative genome size and ploidy of *Kalmia* (Table 1). Values for multiple subsamples of each replicate were consistent with SE for 2C values ranging from less than 0.01 to 0.1 pg demonstrating a high level of precision and repeatability and clearly distinguishing between ploidy levels. Results from flow cytometry revealed that genome sizes were relatively conserved with 1Cx values ranging from 0.57 pg for *K. carolina* to 0.70 pg for *K. latifolia*. These values are similar to other Ericaceous plants, including *Rhododendron* that had 1Cx values ranging from 0.63 to 0.83 pg (Jones et al., 2007). Cytometry results also showed there to be considerable variation in ploidy both between and within species (Table 1). Most species of *Kalmia* were predominantly diploid including *K. buxifolia*, *K. carolina*, *K. cuneata*, *K. hirsuta*, *K. latifolia*, and *K. microphylla*. In the case of *K. latifolia*, there were some exceptions. The cultivar *K. latifolia* Big Boy was a triploid, most likely the result of an unreduced gamete from one parent. There were also two mixoploid/cytochimeras found: *K. latifolia* 'Showtime' had both diploid and tetraploid tissues, whereas 'Silver Dollar' had a mixture of triploid and hexaploid tissues. These mixoploids most likely arose from mitotic irregularities (endoreduplication) in at least one of the histogenic layers resulting in stable cytochimeras (Joubès and Chevalier, 2000). Naturally occurring mixoploids have been documented in other Ericaceous plants including *Rhododendron* (De Schepper et al., 2001; Jones et al., 2007; Sakai et al., 2006). In the case of 'Silver Dollar', this apparently happened in a triploid plant that most likely resulted from the union of an unreduced gamete from one of the parents. Two additional plants of *K. latifolia*, H2014-222-002 and H2014-222-004, were confirmed to be homogeneous tetraploids that resulted from treatment of seedlings with the mitotic inhibitor oryzalin at North Carolina State University.

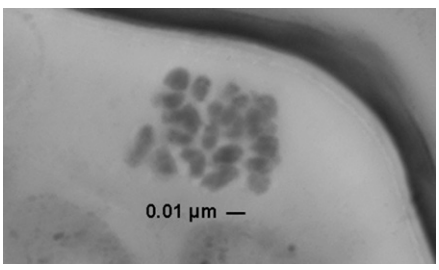

Fig. 1. Condensed chromosomes of *Kalmia latifolia* 'Elf' viewed at 1000× using oil immersion.

As previously reported by Jaynes (1969), under the epitaph *K. angustifolia* var. *carolina*, the southern sheep laurel, *K. carolina* was confirmed to be diploid. However, samples of *K. angustifolia,* the northern sheep laurel, were all tetraploid, including 'Hammonasett', 'Poke Logan', 'Royal Dwarf', and 'Wintergreen'. The cultivars Hammonasett and Poke Logan are known to be wild collected selections from Connecticut and Maine, respectively (Jaynes, 1997). In addition, a sample of *K. angustifolia* f. *candida*, a white-flowered form, was triploid. Most likely this particular plant is an oddity and not necessarily representative of the white-flowered form in general.

Kalmia polifolia also varied in ploidy. A sample of *K. polifolia* obtained from Hidden Valley Nature Center in Jefferson, ME, was tetraploid. However, wild collected samples from Alburg, VT, had genome sizes consistent with pentaploids, though this could not be confirmed with cytology. The existence of putative pentaploid *K. polifolia* in the wild implies that there is a natural occurring ploidy series within the species, potentially including tetraploid, pentaploid and hexaploid individuals or populations. Additional sampling in this location would be warranted. Kron and King (1996) postulated that *K. polifolia* may be a hybrid between two lineages of *Kalmia* ancestors, which are no longer extant. It has often been found that interspecific hybrids have a greater propensity to form unreduced gametes leading to the formation of allopolyploids (Arnold, 2006; Ramsey and Schemske, 1998) and this may be the case for *K. polifolia*. The sample of the hybrid (*K. polifolia* × *K. microphylla* var. *microphylla*) 'Rocky Top' was a tetraploid (2C = 2.37 pg), suggesting that it formed from the union of an unreduced gamete from the *K. microphylla* parent.

This survey provides new information on the genome sizes and ploidy levels for species, cultivars, and hybrids of *Kalmia.* The discovery and confirmation of new natural and induced polyploids within the genus provides basic information for how to best use these plants in breeding programs. Although development of interspecific hybrids in *Kalmia* has been challenging (Jaynes, 1997), attempting these crosses at the tetraploid level may result in allopolyploids (amphidiploids) with greater fertility because of disomic chromosome pairing in meiosis (Ranney, 2006), allowing for advanced generations. Alternatively, the development of new triploid hybrids of *K. angustifolia* and *K. carolina* has the potential to help reduce fertility and invasiveness where they have become problematic in European forests (Inderjit and Mallik, 1996).

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