HortScience 44(1):44–48. 2009.

Crossability, Cytogenetics, and Reproductive Pathways in *Rudbeckia* Subgenus *Rudbeckia*

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Additional index words. apomixis, coneflower, cytology, DNA content, flow cytometry, relative genome size, plant breeding, polyploidy, pseudogamy

***Abstract. Rudbeckia* L. are valuable nursery crops that offer broad adaptability and exceptional ornamental merit. However, there is little information on interspecific and interploid crossability and ploidy levels of specific cultivars. The objectives of this study were to determine the ploidy levels and relative DNA contents (genome sizes) of selected species and cultivars, to evaluate self-compatibility and crossability among species and ploidy levels, and to explore reproductive pathways in triploid *R. hirta* L. with the goal of facilitating future breeding endeavors and development of new hybrids. Reciprocal interspecific crosses were performed between *R. hirta* cultivars and *R. fulgida* Ait., *R. missouriensis* Engelm. ex C.L. Boynton & Beadle, and *R. subtomentosa* Pursh. as well as reciprocal interploid crosses among four *R. hirta* cultivars. A combination of relative DNA content analysis and chromosome counts was used to test for hybridity and to determine ploidy levels for selected species, cultivars, and interploid *R. hirta* F_1 hybrids. Of the specific clones tested, *R. subtomentosa* and *R. missouriensis* were diploid, *R. fuligida* varieties were tetraploid, and *R. hirta* include both diploid and tetraploid cultivars. Mean 1Cx DNA content varied over 320% among species. The interploid *R. hirta* crosses produced triploids as well as pentaploids and hexaploids. Seedlings from open-pollinated triploid *R. hirta* appeared, based on diverse phenotypes and DNA contents, to be aneuploids resulting from sexual fertilization, not apomixis. Of the 844 seedlings from interspecific F_1 crosses, only one individual, *R. subtomentosa* × *R. hirta*, had a DNA content intermediate between its parents and was confirmed as the only interspecific hybrid. Although most taxa had low self-fertility, seedlings (with genomic sizes similar to their maternal parent) resulted after interspecific crosspollination, indicating that pseudogamy is one reproductive pathway in *Rudbeckia species*.**

The genus *Rudbeckia* contains ≈30 species of annuals, biennials, and perennials known for their colorful, golden ray corollas and prominent disk-shaped receptacles that can bloom from midsummer through October. *Rudbeckia* are popular crops and are valued for their ornamental diversity, low-maintenance requirements, and heat and drought resistance.

Rudbeckia include two genetically and morphologically distinct subgenera: *Macrocline* and *Rudbeckia* (Urbatsch et al., 2000). Species in the subgenus *Rudbeckia* include many desirable ornamentals and range from opportunistic annuals to persistent perennials. These species generally flourish in full sun and well-drained soils and are commonly found in recently disturbed habitats such as roadsides or old fields and thrive during primary and secondary succession. The annual species *R. hirta* includes cultivars with a diverse range of flower colors and forms, including bicoloration and double inflorescences (greater number of ray florets). Petal colors range from a lemon yellow and gold exhibited by 'Prairie Sun' to the deep red and mahogany expressed by 'Cherokee Sunset'. However, *R. hirta* is short-lived and susceptible to diseases, including rhizoctonia blight (*Rhizoctonia* sp.) and cercospora leaf spot (*Cercospora* sp.) (Fulcher et al., 2003; Harkess and Lyons, 1994). Other *Rudbeckia* species, including *R. fulgida*, *R. missouriensis*, and *R. subtomentosa*, are reliable perennials with superior disease resistance and good performance in southern climates (Armitage, 1997). Interspecific hybridization between durable perennial species and showier annual species could lead to development of valuable new cultivars.

There has been little published on the genetics and breeding of *Rudbeckia*. Species in *Rudbeckia* subgenus *Rudbeckia* have a base chromosome number of $x = 19$ and polyploids have been reported to exist in *R. hirta*, *R. fulgida* var. *speciosa*, and *R. triloba* L. (McCrea, 1981; Urbatsch et al., 2000). However, there is little information available on ploidy levels of specific cultivars. Pseudogamy, a form of apomixis, has also been implicated as the sole means of reproduction in triploid *R. triloba* (McCrea, 1981). A greater understanding of reproductive pathways and occurrence of apomixis would provide valuable information for both breeding and commercial propagation of *Rudbeckia*.

The objectives of this research were to determine ploidy levels and relative DNA contents of selected species and cultivars, to evaluate self-compatibility and crossability between species, to determine interploid crossability among *R. hirta* cultivars, and to assess reproductive pathways in triploid *R. hirta* to better facilitate future *Rudbeckia* breeding projects.

Table 1. Relative DNA content, ploidy level, and chromosome number of selected *Rudbeckia* taxa from subgenus *Rudbeckia*.

Taxa	2C DNA content (pg)	1Cx DNA content (pg)	Ploidy level	2*n* chromosome number
R. hirta 'Goldilocks'	6.8[z] ± 0.10 a	3.4[z] ± 0.05 a	2*x*	38
R. hirta 'Marmalade'	7.1 ± 0.03 a	3.5 ± 0.02 ab	2*x*	38
R. hirta 'Toto Gold'	7.2 ± 0.07 a	3.6 ± 0.08 ab	2*x*[y]	38[y]
R. hirta 'Toto Rustic'	7.2 ± 0.14 a	3.6 ± 0.16 ab	2*x*	38
R. hirta 'Sonera'	13.7 ± 0.34 b	3.4 ± 0.16 a	4*x*	76
R. hirta 'Cherokee Sunset'	14.0 ± 0.17 bc	3.5 ± 0.08 ab	4*x*	76
R. hirta 'Autumn Colors'	14.8 ± 0.25 bc	3.7 ± 0.13 ab	4*x*	76
R. hirta 'Prairie Sun'	15.4 ± 0.22 bcd	3.9 ± 0.11 abc	4*x*	76
R. hirta 'Indian Summer'	15.8 ± 0.31 bcd	4.0 ± 0.15 abc	4*x*[y]	76[y]
R. hirta 'Tetraploid'	16.1 ± 0.43 cd	4.0 ± 0.21 bc	4*x*	76
R. missouriensis	17.6 ± 0.02 d	8.8 ± 0.01 cd	2*x*[y]	38[y]
R. subtomentosa	21.9 ± 0.17 e	11.0 ± 0.09 e	2*x*[y]	38[y]
R. fulgida var. *fulgida*	33.9 ± 1.06 f	8.5 ± 0.53 f	4*x*	76
R. fulgida var. *speciosa*	35.4 ± 0.15 f	8.9 ± 0.07 f	4*x*	76
R. fulgida var. *sullivantii* 'Goldsturm'	35.8 ± 0.54 f	8.9 ± 0.27 f	4*x*[y]	76[y]

[z]Values are means, n = 2 to 5 ± SEM. Values followed by a common letter, within a column, are not significantly different ($P \leq 0.05$).
[y]Confirmed through cytology.

Received for publication 7 Oct. 2008. Accepted for publication 19 Nov. 2008.
Thanks are expressed to Bluebird Nursery, Clarkson, NE and North Creek Nurseries, Landenberg, PA, for providing seeds and plants for this study. The authors gratefully acknowledge excellent technical assistance of Tom Eaker and Joel Mowery at the Mountain Horticultural Crops Research and Extension Center as well as the staff of the Mountain Horticultural Crops Research Station.
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Table 2. Seedlings per inflorescence and reproductive pathways of self-pollinated and interspecific crosses varying in ploidy level.

Pistillate parent		Staminate parent			
Taxa	Ploidy	Taxa	Ploidy	Seedlings per inflorescence	Reproductive pathway
		Self-pollinations			
R. hirta 'Goldilocks'	2*x*	Self	2*x*	0.0[z]	SI[y]
R. hirta 'Toto Gold'	2*x*	Self	2*x*	1.2	SP/apomixis
R. hirta 'Toto Rustic'	2*x*	Self	2*x*	0.0	SI
R. hirta 'Cherokee Sunset'	4*x*	Self	4*x*	0.0	SI
R. fulgida var. *fulgida*	4*x*	Self	4*x*	0.3	SP/apomixis
R. fulgida 'Goldsturm'	4*x*	Self	4*x*	0.3	SP/apomixis
R. fulgida var. *speciosa*	4*x*	Self	4*x*	0.0	SI
R. subtomentosa	2*x*	Self	2*x*	0.0	SI
		Interspecific crosses			
R. hirta 'Goldilocks'	2*x*	*R. subtomentosa*	2*x*	21.5	SP/apomixis
R. hirta 'Marmelade'	2*x*	*R. subtomentosa*	2*x*	4.0	SP/apomixis
R. hirta 'Toto Gold'	2*x*	*R. missouriensis*	2*x*	0.0	SI/CI
R. hirta 'Toto Gold'	2*x*	*R. subtomentosa*	2*x*	0.3	SP/apomixis
R. hirta 'Toto Rustic'	2*x*	*R. missouriensis*	2*x*	3.5	SP/apomixis
R. hirta 'Toto Rustic'	2*x*	*R. subtomentosa*	2*x*	0.0	SI/CI
R. missouriensis	2*x*	*R. hirta* 'Marmelade'	2*x*	0.0	SI/CI
R. missouriensis	2*x*	*R. hirta* 'Toto Gold'	2*x*	0.5	SP/apomixis
R. missouriensis	2*x*	*R. hirta* 'Toto Rustic'	2*x*	22.5	SP/apomixis
R. subtomentosa	2*x*	*R. hirta* 'Goldilocks'	2*x*	0.3	SP/apomixis
R. subtomentosa	2*x*	*R. hirta* 'Marmelade'	2*x*	0.0	SI/CI
R. subtomentosa	2*x*	*R. hirta* 'Toto Gold'	2*x*	0.3	Hybrid + SP/apomixis
R. subtomentosa	2*x*	*R. hirta* 'Toto Rustic'	2*x*	0.16	SP/apomixis
R. hirta 'Goldilocks'	2*x*	*R. fulgida* var. *fulgida*	4*x*	1.3	SP/apomixis
R. hirta 'Toto Gold'	2*x*	*R. fulgida* 'Goldsturm'	4*x*	0.0	SI/CI
R. hirta 'Toto Rustic'	2*x*	*R. fulgida* 'Goldsturm'	4*x*	0.0	SI/CI
R. missouriensis	2*x*	*R. hirta* 'Cherokee Sunset'	4*x*	38	SP/apomixis
R. missouriensis	2*x*	*R. hirta* 'Sonora'	4*x*	0.0	SI/CI
R. missouriensis	2*x*	*R. hirta* 'Tetraploid'	4*x*	21.8	SP/apomixis
R. subtomentosa	2*x*	*R. hirta* 'Indian Summer'	4*x*	0.2	SP/apomixis
R. subtomentosa	2*x*	*R. hirta* 'Prairie Sun'	4*x*	0.0	SI/CI
R. subtomentosa	2*x*	*R. hirta* 'Sonora'	4*x*	0.0	SI/CI
R. subtomentosa	2*x*	*R. hirta* 'Tetraploid'	4*x*	0.0	SI/CI
R. hirta 'Cherokee Sunset'	4*x*	*R. missouriensis*	2*x*	9.5	SP/apomixis
R. hirta 'Indian Summer'	4*x*	*R. fulgida* var. *fulgida*	4*x*	18.4	SP/apomixis
R. hirta 'Indian Summer'	4*x*	*R. fulgida* 'Goldsturm'	4*x*	0.0	SI/CI
R. hirta 'Indian Summer'	4*x*	*R. fulgida* var. *speciosa*	4*x*	11.3	SP/apomixis
R. hirta 'Indian Summer'	4*x*	*R. subtomentosa*	2*x*	0.0	SI/CI
R. hirta 'Prairie Sun'	4*x*	*R. fulgida* var. *speciosa*	2*x*	2.0	SP/apomixis
R. hirta 'Prairie Sun'	4*x*	*R. fulgida* 'Goldsturm'	4*x*	0.0	SI/CI
R. hirta 'Prairie Sun'	4*x*	*R. subtomentosa*	2*x*	0.0	SI/CI
R. hirta 'Sonora'	4*x*	*R. missouriensis*	2*x*	0.3	SP/apomixis
R. hirta 'Sonora'	4*x*	*R. subtomentosa*	2*x*	0.0	SI/CI
R. hirta 'Tetraploid'	4*x*	*R. missouriensis*	2x	0.0	SI/CI
R. hirta 'Tetraploid'	4*x*	*R. subtomentosa*	2*x*	0.7	SP/apomixis
R. fulgida var. *fulgida*	4*x*	*R. hirta* 'Indian Summer'	2*x*	0.2	SP/apomixis
R. fulgida var. *fulgida*	4*x*	*R. hirta* 'Goldilocks'	2*x*	0.5	SP/apomixis
R. fulgida 'Goldsturm'	4*x*	*R. hirta* 'Indian Summer'	2*x*	0.8	SP/apomixis
R. fulgida 'Goldsturm'	4*x*	*R. hirta* 'Prairie Sun'	4*x*	0.3	SP/apomixis
R. fulgida 'Goldsturm'	4*x*	*R. hirta* 'Toto Gold'	2*x*	0.5	SP/apomixis
R. fulgida 'Goldsturm'	4*x*	*R. hirta* 'Toto Rustic'	2*x*	0.0	SI/CI
R. fulgida var. *speciosa*	4*x*	*R. hirta* 'Indian Summer'	4*x*	0.2	SP/apomixis
R. fulgida var. *speciosa*	4*x*	*R. hirta* 'Prairie Sun'	4*x*	2.2	SP/apomixis

[z]Means based on four or more inflorescenses.
[y]SI = self-incompatible; SP = self-pollinated; CI = cross-incompatible.

Materials and Methods

Cytology. Chromosome counts were performed on *R. hirta* 'Toto Gold', *R. hirta* 'Indian Summer', *R. missouriensis*, *R. subtomentosa*, and *R. fulgida* var. *sullivantii* 'Goldsturm' to calibrate relative DNA content with ploidy level for each species. Root tips were collected and placed in 2 mM 8-hydroxyquioline for 3 to 5 h at 12 °C. Roots were then rinsed with 4 °C distilled water and placed in a 6:3:1 solution of 95% ethanol: chloroform:glacial acetic acid fixative for 24 h at 20 °C. Samples were rinsed and stored in 70% ethanol solution at 4 °C. The next week, samples were removed from storage and transferred to a 30% aqueous ethanol solution for 12 min followed by two 15-min rinses in distilled water. Roots were then hydrolyzed for 1 h at 20 °C in 1 N HCl, followed by a quick rinse in distilled water, and were placed in Feulgen stain for 2 h at room temperature. Root tips were excised and placed on a glass microscope slide with a drop of 1% aceto-carmine stain, squashed with a coverslip, and viewed at ×1500.

Flow cytometry. Holoploid, 2C DNA contents (i.e., DNA content of the entire nonreplicated, chromosome complement regardless of ploidy level) were determined through flow cytometry for all the parental taxa and progeny (Doležel et al., 1998). Approximately 1.5 cm^2 of leaf or petal tissue was chopped with a razor blade in a petri dish containing 400 µL of extraction buffer (CyStain ultraviolet Precise P; Partec, Münster, Germany). The suspension was filtered through 50 µm nylon mesh and nuclei were stained using 1.6 mL staining buffer containing 4′, 6-diamidino-2-phenylindole (DAPI) (CyStain ultraviolet Precise P; Partec). The suspension was analyzed using a flow cytometer with fluorescence excitation provided by a mercury arc lamp (PA-I Ploidy Analyzer; Partec). The mean fluorescence of each sample was

compared with an internal standard of known genome size (*Pisum sativum* L. 'Ctirad', 2C = 8.76 pg; Greihuber et al., 2007). Base 1Cx monoploid DNA content (i.e., DNA content of the nonreplicated base set of chromosomes with $x = 19$) was calculated for each species as 2C DNA content/ploidy level. DNA content data were subjected to analysis of variance and means were separated using Tukey's honestly significant difference procedure.

Breeding and pollinations. Taxa selected for breeding included 10 *R. hirta* cultivars, three *R. fulgida* varieties, *R. missouriensis*, and *R. subtomentosa* (Table 1). Plants were grown in a glass-covered greenhouse in 2006, 2007, and 2008 using standard horticultural practices. Forty-three interspecific crosses were completed in a greenhouse with at least four pollinated inflorescences per cross. The greenhouse was screened to exclude insect pollinators. All pollinations were performed daily by hand and each individual plant was involved in only one reciprocal cross. Pollinations included reciprocal crosses between *R. hirta* cultivars and the remaining species. Two reciprocal interploid crosses were also performed between *R. hirta* 'Cherokee Sunset' × *R. hirta* 'Toto Rustic' and *R. hirta* 'Prairie Sun' × *R. hirta* 'Goldilocks'. Self-pollinations were also performed on separate inflorescences of selected representative taxa: *R. hirta* 'Goldilocks', *R. hirta* 'Toto Gold', *R. hirta* 'Toto Rustic', *R. hirta* 'Cherokee Sunset', *R. fulgida* var. *fulgida*, *R. fulgida* var. *speciosa*, *R. fulgida* var. *sullvantii* 'Goldsturm', and *R. subtomentosa*. Inflorescences were pollinated daily until all disk florets passed anthesis. Achenes were collected after flower senescence and sown. Triploid, F_1 interploid progeny were selected and subjected to open-pollination. Achenes from five inflorescences per clone were collected and sown. The reproductive pathway (i.e., apomixis versus sexual) of this population was determined based on relative DNA content and phenotypic variation.

Results and Discussion

Chromosome counts documented that *R. hirta* 'Toto Gold' was a diploid ($2n = 2x = 38$) and *R. hirta* 'Indian Summer' was a tetraploid ($2n = 4x = 76$) with an average 1Cx value of 3.8 pg (Table 1). Seedlings of *R. missouriensis* and *R. subtomentosa* were confirmed to be diploids with 1Cx values of 8.8 pg and 11.0 pg, respectively. *Rudbeckia fulgida* var. *sullivantii* 'Goldsturm' was found to be tetraploid with a 1Cx value of 8.9 pg. Based on these standards, ploidy levels of the remaining cultivars were then estimated for each species (Table 1). Although we did not find any diploid *R. fulgida*, McCrea (1981) reported finding diploid populations of *R. fulgida*, particularly in the southern portion of its range.

Mean 1Cx DNA content was similar among cultivars of *R. hirta* regardless of ploidy level (i.e., there was no apparent genome downsizing at higher ploidy levels). However, 1Cx DNA content varied over 320% among species. This considerable variation in 1Cx DNA content among closely related species suggests that recent and substantial changes in genome size have occurred in this subgenus. Accumulation of transposable elements, particularly long terminal repeat retrotransposons, can rapidly accumulate over short evolutionary timeframes (Hawkins et al., 2008) and may have occurred in some species of *Rudbeckia*. This large variation in DNA content also emphasizes the need to calibrate DNA content with ploidy level separately for each species within this subgenus and provides a convenient way to confirm hybridity in seedlings of interspecific crosses.

Self-compatibility was generally found to be low for all taxa selected for self-pollinations. *Rudbeckia hirta* 'Goldilocks', 'Toto Rustic', 'Cherokee Sunset', and *R. fulgida* var. *speciosa* produced no viable seedlings after selfing. *Rudbeckia hirta* 'Toto Gold' produced 1.2 seedlings per inflorescence, whereas *R. fulgida* var. *fulgida* and *R. fulgida* var. *sullvantii* 'Goldsturm' both produced 0.3 seedlings per inflorescence (Table 2) after selfing. It could not be determined if the limited number of seedlings produced after self-pollinations resulted from low levels of self-compatibility or apomixis.

Crosses between species yielded a mean of zero to 23 seedlings per inflorescence with a total of 844 seedlings (Table 2). However, 2C DNA contents of all but one of these seedlings were similar to the maternal parent, suggesting these plants arose through either self-fertilization or apomixis. In some crosses (e.g., *R. hirta* 'Goldilocks × *R. subtomentosa*, *R. hirta* 'Cherokee Sunset' × *R. missouriensis*), numerous seedlings, with DNA contents similar to the maternal parent, were recovered, although these taxa had very low fertility when selfed. Pseudogamy, a process whereby crosspollination from a different

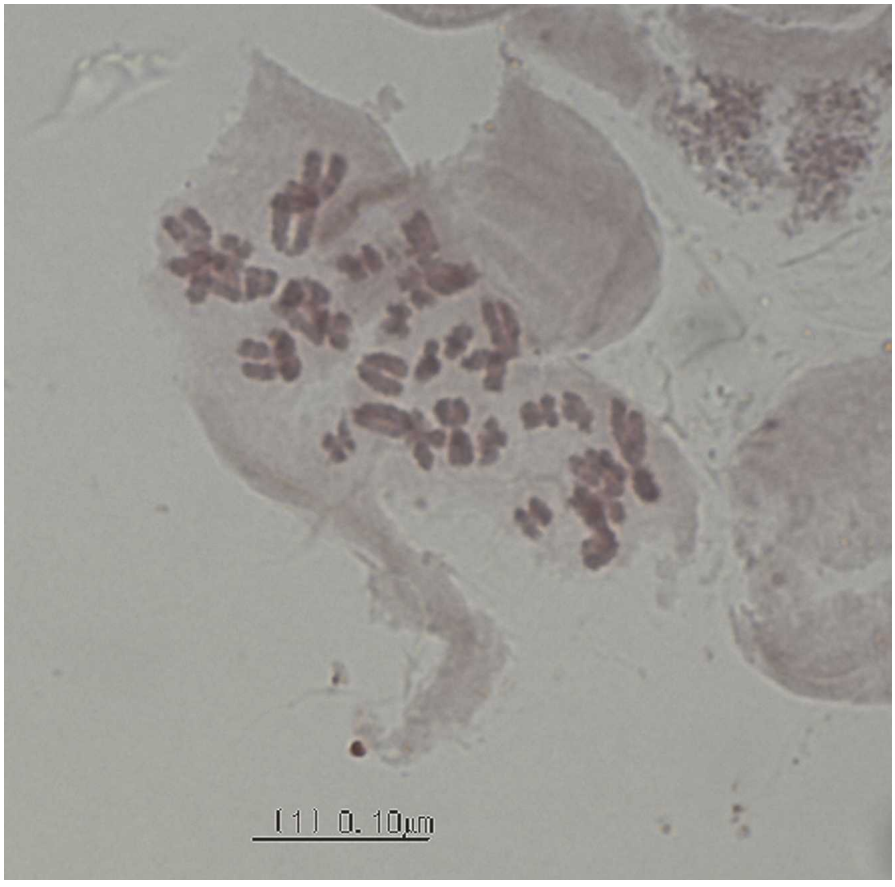

Fig. 1. Photomicrograph of *Rudbeckia subtomentosa* (larger chromosomes) × *R. hirta* 'Toto Gold' (smaller chromosomes) H2007-062-001 nucleus with $2n = 2x = 38$.

Table 3. Ploidy levels of progeny resulting from interploid crosses.

Pistillate parent		Staminate parent		Progeny[z]			
Cultivar	Ploidy	Cultivar	Ploidy	3*x*	4*x*	5*x*	6*x*
R. hirta 'Cherokee Sunset'	4*x*	*R. hirta* Toto Rustic	2*x*	2	39	2	1
R. hirta 'Toto Rustic'	2*x*	*R. hirta* Cherokee Sunset	4*x*	18	3	0	0
R. hirta 'Prairie Sun'	4*x*	*R. hirta* Goldilocks	2*x*	17	0	0	0
R. hirta 'Goldilocks'	2*x*	*R. hirta* Prairie Sun	4*x*	16	0	0	0

[z]Based on five inflorescences per cross.

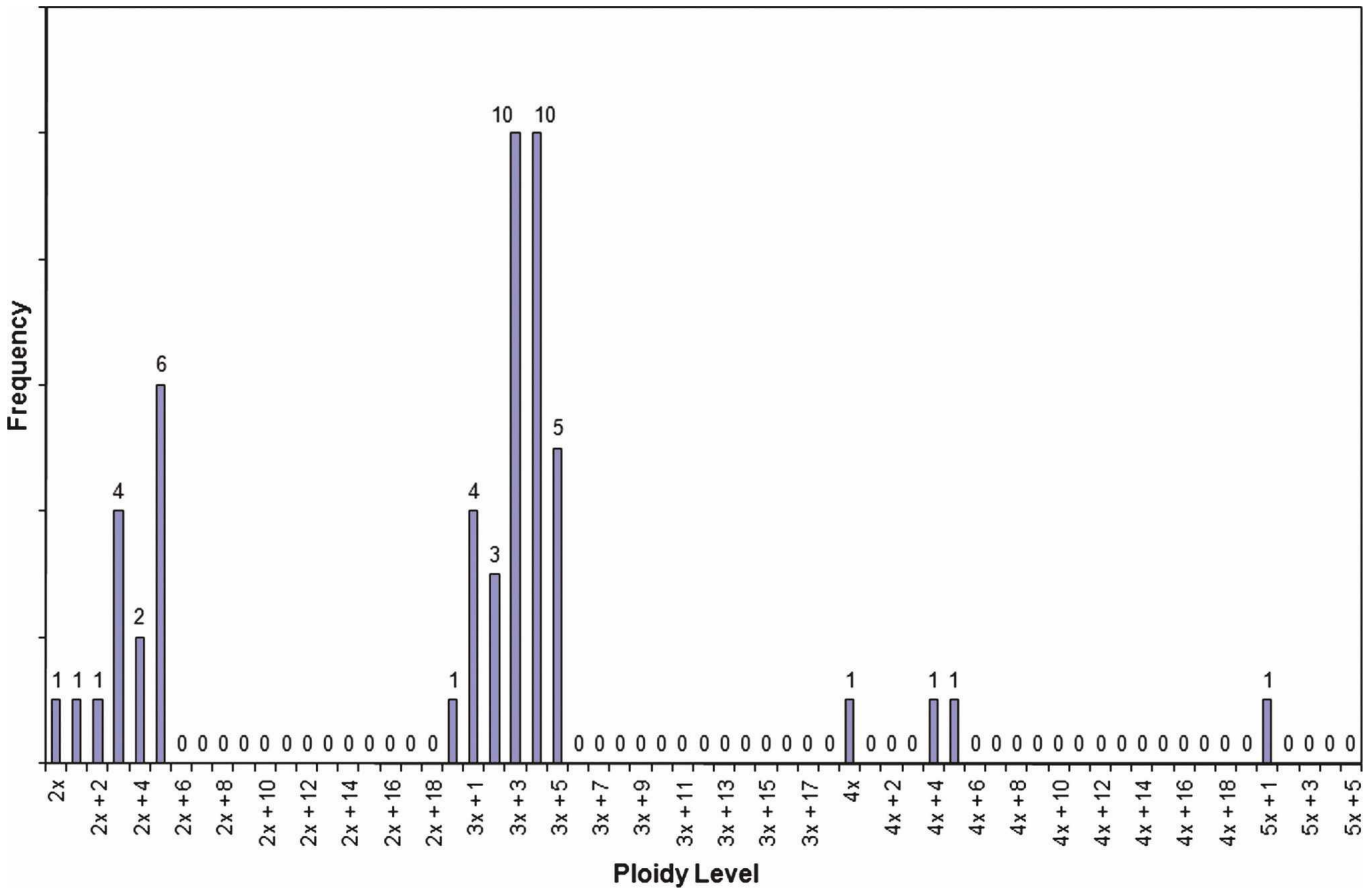

Fig. 2. Frequency distribution of ploidy levels of progeny derived from open-pollinated, triploid *Rudbeckia hirta*. Ploidy level was estimated based on relative DNA content with each chromosome averaging 0.19 ρg.

genotype stimulates apomixis, may be involved and has been recorded previously in *R. triloba* L. (3*x*) and *R. fulgida* (4*x*) (McCrea, 1981). Overall, interspecific crossability among these species was found to be extremely low with production of only one successful hybrid: *R. subtomentosa* (2C = 21.9 ρg) × *R. hirta* 'Toto Gold' (2C = 7.2 ρg), which yielded a seedling with an intermediate 2C DNA content of 14.7 ρg supporting hybridity (Table 2). Cytology also documented two sets of 19 chromosomes of disparate size, consistent with both parents (Fig. 1). McCrea (1981) also found poor crossability among species in this subgenus and reported that among interspecific crosses attempted with six species, the only successful hybrids were between *R. missouriensis* and *R. fulgida*.

Interploid crosses among *R. hirta* cultivars resulted in 98 viable seedlings, 53 of which were triploids (Table 3). *Rudbeckia hirta* 'Cherokee Sunset' (4*x*) × *R. hirta* 'Toto Rustic' (2*x*) produced two triploid, 39 tetraploid (most likely from pseudogamy), two pentaploid, and one hexaploid offspring. *Rudbeckia hirta* 'Toto Rustic' (2*x*) × *R. hirta* 'Cherokee Sunset' (4*x*) produced 18 triploids and three tetraploids. The larger than expected ploidy in some individuals may be the result of unreduced gametes in one or both parents. *Rudbeckia hirta* 'Prairie Sun' (4*x*) × *R. hirta* 'Goldilocks' (2*x*) resulted in 17 triploids, and the reciprocal cross generated 16 triploids. Interploid cross progeny exhibited intermediate traits.

Open-pollination of five inflorescences from each of 13 triploid selections resulted in recovery of 51 seedlings with a broad range of DNA contents (Fig. 2). One population of progeny ranged in ploidy from 2*x* to 2*x* + 5 (≈5 extra chromosomes based on DNA content) and most likely resulted from sexual reproduction of triploids crossing with diploid *R. hirta* plants in the vicinity. A second population of progeny ranged from 3*x* to 3*x* + 5 and most likely resulted from either apomixis or sexual reproduction among triploids resulting in aneuploids. There were also four progeny with ploidy levels of 4*x* or higher and most likely resulted from the union of unreduced gametes (from one or both parents, including outcrosses with diploids or tetraploid *R. hirta* in the vicinity). Progeny from triploid parents exhibited a broad range of unique phenotypic characteristics, distinct from their respective maternal female parents, further suggesting these plants arose primarily from sexual reproduction and not apomixis.

Results from the cytology and cytometry component of this research documented ploidy levels and relative DNA contents of selected species and cultivars of *Rudbeckia*. Breeding and crossability components determined a high level of self-incompatibility among these taxa and found that apomixis (pseudogamy) appears to be more prevalent after interspecific pollination in some taxa. Interploid crosses between 2*x* and 4*x R. hirta* produced 3*x* progeny regardless of cross direction. Triploid plants maintained limited fertility, including sexual reproduction through the formation of both unreduced and aneuploid gametes. Although successful interspecific hybridization was rare and difficult to achieve, one diploid interspecific hybrid of *R. subtomentosa* × *R. hirta* was verified. Additional efforts to produce interspecific hybrids may lead to development of improved cultivars.

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