Karyologic and Heterosis Studies of the Artificial Inter- and Intraspecific Hybrids of *Viola ×wittrockiana* and *Viola cornuta*

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Abstract. An artificial crossing was made between four *Viola ×wittrockiana* (2n = 48) lines and one *Viola cornuta* inbred (2n = 26) line as well as among *V. ×wittrockiana* lines to investigate the cytologic characteristics, fertility, and heterosis of hybrids. The sizes of flowers and stomata and the leaf areas of the *V. ×wittrockiana* plants were larger than those of the *V. cornuta* plants. The karyologic stability of *V. cornuta* was greater than that of *V. ×wittrockiana*, and ≈20% of individuals in the latter deviated from the given 2n = 48. Capsule setting after interspecific hand pollination ranged from 28% to 94%, and F₁ seed germination ranged from 16% to 88%, revealing no obvious pre- or postzygotic selection mechanisms. The chromosome numbers in *V. ×wittrockiana* × *V. cornuta* F₁ seedlings varied (2n = 34–38), with about half the cells exhibiting the intermediate (2n = 36) value. These interspecific hybrids exhibited positive heterosis in flower size (range, 11% to 66%) and negative heterosis in plant height (range, –12% to –57%). Both of these traits are desirable in pansy breeding programs.

Breeding methods for ornamental plants range from traditional selection breeding programs to modern biotechnological and genetic engineering methods. Many ornamental plants, including geraniums, impatiens, begonias, and primulas, originated from interspecific and intergeneric crosses, which leads to a high degree of heterozygosity in the resulting hybrids, with polyploidy and aneuploidy often occurring. In seed-propagated ornamentals with a considerable seed market, cultivars will normally be bred as hybrids based on inbred parental lines. The main emphasis in ornamental plant breeding is to improve the variety traits, novel color, form, size, number of flowers, flower vase life, repeat blooming, disease resistance, nutrient uptake capacity, and growth habits (De, 2017). Commercially available pansies

are F₁ hybrids produced by hand crossing. Currently, such production of *Viola tricolor maxima* with hermaphrodite flowers requiring emasculation occasionally occurs at the expense of limited profitability (De, 2017; Reimann-Philipp, 1983).

Heterosis is a phenomenon whereby the phenotype of F₁ hybrids is superior to that of their parents. Intralocus interactions between alleles, complementation of dominant alleles, or interloci epistatic interactions are genetic mechanisms that may cause nonadditive phenotypic inheritance in hybrids. However, different views exist on what portion of the heterotic variation is modulated by each of these mechanisms (Fridman, 2015).

Viola ×wittrockiana Gams ex Kappert., known as garden pansy, is one of the most common bedding plants worldwide, exhibiting rich flower colors, cold tolerance, and other charming characteristics, such as odor and taste (Fernandes et al., 2017; Lagibo et al., 2005; Li et al., 2014, and literature cited therein). This species is also studied as a promising source of natural antioxidants (Vukics et al., 2008). Present-day garden pansies are horticultural hybrids rather than a natural species. Initially, the species was selected as an interspecific hybrid resulting

from the crossing of *Viola* L. sect. *Melanium* Ging. species (pansies), *V. tricolor* L., *V. lutea* Hudson, and *V. altaica* Ker-Gawler (Kroon, 1972). The species differs from wild pansies based on the absence of dark lines on the flower petals (nectar guides for pollinators) and the presence of huge blocks of color on the lower (anterior) and lateral petals called a “face.” This pansy and its progeny have become popular with gardeners and breeders throughout Europe. At the turn of the 20th century, Charles Stewart bred clear color pansies without faces that were welcomed by North American gardeners. Currently, *V. ×wittrockiana* is one of the dominant hybrids among ornamental bedding plants in the U.S. market (Janick, 1998).

During the past 60 years, much of the innovative pansy breeding has been performed in Germany, the United States, and Japan (Zhang et al., 2010). New pansy varieties with different colors, such as shades of pink, rose, or orange, and unusual bicolor designs have been introduced and are available to gardeners. Studies using a simple selection method to reduce the ploidy level by crossing with diploids to improve uniformity, as well as an increase of polyploidy by mutagenesis to maximize plant morphologic and physiologic characters, have already been performed (Dalbato et al., 2013; Kroon, 1972; Lagibo et al., 2005). Lagibo et al. (2005) demonstrated the positive influence of increased polyploid level on frost tolerance and winter survival of garden pansy.

Along with the increasing popularity of pansy cultivation worldwide, breeding goals continue to develop. Recently, *V. cornuta* L. (horned pansy) has been crossed successfully with *V. ×wittrockiana* for the creation of new desirable phenotypic traits, such as increased flowering, plant compactness, and tolerance to environmental stresses (Li, 2010; Liu, 2008; Zhang and Gu, 2009). Currently, some hybrid cultivars of these two species—e.g., ‘Nature Series’ and ‘Power Mini’—are bred by the Japanese seed company Takii Seed (Kyoto, Japan) and Sakata Seed (Japan) respectively. To meet the ever-growing expectations of customers (practical approach) and to increase the predictability of the crossing treatments (basic science approach), we adopted four inbred lines of *V. ×wittrockiana* and one inbred line of *V. cornuta* to investigate the cytologic basis of hybridization and heterosis in their hybrids.

Materials and Methods

Derivation of inbred lines of *V. ×wittrockiana* and *V. cornuta* and their morpho-anatomic description. The breeding experiments were performed in the garden field of the Henan Institute of Science and Technology (Xinxiang, China) from 2015 to 2017. Four *V. ×wittrockiana* inbred lines and one *V. cornuta* inbred line (Table 1; Fig. 1A–E) were generated from commercially available seeds by self-crossing for five or six generations, given accession numbers, and used as parental lines. Plants were grown in a closed plastic

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Table 1. The pedigrees, accession numbers, and origins of *Viola cornuta* and *Viola ×wittrockiana* lines used for inter- and intraspecific crosses.

No.	Name	Pedigree	Species	Origin
1	JB-1-1-2	Inbred line derived from Penny Blue	<i>V. cornuta</i>	Goldsmith seed
2	DFM-11-2-1	Inbred line derived from Frühblühende Mischung	<i>V. ×wittrockiana</i>	Gartenland GmbH Aschersleben
3	DFM-11-1-3	Inbred line derived from Frühblühende Mischung	<i>V. ×wittrockiana</i>	Gartenland GmbH Aschersleben
4	DFM-8-4-3	Inbred line derived from Frühblühende Mischung	<i>V. ×wittrockiana</i>	Gartenland GmbH Aschersleben
5	XXL-YB-1-1-1-1	Inbred line derived from extra flower size	<i>V. ×wittrockiana</i>	PanAmerican seed

Fig. 1. (A) Plants of inbred lines of *Viola cornuta* during flowering. (B–E) Plants of inbred lines of *Viola ×wittrockiana* during flowering. (F) Plants of hybrid of *V. cornuta* and *V. ×wittrockiana* during flowering. (A) JB-1-1-2, (B) DFM-11-2-1, (C) DFM-11-1-3, (D) DFM-8-4-3, (E) XXL-YB-1-1-1-1, and (F) XXL-YB-1-1-1-1 × DFM-8-4-3.

chamber from Oct. 2015 to Mar. 2016 and then under insect netting from Apr. to June 2016 to avoid high temperatures and insect pollinators. Five plants of each line were selected for detailed morpho-anatomic analyses. The leaf stomata size (length and width) and density of each plant were measured and observed under a Nikon Eclipse 50i microscope (Nikon Corporation, Shanghai, China). The length and width of a completely developed leaf and the height (flower size) and width of a randomly selected flower were measured. Then, leaf and flower shape values were calculated (length–width and height–width ratios, separately). The leaf area was measured using the transparent square grid method (Qiao et al., 2004).

Artificial crossing of inbred lines of *V. cornuta* and *V. ×wittrockiana* to obtain *F*₁ progeny. Our artificial crossing design among five inbred lines adopted Griffing's method III of a complete diallelic cross (Griffing, 1956). Flower buds to be opened the next day were chosen and isolated with sulfuric acid paper bags. The buds used as maternal flowers were emasculated by the removal of all stamens. On the first day of flowering, pollen grains collected from male donor plants with freshly opened anthers were transferred by hand pollination to the stigmas of female donor plants. The pollinated flowers were then isolated with sulfuric

acid paper bags and labeled immediately. One week later, the isolation bags were removed from the enlarged ovaries, and plants were housed under insect nets to avoid any repeated pollinations, which are possible in pansies as a result of stigma receptivity longer than 1 week (Miyajima, 2006). One month after crossing, mature capsules were collected, and the capsule settings were counted with respect to undeveloped ovaries. Five to six flowers per plant were emasculated and pollinated. A total of 24 plants were pollinated in *V. cornuta* × *V. ×wittrockiana* and 36 plants in *V. ×wittrockiana* × *V. ×wittrockiana*.

***F*₁ germination and morphologic characteristics (heteroses).** In Oct. 2016, selected, mature, well-developed seeds of the crosses and of parental plants were sowed in individual pots of planting trays filled with a peat-to-vermiculite ratio (v/v) of 2:1 and were maintained in plastic chambers. Seeds were watered regularly. Two weeks after sowing, the number of germinated hybrid seeds was counted. Only those seedlings that were germinated 2 weeks after cultivation were taken into further consideration. The seedlings of inbred lines of *V. ×wittrockiana* and *V. cornuta* and crosses were transplanted into 10-cm (diameter) × 10-cm (high) pots when they exhibited two to three true leaves. Plants were transplanted into the field in Mar. 2017, and were maintained for

further growth and blossoming (Fig. 1F). Ten randomly selected plants of each accession were chosen in Apr. 2017 to investigate plant height, plant spread (width), flower number and size (height), flower shape (height–width ratio), leaf shape (length–width ratio), and area. A fully developed leaf and an open flower were measured per plant. Mid-parent heterosis of these characters was calculated according to the Eq. $100 \times [F_1 - (P_1 + P_2)/2] / (P_1 + P_2)/2$ (Marame et al., 2009).

Chromosome counting in inbred lines and their *F*₁ hybrid progeny. The seeds of inbred lines and their *F*₁ progeny were germinated on filter paper soaked with distilled water at room temperature. Roots 1 to 2 cm in length were sampled, and their root tips were dissected under a stereomicroscope. The seedlings were pretreated for 24 h at 0 °C and fixed in a mixture of glacial acetic acid and absolute ethanol (3:1 ratio, v/v) for 24 h. The fixed root tips were then macerated with 1 mol·L⁻¹ hydrochloric acid at 60 °C for 6 to 8 min. Finally, the material was stained with carbol fuchsin, flattened with a cover slip, and observed under a Nikon Eclipse 80i microscope (Nikon Corporation) with a magnification of 10 (eye lens) × 100 (field lens). The images were generated using CellSens (ver. 1.12) Imaging Software (Olympus Corporation, Tokyo, Japan). At least 30 plates from 10 plants per accession were observed.

Results

Morpho-anatomic and cytologic characteristics of the inbred lines of *V. cornuta* and *V. ×wittrockiana*. Stomata length, stomata width, leaf area, and flower size of the *V. cornuta* inbred line (118.1 μm, 70.0 μm, 2.5 cm², and 2.1 cm, respectively) were reduced significantly compared with those of the *V. ×wittrockiana* inbred lines (range, 140.4–174.1 μm, 85.4–116.2 μm, 4.4–8.4 cm², and 3.5–5.8 cm, respectively) (Table 2). The *V. cornuta* inbred line exhibited increased stomatal density compared with *V. ×wittrockiana* inbred lines, but the leaf and flower shapes did not differ significantly between the *V. cornuta* inbred line and the *V. ×wittrockiana* inbred lines (Table 2). The *V. cornuta* line (JB-1-1-2) was consistently 2n = 26 in all investigated plants (Fig. 2A). More than 80% of plants in each inbred line of *V. ×wittrockiana* were 2n = 48 (Fig. 2B–E). The remaining plants were 2n = 44–46 (Fig. 2F–H). No intraindividual variability in chromosome number in inbred lines of either species was observed.

Capsule setting after inter- and intraspecific artificial pollination and seed germination of *F*₁ hybrids. Some obstacles, both prezygotic and postzygotic, are typically encountered in interspecific crossing, such as pollen–stigma incompatibility and hybrid embryo abortion. Even if these obstacles are overcome, *F*₁ interspecific hybrids can exhibit reduced fertility, which can be measured as reduced seed setting and germination. The percentage of capsule setting after interspecific crossing ranged from 28% to 94% and from 18% to

Table 2. Chromosome numbers, leaf microstructural characteristics, and flower parameters of *Viola ×wittrockiana* and *Viola cornuta* inbred lines.

No.	Name	Chromosome no.	Stomata length (μm) ^z	Stomata width (μm) ^z	Stomata density (mm ²) ^z	Leaf shape (length/width) ^z	Leaf area (cm ²) ^z	Flower ht (cm) ^z	Flower shape (ht/width) ^z
1	JB-1-1-2	2n = 2x = 26	118.1 (116.4, 123.9)	70.0 (64.1 75.7)	24 (23, 25)	2.3 (2.0, 2.9)	2.5 (2.3, 2.6)	2.1 (2.0, 2.5)	0.9 (0.8, 0.9)
2	DFM-11-2-1	2n = 8x = 48	147.6 (140.0, 152.1)	102.1 (98.8, 102.6)	19 (18, 20)	1.3 (1.2, 1.3)	4.4 (4.3, 4.8)	3.5 (3.3, 3.6)	0.9 (0.8, 0.9)
3	DFM-11-1-3	2n = 8x = 48	155.0 (154.0, 160.1)	116.2 (104.3, 116.5)	18 (16, 19)	1.4 (1.4, 1.5)	4.9 (4.8, 5.3)	3.9 (3.5, 4.1)	1.0 (0.7, 1.0)
4	DFM-8-4-3	2n = 8x = 48	174.1 (163.3, 181.4)	85.4 (79.2, 98.4)	13 (12, 21)	2.1 (2.0, 2.3)	6.9 (6.5, 7.0)	4 (3.8, 4.1)	0.9 (0.9, 1.0)
5	XXL-YB-1-1-1-1	2n = 8x = 48	140.4 (139.0, 145.8)	91.3 (83.2, 106.4)	15 (12, 20)	2.6 (2.0, 2.8)	8.4 (8.3, 8.8)	5.8 (5.2, 6.0)	1.0 (0.9, 1.0)

^zx (y, z), where x is the median value recorded for the trait, y is the smallest value, and z is the largest value.

Fig. 2. (A–F, H) The euploid metaphase plates of *Viola ×wittrockiana* and *Viola cornuta* inbred lines. (G) The aneuploid metaphase plates of *V. ×wittrockiana* and *V. cornuta* inbred lines. Original magnification was 10 × 100, and the image was generated using CellSens (ver. 1.12) Imaging Software (Olympus Corporation, Tokyo, Japan). (A) JB-1-1-2 (2n = 26), (B) DFM-11-2-1 (2n = 48), (C) DFM-11-1-3 (2n = 48), (D) DFM-8-4-3 (2n = 48), (E) XXL-YB-1-1-1-1 (2n = 48), (F) XXL-YB-1-1-1-1 (2n = 44), (G) DFM-11-1-3 (2n = 45), and (H) DFM-11-2-1 (2n = 46).

90% after intraspecific crossing (Table 3). The percentages of seed germination ranged from 16% to 88% for the F₁ hybrids of *V. ×wittrockiana* × *V. cornuta* and from 9% to 98% for *V. ×wittrockiana* × *V. ×wittrockiana*. The differences in the percentages of capsule setting and seed germination were reflected mainly in crossing direction rather than between interspecies or intraspecies (Table 3).

Variation in chromosome numbers of intra- and interspecific F₁ hybrids. High diversity in chromosome number was noted among inter- and intraspecific artificial hybrids. The chromosome number in F₁ hybrids between *V. cornuta* and *V. ×wittrockiana* was 2n = 34–38, whereas that in the hybrids between inbred lines of *V. ×wittrockiana* was 2n = 39–48 (Fig. 3). Both intra- and interindividual variability in chromosome number were observed. However, the most frequent chromosome numbers were close to the middle value of the chromosome number ranges. The most frequent value was 2n = 36 (49.4% of cells) for interspecific crossing and 2n = 46 (43.3% of cells) for intraspecific crossing. The percentage of cells with extreme values was relatively low (Figs. 3 and 4).

Heterosis of inter- and intraspecific F₁ hybrids. Hybrids typically exhibit heterosis in plant vigor and tolerance to environmental conditions. The inter- and intraspecific F₁ hybrids in this study exhibited various heterosis with respect to different characters. We chose to analyze those characters that were the most discriminative for the two species

(leaf area and flower size) and those characters most desired by breeders (e.g., flower number, plant height). With regard to flower size, all the F₁ hybrids yielded positive heterosis, with interspecific hybrids varying from 11% to 66% (flower size range, 3.3–6.4 cm) and intraspecific hybrids varying from 23% to 75% (flower size range, 4.3–7.6 cm). The hybrids became shorter, yielding negative heterosis in interspecific hybrids (from –12% to –57%) and in intraspecific hybrids (from –15% to –42%) compared with parental lines. The leaf area and plant width of the hybrids displayed significant positive or negative heterosis depending on crossed lines and the direction of crossing. With regard to flower number, interspecific hybrids exhibited positive heterosis when *V. cornuta* served as the male parent but negative heterosis when *V. cornuta* served as the female parent. Most intraspecific hybrids exhibited negative heterosis in flower number (Table 3).

Discussion

Based on cytologic and molecular analyses, most pansies from the section *Melanium* originate from hybridization events leading to explosive, rapid reticulate radiation (Ko et al., 1998; Yockteng et al., 2003). High genetic similarity and the lack of interspecific barriers ensure the generation of fertile hybrids with only slightly reduced pollen viability, even between species at different ploidy levels (Słomka et al., 2018). In addition, our results

support this claim given that the frequency of capsule setting after interspecies crossing and seed germination of F₁ hybrids was generally high. The revealed differences resulted mostly from crossing combination and direction.

The current large-flowered garden pansy (*V. ×wittrockiana*) originated from a cross between *V. tricolor* (n = 13), *V. lutea* (n = 24), and *V. altaica* (n = 11) (Kroon, 1972). The chromosome number of the majority of pansy varieties is 2n = 48 according to Horn (1956), whereas Kroon (1972) reported 2n = 52 in the group Hollandse Reuzen (Hemelsblauw, Goudgeel, Wit). All of the inbred lines of *V. ×wittrockiana* in our study were 2n = 48. In general, garden pansies with 48 chromosomes are commonly recognized as octaploid, with x = 6 as the base chromosome number (Ajalín et al., 2002), whereas *V. cornuta* have x = 13. This value is different from earlier counts demonstrating x = 11 (Marcussen et al., 2010, and literature cited therein), which could be recognized as diploid. However, this chromosome number seems to be secondary. Clausen (1927) argued that the genome of 13 chromosomes is not primary for *V. tricolor*. Early cytotaxonomic articles postulated x = 11 or x = 5 (Clausen, 1931), whereas the most recent phylogenetic studies indicate x = 7 (Yockteng et al., 2003) and x = 12 (Marcussen et al., 2015) as the base chromosome number for the *Melanium* section.

A crossing between *V. ×wittrockiana* and *V. cornuta* should, theoretically, yield plants with 2n = 37. In fact, the majority of cells were 2n = 36; however, this number deviated in both directions and ranged from 34 to 38. The variability of hybrid derivatives within such a broad range of chromosome numbers, including those with odd numbers, is not rare in pansies and was described in several species (Krahulcová et al., 1996, and literature cited therein). In our experiment, only F₁ progeny were analyzed, and these progenies typically exhibit increased chromosome variability compared with the following generations of hybrids. However, the relatively high karyologic stability of F₁ is a good prognosis for future breeding treatments of these plants. The variability in chromosome number in interspecific hybrids may result from the karyologic variability of *V. ×wittrockiana* (≈20% of plants with chromosome numbers other than 2n = 48) and/or from incomplete genomic stability of one or two parental species. As an indicator of regular meiosis and genome stability, pollen viability results indicate that the garden pansy exhibits a less stable genome compared with

Table 3. Capsule setting in inbred lines of *Viola cornuta* and *Viola xwittrockiana* after artificial intra- and interspecies crossing and seed germination, phenotype parameters, and heterosis of F₁ intra- and interspecific hybrids.

Direction of crossing	Capsule setting (%) ^z	Seed germination (%) ^z	Leaf area		Flower size (lt)		Flower shape (lt/width)		Plant ht		Plant spread (width)		No. of flowers	
			(cm ²) ^y	Heterosis (%)	(cm) ^y	Heterosis (%)	z	Heterosis (%)	(cm) ^y	Heterosis (%)	(cm) ^z	Heterosis (%)	z	Heterosis (%)
<i>V. cornuta</i> × <i>V. xwittrockiana</i>														
1 × 2	40 (30, 50)	26 (24, 34)	4.0 (3.9, 4.2)	5	4.2 (4.1, 4.3)	52	1.1 (1.1, 1.1)	9	12.0 (10.0, 13.0)	-20	12.5 (12.0, 12.5)	23	8 (7, 9)	-15
1 × 3	60 (50, 70)	51 (41, 55)	5.1 (4.9, 5.3)	33	4.1 (4.1, 4.2)	47	1.1 (1.1, 1.1)	3	11.0 (10.0, 12.0)	-20	8.5 (8.0, 9.5)	-7	4 (3, 7)	-20
1 × 4	28 (20, 30)	42 (40, 53)	4.0 (3.8, 4.3)	-14	3.8 (3.7, 4.1)	32	1.1 (1.1, 1.1)	4	11.0 (8.5, 14.0)	-34	10.0 (9.5, 12.0)	-4	5 (3, 6)	-56
1 × 5	47 (40, 50)	63 (48, 69)	10.0 (9.0, 12.0)	88	6.4 (6.1, 6.5)	66	1.1 (1.1, 1.1)	4	11.0 (10.0, 12.0)	-12	12.5 (12.0, 13.0)	24	3 (3, 4)	-44
<i>V. xwittrockiana</i> × <i>V. cornuta</i>														
2 × 1	50 (40, 60)	70 (54, 74)	5.0 (4.0, 6.0)	43	3.3 (2.1, 4.2)	16	0.9 (0.9, 0.9)	7	12.0 (11.0, 13.5)	-15	12.5 (12.0, 13.0)	24	9 (7, 10)	11
3 × 1	30 (20, 40)	83 (81, 94)	5.0 (4.0, 5.2)	24	3.5 (2.1, 3.9)	13	0.9 (0.7, 0.9)	-6	12.0 (10.0, 13.0)	-57	6.8 (6.0, 9.5)	-20	6 (5, 9)	15
4 × 1	40 (30, 50)	88 (85, 91)	5.4 (5.0, 7.0)	22	3.6 (2.1, 4.0)	11	0.9 (0.9, 0.9)	-1	13.0 (12.0, 14.0)	-23	14.0 (13.5, 14.3)	27	12 (9, 13)	6
5 × 1	94 (90, 100)	16 (10, 19)	—	—	—	—	—	—	—	—	—	—	—	—
<i>V. xwittrockiana</i> × <i>V. xwittrockiana</i>														
2 × 3	40 (30, 50)	46 (44, 55)	4.1 (4.0, 4.2)	-16	4.5 (4.3, 4.6)	29	1.1 (1.1, 1.2)	5	12.0 (10.0, 13.0)	-33	10.3 (9.5, 10.5)	1	7 (4, 8)	51
2 × 4	21 (20, 24)	80 (74, 86)	5.0 (4.0, 6.0)	-12	4.3 (4.2, 4.6)	23	1.1 (1.1, 1.1)	0	14.0 (13, 15)	-31	11.0 (10.5, 12.0)	-4	5 (3, 5)	-52
2 × 5	70 (60, 80)	79 (69, 80)	7.2 (7.0, 8.0)	13	6.2 (5.9, 6.4)	37	1.0 (1.0, 1.1)	8	10.0 (9.0, 12.0)	-34	12.0 (11.0, 12.5)	9	2 (1, 4)	-47
3 × 4	70 (60, 80)	83 (79, 90)	5.0 (4.0, 6.0)	-15	5.3 (5.1, 5.5)	46	1.1 (1.0, 1.1)	3	13.0 (9.0, 14.0)	-39	12.0 (11.5, 12.5)	10	6 (5, 7)	-15
3 × 5	18 (10, 20)	19 (15, 26)	1.4 (1.2, 1.5)	23	6.4 (6.2, 6.7)	42	1.1 (1.0, 1.1)	3	13.0 (11.5, 14.0)	-15	11.2 (11.0, 12.5)	14	2 (1, 3)	-17
4 × 5	60 (50, 70)	94 (92, 96)	8.0 (7.0, 10.0)	9	6.5 (6.4, 6.6)	40	1.1 (1.1, 1.2)	-7	13.0 (11.0, 13.2)	-33	11.8 (11.0, 12.0)	-1	2 (1, 2)	-77
<i>V. xwittrockiana</i> × <i>V. xwittrockiana</i> (reciprocal)														
3 × 2	20 (10, 30)	98 (96, 100)	6.0 (5.0, 7.0)	26	4.4 (4.3, 4.5)	28	0.9 (0.9, 1.0)	7	13.0 (12.0, 13.5)	-24	10.5 (8.5, 11.0)	1	3 (2, 6)	-13
4 × 2	70 (60, 80)	9 (8, 13)	8.0 (6.0, 9.0)	36	4.6 (4.3, 4.8)	29	0.9 (0.9, 1.0)	5	12.3 (12.0, 13.8)	-37	11.5 (10.0, 12.0)	-4	5 (2, 6)	-49
5 × 2	90 (80, 100)	81 (74, 82)	—	—	—	—	—	—	—	—	—	—	—	—
4 × 3	58 (50, 60)	91 (86, 93)	6.0 (5.0, 6.3)	-4	6.2 (6.0, 6.7)	75	1.0 (0.9, 1.0)	4	11.3 (11.0, 12.0)	-42	13.0 (12.0, 13.2)	16	3 (2, 4)	-62
5 × 3	60 (50, 62)	92 (85, 93)	11.0 (8.0, 12.0)	53	6.5 (6.0, 6.8)	43	1.0 (0.9, 1.0)	3	13.0 (12.0, 16.0)	-10	13.5 (12.0, 14.0)	30	2 (1, 2)	-31
5 × 4	37 (30, 40)	72 (63, 74)	16.0 (15.0, 17.0)	110	7.6 (7.2, 8.0)	65	1.0 (0.9, 1.0)	2	16.0 (15.0, 19.0)	-9	15.0 (14.0, 17.0)	31	2 (1, 2)	-77

(v, z), where x is the median value recorded for that trait, v is the smallest value, and z is the largest value.

^zx (y, z), where x is the median value recorded for that trait, y is the smallest value, and z is the largest value.

the horned pansy. The pollen viability of *V. xwittrockiana* is typically reduced compared with *V. cornuta* (Szczytło, 2004; personal observation). Furthermore, in crosses of *V. xwittrockiana* inbred lines, descending aneuploidy (2n = 39–48) was clearly visible.

Polyploids typically exhibit exuberant characteristics, such as thick, broad leaves with large stomata and magnificent flowers. The same finding applies to hybrids (including allopolyploids) (Chen, 2010). Ajalin et al. (2002) found that the stomata size of hexadecaploid plants was increased compared with octaploid plants of *V. xwittrockiana*, but leaf indices of hexadecaploids were reduced compared with octaploid plants. In this study, we observed that the leaf area and stomata and flower size of the *V. xwittrockiana* inbred lines were increased significantly compared with the *V. cornuta* inbred line, but we observed no distinct differences in leaf shape corresponding to the leaf index between the *V. xwittrockiana* inbred and the *V. cornuta* lines. In *V. xwittrockiana*, additive genetic variance in the transmission of parental petal characteristics (superior and lateral petal ratio and area) to the progeny is extremely important and should be taken into account by breeders (Yoshioka et al., 2006).

The hybrids between *V. cornuta* and *V. xwittrockiana* and between *V. xwittrockiana* lines exhibited positive heterosis in flower size. These findings are consistent with the results of Li (2010). However, negative heterosis in plant height was noted, which is in contrast to the results of Li (2010) and likely a result of the different genetic backgrounds of the plant material we used. Thus, the flowers of F₁ hybrids between *V. cornuta* and *V. xwittrockiana* used in this experiment have a tendency to become larger, and the plants tend to become more compact. These features of the hybrid are ideal for using large-flower pansies as bedding plants. Given that the experiment was not replicated across several environments, the phenotypic results we recorded may not be representative. However, the results are consistent with the description of the natural hybrid *V. cornuta* × *V. xwittrockiana* (*V. xwilliamsii* Witt.), which differs from *V. cornuta* based on wider, slightly overlapping petals, with lateral petals directed outward or upward (Marcussen et al., 2010). The positive heterosis with respect to plant height and flower size was also described in other F₁ ornamental hybrids, such as hybrids of inbred lines of *Helianthus annuus*, *Petunia hybrida*, *Dendranthema morifolium* × *D. nankingense* (Bayat et al., 2012; Cheng et al., 2010; Goksoy et al., 2000).

To conclude, intra- (*V. xwittrockiana*) and interspecific (*V. cornuta* and *V. xwittrockiana*) plants exhibited relatively high stability in chromosome numbers of F₁ individuals, which serves as a good prognosis for future breeding treatments of these plants. Positive heterosis in flower size and negative heterosis in plant height was noted in both breeding treatments. However, these findings require

Fig. 3. The distribution of chromosome numbers in cells of interspecific hybrids between *Viola* $\times$ *wittrockiana* and *Viola cornuta* (*V.C* $\times$ *V.W*, *V.W* $\times$ *V.C*) and intraspecific hybrids between *V.* $\times$ *wittrockiana* lines (*V.W* $\times$ *V.W*).

Fig. 4. (A–F) The selected metaphase plates of hybrids between *Viola* $\times$ *wittrockiana* and *Viola cornuta* inbred lines. (G–P) The selected metaphase plates of hybrids between *V.* $\times$ *wittrockiana* inbred lines. Original magnification was 10×100 , and the image was generated using CellSens (ver. 1.12) Imaging Software (Olympus Corporation, Tokyo, Japan). (A) 1×2 ($2n = 36$), (B) 2×1 ($2n = 36$), (C) 1×3 ($2n = 34$), (D) 3×1 ($2n = 36$), (E) 1×4 ($2n = 35$), (F) 1×5 ($2n = 36$), (G) 2×3 ($2n = 44$), (H) 3×2 ($2n = 46$), (I) 2×4 ($2n = 44$), (J) 4×2 ($2n = 46$), (K) 2×5 ($2n = 46$), (L) 3×4 ($2n = 46$), (M) 3×5 ($2n = 46$), (N) 5×3 ($2n = 45$), (O) 4×5 ($2n = 46$), and (P) 5×4 ($2n = 39$).

further study, including block design and season/space replications, to eliminate environmental effects and to open up new possibilities for pansy breeding programs. Although seeds of *V. x williamsii* (described as a hybrid between *V. cornuta* and *V. x wittrockiana*) are commercially available, basic and applied knowledge of this ornamental plant was not known before this study.

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