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Production and evaluation of a synthetic hexaploid in blueberry *

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Summary. One synthetic hexaploid clone (Hex-DT) was produced by placing 3-node stem cuttings of triploid blueberry clone FL 81-19 [*V. corymbosum* (4*x*) × *V. elliottii* (2*x*)] on blueberry micropropagation medium supplemented with 0.6% agar, 24.6 μ*M* 2ip, and 0.02% colchicine. The most effective chromosome-doubling was achieved with colchicine over 6 days, for 6 h per day. Pollen stainability and pollen germination in the hexaploid Hex-DT were 42.2% and 13.0%, respectively, versus 87.9% and 50.9% in Hex-F_1, a hexaploid F_1 hybrid between *V. ashei* (6*x*) × FL 81-19 (3*x*). The number of seedlings produced per flower pollinated with *V. ashei* pollen in Hex-F_1 was similar to the number obtained from *V. ashei* × *V. ashei* crosses, and at least twice the number produced by Hex-DT pollinated with *V. ashei* pollen. Observed mean numbers of univalents, bivalents, trivalents, quadrivalents, and hexavalents per PMC at metaphase I in Hex-DT were 1.29, 20.30, 2.31, 2.63, and 2.11, respectively. No univalents were observed in Hex-F_1, but the mean frequencies of bivalents, trivalents, quadrivalents, and hexavalents were 29.14, 0.87, 1.91, and 0.58, respectively. Irregularities such as chromosome lagging and unequal disjunction were observed at both anaphase I and II in Hex-DT. Anaphases I and II in Hex-F_1 were normal with few irregularities. The high fertility observed in Hex-F_1 suggests a high level of homology among the three species contributing to its makeup.

Key words: *Vaccinium* section Cyanococcus – Chromosome doubling – Interspecific hybridization-Bridging

Introduction

Vaccinium ashei Reade, rabbiteye blueberry ($2n = 6x = 72$), is one of three cultivated species in *Vaccinium* section *Cyanococcus*. The other two are tetraploid ($2n = 4x = 48$), highbush *V. corymbosum* L. and lowbush *V. angustifolium* Ait. Rabbiteye blueberry is grown mostly in the southeastern United States, but rabbiteye acreage is small compared to acreage of the tetraploid species. Factors that have limited the expansion of the rabbiteye blueberry industry include late fruit ripening and low winter hardiness. The species is adapted from southeastern Virginia across the piedmont and coastal plain of the southeastern United States to east Texas and central Arkansas.

The germplasm comprising the released *V. ashei* cultivars is derived from a narrow genetic base consisting mainly of four wild selections (Lyrene 1983). *V. ashei* is an outcrossing species with considerable inbreeding depression, and the narrow genetic base of the improved breeding populations is of great concern to rabbiteye breeders. Crosses have been made between *V. ashei* and *V. corymbosum* to broaden the gene pool and to transfer genes for earlier ripening and larger fruit from highbush to rabbiteye. Most of the progeny from these crosses are pentaploid (Chandler et al. 1985; Jelenkovic and Draper 1973; Moore et al. 1964). The pentaploids produced are partially fertile, but progenies from backcrossing of the pentaploids to both *V. ashei* and *V. corymbosum* species tend to be less vigorous than the pentaploids.

Other sources of germplasm available for broadening the genetic base of the rabbiteye are the wild diploid blueberry species. Direct crosses between *V. ashei* and these diploids result mostly in pentaploids (Goldy and Lyrene 1984) and possibly a small number of tetraploid seedlings (Darrow et al. 1949). Crosses between tetra-

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ploid and diploid species sometimes give rise to tetraploid seedlings as a result of 2*n* gamete formation (Sharpe and Sherman 1971). Lyrene and Sherman (1983) were able to produce three triploid clones among tetraploid, pentaploid, and aneuploid progeny when they crossed tetraploid *V. corymbosum* to diploid *V. elliottii* Chapm. These triploids produced a few seedlings when crossed to *V. ashei* cultivars. Fewer than 0.01 hexaploid seedling per pollination was produced from these crosses (Dweikat and Lyrene 1988).

Chromosome doubling has been used to overcome crossing barriers between species (Dewey 1980). However, chromosome doubling of woody perennial species using colchicine is often difficult (Darrow 1949; Dermen and Bain 1941). Colchicine treatment in vitro has been successful in producing doubled blueberry plants. Perry and Lyrene (1984) obtained autotetraploid shoots by treating 3-node stem segments with 0.01% colchicine for 2 weeks in vitro.

Chromosome associations at meiosis in diploid and hexaploid *Vaccinium* species are predominantly bivalents (Longley 1927; Rousi 1967). Some multivalents and secondary associations at diakinesis and metaphase I (MI) have been reported in tetraploid *V. corymbosum* (Jelenkovic and Hough 1970). Rousi (1967) studied meiosis in the intersectional hybrid between tetraploid *V. corymbosum* and tetraploid *V. uliginosum*. Pairing was surprisingly regular, 24 bivalents being the commonest situation at metaphase I. The most likely explanation is that the two sets of chromosomes introduced into the hybrid from the *V. corymbosum* parent were pairing autosyndetically, as were the two sets from the *V. uliginosum* parent. Chromosome pairing in this hybrid strongly supports the hypothesis that *V. corymbosum* is autotetraploid, with all four chromosome sets sufficiently homologous for good chromosome pairing. Vorsa (1987) suggested that a minimum of ⅔ of the *V. ashei* chromosomes can pair and recombine with *V. corymbosum* chromosomes in *V. corymbosum/ashei* first backcross derivatives, suggesting that interspecific genome homology is extensive within section Cyanococcus.

The objectives of this study were to evaluate the feasibility of gene transfer from *V. elliottii* and *V. corymbosum* to *V. ashei* by doubling the chromosome number of a derived triploid (Lyrene and Sherman 1983; Dweikat and Lyrene 1988), and to study genomic relationships between the three species by using an F_1 hybrid derived from hexaploid *V. ashei* crossed to the triploid.

Materials and methods

Shoot-tip explants of triploid FL 81-19 (*V. corymbosum* × *V. elliottii*) (Lyrene and Sherman 1983; Dweikat and Lyrene 1988) were collected from actively growing shoots, immersed in 95% ETOH for 1 min, transferred to a 1.3% sodium hypochlorite solution for 20 min, and rinsed three times in sterilized distilled water. Shoot tips of 2-cm length were transferred to 35-ml vials containing 10 ml of blueberry micropropagation medium (Lyrene 1980) supplemented with 24.6 μ*M* 2ip (6-gamma-gamma-dimethylallyl amino purine). The vials were incubated at 22 ± 2 °C under at 16-h photoperiod (38–43 $\mu mol \cdot s^{-1} \cdot m^{-2}$ at the level of culture vials) using cool-white fluorescent bulbs. After 8 weeks, 3-node stem cuttings from the newly proliferated shoots were placed horizontally in vials containing the same medium for a duration of 3 days. Five cuttings were placed in each of 70 vials. The vials were divided into 7 groups, each containing 50 explants. Group 1 was maintained as a control, whereas the others were placed on a medium supplemented with 0.02% colchicine for 6 h per day for either 1, 2, 3, 4, 5, or 6 consecutive days, depending on the treatment. Between colchicine treatments, the explants were returned to colchicine-free medium.

After 8 weeks, the cuttings were examined visually for shoots of unusually thick diameter, characteristic of chromosome doubling (Perry and Lyrene 1984). The thick shoots were cut into 3-node explants and used to establish new colonies. If thick shoots were produced from the daughter colonies, the shoots were rooted in peat moss under intermittent mist. Shoot tips were examined microscopically to determine ploidy.

After these plants reached the age of flowering, flower buds were collected and fixed in 1:1 absolute ethanol:glacial acetic acid for 24 h at room temperature. The buds were then placed in fresh fixative and stored at −20 °C. To study meiotic chromosome behavior, fixed buds were rinsed in tap water and placed in about 10% pectinase in H_2O for 24 h to soften the tissue. Individual buds were separated and squashed in 1% acetocarmine and observed at 1000 × using a phase contrast microscope. Flower buds were also collected from *V. ashei* clone 1, and from a hexaploid F_1 hybrid, Hex-F_1, obtained by crossing *V. ashei* cultivar 'Powderblue' with triploid 81-19 (Dweikat and Lyrene 1988).

Fertility of the synthetic hexaploid designated Hex-DT and the F_1 hexaploid (Hex-F_1) was evaluated by pollen stainability, pollen germination, and crossability to an unrelated *V. ashei* clone (*V. ashei* clone 1). Crossability to *V. ashei* was defined as percentage of fruit set, number of seeds per berry, and number of seedlings per pollinated flower. Pollen stainability was estimated by staining about 500 pollen grains from each clone using 1% acetocarmine for 1 h. The percentage of pollen germination was measured, using approximately 700 pollen grains from each clone placed on an agar medium supplemented with sugar and other nutrients (Goldy and Lyrene 1983).

Results

Six thickened shoots, two derived from a colchicine treatment of 30 h over 5 days and four from a colchicine treatment of 36 h over 6 days, were produced. All had 72 chromosomes, twice the normal chromosome number of the triploid plant from which the cuttings were derived (Fig. 1 A). These doubled triploid plants, or synthetic hexaploids (Hex-DT), produced black fruit and dark green leaves.

Pollen stainability in the synthetic hexaploid plants averaged about 40% (Fig. 2 B) versus 88% and 91% in the *V. ashei* × triploid (Hex-F_1) and *V. ashei* clone 1, respectively (Table 1 and Fig. 2 A). Pollen germination

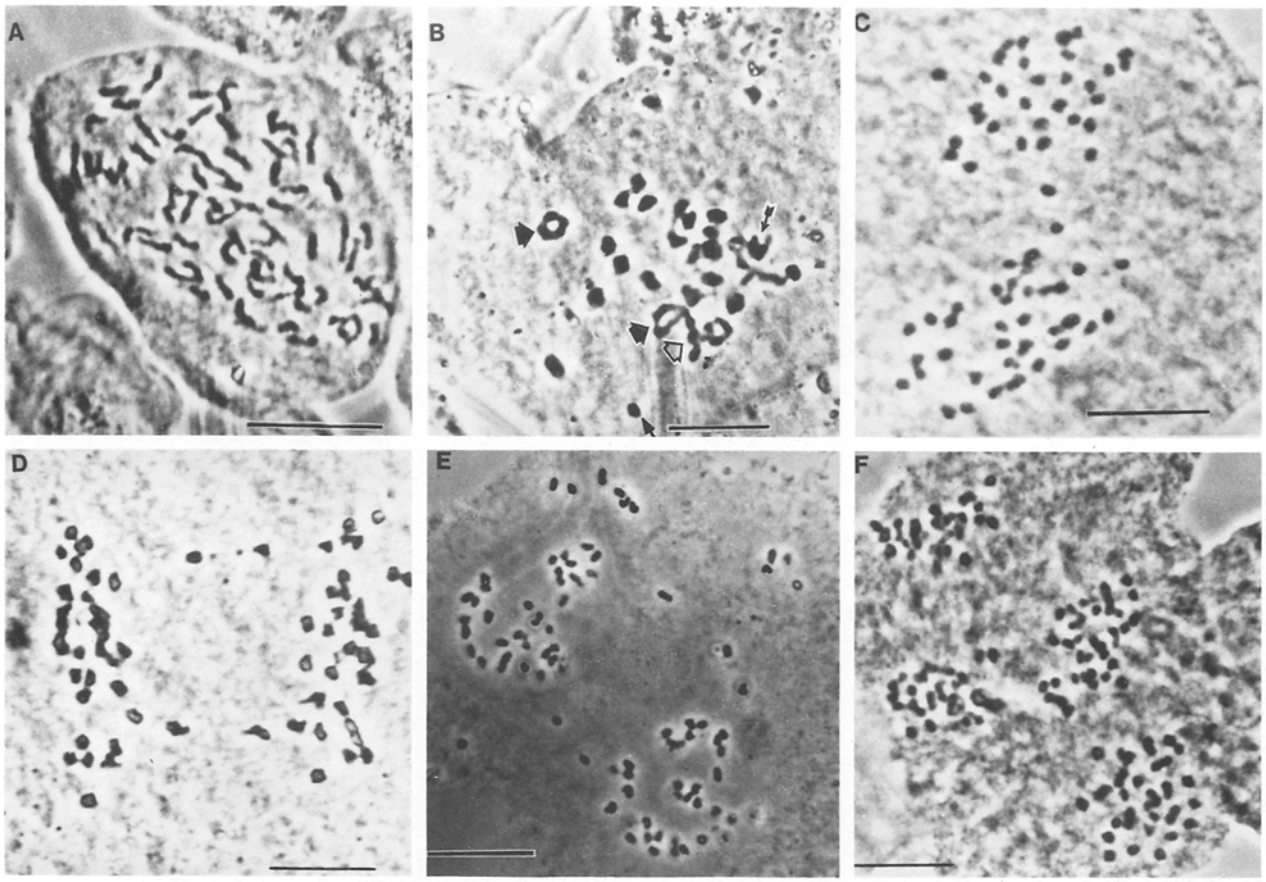

Fig. 1. A–F. Mitotic and meiotic chromosomes in the synthetic hexaploid Hex-DT. **A.** Somatic cell with 72 chromosomes. **B.** Metaphase I with 23 II+1 I (*small arrowhead*)+1 III (*small arrow*)+1 IV (*no arrow*)+3 VI (*big arrow head*). **C.** Anaphase I with unequal chromosome disjunction, 34 (upper side): 38. **D.** Anaphase I with 4 lagging chromosomes. **E.** Anaphase I cell with unorganized chromosome disjunction. **F.** Anaphase II with 34:34:38:38 chromosome distributions. (*Scale bar* represents 10 μm)

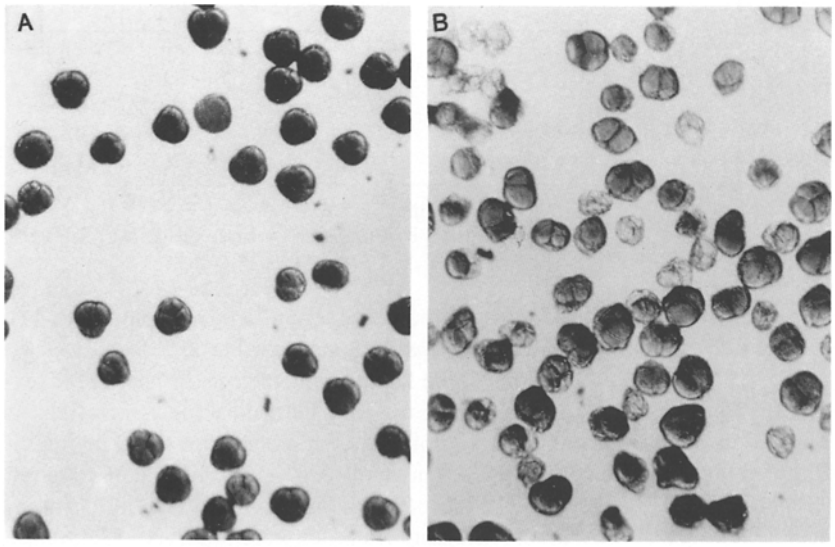

Fig. 2 A and B. Comparison of pollen from *V. ashei* clone 1 and Hex-DT. **A.** Normal staining pollen from clone 1. **B.** Much-aborted and irregular pollen from Hex-DT

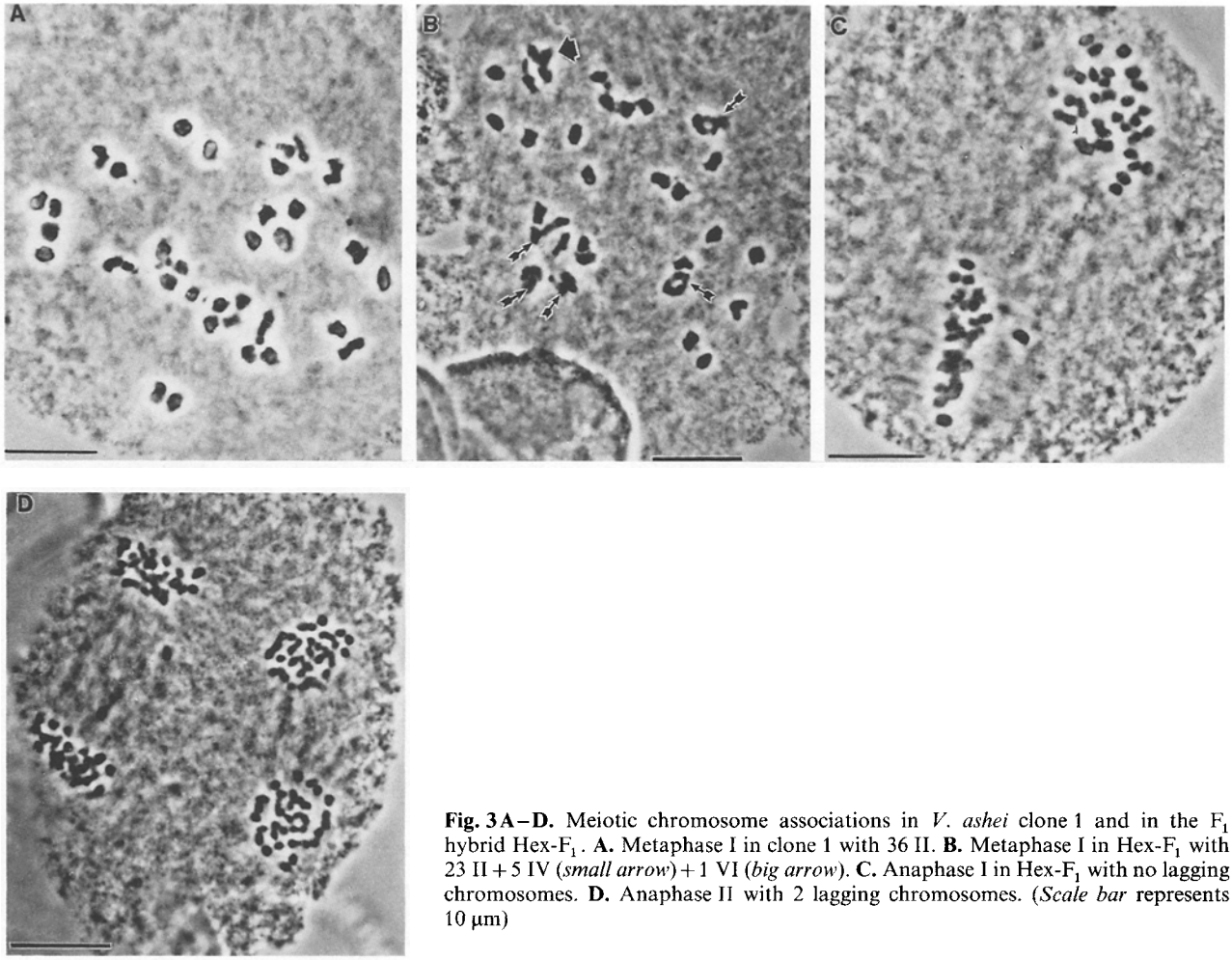

Fig. 3A–D. Meiotic chromosome associations in *V. ashei* clone 1 and in the F_1 hybrid Hex-F_1. **A.** Metaphase I in clone 1 with 36 II. **B.** Metaphase I in Hex-F_1 with 23 II + 5 IV (*small arrow*) + 1 VI (*big arrow*). **C.** Anaphase I in Hex-F_1 with no lagging chromosomes. **D.** Anaphase II with 2 lagging chromosomes. (*Scale bar* represents 10 μm)

Table 1. Pollen stainability and germination of Hex-DT, Hex-F_1, and *V. ashei* clone 1

Clone	Stainability (%)	Germination (%)
Hex-DT[a]	40.2 a[d]	13.0 a
Hex-F_1[b]	87.9 b	50.9 b
V. ashei clone 1[c]	91.0 b	52.0 b

[a] Synthetic hexaploid derived by colchicine doubling of the triploid hybrid obtained by crossing tetraploid *V. corymbosum* × diploid *V. elliottii*
[b] A hexaploid hybrid from the cross of hexaploid *V. ashei* cultivar 'Powderblue' × triploid hybrid FL 81-19
[c] *Vaccinium ashei* line
[d] Mean separation within columns by Duncan's multiple range test, 5% level

Table 2. Crossability of Hex-DT and Hex-F_1 to *V. ashei* (clone 1)

Parental clone	No. flowers pollinated	Fruit set (%)	No. seed/ berry	No. seedling/ flower
Hex-DT × clone 1	220	59.3	14.6	5.9 a[b]
Clone 1 × Hex-DT	237	40.5	9.7	5.0 a
Hex-F_1 × clone 1	400	73.0	18.3	10.9 b
Clone 1 × HexF_1	370	69.8	17.6	10.7 b
Clone 1 × F 87-50[a]	100	74.2	18.7	11.2 b

[a] *Vaccinium ashei* line
[b] Mean separation by Duncan's multiple range test, 5% level

was 13%, 50.9%, and 52.0% in Hex-DT, Hex-F_1, and in *V. ashei* clone 1, respectively (Table 1). The number of seedlings produced by Hex-F_1 when pollinated by *V. ashei* clone 1 was not significantly different from *V. ashei* × *V. ashei* crosses, but was nearly twice the number of seedlings produced by the synthetic hexaploid Hex-DT pollinated by *V. ashei* clone 1 (Table 2).

Chromosome behavior at meiosis in Hex-DT, Hex-F_1, and in clone 1 differed. Chromosome associations at MI in the hexaploid *V. ashei* clone 1 consisted mainly of bivalents with a mean of only 0.42 quadrivalents per cell (29 PMCs) (Table 3 and Fig. 3A). No lagging chromosomes or other abnormalities were observed. Metaphase

Table 3. Chromosome associations at metaphase I in hexaploid clones Hex-DT, Hex-F_1, and *V. ashei* clone 1

Clone	No. of cells examined	Chromosome associations at MI					Bivalents (%)
		Univalents	Bivalents	Trivalents	Quadrivalents	Hexavalents	
Hex-DT	36	0–2[a] (1.29)[b]	14–23 (20.30)	0–4 (2.31)	2–4 (2.63)	1–4 (2.11)	56.4
Hex-F_1	71	0 (0)	23–31 (29.14)	0–2 (0.87)	1–3 (1.91)	0–2 (0.58)	80.9
Clone 1	29	0 (0)	32–36 (35.16)	0 (0)	0–2 (0.42)	0 (0)	97.7

[a] Range among cells
[b] Mean for all cells

I in Hex-DT was irregular, with more than 40% of the chromosomes in 27 PMCs involved in non-bivalent formations, including hexavalents, quadrivalents, trivalents, and univalents (Table 3 and Fig. 1 B). Anaphase I was studied in 31 PMCs and 75% showed from 1 to 5 lagging chromosomes (Figs. 1 D and 1 E). Other abnormalities were also observed in anaphase I. Abnormalities such as unequal disjunction and numerically unbalanced distribution of chromosomes were observed in 80% of the PMCs examined (Fig. 1 C). These abnormalities resulted in unequal chromosome distribution in nearly 80% of the PMCs observed at anaphase II (Fig. 1 F).

The hexaploid F_1 hybrid between *V. ashei* and the triploid displayed fewer abnormalities than the doubled triploid (Fig. 3 A). The hybrid (Hex-F_1) had an average of 29.14 bivalents per cell in 71 PMCs observed (Fig. 3 B). Multivalents accounted for less than 20% of the total number of chromosomes observed in 71 PMCs. Hexavalents, quadrivalents, and trivalents were observed at means of 0.58, 1.91, and 0.87 per cell, respectively (Table 3). Anaphase I cells displayed normal chromosome disjunction with only 2 of 29 PMCs showing lagging chromosomes (Fig. 3 C). Anaphase II cells observed were mostly regular (Fig. 3 D).

Discussion

In the present study, treatment of shoot segments with 0.02% colchicine for 6 days at 6 h per day was most effective for producing plants with a doubled chromosome number. Perry and Lyrene (1984) found 0.01% colchicine in a solid medium for 2 weeks of continuous exposure to be most effective. In this experiment, a 6 h duration was chosen in order to give the dividing cells a chance to recover from the toxic effect of colchicine.

The derived synthetic hexaploid plants showed a marked increase in pollen stainability over the original triploid, which had less than 2% stainability (Dweikat and Lyrene 1988). Lack of uniformity of pollen in the doubled triploid (Fig. 2 B) was probably mainly due to microspore aneuploidy, which resulted from lagging chromosomes and unequal disjunction during anaphase I and II. On the other hand, the F_1 hexaploid hybrid (*V. ashei* × triploid) showed more regular meiosis and produced pollen with high stainability and germination. The number of seedlings produced by Hex-DT pollinated with *V. ashei* was over 300 times higher than the number produced by the original highly sterile triploid.

The F_1 hybrid (Hex-F_1), obtained from *V. ashei* × triploid as a result of unreduced gamete formation by the triploid, is a composite of three species with $^3/_6$ of the genome derived from *V. ashei*, $^2/_6$ from *V. corymbosum*, and $^1/_6$ from *V. elliottii*. High pollen viability and meiotic regularity in Hex-F_1 is an indication of strong genome homology among these three species. Vorsa (1987) suggested a high degree of homology between at least $^2/_3$ of the *V. ashei* genome with that of *V. corymbosum*. High fertility and vigor in F_1 and backcross tetraploids derived from *V. corymbosum* × *V. elliottii* (P.M. Lyrene, unpublished results) also suggest homology between these species.

The results of this study suggest that the transfer of genes between these three species is feasible. The main barrier to gene flow between species of different ploidy levels in *Vaccinium* may be the differences in ploidy rather than low genome homology.

References

Chandler CK, Draper AD, Galletta GJ (1985) Crossability of a diverse group of polyploid blueberry interspecific hybrids. J Am Soc Hortic Sci 110:878–881

Darrow GM (1949) Polyploidy in fruit improvement. Proc Am Soc Hortic Sci 54:523–532

Darrow GM, Morrow GB, Scott DH (1949) Tetraploid blueberry from a cross of diploid and hexaploid species. J Hered 40:304–306

Dermen H, Bain HF (1941) Periclinal and total polyploidy in cranberries induced by colchicine. Proc Am Soc Hortic Sci 38:400

Dewey DR (1980) Some applications and misapplications of induced polyploidy to plant breeding. In: Lewis WH (ed) Polyploidy: Biological relevance. Plenum Press, New York, pp 445–470

Dweikat IM, Lyrene PM (1988) Production and viability of unreduced gametes in triploid interspecific blueberry hybrids. Theor Appl Genet 76:555–559

Goldy RG, Lyrene PM (1983) Pollen germination in interspecific *Vaccinium* hybrids. Hort Science 18:54–55

Goldy RG, Lyrene PM (1984) Meiotic abnormalities of *Vaccinium ashei* × *V. darrowi* hybrids. Can J Genet Cytol 26:146–151

Jelenkovic G, Draper AD (1973) Breeding value of pentaploid interspecific hybrids of *Vaccinium*. J Yugoslov Pomol 7:237–244

Jelenkovic G, Hough LF (1970) Chromosome associations in the first meiotic division in three tetraploid clones of *Vaccinium corymbosum* L. Can J Genet Cytol 12:316–324

Longley AE (1927) Chromosomes in *Vaccinium*. Science 66:566–567

Lyrene PM (1980) Micropropagation of rabbiteye blueberries. Hort Science 15:80–81

Lyrene PM (1983) Inbreeding depression in rabbiteye blueberries. Hort Science 18:226–227

Lyrene PM, Sherman WB (1983) Mitotic instability and 2*n* gamete production in *Vaccinium corymbosum* × *V. elliottii* hybrids. J Am Soc Hortic Sci 108:339–342

Moore JN, Scott DH, Dermen H (1964) Development of a decaploid blueberry by colchicine treatment. J Am Soc Hortic Sci 84:274–279

Perry JL, Lyrene PM (1984) *In vitro* induction of tetraploidy in *Vaccinium darrowi, V. elliottii,* and *V. darrowi* × *V. elliottii* with colchicine treatment. J Am Soc Hortic Sci 109:4–6

Rousi A (1967) Cytological observation on some species and hybrids in *Vaccinium*. Zuechter 36:352–359

Sharpe RH, Sherman WB (1971) Breeding blueberries for low chilling requirement. Hort Science 6:145–147

Vorsa N (1987) Meiotic chromosome pairing and irregularities in blueberry interspecific backcross-1 hybrids. J Hered 78:395–399