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Triploid Hybrids of 2*x* Lingonberry (*Vaccinium vitis-idaea*) by 2*x* Black Highbush Blueberry (*V. fuscatum*) and 2*x* Elliott's Blueberry (*V. elliottii*) as Evidence of a Genome Balance Requirement for Hybridization Success

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Abstract: Hybridizations were made between a 2*x V. vitis-idaea* (sect. *Vitis-idaea*, lingonberry) and 2*x V. fuscatum* (sect. *Cyanococcus*, Black Highbush Blueberry) as part of a project aimed at understanding the crossability and compatibility of these but specifically aimed at assessing the possibilities for improvement and utilization of lingonberry. The crosses succeeded at a low level, and six hybrids were produced and genetically verified. When tested for ploidy level, five of the six hybrids were found to be triploids and one was found to be a tetraploid. Hybrids were intermediate in morphology and only fertile at very low levels, largely due to their triploid nature. Several of these hybrids produced progeny when used as females and pollinated with 6*x V. virgatum*-derived males, indicating viable unreduced 2*n* ovules. Similar crosses were made between lingonberry and 2*x V. elliottii* (sect. *Cyanococcus*, Elliott's Blueberry). These crosses produced two genetically verified hybrids, which were also determined to be triploids. These hybrids were effectively sterile. The production of triploids from 2*x* × 2*x* crosses indicates that there is a natural selection for a reproductive genome balance of two *V. vitis-idaea*:one *Cyanococcus*. The success of secondary hybridizations with hexaploid materials suggests that the triploid hybrids may be used to advance the utilization and recombination of lingonberry germplasm.

Keywords: *V. corymbosum* forma *atrococcum*; intersectional hybrid; genome strength; EBN (Endosperm Balance Number)

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1. Introduction

Lingonberry, *V. vitis-idaea* L., is a circumboreal, dwarf evergreen shrub in the *Vitis-idaea* (Moench) W. Koch section [1]. Lingonberry plants are semi-woody with numerous shoots and have simple, petiolate leaves that are evergreen and coriaceous. Their leaves are obovate and dark green with pale green lower surfaces. Lingonberry plants reproduce by seeds and rhizomes. Their flowers are produced singly or in clusters in terminal racemes with four locules per ovary, four sepals, a bell-shaped corolla, and eight stamens with non-spurred anthers. Their fruits are red and 8–10 mm in diameter [2,3].

Lingonberry has long been harvested from the wild in Scandinavia [4]. In more recent times, it has gone through phases of interest by US growers hoping to enter the market, but in many US areas, it has suffered from a lack of adaptation and a susceptibility to fungal diseases causing dieback and often killing entire plants [5].

Lingonberry has long been considered reproductively isolated from mainline *V*. sect. *Cyanococcus* species and other blue-fruited berries, but more recent research has shown multiple possibilities for crossing lingonberry to other *Vaccinium* species with varying degrees of success and varying degrees of resultant fertility [6–9].

V. fuscatum Ait. (syn. *V. atrococcum* Gray) is a deciduous diploid species of sect. *Cyanococcus* that forms shrubs 1–3 m tall. Plants can be straggly to crown-forming and monopodial to fastigiate stemmed with bark from dark brown to gray, often shredding. Young twigs are olive-green with close, dingy, incurved, or shaggy hairs. Leaves of *V. fuscatum* are membranaceous, narrowly to broadly elliptic, dull deep green on the upper surface and gray-green on the lower surface. Leaves are pubescent, especially on the lower surface, and usually marginally ciliate. Flowers are cylindric–urceolate with corollas 5–8 mm long with greenish or yellowish-white coloring and pink tinges. Plants bloom relatively early in spring (late March–April). Fruits are shiny black, insipid, 5–8 mm in diameter, and ripen in late May to July [1,3].

V. fuscatum is most often considered a diploid form of *V. corymbosum* L. and has similar distribution and adaptation. Forms of *V. fuscatum* are widely distributed and found from Maine (USA) to Southern Ontario (CAN), Indiana to Florida, Tennessee, Arkansas, and east Texas (USA). Typical habitats are flatlands, wooded hillside seeps, swales, swamp hummocks, stream banks, pocosins, and edges of bogs and bays.

Vaccinium elliottii Chapm., also a diploid species of sect. *Cyanococcus*, is a crown-forming species, 2–4 m tall. Its leaves are elliptic to ovate, ranging from 0.7 to 3.5 cm × 0.3 to 1.5 cm, and are green and lustrous on the upper surface to gray-green on the lower surface. Corollas are narrowly cylindric–urceolate, 4–7 mm long, white, and often pink-tinged. Flowering occurs in late March to early April, and then ripening occurs in May to a dull to shining blackberry (5–8 mm) of variable quality with thin skins and small seeds. Accessions of *V. elliottii* have been noted to possess drought tolerance, adaptation to high pH, tolerance to mineral soils, low chilling requirement, early ripening, small fruit scar, and various disease and insect resistances [10]. *V. elliottii* is deciduous and native to the southeastern United States, ranging from southeastern Virginia, south to the northern Florida peninsula, and west to Louisiana and Arkansas [1]. *V. elliottii* is typically found in habitats such as wooded flood plains, wooded streambanks, ravines, and sandy flatlands.

These crosses were part of a project aimed at understanding the crossability and compatibility of the three species involved, but most were specifically aimed at assessing the crossing possibilities of lingonberry (*V. vitis-idaea*). They also aimed to investigate the potential of melding the climatic adaptation between these species, primarily with the goal of broadening the temperate adaptation of *V. vitis-idaea*. The three species utilized *V. vitis-idaea*, *V. fuscatum*, and *V. elliottii*, which are all diploids [11]. As members of sect. *Cyanococcus*, *V. fuscatum* and *V. elliottii* have been successfully used on a limited basis in *V. corymbosum* breeding, and at least two highbush cultivars possess ancestry of either *V. fuscatum* or *V. elliottii* ('Hannah's Choice' (*V. fuscatum*) [12]) and ('Carteret' (*V. elliottii*) [13]).

2. Results

2.1. Primary Hybrids

Typically, we assume good, good–fair, and fair seeds to have the potential to germinate and produce hybrids. Crosses of *V. vitis-idaea* 'Red Sunset' × *V. fuscatum* US 2028 resulted in a modest fruit set (38/86, or 44%) and an average of 1.5 seeds/fruit (58 seed, good, good/fair, and fair seed only). At least some of these seeds were rated as 'good but small'. Similarly, *V. vitis-idaea* 'Sanna' × *V. fuscatum* US 2028 resulted in a modest fruit set (22/46, or 48%) and an average of 1.5 seeds/fruit (34 seeds, g to f). These crosses produced four and two viable hybrids, respectively (Table 1). Crosses using *V. fuscatum* US 2029 as a male had both lower fruit-set and lower seed-set rates and failed to generate any viable hybrids. Thus, despite relatively large numbers of reasonable quality seed (147 g + gf + f), only six hybrids in total were produced from these crosses using *V. vitis-idaea* as female. Limited

crosses of the reciprocals *V. fuscatum* (US 2028, US 2029) × *V. vitis-idaea* ('Red Sunset') yielded a low fruit set (16.7%/2 fruit) and no seed (Table 1).

Table 1. Crosses generating primary *V. vitis-idaea* × *V. fuscatum* hybrids and *V. vitis-idaea* × *V. elliottii* hybrids.

Cross					Seed Quality					Extant
Female		**Male**	**Pollinations**	**Fruit**	**G**	**GF**	**F**	**FP**	**P**	**Hybrids**
V. vitis-idaea 'Red Sunset'	×	*V. fuscatum* US 2028	86	38	45 *	3	10	10	4	4
V. vitis-idaea 'Red Sunset'	×	*V. fuscatum* US 2029	40	13	33	-	-	-	1	-
V. vitis-idaea 'Sanna'	×	*V. fuscatum* US 2028	46	22	21	7	6	1	-	2
V. vitis-idaea 'Sanna'	×	*V. fuscatum* US 2029	57	15	7	14	1	1	-	-
		Totals	**229**	**88**	**106**	**24**	**17**	**12**	**5**	**6**
V. fuscatum US 2028	×	*V. vitis-idaea* 'Red Sunset'	31	2	-	-	-	-	-	-
V. fuscatum US 2029	×	*V. vitis-idaea* 'Red Sunset'	29	-	-	-	-	-	-	-
		Totals	**60**	**2**	**0**	**0**	**0**	**0**	**0**	**0**
V. vitis-idaea 'Red Sunset'	×	*V. elliottii* NJ 88-04-10	**38**	**28**	**112 ** **	**2**	**7**	**7**	**1**	**2**

* Most of these seed were 'good', but considered small. ** Many of these seed were overgrown and had split the seedcoat.

Crosses of *V. vitis-idaea* 'Red Sunset' × *V. elliottii* NJ 88-04-10 resulted in a good fruit set (28/38, or 74%) and an average of four seeds/fruit (121 seed). Again, despite the relatively high seed number, only two hybrids were produced. An interesting observation in the crosses with *V. elliottii* was that many seeds were split with the appearance of the combined embryo–endosperm having outgrown the seedcoat. In other interspecific *Vaccinium* crosses, we have interpreted the overgrowth of the embryo, rupturing the seed coat, as potentially indicative of male-excess genomic dosage in the endosperm. This follows the observed pattern of seed development/failure described by Cooper and Brink [14] and Haig and Westoby [15]. The validity of such an inference in this specific case is uncertain and awaits further testing and evaluation.

2.2. Flow Cytometry/Ploidy Levels

Diploid, tetraploid, and hexaploid species standards were measured for DNA content, and ploidies regressed to allow the calculation of standardized DNA values for triploids and pentaploids. Although all of the hybrids were derived from $2x \times 2x$ crosses, all were confirmed as triploids ($2n = 3x = 36$) with one exception (Table 2). The mean DNA value of these hybrids was 1.60 pg. (s.d. 0.06 pg.). The single exception to these results was US 2524-D, a hybrid of *V. vitis-idaea* 'Red Sunset' × *V. fuscatum* US 2028, which was determined to be $4x$ ($2n = 4x = 48$), with a DNA value of 2.36 pg. Pending molecular corroboration, we inferred the triploids to have arisen from $2n + n$ gametic fusions since we previously encountered low levels of $2n$ ovule production in crosses utilizing 'Red Sunset'. We believe the same process occurred in crosses utilizing 'Sanna'. The tetraploid hybrid, US 2524-D, appeared to result from a $2n + 2n$ gametic fusion.

2.3. SSR Hybrid Identification

The parental screen (Table 3) shows the SSR fragment analysis using six cross-transferable SSR markers for *Vaccinium*. The resolution of the SSR scores is robust (locus variation = 0.5–0.9 bps). The markers are shown to be polymorphic at times within species and at times across species. Four progenies of *V. vitis-idaea* 'Red Sunset' (RS) × *V. fuscatum* US 2028 (US 2524-A to US 2524-D) are likely F_1 hybrids based on the SSR scores presented in Table 3. For five of the six markers, RS × US 2028 progenies are shown to be a triploid. Two progenies are likely triploids (US 2524-A and US 2524-D), with the lingonberry parent contributing twice the genetic material as US 2028, as evidenced by both RS alleles being detected in the progeny when two alleles are available. Two F_1 hybrids of *V. vitis-idaea* 'Red

Sunset' (RS) × *V. elliottii* NJ 88-04-10 were detected (Table 3). RS also contributed two alleles in crosses with NJ 88-04-10, making the RS × NJ 88-04-10 a triploid as well (US 2525-A and US 2525-B). The results of the RS × US 2028 were recapitulated in crosses using *V. vitis-idaea* 'Sanna' (SAN) in place of RS. Although some alleles and inconsistencies were observed, two F_1 hybrids were detected in this cross as well. Taken as a whole, the SSR panel used in this study demonstrates F_1 hybrid likelihood in these diverse *Vaccinium* crosses. Some of the missing alleles and inconsistencies could be due to stuttering and allelic dropout in the hybrid progeny.

Table 2. Flow cytometry DNA measurements of standards, parents, and hybrids.

Group	Genotype	DNA Actual (Regressed) (pg)	Ploidy	Comments
Standards				
	V. darrowii 'Fla 4B'	1.17 (1.13)	2*x*	
		(1.72)	3*x*	by regression
	V. corymbosum 'Duke'	2.22 (2.30)	4*x*	
		(2.89)	5*x*	by regression
	V. virgatum 'Powderblue'	3.51 (3.47)	6*x*	
Parents				
	V. vitis-idaea 'Red Sunset'	1.05	2*x*	
	V. vitis-idaea 'Sanna'	1.10	2*x*	
	V. fuscatum US 2028	1.11	2*x*	
	V. elliottii NJ 88-04-10	1.05	2*x*	
Hybrids				
V. vitis-idaea 'Red Sunset' × *V. fuscatum* US 2028				
	US 2524-A	1.50	3*x*	from 2*n* + *n*
	US 2524-B	1.59	3*x*	from 2*n* + *n*
	US 2524-C	1.62	3*x*	from 2*n* + *n*
	US 2524-D	2.36	4*x*	from 2*n* + 2*n*
V. vitis-idaea 'Sanna' × *V. fuscatum* US 2028				
	US 2526-A	1.70	3*x*	from 2*n* + *n*
	US 2526-B	1.65	3*x*	from 2*n* + *n*
V. vitis-idaea 'Red Sunset' × *V. elliottii* NJ 88-04-10				
	US 2525-A	1.60	3*x*	from 2*n* + *n*
	US 2525-B	1.54	3*x*	from 2*n* + *n*

Table 3. Simple sequence repeat (SSR) marker panel and capillary DNA fragment weights (in bps) tested on *Vaccinium* hybrids of *V. vitis-idaea* × *V. fuscatum*, and *V. vitis-idaea* × *V. elliottii*.

Plant ID	SCF275d	SCF804	SCF9815	SCF37628	SCF132922	172672K70
V. fuscatum US 2028	153, 157	234	179	271	183	349
V. elliottii NJ 88-04-10	141, 143	218, 224	179	263, 265	174	355, 355
V. vitis-idaea 'Red Sunset' (RS)	151, 169	244	187, 189	253	169, 171	331, 333
V. vitis-idaea 'Sanna' (SAN)	171, 173	223, 225	187, 195	253	176	331, 333
RS × US 2028						
US 2524-A	151, 153, 169	234, 244	179, 187, 189	253 *	169, 171, 183	331, 333, 349
US 2524-B	151, 157	234, 244	179, 187, 189	253, 271	169, 171 *	333, 349
US 2524-C	151, 157	234, 244	179, 187	253, 271	171, 183	331, 347 *
US 2524-D (4*x*)	151, 153, 169	234, 244	179, 187, 189	253 *	169, 171 *	333, 347 *

Table 3. *Cont.*

Plant ID	SCF275d	SCF804	SCF9815	SCF37628	SCF132922	172672K70
SAN × US 2028						
US 2526-A	157, 173	223, 247 *	179, 195	253 *	183 *	333, 349
US 2526-B	153, 171	223, 247 *	179, 195	253, 271	F	F
RS × NJ 88-04-10						
US 2525-A	143, 151, 169	224, 244	179, 187, 189	253, 263	169, 171, 185 *	333, 355
US 2525-B	143, 151, 169	224, 244	179, 187, 189	253, 265	169, 171, 174	333, 355

Freeze-dried leaf material (plant ID) was tested against six cross-transferable SSR primers (SCF275d, SCF804, SCF9815, SCF37628, SCF132922, 172672_K70). Primer sequences are described in Rodriguez-Bonilla et al. 2019. The values denote PCR fragment weights (in bps). The parental DNA fragment values, in the first four rows, are necessary to determine parentage in the progeny. 'F' denotes reaction failure. * = the fragment value not matching expectations.

2.4. Hybrid Morphology

For both sets of hybrids, plants generally appeared intermediate to the two parents (Figure 1A,B). The most notable deviation from general plant types was the single 4*x* F_1 hybrid US 2524-D (=*V. vitis-idaea* 'Red Sunset' × *V. fuscatum* US 2028) (Figure 2C).

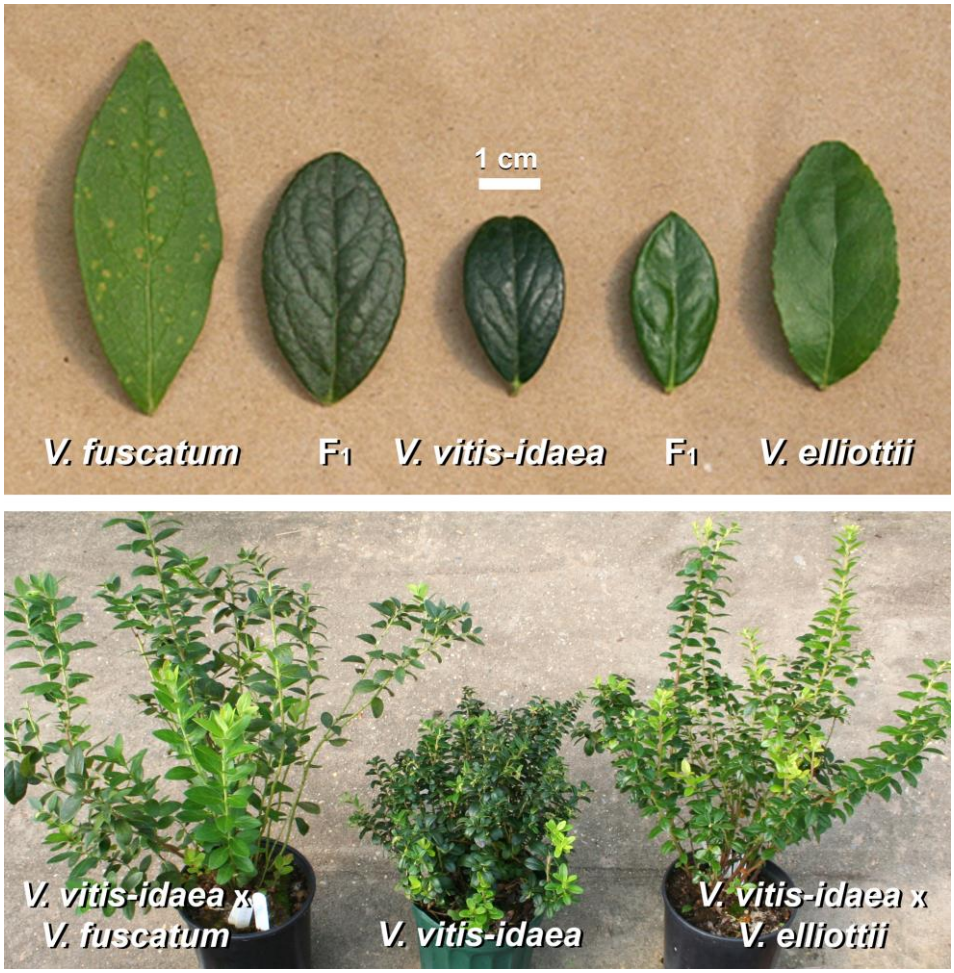


Figure 1. (**A**) (left to right) Leaves of *V. fuscatum*, F_1 US 2524-A (=*V. vitis-idaea* × *V. fuscatum*), *V. vitis-idaea*, F_1 US 2525-A (=*V. vitis-idaea* × *V. elliottii*), and *V. elliottii*. Scale bar = 1 cm. (**B**) Plants of F_1 US 2524-A (=*V. vitis-idaea* × *V. fuscatum*), *V. vitis-idaea* 'Red Sunset', and F_1 US 2525-A (=*V. vitis-idaea* × *V. elliottii*).

(**A**) (**B**) (**C**)

Figure 2. (**A**) Flowers of F_1 US 2524-A (=*V. vitis-idaea* × *V. fuscatum*); (**B**) F_1 US 2525-A (=*V. vitis-idaea* × *V. elliottii*); and (**C**) small statured plants of 4*x* F_1 US 2524-D (=*V. vitis-idaea* × *V. fuscatum*).

2.5. General Morphology of V. vitis-idaea × V. fuscatum Hybrids

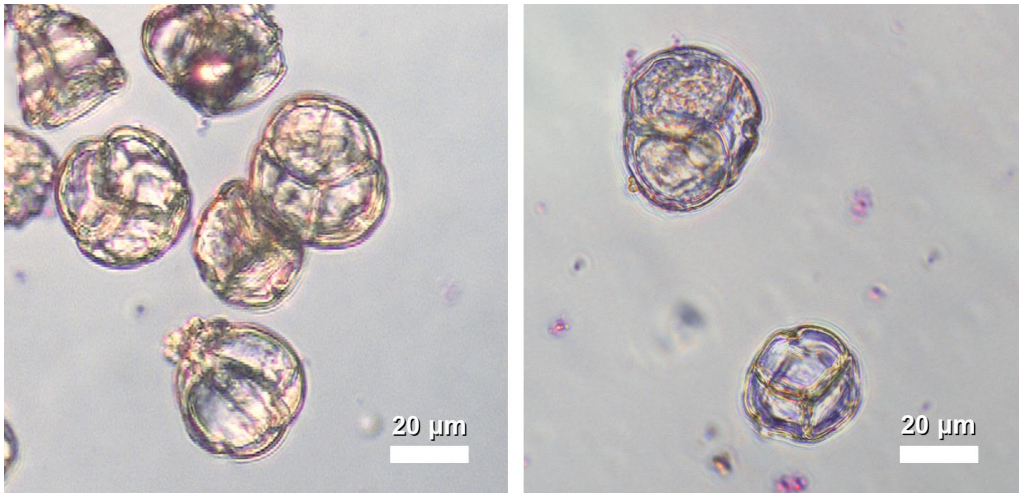
Plants: upright, multi-stemmed, non-rhizomatous, deciduous to non-deciduous, depending on individual genotype, and heights ranging from 13 to 46 cm. **Stems:** short internodes, 1 cm or less; slender and cylindrical to slightly flattened twigs with short white hairs. **Foliage:** textured, dark green, often with hints of purple when mature; new foliage is bright green. Leaves are elliptic to ovate-elliptic, ranging from 12 to 36 × 5 to 22 mm in width, depending on genotype and maturity. Leaf apices are broadly acute or obtuse, often terminating in a knob or apiculus without glandular setae. **Flowers:** (Figure 2A) campanulate, white, 5 mm (L) × 6 mm (diam.), with stigma extending beyond corolla for a total stigma length of approx. 7 mm; 8–11 flowers per inflorescence. **Fruits** of hybrids, where observed, were red and shiny, approximately 5–6 mm in diameter. (as most fruits were seedless or had very few seeds, this value may not be fully representative of fruit size potential). Plants were not evergreen; however, they exhibited various levels of leaf retention when overwintered in a cold but non-freezing greenhouse.

2.6. General Morphology of V. vitis-idaea × V. elliottii Hybrids

Plants: upright, multi-stemmed, non-rhizomatous, mostly deciduous, and 19–28 cm tall. Stems: short internodes, 1 cm or less; subterete twigs with dense short white hairs. Foliage: mildly coriaceous leaves and elliptic, ranging from 12 to 26 × 7 to 17 mm, depending on genotype and maturity. Leaves display a dark glossy green shade and a slight ventral concavity. Basal leaf areas can be obtuse or broadly acute to rounded, while the apical end transitions from acute to a broader acute to rounded shape with an early deciduous apiculus. Remote crenations adorn the leaf margins, each terminated with a glandular-tipped seta. The upper leaf surfaces are mainly glabrous but have scattered glandular hairs beneath. They have a moderate leaf texture that is medium green, new foliage is light green, and the leaves are smaller than *V. vitis-idaea* × *V. fuscatum* hybrids. Flowers: (Figure 2B) narrowly campanulate, white, pinkish cast before opening, and there are up to eight flowers per inflorescence (see Supplemental Table S1 for morphological observations on specific *V. vitis-idaea* × *V. fuscatum* hybrids and *V. vitis-idaea* × *V. elliottii* hybrids).

2.7. Hybrid Fertility

All but one of the hybrids were confirmed as triploids. In triploids, unbalanced meiosis resulted in low fertility and poor pollen shed. Flowers of these hybrids shed considerable anther fragment debris. Overall, pollen viability was poor, but apparently viable at 1*n*, and occasionally 2*n* microspores were observed (Figure 3A,B).



(**A**) (**B**)

Figure 3. (**A**) Pollen of F_1 US 2524-A (=*V. vitis-idaea* × *V. fuscatum*); (**B**) F_1 US 2525-A (=*V. vitis-idaea* × *V. elliottii*) (200×).

Female fertility was expected to generally mirror pollen fertility and to be low to non-existent. A logical test of potentially usable female fertility in triploid hybrids is used to evaluate the presence or lack of 2*n* gametes. This was performed in the case of triploids by pollinating with rabbiteye-derived (*V. virgatum* Ait.) hexaploids (Table 4). In our sole tetraploid F_1, pollinations were made with both 4*x* and 6*x* males. In the evaluations conducted to this point, very low levels of 2*n* gamete production were seen in three of the hybrids from *V. vitis-idaea* × *V. fuscatum* hybrids (two triploids and one tetraploid). These crosses have the potential to yield 6*x* and 5*x* progencies, respectively. The 3*x* *V. vitis-idaea* × *V. elliottii* hybrid crosses detected no 2*n* gametes; however, this hybrid bloomed at a low level and may not have a high enough frequency of 2*n* ovules to be detected by our limited testing. Notably, the 4*x* *V. vitis-idaea* × *V. fuscatum* hybrid produced two seeds in crosses with 4*x* *V. corymbosum* 'Bluecrop', suggesting that these seeds may possibly yield fertile 4*x* advanced generation hybrids.

Table 4. Crosses evaluating 2*n* gamete production of 3*x* and 4*x* *V. vitis-idaea* × *V. fuscatum* hybrids (US 2524-A through–D, US 2526-A) and 3*x* *V. vitis-idaea* × *V. elliottii* hybrids (US 2525-A).

Ploidy	Female		Male	Pollinations	Fruit	Total Seed
3*x*	US 2524-A	×	ARS 07-97	52	1	1g
3*x*	US 2524-A	×	ARS 07-97	13 + GA	11	-
3*x*	US 2524-A	×	Nocturne	26	-	-
3*x*	US 2524-B	×	6*x* various	13	-	-
3*x*	US 2524-C	×	ARS 07-97	75	-	-
3*x*	US 2524-C	×	ARS 07-97	8 + GA	20	1g, 1f
3*x*	US 2524-C	×	Nocturne	51	-	-
4*x*	US 2524-D	×	ARS 07-97	8	-	-
4*x*	US 2524-D	×	ARS 07-97	4 + GA	4	1g, 1p
4*x*	US 2524-D	×	Nocturne	6	1	1g
4*x*	US 2524-D	×	Bluecrop	5	1	2g
3*x*	US 2526-A	×	ARS 07-97	74	-	-
3*x*	US 2526-A	×	ARS 07-97	2 + GA	1	-
3*x*	US 2525-A	×	6*x* various	41	-	-

2.8. Specific Observations of Ploidy and Fertility for Individual Hybrids

2.8.1. *V. vitis-idaea* 'Red Sunset' × *V. fuscatum* US 2028 Hybrids

US 2524-A—Ploidy: 3*x*. Pollen: low shed and reasonable tetrad formation, but most microspores were judged to be aborted/non-viable. A few single microspores (1*n*, 1 of 4 in tetrad) were observed that were rated as possibly viable (19 flowers sampled). Female fertility: indications of viable 2*n* ovule production (Table 4).

US 2524-B—Ploidy: 3*x*. Pollen: poor quality, no perfect tetrads, indications of one, or rarely two, viable microspores per tetrad. Female fertility: very low bloom and no assessment.

US 2524-C—Ploidy: 3*x*. Pollen: low shed and poorly formed tetrads; rated as totally non-viable (16 flowers sampled). Female fertility: indications of viable 2*n* ovule production (Table 4).

US 2524-D—Ploidy: 4*x*. Pollen: bloomed moderately; no pollen shed (10 flowers sampled). Female fertility: indications of viable 2*n* ovule production (Table 4).

2.8.2. *V. vitis-idaea* 'Sanna' × *V. fuscatum* US 2028 Hybrids

US 2526-A—Ploidy: 3*x*. Pollen: low shed and reasonable tetrad formation; a few 2*n* microspores within triads observed (11 flowers sampled). Female fertility: no indication of 2*n* ovule production (Table 3).

US 2526-B—Ploidy: 3*x*. Pollen: no bloom; no pollen collected. Female fertility: no bloom and no assessment.

2.8.3. *V. vitis-idaea* 'Red Sunset' × *V. elliottii* NJ 88-04-10 Hybrids

US 2525-A—Ploidy: 3*x*. Pollen: slightly better shed than the best *V. vitis-idaea* × *V. Fuscatum* hybrid; reasonable tetrad formation; rated to have some potentially viable 1*n* (1/4 of tetrad) microspores; and observed to have a few potentially viable 2*n* microspores (coupled with two aborted microspores, i.e., triads) and a few potentially balanced dyads (eleven flowers sampled). Female fertility: no indication of 2*n* ovule production (Table 3).

US 2525-B—Ploidy: 3*x*. Pollen: no bloom; no pollen collected. Female fertility: a weak plant, no bloom, and no assessment.

3. Discussion

These seven hybrids, with one exception, proved to be triploids and were, for practical purposes, male sterile. Such sterility is the typical outcome of unbalanced meiosis in triploids [16]. The one ploidy exception was a tetraploid hybrid that also proved to be essentially male sterile. Testing these plants as females, however, against both 6*x* and 4*x* sect. *Cyanococcus* males suggested that low but usable levels of 2*n* (unreduced) gametes may occur, and this germplasm might be advanced into secondary hybrids.

We feel that the most important fact regarding these plants is that although only a very few hybrids were produced, they were, with a single exception, triploids. Although both parents were diploids, the successful combinations were triploids. The probable composition of all of these triploids was two *V. vitis-idaea*:one sect. *Cyanococcus* genome. This is substantiated by the SSR marker analysis as well (Table 4). It is also notable that two different 2*x* lingonberry parents both produced the same result. Both 'Red Sunset' and 'Sanna' have been used in our program for their ability to retain and develop fruit, even if only a single viable seed has been produced. Such fruit can be as small as 4 mm. Previous empirical evidence in our program suggested that 'Red Sunset' has a low but quantifiable level of 2*n* gamete production. The current results suggest the 2*n* gametes are also produced in 'Sanna' but at an even more reduced rate. The triploid production in these crosses suggests that for proper embryo/endosperm development, a dosage of two lingonberry genomes is needed to balance a 'typical' sect. *Cyanococcus* genome. Thus, there is a functional equivalence with respect to the pollination and fertilization of two lingonberry genomes for each sect. *Cyanococcus* genome.

The 2:1 reproductive balance is suggestive of a possible Endosperm Balance Number (EBN) system in *Vaccinium* similar to *Solanum* [17,18]. In the current case, the results suggest that *V. vitis-idaea* possesses a lower EBN value than either *V. fuscatum* or *V. elliottii*, as two *V. vitis-idaea* genomes are needed to achieve balance. In tuber-bearing *Solanum*, where the EBN system was first elaborated, EBN values were assigned as integral whole numbers based on cross outcomes utilizing 4*x S. tuberosum* and 2*x S. phureja* or 2*x S. chacoense* genomes. Unlike *Solanum*, we believe that species of *Vaccinium* beyond those in sect. *Cyanococcus* have shown indications of possessing non-integral genomic values (unpublished data). Such investigations are still underway, and we will present this topic in a future publication. Indeed, we think that any genus with a large number of geographically distributed species at multiple ploidy levels has the potential to have non-integral EBN values. One of our authors (M.E.) determined that three genes with additive dosage effects controlled EBN in an *S. commersonii* (1EBN)/*S. chacoense* (2EBN) system [19]. Thus, if the same or similar parameters apply to EBN function in *Vaccinium*, it is not improbable that stepwise EBN dosages might be both feasible and observable.

The one exception among our hybrids was a tetraploid, from what we believe to be an even rarer pollination/fertilization event. Although the precise genome composition of this hybrid is potentially difficult to determine, its morphology and molecular signature are sufficient to indicate that it is a lingonberry × *V. fuscatum* hybrid, and based on its sibling hybrids, it is a viable assumption that this hybrid has a composition of two *V. vitis-idaea*:two *V. fuscatum* genomes. The poor growth quality of this plant makes it tantalizing to suggest that a 2:2 balance is also less somatically compatible than the previously observed 2:1 ratio, but such speculation is unsupportable based on a single rare hybrid. The advantage of this hybrid, if any, is the potential for tetraploid fertility, which has been preliminarily indicated, and the subsequent likelihood that this fertility will allow the germplasm of this plant to be introgressed into commercial tetraploid highbush materials.

The *V. vitis-idaea* genome appears to be relatively compatible. Earlier studies hybridized lingonberry with cranberry (*V. macrocarpon* Ait.) based on its similar morphology (fruit color, fruit texture) as indicators of compatibility [20]. However, it has also been suggested that lingonberry is more affiliated with blueberry than cranberry [10]. In this vein, lingonberry has been implicated as one parent of the naturally occurring hybrid species *V.* × *intermedium*, with *V. myrtillus* L. (European blueberry) being the corresponding parent [21,22]. Our group has recently successfully re-synthesized this *V. myrtillus*–*V. vitis-idaea* combination through controlled crosses (unpublished results). In more recent years, lingonberry has also been shown to hybridize with *V. meridionale* (Andean blueberry) [9], *V. darrowii* [23], *V. uliginosum* [7], and *V. reticulatum* [6].

4. Materials and Methods

4.1. Plant Materials

The plant materials used in hybridizations and test pollinations for these studies are listed in Table 5.

Table 5. *Vaccinium* genotypes used in the experiment.

Genotype	Ploidy	Source/Pedigree
V. vitis-idaea 'Red Sunset'	2*x*	Hartmans Plant Nursery, Lakota, MI/'Koralle' O.P.
V. vitis-idaea 'Sanna'	2*x*	wild selection, Sweden [24]
V. fuscatum US 2028, US 2029	2*x*	wild selections, Burlington County, NJ
V. elliottii NJ 88-04-10	2*x*	wild selection, Rutgers University
US 2524-A to -D	3*x*, 4*x*	'Red Sunset' × US 2028
US 2525-A to -B	3*x*	'Red Sunset' × NJ 88-04-10
US 2526-A to -B	3*x*	'Sanna' × US 2028
V. × *virgatum* 'Nocturne'	6*x*	US 874 × 'Premier'
V. × *virgatum* ARS 07-97	6*x*	T 451 × 'Nocturne'
V. corymbosum 'Bluecrop'	4*x*	USDA cultivar, GM-37 × CU-5 [25]

4.2. Pollinations

For all plant material, pollen was extracted from open flowers by manual manipulation and collected on glassine weighing paper. If pollen was needed for longer-term work, it was stored for up to a month under refrigerated (3 °C), desiccated conditions.

To perform pollinations, a graphite pencil tip was dipped into the collected pollen and then used to apply the pollen to the stigmas of unemasculated flowers in an insect-free greenhouse. Pollinations were made on what were judged to be mature stigmas. For the testing of female fertility of hybrids, pollinations were conducted using both 6*x* and 4*x* cultivars as pollen sources. To enhance the likelihood of retaining low-frequency fertilization events, some further pollinations, as available, were supplemented with ProGibb^{TM} (GA) treatment (130 ppm) at 6–8 days following pollination.

Because all pollinations were performed in an insect-free greenhouse and because it was expected that hybrids would be morphologically recognizable, the female cultivar parents were not emasculated.

4.3. Ploidy Determinations

Because previous crosses with *V. meridionale* had produced anomalous triploids, the ploidy of F_1 hybrids was verified using flow cytometry. Ploidy determinations were performed as described by Ehlenfeldt and Luteyn [26] using the following procedures. Sampled leaf material (1 cm^2/20 to 50 mg) together with leaf material of an internal standard with known DNA content (*Zea mays* L.) was chopped with a sharp razor blade in 500 mL of an extraction buffer (CyStain PI absolute P buffer, catalog number 05-5502; Partec, Münster, Germany) containing RNA-se, 0.1% dithiothreitol (DTT), and 1% polyvinylpyrrolidone (ice cold) in a plastic Petri dish. After 30 to 60 s of incubation, a 2.0 mL staining buffer (CyStain PI absolute P buffer) containing propidium iodide (PI) as a fluorescent dye, RNA-se, 0.1% DTT, and 1% polyvinylpyrrolidone was added. The sample containing cell constituents and large tissue remnants of the sample and the internal standard was then filtered through a 50 µm mesh nylon filter. After an incubation of at least 30 min at room temperature, the filtered solution with stained nuclei was measured with a flow cytometer (CyFlow ML (Partec) with a green diode laser 50 mW 532 nm (for use with PI); software: Flomax Version 2.4 d (Partec)). The DNA amount of the unknown samples was calculated by multiplying the DNA amount of the internal standard with the DNA ratio of the relative DNA amount of the unknown sample and the internal standard. DNA amounts were measured and compared to a set of standards covering a diploid to hexaploid range (2*x* *V. darrowii* 'Fla 4B', 4*x* *V. corymbosum* cv. 'Duke', and 6*x* *V. virgatum* cv. 'Powderblue') to determine basic ploidy levels.

4.4. Plant Material and DNA Isolation

Progenitor plants and hybrid seedlings were grown in a greenhouse at the P.E. Marucci Center for Blueberry & Cranberry Research in Chatsworth, NJ. Plant material was shipped overnight to the USDA Cranberry Genetics and Genomics Laboratory (CGGL) in Madison, WI for genetic testing. At the CGGL, plant tissue was processed using a BenchTop lyophilizer (Virtis, Gardiner, Solon, OH, USA), and DNA was extracted from dry plant matter samples.

A total of 0.03–0.04 g of freeze-dried leaf tissue per sample was pulverized to extract DNA via a modified CTAB method [27] with added beta-mercaptoethanol (2 µL in 750 µL CTAB) and incubated at 65 °C for 1 h. Solubilized DNA from each sample was retrieved from the aqueous layer after adding chloroform:isoamyl alcohol [24:1] and centrifugating at 14,000 rpm for 6 min. DNA was precipitated from the aqueous layers by adding cold isopropanol, placing it in a freezer at −20 °C overnight, and then centrifugating at 14,000 rpm for 22 min to pellet the DNA. The DNA pellet was washed twice in cold 70% ethanol and then resuspended in 50 µL of a 1× TE buffer (10 mM TrisHCl pH 8.0, 1 mM EDTA pH 8.0) with 3 µL of RNase-A. The DNA in 1× TE and RNase-A was incubated at 36 °C for 3 h to remove any RNA. DNA was stored at 4 °C until use.

4.5. DNA Amplification, Fragment Analysis, and Validation of SSR Polymorphisms

Polymerase chain reactions (PCRs) for each plant sample were assembled in duplicate, and each reaction had a total volume of 8 µL. Individual reactions comprised a 5 µL 1× JumpStart REDTaq ReadyMix (Sigma, St. Louis, MO, USA), 1.0 µL of plant DNA in a 1× TE buffer, 0.5 µL of a Betaine PCR reagent (5M, MilliporeSigma, Burlington, MA, USA), 0.5 µL of a 5 µM hexachlorofluorescein (HEX) M13 primer, 0.5 µL of a 5 µM forward simple sequence repeat (SSR) primer appended with the M13 5′-CACGTTGTAAAACGAC-3′ sequence, and 0.5 µL of a 50 µM reverse SSR primer appended with 5′-GTTTCTT-3′. The modifications to the SSR forward [28] and reverse [29] primers serve to facilitate the fluorescent labeling of PCR fragments and promote non-templated adenylation, respectively.

4.6. Male Fertility

Pollen samples were stained with acetocarmine jelly (75% acetic acid with iron acetate) and prepared according to the recipe of Jensen [30]. Pollen samples were assayed for quantity, stainability, and general condition. For evaluating general pollen condition, our ratings were as follows: very good = almost all tetrads, good = tetrads and triads, fair = almost exclusively triads, and poor = mostly aborted grains.

4.7. Female Fertility

The number of flowers pollinated to evaluate female fertility varied depending on flower availability. Pollinations and fruit sets were recorded. Fruit was collected when ripe and measured for fruit size (mm) at the time of seed extraction. Extraction was performed manually under a dissecting microscope, and the seeds were evaluated for number and quality. For our purposes, seeds were classified as good (g), good/fair (g/f), fair (f), fair/poor (f/p), poor (p), or aborted. 'Good' and 'fair' described seeds that subjectively ranged from those considered fully normal to those somewhat reduced in size and/or development, but nonetheless were judged likely to be capable of germination. 'Poor' described seeds that displayed reduced size and/or development, often flattened or brown, and judged less likely to be capable of germination. 'Aborted seeds' were those that were flat, brown, and generally translucent. Notes were made of the size of aborted seeds. For the purpose of this paper, we report only seed totals that combine categories of good and good/fair.

Seeds were planted directly from the fruit and germinated on a greenhouse mist bench in a soil mix composed of a 50:50 peat:sand mixture. At approximately a 3 true-leaf stage, seedlings were transplanted to 36-cell flats. All primary hybrids were transferred to 3L pots in their second season.

PCR was completed on S1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA) machines following a program of a single melting step at 94 °C for 3 min, followed by 33 cycles of 94 °C, 55 °C, and 72 °C for 15 s, 90 s, and 2 min, respectively, and finally, an extension step at 72 °C for 30 min.

To analyze the PCR fragments, 1 µL of the HEX-labeled PCR product was added to a 10 µL mix of formamide and carboxy-X-rhodamine ladder (Custom MapMarker ROX 75–375 bp, BioVentures, Murfreesboro, TN, USA) at a ratio of 1000 µL:25 µL. The PCR–formamide mixes were sent to Functional Biosciences, Inc. in Madison, WI, for fragment analysis. Samples were run on an Applied Biosystems 3730 fluorescent sequencer with a 50 cm microcapillary array. The raw results were sent back to the CGGL, and genotyping calls were determined using GeneMarker software version 1.91 (Soft-Genetics LLC, State College, PA, USA).

The SSR primers were selected from a validated polymorphic SSR marker library developed for *Vaccinium macrocarpon* [31]. A subset of this SSR marker library was shown to be cross-transferable to many other *Vaccinium* species [32]. The SSR marker panel set in this study was assembled by systematically testing the subset of primers on the plant progenitor DNA. A total of six markers were identified with the ability to discern the parentage of progeny (SCF275d, SCF804, SCF9815, SCF37628, SCF132922, 172672_K70) [32].

5. Conclusions

Our goal with all such hybrids as the ones produced is the introgression and evaluation of non-sect. *Cyanococcus* germplasm into mainline *V. corymbosum* materials, but at the same time, we are working to derive a deeper understanding of *Vaccinium* species relationships and develop a rational and systematic methodology for approaching *Vaccinium* germplasm utilization. These crosses represent a previously unrecognized hybridization relationship in *Vaccinium* and promise to facilitate germplasm transfer into usable forms for mainstream *Vaccinium* breeding.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae9121308/s1, Table S1. Morphological observations on specific *V. vitis-idaea* × *V. fuscatum* hybrids and *V. vitis-idaea* × *V. elliottii* hybrids in spring 2023. Notes on deciduous vs. non-deciduous appearance are based on winter storage in a cold greenhouse maintained at above freezing temperatures.

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