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Highly Fertile Intersectional Blueberry Hybrids of *Vaccinium padifolium* Section *Hemimyrtillus* and *V. corymbosum* Section *Cyanococcus*

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Abstract. The primary gene pool of *Vaccinium* species used by blueberry breeders has traditionally been the North American *Vaccinium* species of section *Cyanococcus*. Blueberries in commercial production represent three primary *Vaccinium* species and two ploidy levels. Significant use has been made of the secondary gene pool of *Vaccinium*, especially in the development of southern highbush blueberry (*Vaccinium* ×*corymbosum*) cultivars. Section *Hemimyrtillus* species are distantly related and are best considered part of the tertiary gene pool of *Vaccinium*. *Vaccinium padifolium*, a member of section *Hemimyrtillus* and native to the Madeira Islands, Portugal, has features of notable value to conventional blueberry development, among these: upright structure, strong growth, abundant flowering and fruiting, good self-fertility, inflorescence structure suited to mechanical harvesting, and indeterminate/repeat flowering. Our objective was to incorporate germplasm from this section into cultivated materials and transfer the desirable traits these species possess for commercial production. We used *V. padifolium* as a female in crosses with *V. corymbosum* and generated two highly fertile hybrids. These hybrids are intermediate in morphology, phonological, and their hybridity has been confirmed through DNA testing. These hybrids were used in further crosses to a variety of section *Cyanococcus* selections and have generated numerous second-generation hybrids. We have also determined by flow cytometry the ploidy levels of the hybrids and several previously unevaluated section *Hemimyrtillus* species.

Blueberries (family Ericaceae, species *Vaccinium*, commonly section *Cyanococcus*) are a diverse taxonomic group, and blueberries currently in commercial production represent three major *Vaccinium* species and two ploidy levels: 4*x* *V. angustifolium* (lowbush blueberry), 4*x* *V. corymbosum* (highbush blueberry), and 6*x* *V. ashei* (rabbiteye blueberry). As such, these three types may be considered the primary gene pool of blueberry. Two other commercial types of blueberry are more distinctly true mixtures of species: half-high blueberry cultivars and southern highbush cultivars. Half-highs have been produced by hybridization of 4*x* *V. corymbosum* with *V. angustifolium* and retain a significant but variable percentage contribution from each species. Southern highbush cultivars have been developed by the introgression of the low-chilling-requirement species 2*x* *V. darrowii* into 4*x* *V. corymbosum* at contribution levels averaging ≈25%. Several additional species from the secondary gene pool have also contributed small amounts of germplasm to named blueberry cultivars, among them 2*x* *V. elliottii*, 6*x* *V. constablaei*, and 4*x* *V. tenellum*.

Blueberry species are rather promiscuous, and as a result, most of the breeding of the previously mentioned species has been relatively straightforward. Diploid species often produce unreduced gametes at low frequencies that have allowed their use with cultivated tetraploid partners (Ortiz et al., 1992). Tetraploid and hexaploid species typically can hybridize freely but give rise to pentaploid hybrids. Often, the greatest difficulty with interspecific offspring has been reduced fertility resulting from the production of odd-ploid hybrids (e.g., pentaploids from $6x \times 4x$ crosses). The ability to transfer germplasm through conventional crosses led Hall and Galletta (1971) to hypothesize a highly conserved basic genome in the genus *Vaccinium*. A problem of equal or greater importance in the breeding of exotic species hybrids is the recovery of fruit morphology that is neither too small nor too dark to meet commercial standards.

Section *Hemimyrtillus* and its species. Vander Kloet and Dickinson (1992) considered the species of section *Hemimyrtillus* to be the remnants of a once much more widely distributed taxon. Today, only six species occupy section *Hemimyrtillus*, and three of these are found in very limited localities. The species and their ranges are: *V. cylindraceum* (Açores, Portugal), *V. padifolium* (Madeira Islands, Portugal), *V. arctostaphylos* (Caucasus Region), *V. smallii* (Korea, Japan, Russia), *V. yakushimense* (Yakushima Island, Kyushu, Japan), and *V. hirtum* (Japan, South Korea).

The morphological feature that unifies *Hemimyrtillus* is a set of paired prophylls covering perenniating buds. In section *Hemimyrtillus*, the fused prophylls result in a distinctive, narrow bud, looking like a much-reduced, flattened nail of a bird claw. Vander Kloet and Dickinson (1992) also state that among species of section *Hemimyrtillus*, twigs are typically smooth or pitted (but not verrucose). In a morphometric study, Vander Kloet and Dickinson (1992) concluded that the western *Hemimyrtillus* species (*V. cylindraceum*, *V. padifolium*, and *V. arctostaphylos*) could be shown to be distinct from the Northeast Asian section *Hemimyrtillus*. Similarly, an extensive DNA-based evaluation of *Vaccinium* species by Powell and Kron (2002) using nuclear and chloroplast markers supported this distinction and showed that the Northeast Asian

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Hemimyrtillus (*V. smallii*, *V. yakushimense*, and *V. hirtum*), and the "Tethyan" *Hemimyrtillus* (*V. cylindraceum*, *V. padifolium*, and *V. arctostaphylos*) form well-supported but separate clades, and led them to suggest that the Northeast Asian species should be removed from section *Hemimyrtillus* (Fig. 1). These Northeast Asian species appear more closely related to sections *Praestantia* and *Oxycococcoides* (Powell and Kron, 2002).

The following summation of these species is taken largely from Ehlenfeldt and Ballington (2012) and Vander Kloet and Dickinson (1992) and is supplemented with other sources and our personal observations.

Vaccinium padifolium, our primary species of concern, was first collected in 1777 by Francis Masson, an early plant explorer for the Royal Kew Gardens who transmitted specimens to Linneaus. *V. padifolium* is native only to the Madeira Islands and is typically found in sub-alpine shrubberies, degraded pastures, draws, and edges of coniferous (Coniferae) woods and pine (*Pinus*) barrens at altitudes from 1220 to 1700 m. Plants are bushy to tree-like and typically 1 to 4 m tall. Under native conditions, plants possess an evergreen habit. In general, our specimens have tough, semiglossy leaves with a finely reticulated texture. Plants produce creamy, pink-tinged, bell-shaped flowers that give rise to medium blue, ovate fruit. *V. padifolium* is notable for its profuse flowering, repeat flowering, and its high numbers of flower buds located on both young and old wood. Repeat flowering is a trait which, if introgressed into *V. corymbosum*, may allow multiple crops or continuous cropping. Vander Kloet termed the development of a floral axis outside a perenniating bud as it occurs in

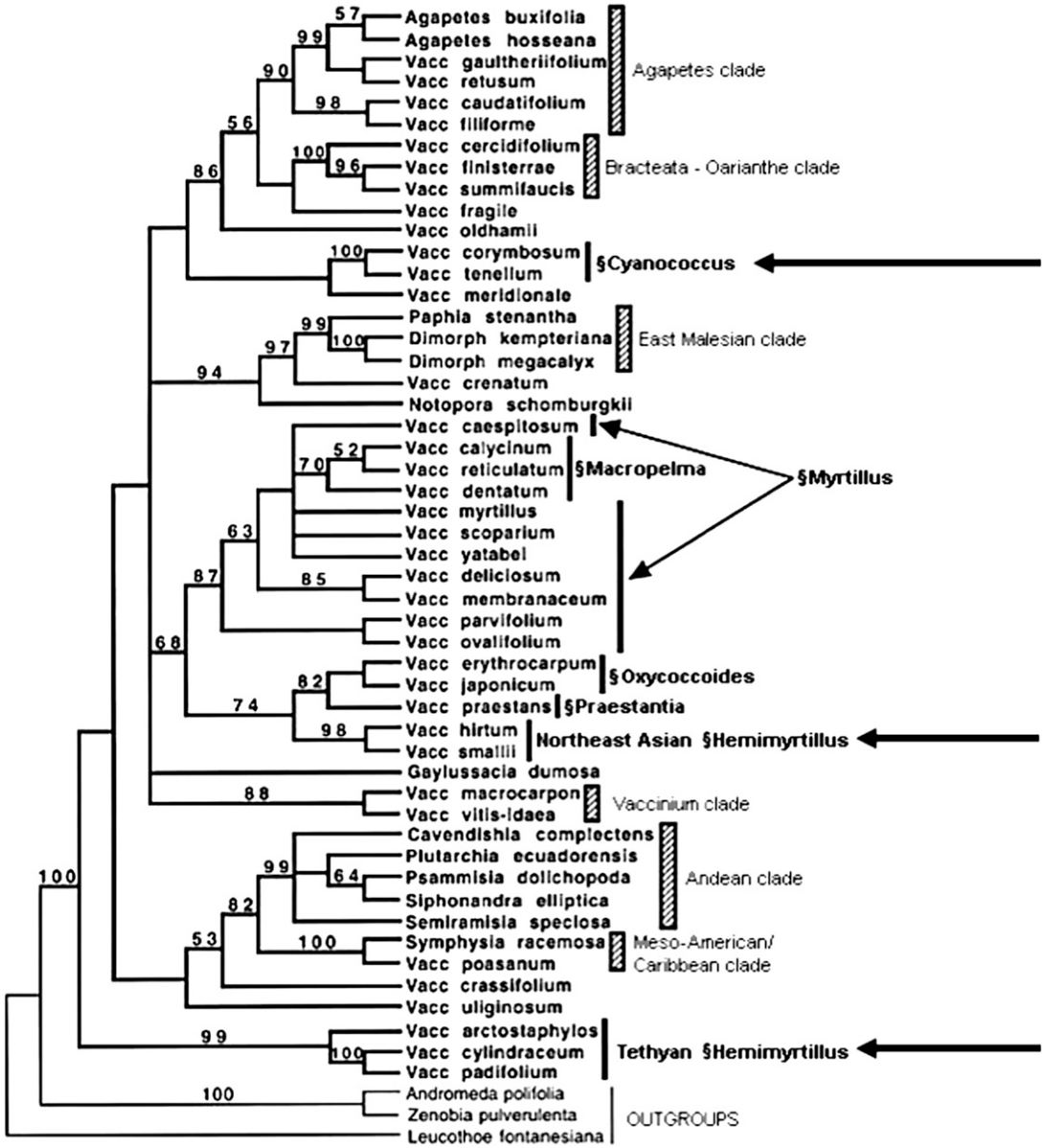

Fig. 1. DNA-based taxonomic tree of *Vaccinium* species of 50 species from tribe *Vaccinieae* and three outgroups from Powell and Kron (2002). Section *Cyanococcus*, Northeast Asian section *Hemimyrtillus*, and Tethyan section *Hemimyrtillus* are highlighted with arrows (reproduced by permission of the American Society of Plant Taxonomists).

V. padifolium indeterminate flowering (Vander Kloet and Dickinson, 2005). Under our conditions, *V. padifolium* is insufficiently cold-hardy to thrive outdoors. The potted plants that we have kept under semiprotected conditions flower most typically in fall. Indeterminate flowering has implications in the season of flower bud development, the differentiation time of flowers within a bud, and the location of indeterminate flower buds (often developing from adventitious buds on older wood). Other useful traits include its general vigor, large mature plant size, excellent fertility, good fruit size, and general self-fruitfulness. Its evergreen nature may be a useful trait under some conditions, but might also present management problems in terms of physiology and/or allowing the plants to be an insect or pathogen reservoir under field conditions.

Vaccinium cylindraceum is found natively on Faial, Pico, São Miguel, and Terceira Islands in the Açores, Portugal, on volcanic slopes of 350 to 1550 m. Plants are drought-tolerant and succeed in neutral soils that are not too heavy (Trehane, 2004). Leaves are variable with some clones possessing relatively large, lance-shaped leaves and others having smaller leaves with a pronounced serrated edge. The leaves are tough, semiglossy, and have a distinct, net-like patterning. Plants flower in profusion and, like *V. padifolium*, both flowers and ripe fruit can be present on the plant simultaneously (Trehane, 2004). Flowers are typically deep pink at anthesis, fading to cream or white as they age. Flowers are cylindrical and elongated, hence the name *V. cylindraceum*. *V. cylindraceum* has a long inflorescence, primarily attributable to its long pedicels. This trait may be of considerable value for mechanical harvesting if it can be incorporated into commercial blueberries. Fruit is medium blue and ovate. A main difference between *V. cylindraceum* and *V. padifolium* is the deciduous habit of *V. cylindraceum* under native conditions. Under our conditions, *V. padifolium* appears more vigorous and more free-flowering than *V. cylindraceum*. Ploidy levels of *V. cylindraceum* and *V. padifolium* are unknown.

The third Tethyan *Hemimyrtillus* species, *V. arctostaphylos*, is a disjunct species with populations in Bulgaria, Turkey, and Anatolia as well as a relict population near the Caspian Sea. It is a mesophytic understory shrub found in open beech (*Fagus*) or oak (*Quercus*) woods as well as in coniferous (Coniferae) stands from 500 to 2400 m. Plants are deciduous, 1 to 6 m tall, and have a relatively smooth bark. The selections of *V. arctostaphylos* that we have observed have finer (less coarse) and overall smoother leaves than the previous two species, but the texture can vary considerably. Flowering is less prolific than the previous two species, but like them, the plants may have a second flush of flowers (Trehane, 2004). Flowers normally have a distinct red coloration at anthesis that fades to shades of pink and cream. Its fruit is black, round, and glossy. *V. arctostaphylos* is a valuable complement to the other two species in that it has greater cold-hardiness, and it appears to have a tolerance to upland soils (Trehane, 2004). Ehlenfeldt and Ballington (2012) have indicated that it freely hybridizes with *V. cylindraceum* and *V. padifolium*. Darrow et al. (1944) determined a single specimen of *V. arctostaphylos* to be 4*x*. Together, the three species represent a considerable range of diverse and desirable germplasm.

Utilization value of the Tethyan *Hemimyrtillus* species. The earliest attempt to use these species in the development of cultivated blueberry was Darrow and Camp (1945) who reported the cross *V. australe* (= *V. corymbosum*) × *V. arctostaphylos*. They did not however subsequently report that any hybrids or advanced selections resulted. Ballington (2000) reported several intersectional crosses of *V. cylindraceum* with both 2*x* and 4*x* germplasm of section *Cyanococcus* and other sections. Ballington generated second-generation hybrids with *V. corymbosum*, several of which were grown under field conditions to observe their performance. These second-generation hybrids used only *V. cylindraceum* germplasm. The observed hybrids exhibited good fertility but were early flowering and often sustained frost damage.

The species of section *Hemimyrtillus* are more distantly related to commercial blueberry than many of the species previously used in breeding and almost certainly would be considered to be positioined in the tertiary gene pool. Powell and Kron (2002) taxonomically place them as being more distantly related to section *Cyanococcus* than is cranberry (*Vaccinium macrocarpon*) or lingonberry (*Vaccinium vitis-idaea*). Nonetheless, our objective in working with these species is to incorporate germplasm from this section into cultivated germplasm and transfer the desirable traits these species possess for mechanical harvesting and commercial production.

Material and Methods

Materials. Specific materials used are listed (Table 1). Unless otherwise noted, other cultivars listed in tables are commonly available commercial cultivars (e.g., 'Duke', 'Hannah's Choice', etc.).

Flow cytometry determination of DNA content of plant nuclei (C-value). Leaf material (≈1 cm^2/20 to 50 mg) together with leaf material of an internal standard (*Vinca minor*) with known DNA content was chopped with a sharp razor blade in 500 µL of 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, 2.0 mL staining buffer (CyStain PI absolute P buffer) containing propidium iodide (PI) as 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 the 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. Flow cytometry determinations were performed by Plant Cytometry Services (Schijndel, The Netherlands).

Crossing. In Fall 2009, several *V. padifolium* plants were observed to be flowering. These were crossed to other species, including those from section *Hemimyrtillus* that were also showing small amounts of flowering at this time. The potted plants of section *Hemimyrtillus* species were brought into a heated insect-free greenhouse and supplied with supplemental artificial illumination.

Most pollinations were made onto section *Hemimyrtillus* species, because other flowering pollen sources were field-planted plants. Crosses were made without emasculation, to

Table 1. Plant materials used in *Vaccinium* section *Hemimyrtillus* investigations.

Section *Hemimyrtillus* species
V. padifolium, US 908, an open-pollinated seedling of CVAC 1153, PI 618126, NCGR[z]
V. cylindraceum, US 1891, São Miguel, Achahinha 3, XII, 99, seed from S. Vander Kloet
V. arctostaphylos, US 1878, US 1879, US 1881, CVAC 1656 (GE-2004-096)/CVAC 1657 (GE-2004-098, Georgia), from seed accessions from NCGR[z]
V. smallii, "clone g," "clone r," seed from S. Vander Kloet, specific geographic origin unknown
Section *Cyanococcus* selections—*V. corymbosum*
US 1814[y] (= 'Sharpblue' × US 1255); clone generated by USDA-ARS program, Chatsworth, NJ US 1255 included 'Duke', 'Hannah's Choice', and *V. boreale* in its ancestry
US 1825[y] (= TH 275 × 'Windsor'); clone generated by USDA-ARS program, Chatsworth, NJ TH 275 included 'Bluecrop' and Fla 4B (*V. darrowii*) in its ancestry
Hybrids
US 1896 (= *V. padifolium* US 908 × *V. corymbosum* US 1825)
US 1897 (= *V. padifolium* US 908 × *V. corymbosum* US 1814)

[z]USDA-ARS, National Clonal Germplasm Repository, Corvallis, OR.
[y]These two selections were clones resulting from one cycle of selection for indeterminate fruiting. Their immediate parents had expressed the trait to some degree as well.

disrupt the flowers as little as possible, with the recognition that selfing might also occur. We believed that selfs and hybrids would be sufficiently different morphologically to be recognizable.

For pollen, cut stems were taken from the field and put in vases in a heated greenhouse and pollen was collected from newly opened flowers over 1 to 2 weeks. Pollen, when not in use, was stored in glassine paper in a refrigerated dessication chamber. Pollen was applied with a sharpened pencil tip. Pollinations were made on what were judged to be mature stigmas.

Subsequent crossing was done primarily in Fall–Winter 2011–12.

Paclobutrazol treatment. Paclobutrazol (Bonzi™; Syngenta, Greensboro, NC) was applied once per week for 3 weeks as a foliar spray (100 mg·mL^{-1} a.i. to runoff) beginning in early September to promote floral bud development (Ehlenfeldt, 1998).

Morphology and DNA fingerprinting. Morphological descriptions were based on the terminology and images of blueberry by Bailey and French (1946). Because sequence data are not yet available for *V. padifolium*, random amplified polymorphic DNA (RAPD) markers were used to fingerprint the parents (US 908, US 1814, US 1825) and the putative F_1 hybrids (US 1896, US 1897). The primers used were prescreened on the parents for the generation of reliable polymorphic bands (*V. padifolium* vs. *V. corymbosum*). Those selected for final analysis were OPA-4, 7, 9, 10, 11, 12, and OPB-1. Primer designations are those of Eurofins MWG Operon (Ebersberg, Germany); however, all primers were synthesized by IDT (Coralville, IA). DNA was isolated using the CTAB procedure (Stewart and Via, 1993) as modified and described in Georgi et al. (2012). Polymerase chain reactions, polyacrylamide gel electrophoresis, and silver staining were as described (Novy et al., 1994). Reliable polymorphic bands were scored as 1 (present) or 0 (absent). Cluster analysis and tree generation using NTSYS-pc (Exeter Software, Setauket, NY) were as described (Polashock and Vorsa, 2002).

Fertility. Pollen was stained with acetocarmine jelly (75% acetic acid with iron acetate) prepared according to the protocols/recipe of Jensen (1962).

Results

Flow cytometry

Almost nothing was known regarding the ploidy of the section *Hemimyrtillus* species. We performed flow cytometric measurements of as much species material of the section as we could locate to address this issue. In our assays, the three Tethyan *Hemimyrtillus* species (*V. padifolium*, *V. cylindraceum*, *V. arctostaphylos*) were all determined to be 4*x* with DNA content ranging from 2.39 pg to 2.67 pg/2C (Table 2). In contrast, a single Northeast Asian *Hemimyrtillus* species tested, *V. smallii*, was determined to be 6*x* with DNA content averaging of 3.86 pg/2C. The two intersectional hybrids of *V. padifolium* × 4*x* *V. corymbosum*, whose generation we subsequently describe (US 1896 and US 1897), were also determined to be tetraploid with DNA contents of 2.51 and 2.54 pg/2C, respectively.

Fertility and crossing of the parents

Female fertility of *V. padifolium*—US 908. Initial crosses were made in a screened, insect-free greenhouse under conditions in which *V. corymbosum* would not normally set spontaneous fruit. Therefore, we did not initially emasculate US 908 or our other *V. padifolium* clone, believing that minimizing handling stress caused by emasculation would optimize cross-pollination success. Most crosses handled in this manner set fruit. Crosses were made with six different *V. corymbosum* selections and cultivars as males, most of which were used because they expressed some degree of fall flowering much like the indeterminate flowering of *V. padifolium*. US 908 flowered more prolifically, set more fruit, and produced more seeds/fruit than the other *V. padifolium* clone (Table 3). When this seed was planted out, many of the seedlings appeared to be selfs based on seedling color and leaf texture. All seedlings were retained, and ultimately only two appeared to be hybrids (Table 3). This is an estimated hybrid success rate of 0.008 seed/pollination. Notably, one of the hybrids, US 1896, resulted from only three pollinations made with the pollen source US 1825, whereas US 1897 resulted from 108 pollinations made with the pollen source US 1814.

After the initial round of crossing, it was recognized that *V. padifolium*, as a species, and particularly our favored parental

Table 2. DNA content and calculated ploidy levels of selected standards, section *Hemimyrtillus* species, and section *Hemimyrtillus* × *Vaccinium corymbosum* hybrids.

Selection	DNA ratio with int. std.[z]	DNA pg/2C[y]	Ploidy
V. darrowii BNJ 95-267-5 (2*x* standard)	0.81	1.22	2*x*
V. corymbosum 'Duke' (4*x* standard)	1.66	2.51	4*x*
V. ashei 'Powderblue' (6*x* standard)	2.60	3.93	6*x*
V. padifolium US 908	1.65	2.49	4*x*
V. cylindraceum US 1891	1.58	2.39	4*x*
V. arctostaphylos US 1881	1.69	2.55	4*x*
V. arctostaphylos US 1878	1.71	2.58	4*x*
V. arctostaphylos US 1879	1.77	2.67	4*x*
US 1896 = *V. padifolium* (US 908) × 4*x* *V. corymbosum* (US 1825)	1.66	2.51	4*x*
US 1897 = *V. padifolium* (US 908) × 4*x* *V. corymbosum* (US 1814)	1.68	2.54	4*x*
V. smallii "clone r"	2.54	3.84	6*x*
V. smallii "clone g"	2.56	3.87	6*x*

[z]*Vinca minor* internal standard with DNA content of 1.51 pg DNA per somatic nucleus.
[y]Picograms of DNA in somatic (2C) tissue.

Table 3. Unemasculated crosses from 2009 producing verified F_1 hybrids with section *Hemimyrtillus* species.

Cross	Pollinations	Fruit	Seeds	Seeds/fruit	F_1	F_1/pollination
	(no.)					
Vaccinium padifolium × *V. corymbosum* (4 selections)	148	22	65[z]	3.0[z]	0	0
V. padifolium US 908 × *V. corymbosum* (5 selections)	250	49	230[z]	4.7[z]	2	0.008

[z]As a result of decayed condition of some fruit, some seed counts included in this total are estimates.

clone, US 908, was very fertile and set fruit spontaneously under greenhouse conditions. The high fruit set of *V. padifolium* appeared to be the result of the occurrence of cleistogamous pollination. Flowers of US 908 were observed to set fruit virtually 100% of the time, in the greenhouse when left undisturbed, and resulted in a few seed per flower. Flowers were observed to shed copious amounts of pollen and could be induced to release pollen before normal bloom by manual opening of the flower tips. Thus, many of our initial cross-pollinations, which in retrospect would not be expected to be successful, resulted in small numbers of self-progeny.

In a second round of crossing, both unemasculated and emasculated crosses were performed as a comparison (Table 4). Flowers were emasculated at an early stage (when flowers were approximately three-fourths of their fully formed size). Among the emasculated flowers (greater than 500), many flowers aborted, and only 14 fruit were produced. These crosses averaged 2.4 seeds/fruit (s/f). Among unemasculated flowers (≈170), there was a higher fruit set but a lower s/f ratio (1.8). During these crosses, ≈15 emasculated flowers were left unpollinated. These unpollinated flowers failed to develop.

If only well-developed seed are counted, the results from the emasculated crosses translate into a success rate of 0.016 seed/pollination (approximately double the value in our first pollinations but within the same order of magnitude). As of this writing, none of this second set of *V. padifolium* × *V. corymbosum* seed has been verified as outcross hybrids. This low set supports the suggestions regarding the expected difficulty of this cross as well as the concerns voiced by Ehlenfeldt and Ballington (2012) of ability of non-identical pollen to grow viably down the long styles of these species.

Male fertility of *V. padifolium*—US 908. *Vaccinium padifolium* (US 908) shed pollen profusely and cleistogamously as previously noted. Pollen appeared highly viable. Pollen grains in US 908 showed mostly normal tetrad structure with a small percentage (≈5%) of grains being triads having one microspore of reduced size, often with a granular interior appearance (Fig. 2). US 1814 and US 1825 also were highly fertile (not shown).

A limited number of pollinations using US 908 as a pollen source onto four different *V. corymbosum* clones had variable results (Table 4). Some crosses of 4*x* highbush × *V. padifolium* failed. Across clones, crosses of 4*x* highbush × US 908 resulted in seed set averaging 1.4 s/f; however, at this point, none of these have been verified as true hybrids. Crosses of US 908 with two non-highbush selections, 'Brunswick' (4*x* lowbush) and NC 86-40-2 (6*x* *V. constablaei*), failed to set fruit.

Parent and hybrid morphology

Notable features of the mature *V. padifolium* clone, US 908, were very uniform, narrow elliptic leaves with narrow tip and basal angles, serrulate margins, and a leaf-to-width ratio approximating 2.3 (Fig. 3). Leaves had a relatively flat surface with fine leaf reticulation. US 908 possessed bell-shaped, cream-colored flowers (Fig. 4).

The *V. corymbosum* parents varied, but both possessed relatively small leaves (possibly as a result of *V. darrowii* and *V. boreale* components in their respective backgrounds) (Fig. 3). US 1825 leaves were oval with a wide to very wide tip and basal angles. US 1814 leaves were ovate with a wide tip and basal angles. For both clones, leaf margins were entire with a slightly reflexed edge typical of much *V. corymbosum*. The average leaf-to-width ratio for both clones was ≈1.7 indicating a relatively broader leaf. Leaf venation differed between the clones but was within the range typical of *V. corymbosum*. The *V. corymbosum* parents in both cases possessed white urn-shaped flowers with relatively small apertures (Fig. 4).

Table 4. Cross-fertility of *Vaccinium padifolium* and F_1 *V. padifolium* × *V. corymbosum* hybrids.

Female × male	Pollinations	Fruit	Seeds[z]	Seeds/fruit[y]
	(no.)			
V. padifolium × 4*x V. corymbosum* (and reciprocal)				
V. padifolium US 908 × 4*x V. corymbosum* (6 selections)	169	12	13/0	1.8
V. padifolium US 908 emasculated × 4*x V. corymbosum* (7 selections)	530	14	9/24	2.4
4*x V. corymbosum* (4 selections) × *V. padifolium* US 908	170	61	87[x]	1.4
V. padifolium × F_1				
V. padifolium US 908 × F_1—US 1896	63	28	131/8	5.0
V. padifolium US 908 emasculated × F_1—US 1897	47	13	82/52	10.3
F_1 × 4*x V. corymbosum*				
F_1—US 1896 × 4*x V. corymbosum* (8 selections)	137	3	22/80	34.0
F_1—US 1896 emasculated × 4*x V. corymbosum* (3 selections)	53	6	76/0	12.7
F_1—US 1897[w] × 4*x V. corymbosum* (4 selections)	89	5	13/24	7.4
F_1 selfs				
F_1—US 1896 × F_1—US 1896	4	3	9/0	3.0
F_1—US 1897[w] × F_1—US 1897	24	0	—	—
F_1 sibmating				
F_1—US 1896 × F_1—US 1897	7	2	0/73	36.5
F_1—US 1897[w] × F_1—US 1896	47	11	167/168	31.4
F_1 × *V. padifolium*				
F_1—US 1896 × *V. padifolium* US 908	6	1	0/8	8.0
F_1—US 1897[w] × *V. padifolium* US 908	11	1	—	—

[z]Seed counts represent counts of seed rated as good and good–fair in quality.
[y]Ratios were calculated using the sum of good and good–fair seed.
[x]Seed of this cross combination were tallied only as viable (i.e., sum of good and good–fair).
[w]Flowering was induced through the use of paclobutrazol.

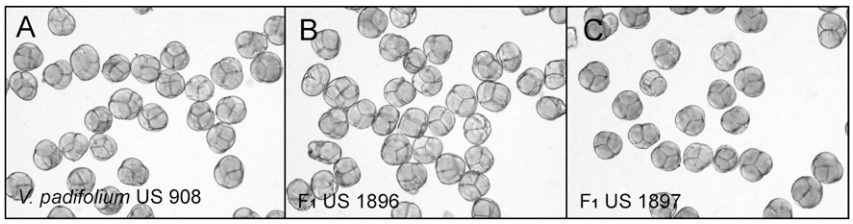

Fig. 2. Pollen of (**A**) US 908 (*Vaccinium padifolium*), (**B**) US 1896 (*V. padifolium* × 4*x V. corymbosum*), and (**C**) US 1897 (*V. padifolium* × 4*x V. corymbosum*). All samples were stained with acetocarmine jelly (75% acetic acid with iron acetate). Approximately 200× magnification.

Hybrids were intermediate in their morphology. Several notable features of selfs of *V. padifolium* vs. hybrid seedlings, even at a very young age (and recognized largely ex post facto), were strongly reflexed midribs, strongly serrulate leaves (especially for their size), textured leaves, and often slightly paler coloration. Despite the recognition of this character complex, the two *V. padifolium* × *V. corymbosum* hybrids went through a considerable phase during which their morphology alone was not considered distinct enough to confirm their hybridity, although they appeared recognizably different from most other seedlings. As seedlings, they had less reflexed leaves and less serrulate edges. As more mature plants, the hybrids possessed leaf serrulation similar to the *V. padifolium* parent (Fig. 3). The leaves of both hybrids were narrow-elliptic like *V. padifolium*. US 1896 had narrow tip and basal angles and a length-to-width ratio of ≈2.6. US 1897 had narrow tip angles, but wide basal angles and a length-to-width ratio of ≈2.3.

US 1896 possessed a leaf surface and netting/reticulation similar to US 908, but less divided in appearance and distinct from the *V. padifolium* type (Fig. 3). US 1896 leaves were relatively coarse/thick. US 1897 had a leaf netting intermediate to the two parents and a relatively smooth surface texture considerably more like *V. corymbosum*, especially that seen in southern highbush (Fig. 3). US 1896 flowered in late summer, and both flower trusses and flower morphology appeared intermediate (Fig. 4). The flowers were very slightly cream-colored. US 1897 was slow to flower, and paclobutrazol was used to induce flower bud development. The resulting flowers were similar to those of the *V. corymbosum* parent but more rounded with a relatively larger opening. Unlike the flowers of US 908, they were bright, pure white. The effects of paclobutrazol precluded any meaningful observations about truss structure. At this writing, the US 1897 hybrid has not gone through a natural flowering and fruiting cycle.

Fruit of US 1896 was deep blue with uneven waxiness and averaged 10.5 mm in diameter with a few fruit reaching diameters as large as 13 mm (Fig. 4). US 1897 fruit were black and somewhat smaller, averaging 9 mm but reaching as large as 14 mm. Size appeared to be strongly correlated with seed set numbers (Ehlenfeldt and Martin, 2010).

DNA fingerprinting

Fifty-six RAPD markers were scored as polymorphic across the *V. padifolium* parent (US 908), the *V. corymbosum* parents (US 1814 and US 1825), and their putative F_1 hybrids (US 1896, US 1897). Approximately two-thirds of the markers scored in the hybrids were those of the *V. padifolium* parent, whereas approximately one-third were derived from the respective *V. corymbosum* parents (data not shown). A phenogram generated using these data shows the progeny grouping

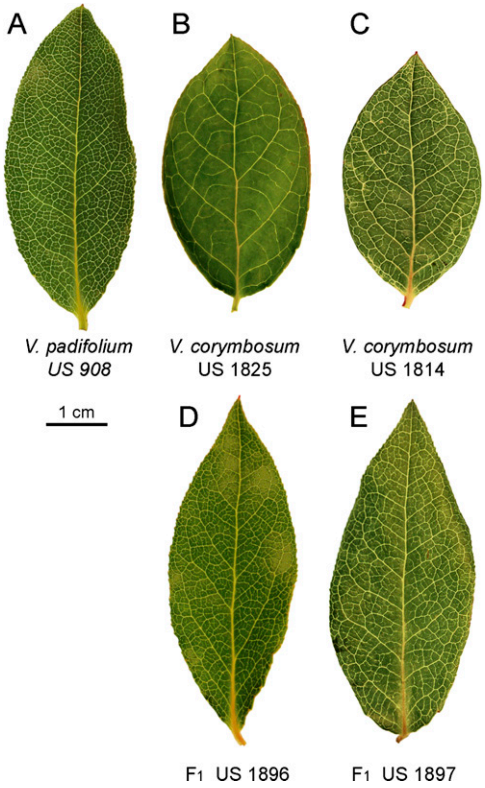

Fig. 3. Leaves of (**A**) US 908 (*Vaccinium padifolium*), (**B**) US 1814 (*V. corymbosum*), (**C**) US 1825 (*V. corymbosum*), (**D**) US 1896 (*V. padifolium* × 4*x V. corymbosum*), and (**E**) US 1897 (*V. padifolium* × 4*x V. corymbosum*). Images have been enhanced to accentuate venation.

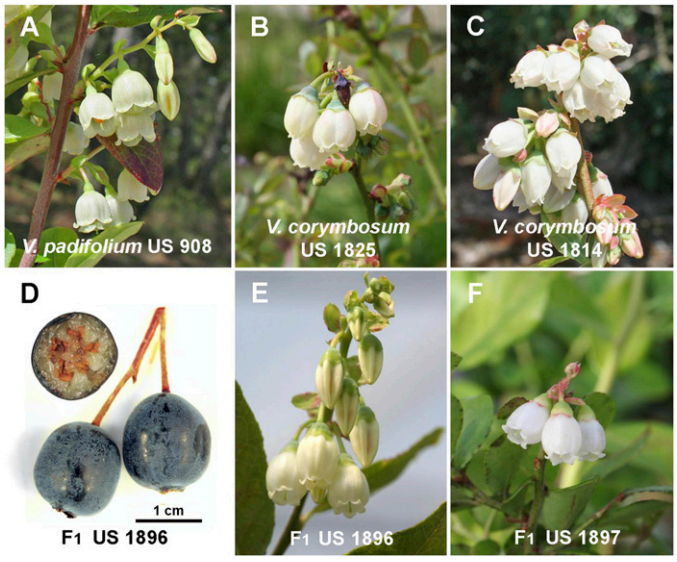

Fig. 4. Flowers (**A–C** and **E–F**) and fruit (**D**) of (**A**) US 908 (*Vaccinium padifolium*), (**B**) US 1814 (*V. corymbosum*), (**C**) US 1825 (*V. corymbosum*), (**D**) fruit of US 1896, (**E**) US 1896 (*V. padifolium* × 4*x V. corymbosum*), and (**F**) US 1897 (*V. padifolium* × 4*x V. corymbosum*).

most closely with the *V. padifolium* parent, whereas the *V. corymbosum* parents group most closely to each other (Fig. 5). RAPD markers tend to be dominant, but can be codominant, and their inheritance patterns are dependent on the state of the

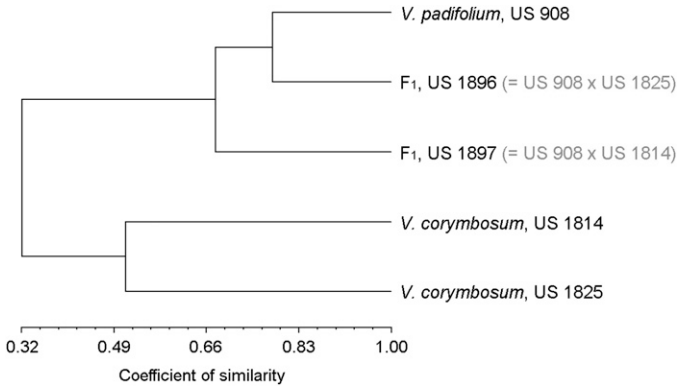

Fig. 5. Dendrogram showing genetic relatedness of *Vaccinium padifolium*, *V. padifolium* × *V. corymbosum* hybrids, and *V. corymbosum* parents as generated using 56 random amplified polymorphic DNA markers.

parents. Given the high self-fertility observed in our crossing experiments, it is likely that the *V. padifolium* parent is more inbred (homozygous) and thus the majority of the selected markers in the progeny are derived from that parent. This may also reflect the fact that the hybrids are in *V. padifolium* cytoplasm. Nonetheless, the presence of *V. corymbosum*-derived bands (scored bands not present in *V. padifolium*) supports the contention that these progeny are true hybrids.

Female fertility of F_1 hybrids

Female cross-fertility was assayed for both US 1896 and US 1897, and much like the crosses with *V. padifolium*, initial pollinations were made onto unemasculated flowers.

US 1896 was successful as a female when pollinated by 4*x* highbush (Table 4). In unemasculated crosses with eight different highbush selections, it produced an average of 34.0 s/f and, in emasculated crosses with three selections, 12.7 s/f. Much like the case with US 908, when these latter seed were germinated, a number of the putative hybrids appeared little different from US 1896 and were presumed to be selfs. Among both of these groups of crosses, a number of hybrids occurred that appeared likely to be true hybrids based on their leaf morphology (i.e., tending toward highbush). Among these, many appear to express variegation, suggesting possible nuclear–cytoplasmic incompatibility in advanced hybrids in *V. padifolium* cytoplasm (Correns, 1909).

The paclobutrazol treatments of US 1897 precluded completely reliable assessments of its female fertility; however, US 1897 also was successful as a female with 4*x V. corymbosum* in unemasculated crosses, yielding low numbers (7.4) of s/f.

Male fertility of F_1 hybrids

As noted, US 1896 initiated a flowering cycle in the greenhouse in late summer/early Fall 2011. Flowering in the greenhouse in late summer is atypical for *V. corymbosum*, suggesting this hybrid possessed some level of indeterminate flowering character of *V. padifolium*. US 1897 flowered in the winter of 2012 as the result of the paclobutrazol treatment. Hybrids in both cases shed pollen profusely and pollen appeared to have high levels of stainability and viability. US 1896 and US 1897 displayed high fertility and, like US 908, had low levels (≈10%) of triad formation (Fig. 2).

US 1896 was used as a male with a variety of blueberry selections and was successful in generating seed (Table 5).

Table 5. Crosses of US 1896 (F_1 *Vaccinium padifolium* × *V. corymbosum*) as male.

	Male: F_1—US 1896				
	Pollinations	Fruit	Seeds	Seeds/fruit	
Female	(no.)				Comments
4*x* *V. corymbosum* (highbush)					
Bluecrop	56	56	762	13.6[z]	
Hannah's Choice	14	12	90	7.5	
4*x* *V. corymbosum* (highbush/southern highbush)					
Cara's Choice	57	45	603	13.4[z]	
Sweetheart	57	46	805	17.5[z]	
4*x* *V. corymbosum* (southern highbush)					
Biloxi	22	19	112	5.9[y]	
Legacy	135	82	1705	20.8[z]	
Sharpblue	41	37	1388	37.5[z]	
TH 622[x]	20	17	374	22.0	
4*x* *V. angustifolium* (lowbush)					
Brunswick	75	3	22	7.3	Small, narrow seed
4*x* *V. angustifolium–V. corymbosum* (half-high)					
Northsky	75	44	392	8.9[z]	
6*x* *V. ashei* (rabbiteye)					
Powderblue	30	22	5	0.2[z]	Small seed, fair quality
6*x* *V. constablaei*					
NC 86-28-3	46	0	—	—	
NC 86-40-2	73	2	9	4.5	
US 831	12	0	—	—	

[z]Calculated on the 20 earliest ripening fruit.
[y]Calculated on the 15 earliest ripening fruit.
[x]TH 622 is a sibling of the southern highbush cultivars Camellia and Gupton.

Using US 1896 as a pollen source in 4*x* × 4*x* crosses resulted in seed set ranging from 5.9 s/f for 'Biloxi' × US 1896 to 37.5 s/f for 'Sharpblue' × US 1896. Interploidy crosses (6*x* × 4*x*) also succeeded at a low rate (ranging from 0.2 s/f with 'Powderblue' to 4.5 s/f with *V. constablaei* NC 86-40-2) (Table 5). US 1897 in pollinations with four 4*x* highbush selections produced an average of 7.4 s/f.

Selfing, sibmating, and backcrosses of F_1 hybrids to *V. padifolium*

Modest attempts were made at selfing, sibmating, and backcrossing the F_1 hybrids (Table 4). Selfing appeared successful at a low level with US 1896, supporting our observation that unemasculated crosses of US 1896 × *V. corymbosum* might be producing self-progeny. Among sibmatings, the cross of US 1897 × US 1896 appeared to produce qualitatively better seed than its reciprocal; however, s/f numbers were approximately equivalent (≈30 s/f) if seed of both categories that we considered likely to be viable ("good" and "good–fair") were considered. These F_2 progeny possessed excellent seedling vigor.

The hybrids could be backcrossed as males onto *V. padifolium* US 908 with relative ease and produced 5.0 and 10.3 s/f for US 1896 and US 1897, respectively. The success of these backcrosses to *V. padifolium* buttress the proof of their hybridity in that these crosses had substantially better fruit set and better s/f ratios than comparable *V. padifolium* × *V. corymbosum* crosses. Limited pollinations from reciprocal crosses (F_1 × *V. padifolium*) prevent drawing any firm conclusions other than that these crosses could be achieved if needed.

Discussion

In this article, we report on crossing and incorporation of the Tethyan *Hemimyrtillus* species into cultivated blueberry (section *Cyanococcus*). These section *Hemimyrtillus* species are distantly related to cultivated blueberry, but possess valuable characters including high fertility and indeterminate flowering.

We elucidated the ploidy level of the Tethyan *Hemimyrtillus* species based on DNA flow cytometry; we showed that *V. padifolium*, *V. cylindraceum*, and *V. arctostaphylos* are all tetraploids. We also determined that at least one of the Northeast Asian section *Hemimyrtillus* species is hexaploid. Our flow cytometry studies verified and expanded the results of Darrow and Camp (1945) and clarifies the crossing results obtained by Ballington (2000). These determinations give further impetus to the suggestion of Powell and Kron (2002) that the Northeast Asian section *Hemimyrtillus* species form a taxonomic clade distinct from that of the Tethyan section *Hemimyrtillus*. Ehlenfeldt and Ballington (2012) reported successful crosses among Tethyan species of section *Hemimyrtillus* in agreement with this knowledge of ploidy levels. These authors similarly reported a putative hybrid of *V. smallii* (determined here to be hexaploid) with a 6*x* rabbiteye-derived hybrid.

We produced *V. padifolium* × *V. corymbosum* species at low frequencies and verified their hybridity by morphology and by DNA fingerprinting. Despite the apparent degree of unrelatedness suggested by molecular markers (Powell and Kron, 2002), the crosses with the Tethyan section *Hemimyrtillus* species succeeded more easily than might be expected. Ballington (2000) had previously noted the production of hybrids with *V. cylindraceum* but gave no indications of ease or difficulty of production. In our previous crossing attempts, we tried a number of crosses on limited flowers available of *V. cylindraceum* and *V. padifolium*, which did not succeed. Ultimately, when

plants grew larger and more flowers were available, hybrids with *V. padifolium* were produced with only modest difficulty and the hybrids produced had excellent fertility. In tetraploid hybrids such as these, a significant degree of fertility resulting from allopolyploidy might be expected (Clausen and Goodspeed, 1925). A critical question for future use might be the subsequent degree of hybrid fertility on crossing to other 4*x V. corymbosum* cultivars.

We evaluated the fertility of the derived hybrids and found them to be male- and female-fertile. All three parents and both hybrids shed abundant, stainable pollen, indicating that meiosis in the parents and hybrids was regular. F_1 hybrids were interfertile on sibmating and readily produced vigorous offspring. We also crossed the hybrids as males to *V. corymbosum* selections and several other section *Cyanococcus* species [*V. angustifolium* (lowbush), *V. angustifolium–V. corymbosum* (half-highs), *V. ashei* (rabbiteye), and *V. constablaei*] with relative ease. These secondary hybrids, although as yet unverified, suggest that further manipulation of this germplasm will not be difficult. A limited number of putative progeny with F_1 hybrids as females (and thus in *V. padifolium* cytoplasm) have exhibited variegation, suggesting possible nuclear–cytoplasmic incompatibilities when advanced hybrids reside in *V. padifolium* cytoplasm.

V. padifolium × F_1 hybrids could be made with relative ease. The quantitative aspects of these backcrosses to *V. padifolium* support their hybridity. These hybrids present another avenue for recombining and exploiting this germplasm, especially if it is desired to use other *V. padifolium* clones that were less successful in primary crosses.

The diversity of germplasm among section *Hemimyrtillus* species for adaptation and useful characteristics as well as the fact that hybrids have been produced among the three species of section *Hemimyrtillus* (Ehlenfeldt and Ballington, 2012) suggest that it should be possible to exercise considerable selection for adaptive and fruiting characteristics before incorporating this material into conventional *V. corymbosum* germplasm. If the other species in section *Hemimyrtillus* are not as easily incorporated with *V. corymbosum* as was *V. padifolium*, our initial hybrids are likely to be an important avenue for incorporation.

Several vital questions exist for this germplasm: 1) Will there be nuclear–cytoplasmic barriers to the development of this germplasm? 2) Will it be easy to recover or maintain desired traits in offspring? 3) What is the expression of desired traits under various regional climatic conditions? At this point, little is known about the expression of section *Hemimyrtillus* genes such as indeterminate flowering in mixed hybrids or the inheritance of these traits. We have many secondary hybrids, however, that should allow determination of these first two questions. Plants of our initial hybrids are being propagated for field testing under regional climatic conditions to answer the final question. Further evaluations of these breeding materials will promote use of *V. padifolium* germplasm in the cultivated blueberry gene pool.

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