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Variation of Anthocyanins, Proanthocyanidins, Flavonols, and Organic Acids in Cultivated and Wild Diploid Blueberry Species

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Abstract. **The flavonoid and organic acid profiles of one cultivated tetraploid and six wild diploid blueberry species (*Vaccinium* spp.) were systematically investigated using high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry (HPLC-ESI-MS-MS). Eighteen individual anthocyanins from five aglycone classes were characterized among species, with malvidin and delphinidin glycosides accounting for 31.4% and 29.1% of total anthocyanins. Twenty-three flavonol glycosides from six aglycone classes were identified, among which quercetin and myricetin glycosides accounted for more than 80% of total flavonols in most species. Both inter- and intraspecies differences in anthocyanin and flavonol composition were observed, as described by principal component analysis. Only B-type proanthocyanidins were found in blueberry species, and highly polymerized molecules with degree of polymerization greater than 10 appeared to be the most abundant fraction. Although overall proanthocyanidin levels varied from 27.7 to 146.3 mg/100 g fruit, all species exhibited similar proanthocyanidin composition. Citric, quinic, and shikimic acid were the major identified blueberry organic acids. However, their relative abundance varied across species. In certain species either citric acid (e.g., *Vaccinium darrowii*) or quinic acid (e.g., *Vaccinium corymbosum*) was lacking.**

Blueberry, as the second most important U.S. berry crop after strawberry, consists of several species that are native to North America (Krewer and NeSmith, 2000). Within the *Vaccinium* genus and *Cyanococcus* section, three blueberry species have been commercialized, including two tetraploid ($2n = 4x = 48$) species: highbush (*Vaccinium corymbosum* L.) and lowbush (*Vaccinium angustifolium* Aiton.) blueberries, and one hexaploid ($2n = 6x = 72$) species: rabbiteye blueberry (*Vaccinium virgatum* Aiton., formerly *ashei* Reade). Highbush blueberry is the major species grown in the United States, accounting for ≈95% of total blueberry production (Du and Rouseff, 2014). Germplasm exists at the diploid ($2n = 2x = 24$) level, which includes the diploid blueberry species: *V. corymbosum* (2*x*), *Vaccinium boreale* Hall & Alders, *Vaccinium darrowii* Camp, *Vaccinium elliottii* Chapm., *Vaccinium pallidum* Ait., *Vaccinium myrtilloides* Michx., and *Vaccinium tenellum* Ait., of which a number are believed to be progenitor species of the North American domesticated polyploid blueberries (Bruederle and Vorsa, 1994).

Besides their characteristic aroma resulting from various volatile constituents (Baloga et al., 1995; Du and Rouseff, 2014; Horvat and Senter, 1985), blueberries also contain different organic acids, including citric, quinic, malic, and succinic acids that contribute to their unique flavor (Ehlenfeldt et al., 1994). Moreover, they are also known for the rich profile of various secondary metabolites with putative health benefits. Many in vitro and in vivo studies have shown blueberry's health-promoting effects to be antioxidants in cardiovascular protection, neurobiology, cancer chemoprevention, and eye health (Kalt et al., 2007). The flavonoid compounds are believed to be the most relevant constituents contributing to blueberry's health benefits. The most abundant classes of blueberry flavonoids are the anthocyanins, flavonols, and proanthocyanidins (PACs). Beside these flavonoids, blueberries also contain high levels of chlorogenic acids, which are nonflavonoid phenolics (Kalt et al., 2007).

Due to the important human health implication of blueberry flavonoid consumption, there has been interest in the characterization and profiling of flavonoids in blueberry cultivars. Studies have identified and/or quantified anthocyanins, flavonol glycosides, or PACs in various cultivars of highbush, lowbush, or rabbiteye blueberries (Barnes et al., 2009; Kalt and McDonald, 1996; Kalt et al., 2001; Lohachoompol et al., 2008; Mi et al., 2004; Prior et al., 2001; Vrhovsek et al., 2012). Other studies were focused on the effect of plant growth conditions or fruit maturity level on the concentration of blueberry flavonoids (Castrejón et al., 2008; Wang et al., 2008; Zheng et al., 2003). By using liquid chromatography and mass spectrometry, many of those studies provided knowledge on the structure and composition of different blueberry flavonoid compounds.

Although a considerable number of studies have been conducted regarding blueberry phytochemicals, most of the analyses focused only on the three commercialized blueberry species, and among these the highbush tetraploid blueberry species *V. corymbosum* has received most of the research interest because of its wide cultivation. Studies focused on the phytochemical analysis of wild blueberry species are limited. Compared with the cultivated tetraploid and hexaploid blueberry species, the diploid wild blueberry species exhibit a number of differences, such as smaller fruit size and different flower phenology.

Because the diploids are considered to be the progenitors of cultivated blueberry species (Camp, 1945), a comprehensive analysis and comparison of their flavonoid and organic acid constituents vs. cultivated blueberry species can provide valuable information on the biosynthesis of such compounds in blueberry and how it can be affected by factors such as genetic background and polyploidy. Furthermore, from a practical standpoint of breeding, the germplasm of diploid species may offer valuable traits of interest, such as insect resistance (Ranger et al., 2006), and different fruit volatile profiles (Baloga et al., 1995). Gene transfer from diploid to tetraploid and hexaploid is facilitated by widespread 2n gamete production in diploid blueberry species (Ortiz et al., 1991, 1992; Qu and Vorsa, 1999).

In the current study, we selected three commercial tetraploid (4*x*) northern highbush blueberry (*V. corymbosum*) varieties 'Bluecrop', 'Duke', and 'Cara's Choice', and clones of six diploid (2*x*) wild blueberry species including two highbush species, *V. corymbosum*, *V. elliottii*, and four lowbush species, *V. boreale*, *V. darrowii*, *V. pallidum*, and *V. tenellum*. By using HPLC and LC-ESI-MS-MS, the objectives of the study were to investigate 1) flavonoid (anthocyanins, flavonols, and proanthocyanidins) and organic acid profiles of different blueberry species, 2) variation of flavonoid and organic acid content among blueberry species, and 3) whether different

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blueberry species can be differentiated based on their phytochemical profile.

Materials and Methods

Blueberry samples. Mature blueberry fruit samples were harvested at the P. E. Marucci Center for Blueberry and Cranberry Research and Extension in 2016 and 2017. Samples were stored at –80 °C until analyzed. The fruit of six diploid wild blueberry species were chosen: *V. corymbosum* (2*x*), *V. darrowii*, *V. boreale*, *V. pallidum*, *V. elliottii*, and *V. tenellum*. Three cultivars of northern highbush blueberry *V. corymbosum* (4*x*) were chosen to represent the cultivated species. Bluecrop and Duke are the most widely planted northern highbush cultivars; and Cara's Choice represents a cultivar selected in the northern climate, but whose pedigree consists of significant amount of southern species ancestry. The fruit from a total of 28 clones was collected and analyzed, as shown in Table 1. NJ85-1 is a wild diploid *V. corymbosum* that lacks anthocyanin pigments in the fruit epidermis resulting in a white fruit.

Reagents. All solvents, including acetone, ethyl acetate, acetonitrile, and methanol were purchased from EMD Millipore (Billerica, MA). Acetic acid was purchased from Avantor Performance Materials (Center Valley, PA), formic acid was purchased from Mallinckrodt Baker (Phillipsburg, NJ), and phosphoric acid was purchased from Amresco (Solon, OH).

Quinic acid, citric acid, shikimic acid, and the anthocyanin standard cyanidin-3-galactoside chloride was obtained from Sigma (St. Louis, MO). Flavonol standards, including myricetin-3-rhamnoside, quercetin-3-rutinoside, quercetin-3-galactoside, quercetin-3-glucoside, and quercetin-3-rhamnoside were purchased from Indofine Chemical Company (Somerville, NJ). Individual B-type PAC standards were kindly provided by Planta Analytica (Danbury, CT).

Blueberry extraction for flavonoid and organic acid identification and quantification. For flavonoid analysis, depending on sample availability, 2 to 8 g of fruits were weighed and crushed in 30 mL 80% aqueous acetone with 0.1% acetic acid in a laboratory blender, followed by 30 min sonication and filtration with filter paper. Liquid extracts were then dried in rotary evaporator under 35 °C water bath with vacuum, and redissolved in 100% methanol to a final volume of 6 mL. Samples were filtered through a 0.45-µm Spin-X centrifuge filter tube before analysis. Injection volume for HPLC and LC-MS analyses were 10 and 5 µL, respectively.

For organic acid analysis, depending on sample availability, 1 to 6 g of fruit was weighed and crushed in 10 to 30 mL distilled water in a laboratory blender and then heated in a 90 °C water bath for 10 min. Samples were then filtered through a 0.45-µm Spin-X centrifuge filter and injected (10 µL) in HPLC.

HPLC analysis for blueberry flavonoids and organic acids. For flavonol, PAC, and organic acid analysis, the HPLC apparatus and conditions were described in our previously published study (Wang et al., 2017). A Gemini 250 × 4.6-mm C18 110-Å, 5-µm LC column was used for organic acid analysis, differing from the previous study.

Table 1. Blueberry species and clones evaluated in the study.

Species (*Vaccinium*)	Clone ID	Origin
V. corymbosum (4*x*)	Bluecrop	NJ
	Duke	NJ
	Cara's Choice	NJ
V. corymbosum (2*x*)	NJOPB-1	NJ
	NJOPB-8	NJ
	NJOPB-15	NJ
	NJ85-1[z]	NJ
	NJ17-9	NJ
V. boreale (2*x*)	NJ88-29	NS (Nova Scotia)
	NJ88-30	NS
V. pallidum (2*x*)	NJ17-1	NJ
	NJ17-4	NJ
	NJ17-5	NJ
	NJ17-6	NJ
V. elliottii (2*x*)	NJ17-7	FL
	NJ17-8	FL
V. darrowii (2*x*)	NC84-6A	FL
	NJ88-05	AL
	NJ88-06	FL
	NJ88-07	FL
	NJ88-10	FL
	NJ88-11	FL
	NJ88-12	FL
	NJ88-13	FL
	NJ88-14	FL
V. tenellum (2*x*)	NJ88-31	GA
	NC78-8	SC
	NC83-9	NC

[z]Clone lacks anthocyanins, "white fruited."

For anthocyanin analysis, a Waters Alliance HPLC system was used, with a Phenomenex Luna 250 × 4.6-mm C18(2) 100-Å, 5-µm LC column. A binary solvent system with solvent A: 95% water + 5% formic acid and solvent B: 100% methanol was used with linear gradient of 5% B to 20% B from 0 to 2 min; isocratic elution of 20% B from 2 to 10 min; linear gradient of 20% B to 30% B from 10 to 15 min; linear gradient of 30% B to 40% B from 15 to 35 min; linear gradient of 40% B to 5% B from 35 to 40 min; and isocratic elution of 5% B from 40 to 50 min at a flow rate of 1 mL/min. Anthocyanins were detected at 520 nm absorbance in PDA detector.

MS conditions for blueberry flavonoid identification. LC-MS analysis was carried out in an Applied Biosystems (Foster City, CA) API 3000 triple-quad LC-MS/MS mass spectrometer coupled with the Dionex (Sunnyvale, CA) UltiMate 3000 LC system. MS data acquired under ESI mode in negative (flavonols, PACs) or positive (anthocyanins) ion mode, with the following parameters: curtain gas: 12 psi, nebulizer gas: 7 psi, collision gas: 6 psi, IonSpray voltage: 4200 V, entrance potential: 10 V, focusing potential: 275 V, declustering potential: 60 V, collision energy: 50 V, collision cell exit potential: 5.0 V, source temperature: 400 °C. The same LC methods were used for compound separation and a flow splitter was used to reduce the flow rate to 0.25 mL/min for proper ionization.

Identification and quantification of blueberry flavonoids. Identification of individual blueberry flavonoids and organic acids was carried out by comparing their HPLC chromatograph (retention time, ultraviolet-Vis spectra, fluorescence spectra) and/or MS fragmentation data with authentic standards and/or previously published data.

For quantification of anthocyanins and polymeric PACs, HPLC standard curves were first prepared using standards. Compound concentrations were then determined by calculating peak area corresponding to relevant standard curves. Cyanidin-3-galactoside chloride was used as standard for all blueberry anthocyanins.

For quantification of flavonols, both standard and blueberry samples were analyzed by LC-ESI-MS-MS in multiple reaction monitoring (MRM) scan mode at a dwell time of 100 ms with ion pairs as follows: myricetin-3-galactoside/glucoside (479/316), myricetin-3-pentoside (449/316), myricetin-3-rhamnoside (463/316), quercetin-3-rutinoside (609/300), quercetin-3-galactoside/glucoside (463/300), quercetin-3-pentoside (433/300), quercetin-3-rhamnoside (447/300), quercetin-3-glucoside acetate (505/300), laricitrin-3-galactoside/glucoside (493/330), laricitrin-3-pentoside (463/330), laricitrin-3-rhamnoside (477/330), kaempferol-3-rutinoside (593/284), kaempferol-3-glucoside (447/284), isorhamnetin-3-galactoside/glucoside (477/314), syringetin-3-galactoside/glucoside (507/344), syringetin-3-pentoside (477/344), syringetin-3-rhamnoside (491/344). Flavonol concentrations were acquired by calculating peak area corresponding to relevant standard curves. Quercetin-3-galactoside was used as standard for flavonol compounds without available authentic standards.

Statistical analysis. SPSS Statistics 19 (IBM, Armonk, NY) was used for one-way analysis of variance with least significant

difference (LSD) mean separation. SIMCA 14 (Umetrics, Umeå, Sweden) was used for principal component analysis (PCA) of the blueberry flavonoid (anthocyanins, flavonols, and PACs) profiles. PCA was conducted to evaluate the effect of genetic background on the flavonoid profile among different blueberry species.

Results

Anthocyanins

Identification and quantification of blueberry anthocyanins. A total of 18 anthocyanins were identified and characterized among the different species, although not every species contained each identified anthocyanin. Figure 1A and Table 2 show the HPLC chromatograph, retention times, ultraviolet-visible absorbance spectra, and MS spectra of individual blueberry anthocyanins. Peaks 1, 2, 3, and 14 all had MS-MS fragment of *m/z* 303, which corresponds to delphinidin. They had molecular ion peaks at *m/z* 465 (peaks 1 and 2), 435 (peak 4), and 507 (peak 14), thus based on comparison with compound molecular weight and previously published data (Barnes et al., 2009; Prior et al., 2001) they were tentatively identified as delphinidin-3-galactoside, -glucoside, -arabinoside, and -(6"-acetyl)galactoside, respectively. Peaks 3, 5, and 7 all had MS-MS fragment of *m/z* 287, indicating their aglycone structure as cyanidin. They showed molecular ion peaks at *m/z* 449 (peaks 3 and 5) and 419 (peak 7), and were tentatively identified as cyanidin-3-galactoside, -glucoside, and -arabinoside, respectively. Similarly, peaks 6, 10, and 16 were identified as petunidin (*m/z* 317) glycosides, peaks 9 and 17 as peonidin (*m/z* 301) glycosides, and peaks 11, 12, 13, 15, and 18 as malvidin (*m/z* 331) glycosides.

Anthocyanins were quantified through HPLC-PDA at 520 nm. The anthocyanin concentrations in different blueberry species are shown in Supplemental Table 1. The white-fruited variant of diploid *V. corymbosum* species, clone NJ85-1, contained no detectable anthocyanins. Although most of the clones showed very low or no detected acetylated anthocyanins, one *V. boreale* clone, NJ 88-30 exhibited a significant content of acetylated anthocyanins (83.16 mg/100 g fruit). To compare the anthocyanin profiles of different species, means of the five anthocyanin classes, delphinidins, cyanidins, petunidins, peonidins, and malvidins, were calculated for each species. Malvidins and delphinidins were the two major anthocyanin classes among blueberry species, accounting for an average 31.4% and 29.1% of total anthocyanins (Fig. 1B). Peonidins were least abundant, only accounting for an average 4% of total anthocyanins.

LSD mean separation analysis revealed significant differences on the relative proportions of malvidin, delphinidin, and cyanidin among species. *V. boreale* clones exhibited the highest proportion of malvidin (45.8%), significantly higher than diploid *V. corymbosum*, *V. pallidum*, *V. elliottii*, and *V. tenellum* (<31%). Diploid *V. corymbosum* clones contained the highest proportion of delphinidins (39.0%), significantly higher than *V. boreale*, *V. pallidum*, and *V. darrowii* (<25%). Cultivated tetraploid *V. corymbosum* clones showed the lowest proportions of cyanidin (4.9%), significantly lower than *V. pallidum*, *V. elliottii*, and *V. darrowii*.

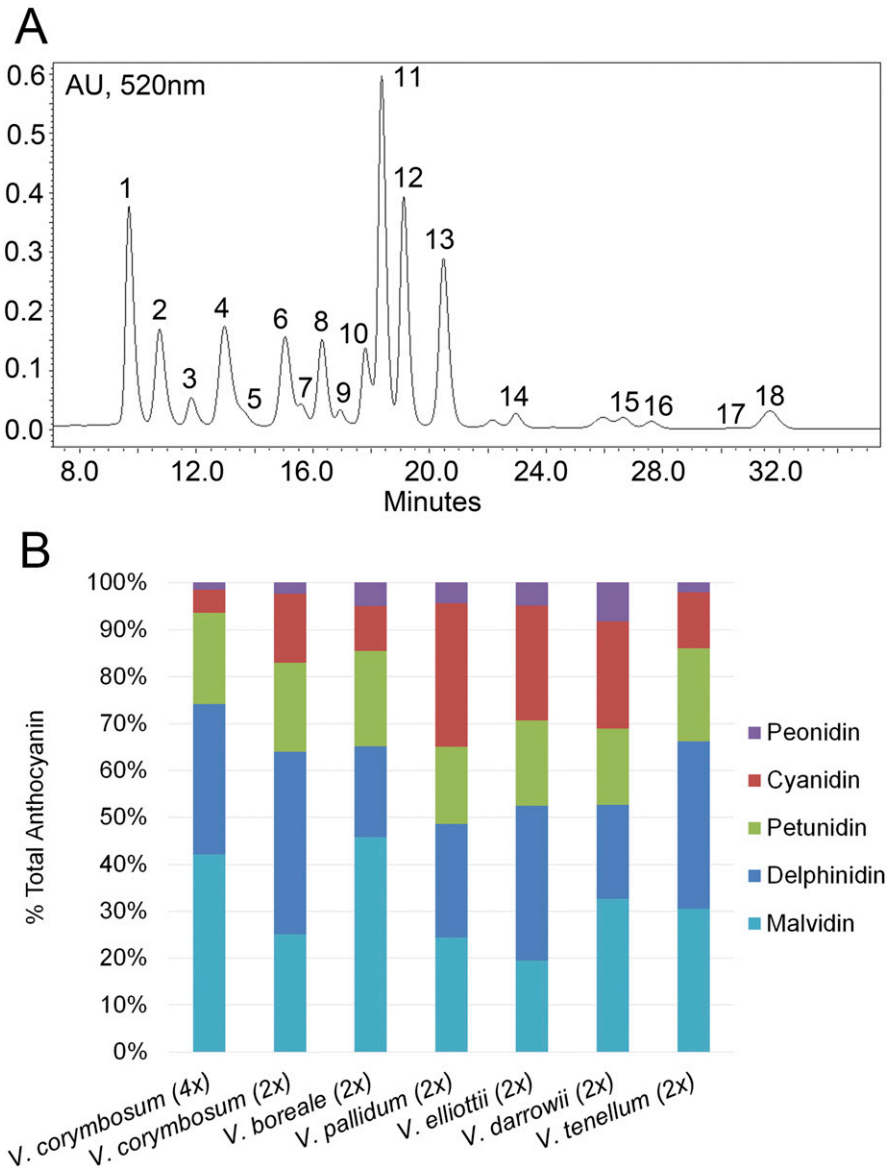

Fig. 1. Blueberry anthocyanin profiles. (**A**) High-performance liquid chromatography chromatograph of blueberry anthocyanins. All peaks were detected at ultraviolet absorbance of 520 nm. Labels 1 to 18 on peaks correspond to peaks 1 to 18 in Table 2. (**B**) Average composition of different anthocyanin groups in blueberry species.

PCA of blueberry anthocyanin contents. In the PCA of the anthocyanins, the first two principal components (PCs) accounted for 44% and 20% of the total variation among the species. Figure 2 shows the PCA score and loading plots on the first two components (PC1 and PC2). In the PC score plot (Fig. 2A), different species were not clearly separated, suggesting that variation on anthocyanin contents is not sufficient for discriminating all blueberry species. However, LSD mean separation suggested that PC1 and PC2 scores were significantly different among species (Supplemental Table 5), and clones from *V. elliottii*, *V. tenellum*, diploid *V. corymbosum,* and *V. pallidum* were found to form clusters. Tetraploid *V. corymbosum* and *V. boreale* clones did not cluster closely, suggesting large intraspecies variation on anthocyanin profiles.

Comparison between the PCA score plot (Fig. 2A) and loading plot (Fig. 2B) suggests that specific anthocyanin groups were preferably produced in certain species. Most notably are the variables (anthocyanins) located on the upper, left corner of the loading plot, consisting of the galactosides and arabinosides of delphinidin, petunidin, and malvidin. Thus the two *V. elliottii* clones, being highest in galactosides and arabinosides (average 606.1 mg/100 g, significantly higher than other species except *V. tenellum*, Supplemental Table 1), are found in the upper left of the score plot. Similarly, all

Table 2. Liquid chromatography retention time, ultraviolet-Vis absorbance, $[M+H]^+$, and fragmentation ions of blueberry anthocyanins.

Peak number	Retention time (min)	λmax (nm)	$[M+H]^+$ and fragment ions in ESI-MS-MS (*m/z*)	Peak identity
1	9.691	276.9, 523.7	465, 303	Delphinidin-3-galactoside
2	10.744	276.9, 524.9	465, 303	Delphinidin-3-glucoside
3	11.837	279.2, 516.4	449, 287	Cyanidin-3-galactoside
4	12.968	275.7, 526.1	435, 303	Delphinidin-3-arabinoside
5	13.441	278.0, 522.5	449, 287	Cyanidin-3-glucoside
6	15.048	276.9, 526.1	479, 317	Petunidin-3-galactoside
7	15.588	281.6, 512.7	419, 287	Cyanidin-3-arabinoside
8	16.316	275.7, 526.1	479, 317	Petunidin-3-glucoside
9	16.941	278.0, 516.4	463, 301	Peonidin-3-galactoside
10	17.798	276.9, 528.5	449, 317	Petunidin-3-arabinoside
11	18.360	276.9, 528.5	493, 331	Malvidin-3-galactoside
12	19.117	275.7, 527.3	493, 331	Malvidin-3-glucoside
13	20.479	276.9, 528.5	463, 331	Malvidin-3-arabinoside
14	22.963	255.5, 527.3	507, 303	Delphinidin-3-(6”-acetyl)galactoside
15	26.655	274.5, 531.0	535, 331	Malvidin-3-(6”-acetyl)galactoside
16	27.612	282.8, 529.8	521, 317	Petunidin-3-(6”-acetyl)glucoside
17	30.418	281.6, 520.0	505, 301	Peonidin-3-(6”-acetyl)glucoside
18	31.667	280.4, 528.5	535, 331	Malvidin-3-(6”-acetyl)glucoside

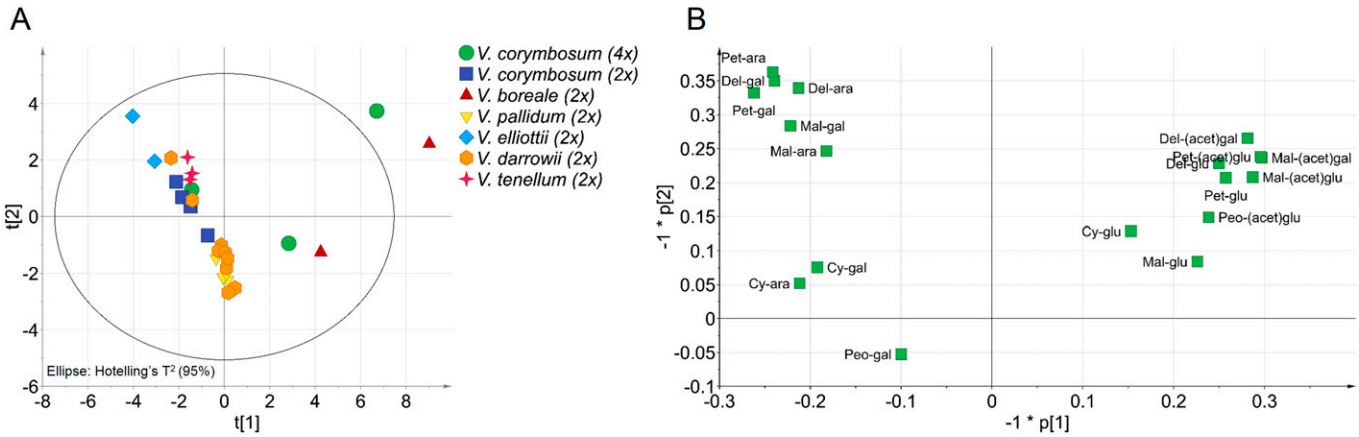

Fig. 2. Principal component analysis (PCA) on blueberry anthocyanin levels. (**A**) PCA score plot; (**B**) PCA loading plot. Del = delphinidin; Cy = cyanidin; Pet = petunidin; Peo = peonidin; Mal = malvidin; gal = galactoside; glu = glucoside; ara = arabinoside

the anthocyanin glucosides were present on the right side of loading plot. *V. boreale* clones, being highest in the glucosides (average 137.9 mg/100 g, significantly higher than other species, Supplemental Table 1), are found in the same location of the score plot.

Proanthocyanidins

Identification and quantification of blueberry proanthocyanidins. Figure 3A and Table 3 show the HPLC chromatograph (in fluorescence detection), retention times, and MS spectra of individual blueberry PACs. All blueberry PAC peaks showed the same fluorescence excitation/emission at 280/308 nm. The molecular weights of oligomeric PACs were analyzed by LC-ESI-MS to determine their degree of polymerization and type of interflavan linkage. Peak 1 showed molecular ion at *m/z* 577.4, corresponding to a B-type PAC dimer (DP-2, DP as degree of polymerization) without double interflavan linkage. Peaks 2 to 4 showed molecular ion at *m/z* 865.5 to 866.0, suggesting their structure as B-type trimers (DP-3). Similarly, peaks 5 and 6 were identified as B-type tetramers (DP-4, molecular ion *m/z* 1153.6–1153.8), peak 7 as B-type pentamer (DP-5, molecular ion *m/z* 1442.2), peak 8 as B-type hexamer (DP-6, molecular ion *m/z* 1730.4). Due to the detection limit of ESI-MS, PAC peaks after DP-6 were identified based on their retention time and elution sequence. Similar identification strategy has been used in previously published studies on cranberry (Wang et al., 2017; Wilson et al., 2008).

The concentrations of individual PAC oligomers (DP-2 to DP-6) and their combinations (DP-7 to 10, DP-11+ [PAC compounds with DP ≥ 11]) are given in Supplemental Table 2. The white-fruited clone NJ85-1 had the lowest amount of PACs (27.7 mg/100 g fruit) among all the samples, and was excluded from species analyses. Figure 3B shows the average composition of PAC fractions in the seven blueberry species. The different blueberry species exhibited similar PAC compositions. The high-polymeric PAC fractions DP-11+ was the most abundant PAC fraction among all species, accounting for 26% to 47% of total quantified PACs (Fig. 3B). They were followed by DP-7 to 10 (19% to 22%), DP-4 (8% to 12%), DP-3 (8% to 12%), DP-5 (7% to 9%), DP-6 (6% to 8%), and DP-2 (5% to 10%).

LSD mean separation analysis revealed significant differences on the relative proportions of DP-5, DP-6, DP-7 to 10, and DP-11+ among species. The diploid *V. corymbosum* clones contained the highest proportion of DP-11+ (47.1%) among species (Fig. 3B), significantly higher than other species except for *V. boreale*. Different species also varied in the content of total quantified PACs. *V. pallidum* (110.5 mg/100 g) and *V. tenellum* (106.0 mg/100 g) contained significantly more PACs than other species (Supplemental Table 2).

PCA of blueberry proanthocyanidin contents. Figure 4 shows the PCA score and loading plots on blueberry PAC contents. Similar to anthocyanins, not all species were clearly separated in the score plot (Fig. 4A), indicating that the variation on PAC profiles is not sufficient for the discrimination of all blueberry species. Significant differences on PC1 score were found among species (Supplemental Table 5), and clones from tetraploid *V. corymbosum*, *V. boreale,* and *V. pallidum* are found to be well separated by their differential PAC profiles. The *V. boreale* clones, located on the top of the score plot, contained the highest level of DP-11+ (average 40.8 mg/100 g, Supplemental Table 2) among all species, significantly higher than tetraploid *V. corymbosum* and *V. boreale*. This observation is consistent with the

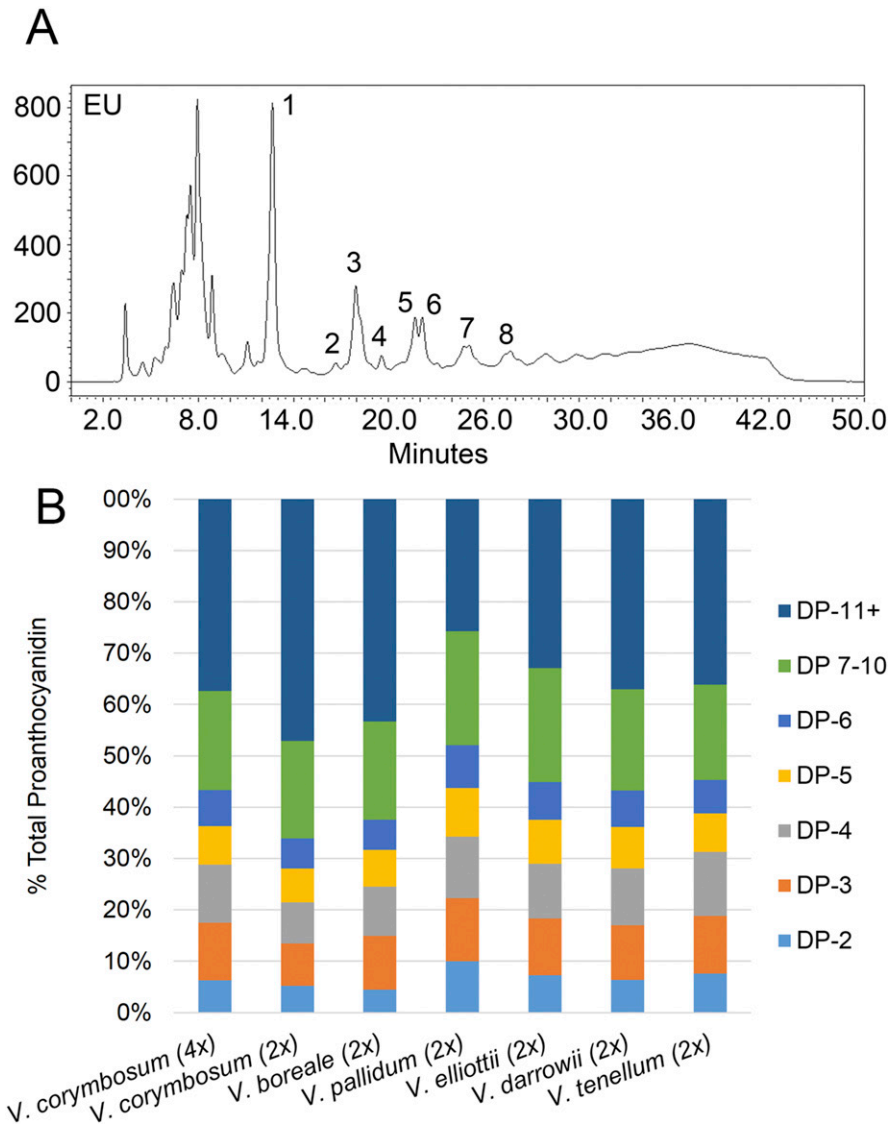

Fig. 3. Blueberry proanthocyanidin profiles. (**A**) HPLC chromatograph of blueberry proanthocyanidins. All peaks were detected by fluorescence detector at 280/308 nm of excitation/emission. Labels 1 to 8 on peaks correspond to peaks 1 to 8 in Table 3. (**B**) Average composition of different proanthocyanidin fractions in blueberry species.

distribution of DP-11+ on the PCA loading plot (Fig. 4B). The *V. pallidum* clones were located on the right side of the score plot (Fig. 4A), and all PAC components were on the right side of the loading plot with positive PC1 loadings (Fig. 4B). Such observations are consistent with the fact that *V. pallidum* contained the highest amount of total PACs (average 110.5 mg/100 g, Supplemental Table 2) among the species. The diploid and tetraploid *V. corymbosum* species appeared similar in the score plot, indicating similar PAC profiles regardless of polyploidy.

Flavonols

Identification and quantification of blueberry flavonols. A total of 23 flavonol glycosides were identified among different blueberry species; they can be divided into six flavonol groups based on their aglycone structures. Figure 5A shows selected LC-MS-MS chromatograph of blueberry flavonols in MRM mode. Table 4 shows the retention times and MS spectra of 23 identified blueberry flavonol glycosides. Peaks 1 to 4 had MS-MS fragment of *m/z* 316, corresponding to myricetin aglycone. Peaks 1 and 2 had molecular ion peak at *m/z* 479, indicating they are myricetin-hexoside. By comparing their retention time with previously published data (Vrhovsek et al., 2012), they were identified as myricetin-3-galactoside and myricetin-3-glucoside, respectively. Peak 3 had molecular ion peak at *m/z* 449, indicating its structure as myticetin-3-pentoside. Peak 4 had molecular ion peak at *m/z* 463 and was identified as myticetin-3-rhamnoside based on comparison with standard. Peaks 5 to 10 all had MS-MS fragment of *m/z* 300, corresponding to quercetin aglycone. Based on their molecular ion peak and retention time, they were tentatively identified as quercetin-3-galactoside, -glucoside, -pentoside, -rhamnoside, and -glucoside acetate. Similarly, we tentatively characterized peaks 11 to 14 as laricitrin glycosides (MS-MS fragment ion at *m/z* 330), peaks 15 and 16 as kaempferol glycosides (MS-MS fragment ion at *m/z* 284), peaks 17 and 18 as isorhamnetin glycosides (MS-MS fragment ion at *m/z* 314), and peaks 19 to 23 as syringetin glycosides (MS-MS fragment ion at *m/z* 344).

Concentrations of individual flavonol glycosides in different blueberry species are given in Supplemental Table 3. Clone NJ85-1 contained considerably less flavonols (0.4 mg total flavonols/100 g fruit) and no detectable laricetrin, kaempferol, isorhamnetin, and syringetin compared with other samples, and was excluded in species analyses. Figure 5B shows the average composition of the six flavonol classes. Quercetin glycosides were the most abundant flavonols in six blueberry species (66.8% to 82.4%), except for diploid *V. corymbosum*, where myricetin glycosides were the primary flavonols.

LSD mean separation analysis revealed significant differences on the relative proportions of aglycones quercetin, myricetin, laricetrin, and kaempferol among species. Most notably, diploid *V. corymbosum* clones contained significantly higher proportions of myricetin (49.2%) and laricitrin (7.4%), and significantly lower proportions of quercetin (34.8%) and kaempferol (lacking) than all other species. Together, quercetin and myricetin glycosides accounted for 79.9% to 95.1% of total quantified flavonols in all species. Isorhamnetin glycosides appeared to be the least abundant flavonols in all species, accounting for only 0.05% to 0.65% of total quantified flavonols.

PCA of blueberry flavonol contents. The first two PCs accounted for 30.3% and 20.1%, respectively, of the total variance of flavonol constituents. Figure 6 shows the PCA score and loading plots on the first two components. LSD mean separation revealed significant differences on PC1 and PC2 scores among species (Supplemental Table 5), which were found to be loosely separated by their PC scores (Fig. 6A), suggesting intraspecific and interspecific variation exist for flavonol profiles.

On the PCA loading plot (Fig. 6B), individual flavonols were found to form three major clusters based on their types of glycosylation. The four flavonol rhamnosides with negative loadings on PC1 were located on the left side of the loading plot. Accordingly, clones of the three species, *V. pallidum*, *V. darrowii*, and *V. tenellum*, were located at the same area in the score plot (Fig. 6A). It appeared that flavonol rhamnosides are preferably produced in these species relative to the other species. Indeed, as shown in Fig. 5C, flavonol rhamnosides accounted for 49% to 66% of total quantified flavonols in the three species, significantly higher than others.

Similarly, most of the clones from diploid and tetraploid *V. corymbosum* and *V. elliottii,* located on the right side of score plot, suggesting they contain higher proportions of flavonols galactosides, and pentosides, which were located at the same area in the loading plot (Fig. 6B). Flavonols galactosides

Table 3. Liquid chromatography retention time, fluorescence excitation/emission, and $[M-H]^-$ of blueberry proanthocyanidins.

Peak number	Retention time (min)	$\lambda_{Ex}/\lambda_{Em}$ (nm)	$[M-H]^-$ in ESI-MS (*m/z*)	Degree of polymerization	Peak identity
1	12.673	280/308	577.4	2	B-type Dimer
2	16.665	280/308	865.5	3	B-type Trimer
3	17.933	280/308	865.8	3	B-type Trimer
4	19.566	280/308	866.0	3	B-type Trimer
5	21.675	280/308	1153.8	4	B-type Tetramer
6	22.118	280/308	1153.6	4	B-type Tetramer
7	25.076	280/308	1442.2	5	B-type Pentamer
8	27.675	280/308	1730.4	6	B-type Hexamer

ESI-MS = electrospray ionization–mass spectrometry.

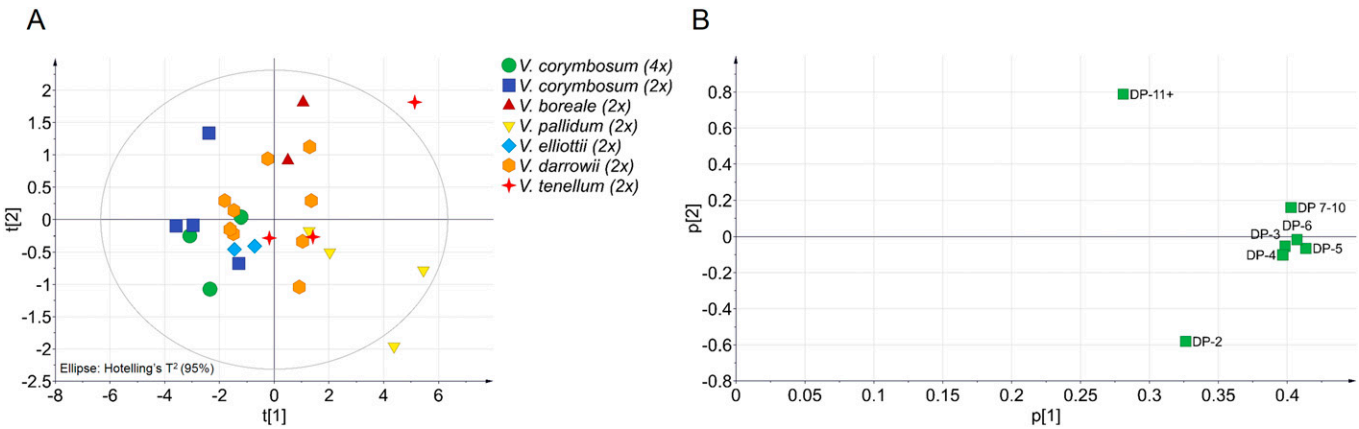

Fig. 4. Principal component analysis (PCA) on blueberry proanthocyanidin levels. (**A**) PCA score plot; (**B**) PCA loading plot.

and pentosides accounted for 60% to 95% of total flavonols among the three species (Fig. 5C), significantly higher than others. The two *V. boreale* clones with the highest PC2 scores were found on the top of the score plot and flavonol rutinosides and glucosides were located at the same area on the loading plot. *V. boreale* contained the highest ratio of flavonol rutinosides (50.9%) (Fig. 5C), significantly higher than all other species. It also possessed the highest proportions of flavonols glucosides (24.9%) among all species (Fig. 5C), significantly higher than diploid *V. corymbosum*, *V. pallidum*, and *V. elliottii*.

Organic acids

Figure 7A shows the differentiated HPLC chromatograms of organic acids from selected blueberry species. Three major organic acids, quinic, shikimic, and citric acids, were identified. Both quinic and shikimic acids have 212 to 214 nm maximum ultraviolet absorbance, whereas citric acid has 208 to 210 nm maximum absorbance.

The concentrations of the three organic acids in different clones are summarized in Supplemental Table 4. Their average levels in different species are shown in Fig. 7B. The blueberry species differ in their organic acid profiles. Diploid *V. corymbosum* clones contained the highest levels of citric acid (10.0 mg/g), significantly higher than other species. Citric acid appeared to be the major component in both diploid and tetraploid *V. corymbosum* clones, accounting for more than 90% of total quantified acids. The *V. corymbosum* clones also possessed the least amount of quinic acid, significantly lower than *V. pallidum* and *V. tenellum*. In fact, four of five diploid *V. corymbosum* clones did not show any detectable quinic acid in the analysis (Supplemental Table 4).

The *V. pallidum* clones contained the highest level of quinic acid (17.6 mg/g), significantly higher than all other species. Together with *V. tenellum* (7.6 mg/g) and *V. darrowii* (2.6 mg/g), these three lowbush species had quinic acid accounting for >85% of their organic acids. They also had the lowest level of citric acid among species (less than 0.70 mg/g), significantly lower than the *V. corymbosum* clones. Specifically, all *V. tenellum* clones and 7 of 9 *V. darrowii* clones did not show any detectable citric acids in their HPLC chromatograms (Supplemental Table 4). Although other species showed only trace levels (<0.1 mg/g) of shikimic acid, the *V. darrowii* (0.4 mg/g) and *V. tenellum* (1.0 mg/g) clones exhibited significantly higher levels of shikimic acid.

Discussion

Berry fruit species, such as grape, strawberry, cranberry, blackberry, and blueberry, are rich sources of phenolic compounds, including flavonoids, phenolic acid, and stilbenes (Cieślik et al., 2006; Puupponen-Pimiä et al., 2005). Among different flavonoid classes, anthocyanins are water-soluble pigments that mainly accumulate in the epidermis of the berry fruit. The identity and quantity of blueberry anthocyanins have been investigated because of their potential human health benefits and important implication on fruit quality. However, most of the studies were focused on the three commercially grown blueberry species: highbush (*V. corymbosum*), lowbush (*V. angustifolium*), and rabbiteye (*V. virgatum*) blueberries. In this study, we explored the flavonoid composition, including anthocyanins, in various diploid wild blueberry species, and compared them with the tetraploid highbush blueberry cultivars. To our best knowledge, there is no comparative study on the flavonoid composition of diploid wild blueberry species.

A total of 18 anthocyanins were identified and quantified in different blueberry species. They belong to five different aglycone groups based on anthocyanidin structure. Yousef et al. (2013) reported very similar anthocyanin profiles in different blueberry selections. In their study, 18 anthocyanins consisting of five acetylated molecules and 13 nonacetylated anthocyanins were identified, consistent with this study. One of the most comprehensive blueberry anthocyanin profiles was reported by Barnes et al. (2009), which characterized 25 individual anthocyanins in an interspecific (*V. darrowii* × *V. corymbosum*) blueberry using ESI–time of flight (TOF)-MS. Compared with Barnes et al. (2009), peonidin-3-glucoside/arabinoside and five other acetylated anthocyanins were not found and characterized in our study, potentially due to low peak intensity, peak overlap, or the low resolution of triple-quadruple MS compared with TOF. Other studies reported less complicated blueberry anthocyanin profiles. Lohachoompol et al. (2008) identified 15 different anthocyanins in highbush and rabbiteye blueberry cultivars, and no acetylated anthocyanins were characterized. Prior et al. (2001) identified 21 anthocyanins in lowbush blueberry and 14 anthocyanins in

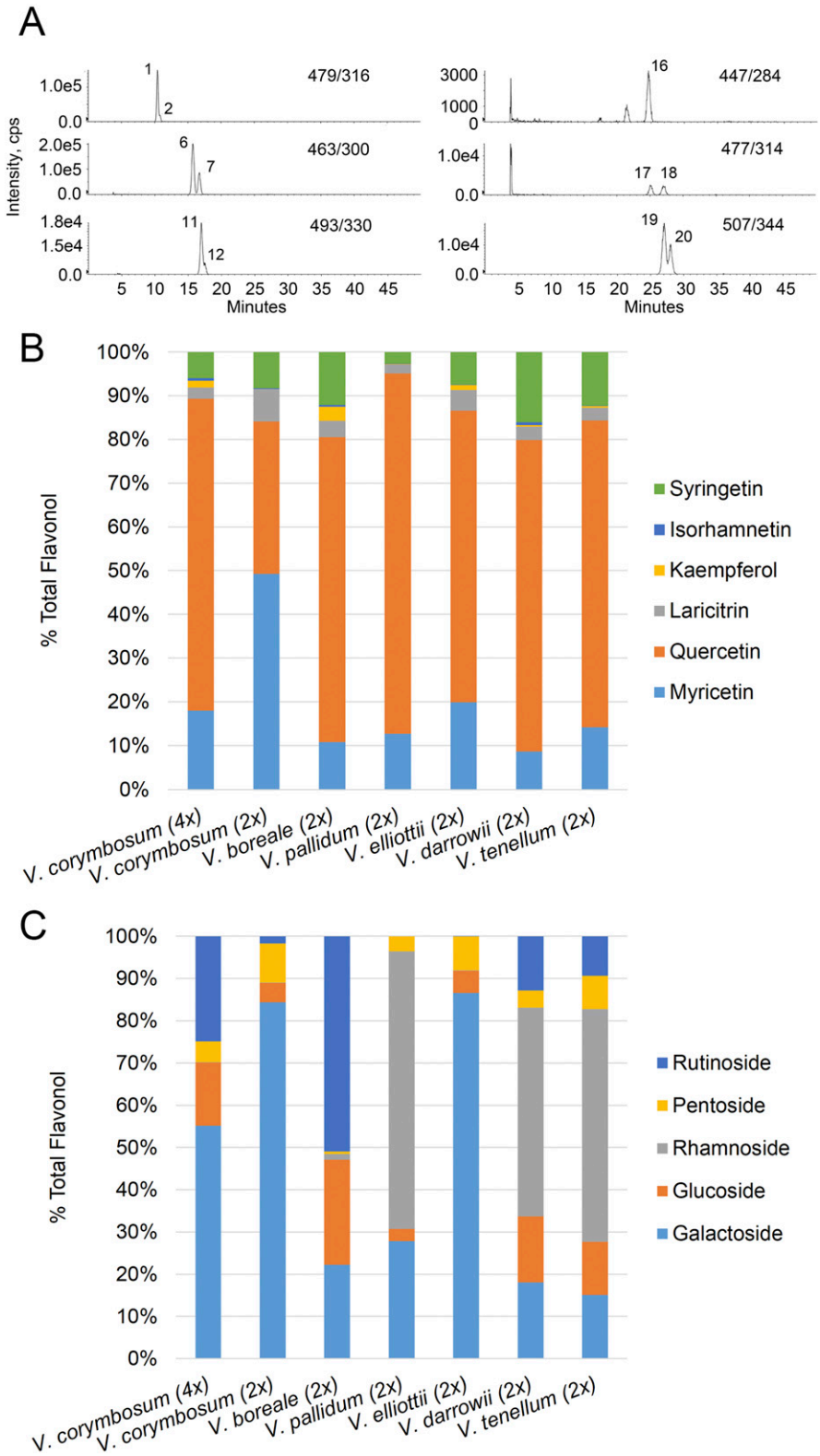

Fig. 5. Blueberry flavonol profiles. (**A**) Extracted-ion chromatograms of blueberry flavonol glycosides. Labels on peaks correspond to peaks with same numbers in Table 4. (**B**) Average composition of different flavonol groups based on aglycone structure. (**C**) Average composition of different flavonol groups based on glycosylation type.

rabbiteye blueberry. It should be noted that in most of the aforementioned studies, compound coelution (HPLC peak overlap) was observed.

PCA was applied in the present study to compare the anthocyanin profile of different blueberry species. Although we observed varying degrees of sample clustering within the same blueberry species, clones from certain species are not well separated from each other. Thus, using solely anthocyanin profiles to differentiate blueberry species is problematic. The loading values of different anthocyanins on PC1 and PC2 revealed potential relationships of their biosynthesis pathways as to aglycone and glycosylation tendencies. Regardless of the anthocyanidin type, all the glucosides positively contribute to PC1. This can be partially explained by the proposed anthocyanin biosynthesis pathway in which the flavonoid 3-glucosyltransferase (3GT) catalyzes the glucosylation of different anthocyanidins into their glucosides (Holton and Cornish, 1995). Similarly, delphinidin, petunidin, and malvidin glycosides, with three hydroxyl/methoxy groups in their B-ring, positively contribute to PC2 based on their loadings, compared with cyanidin and peonidin glycosides (two hydroxyl/methoxy groups in the B-ring). In the proposed anthocyanin biosynthesis pathway, this division occurs during the transformation of precursor compound dihydrokaempferol (one hydroxyl group in B-ring) into either dihydroquercetin (two hydroxyl groups in B-ring) or dihydromyricetin (three hydroxyl groups in B-ring) through the catalyzation of flavonoid 3′-hydroxylase (F3′H) or flavonoid 3′,5′-hydroxylase (F3′5’H) (Holton and Cornish, 1995).

Proanthocyanidins can occur as A-type, which contain double interflavan linkages, or B-type, containing exclusively single interflavan linkages. Cranberry (*Vaccinium macrocarpon*) is well known for rich content of A-type PACs. In this study, we only detected B-type PACs in the blueberry species we surveyed, which is consistent with the previous report of Prior et al. (2001) on highbush, lowbush, and rabbiteye blueberries. Due to interference and coelution of other non-PAC compounds, monomer PACs, presumably identified as epicatechin or catechin by LC-ESI-MS-MS, were not quantified by HPLC in the present study. Monomer PACs were previously reported to account for only 1.0% to 2.2% of total PACs in highbush and lowbush blueberries (Gu et al., 2004), therefore having limited influence on the overall evaluation of blueberry PACs in this study.

Among the three highbush blueberry cultivars, Cara’s Choice exhibited higher level of PACs (62.3 mg/100 g) than the other two cultivars (37.5 and 41.4 mg/100 g) (Supplemental Table 2). Unlike ‘Bluecrop’ and ‘Duke’, ‘Cara’s Choice’ has diploid *V. darrowii* and hexaploid *V. constablaei* and *V. virgatum* (rabbiteye blueberry) southern species background (Ehlenfeldt et al., 2005). Thus, the higher PACs could be a reflection of the *V. darrowii* (average 74.2 mg/100 g) coancestry. In fact, in the PCA score plot, ‘Cara’s Choice’ (–1.1/0.06, x/y) can be found in the cluster of *V. darrowii* clones. Compared with the current study, Gu et al. (2004) reported 179.8 ± 50.8 mg/100 g PACs in highbush blueberry (cultivar unknown) and even higher PAC levels in lowbush species (331.9 ± 14.0 mg/100 g fruit). The highest PAC level observed in this study is 146.3 mg/100 g fruit in one of the *V. tenellum* clones, which is still less than half of the typical cranberry PAC contents reported by us and

Table 4. Liquid chromatography retention time, $[M-H]^-$, and fragmentation ions of blueberry flavonols.

Peak number	Retention time (min)	$[M-H]^-$ and fragment ions in ESI-MS-MS (*m/z*)	Peak identity
1	10.42	479, 316	Myricetin-3-galactoside
2	10.81	479, 316	Myricetin-3-glucoside
3	13.69	449, 316	Myricetin-3-pentoside
4	14.74	463, 316	Myricetin-3-rhamnoside
5	14.96	609, 316	Quercetin-3-rutinoside
6	15.71	463, 300	Quercetin-3-galactoside
7	16.71	463, 300	Quercetin-3-glucoside
8	20.76	433, 300	Quercetin-3-pentoside
9	24.98	447, 300	Quercetin-3-rhamnoside
10	27.09	505, 300	Quercetin-3-glucoside acetate
11	16.90	493, 330	Laricitrin-3-galactoside
12	17.43	493, 330	Laricitrin-3-glucoside
13	22.81	463, 300	Laricitrin-3-pentoside
14	26.94	477, 330	Laricitrin-3-rhamnoside
15	23.74	593, 284	Kaempferol-3-rutinoside
16	23.61	447, 284	Kaempferol-3-glucoside
17	25.05	477, 314	Isorhamnetin-3-galactoside
18	27.25	477, 314	Isorhamnetin-3-glucoside
19	27.04	507, 344	Syringetin-3-galactoside
20	27.97	507, 344	Syringetin-3-glucoside
21	35.01	477, 344	Syringetin-3-pentoside A
22	35.70	477, 344	Syringetin-3-pentoside B
23	36.85	491, 344	Syringetin-3-rhamnoside

ESI-MS-MS = electrospray ionization–tandem mass spectrometry.

others (Gu et al., 2004; Wang et al., 2017), indicating that blueberry species are not the best food sources for dietary PAC intake.

The PCA analysis on blueberry PAC concentration yielded a PC1 that accounts for more than 80% of the total variance. All the PAC components showed considerable positive loadings on PC1, suggesting that variation largely reflected quantity. A similar PAC profile pattern was observed in cranberries (Wang et al., 2017). Indeed, in the correlation matrix from the PCA analysis (data not shown), the correlation coefficients between PAC components (DP-2 to DP-7 to 10) were from 0.67 to 0.99. DP-11+ and DP-2 showed least degree of correlation (correlation coefficient 0.32), which is also suggested by their loadings in PC2.

A total of 23 different flavonol glycosides were identified and quantified in blueberry species. They consisted of six flavonol aglycones, including myricetin, quercetin, laricitrin, kaempferol, isorhamnetin, and syringetin. The blueberry flavonol profiles in our study are highly consistent with the previous report of Vrhovsek et al. (2012) on cultivated highbush blueberry cultivars, except that isorhamnetin 3-rhamnoside was not detected in our study. For maximum recovery of different flavonols, LC-MS-MS in MRM scan mode was used in our study for flavonol quantification, to avoid the negative effect of low peak intensity and peak overlap in HPLC-PDA chromatogram.

In our study, the two highbush cultivars Bluecrop and Duke had total flavonol levels of 19.8 and 19.5 mg/100 g fruit (Supplemental Table 3), similar to the levels reported by Vrhovsek et al. (2012), which were 21.1 and 13.7 mg/100 g fruit, respectively. 'Bluecrop' was reported to contain 22.5 mg/100 g fruit of flavonols in another study (Mi et al., 2004), which suggests that the flavonol levels are consistent over various environments. 'Cara's Choice' had higher levels of flavonols than 'Bluecrop' and 'Duke'. Interestingly, *V. darrowii* clones contain fewer flavonols than the tetraploid cultivars, so the high flavonol content in 'Cara's Choice' could be inherited from its hexaploid ancestry. Rabbiteye blueberry cultivars have been reported to contain more flavonols than tetraploid *V. corymbosum* cultivars, including 'Bluecrop' and 'Duke' (Wang et al., 2012).

Different blueberry species vary in both flavonol concentrations and their profiles. Except for diploid *V. corymbosum*, all the other species have quercetin glycosides as the most abundant flavonol class, which is consistent with a previous report on cultivated highbush blueberries (Vrhovsek et al., 2012). The white-fruited variant NJ85-1 had the lowest level of flavonol glycosides, which is only 6% of the average flavonol content of other diploid *V. corymbosum* clones (Supplemental Table 3). The extremely low flavonol content and total lack of anthocyanin accumulation in NJ85-1 blueberry suggests that this genetic mutation affects the upstream flavonoid biosynthesis pathway, which blocks the production of both anthocyanin and flavonol glycosides. However, NJ85-1 does produce anthocyanins in the leaves (N. Vorsa, unpublished data).

The differences in flavonol profiles among blueberry species are also revealed by PCA. According to the distribution of individual flavonols in the loading plot, PC1 exhibits the variation among blueberry clones with regard to flavonol rhamnosides vs. other glycosides. PC2 mainly represents the variation of flavonol rutinosides and glucosides vs. other glycosides. Concentrations of these three flavonol glycoside groups, especially rhamnosides and rutinosides, differentiated the blueberry species. The sugar conjugate appears to mediate the absorption and clearance of flavonols in humans (Wang et al., 2016), thus affecting their health benefit potential.

Besides the variation on flavonol glycosylation, the blueberry species also varied in their compositions of different flavonol aglycones. Most notably is that the diploid *V. corymbosum* clones had higher proportion of myricetin than the other species and tetraploid *V. corymbosum*. However, compared with the PCA loading plot of blueberry anthocyanins, which revealed relationships of compounds with the same anthocyanidin structure regardless of sugar moiety type (delphinidins, petunidins, and malvidins on PC2), the PCA loadings of flavonols did not exhibit clear relationships based on aglycone, thus suggesting the variation of flavonol biosynthesis among different blueberry species mainly results from differences in glycosylation. The observation that flavonol rhamnosides and rutinosides exhibited highest level of variation among blueberry species may suggest that flavonol rhamnosyltransferases, key enzymes for biosynthesis of flavonol rhamnosides and rutinosides (from glucosides) (Frydman et al., 2004; Saito et al., 2013), are differentially expressed or more efficient in different blueberry species.

Citric, quinic, and shikimic acids were identified as the major blueberry organic acids in the present study. Besides these three acids, other studies also reported low levels of malic, succinic, and tartaric acids in cultivar Bluecrop (Ehlenfeldt et al., 1994; Forney et al., 2012). In our previous study (Wang et al., 2017), malic acid was detected in cranberry using the same fruit extraction and HPLC analysis method. Succinic acid standard was found to have similar ultraviolet absorption profile at 214 nm, but very low absorption coefficient under same HPLC conditions (S. Fong, unpublished observation). Therefore, the lack of these acids in the current report could potentially be due to low acid level, different growth condition, and fruit maturity. *V. boreale* and *V. pallidum* are thought to be probable ancestors of lowbush

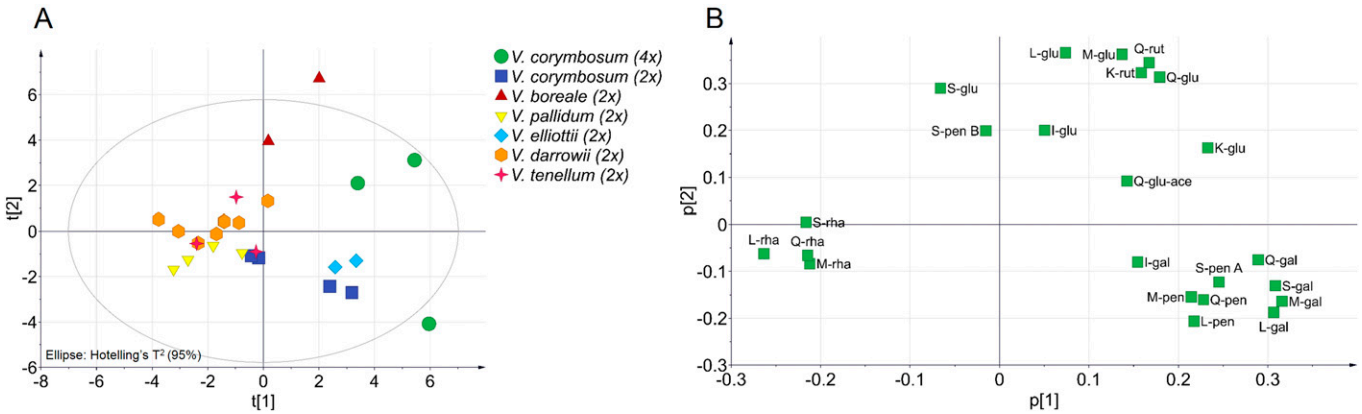

Fig. 6. Principal component analysis (PCA) on blueberry flavonol glycoside levels. (**A**) PCA score plot; (**B**) PCA loading plot. M = myricetin; Q = quercetin; L = laricitrin; K = kaempferol; I = isorhamnetin; S = syringetin; gal = galactoside; glu = glucoside; pen = pentoside; rha = rhamnoside; rut = rutinoside; glu-ace = glucoside acetate.

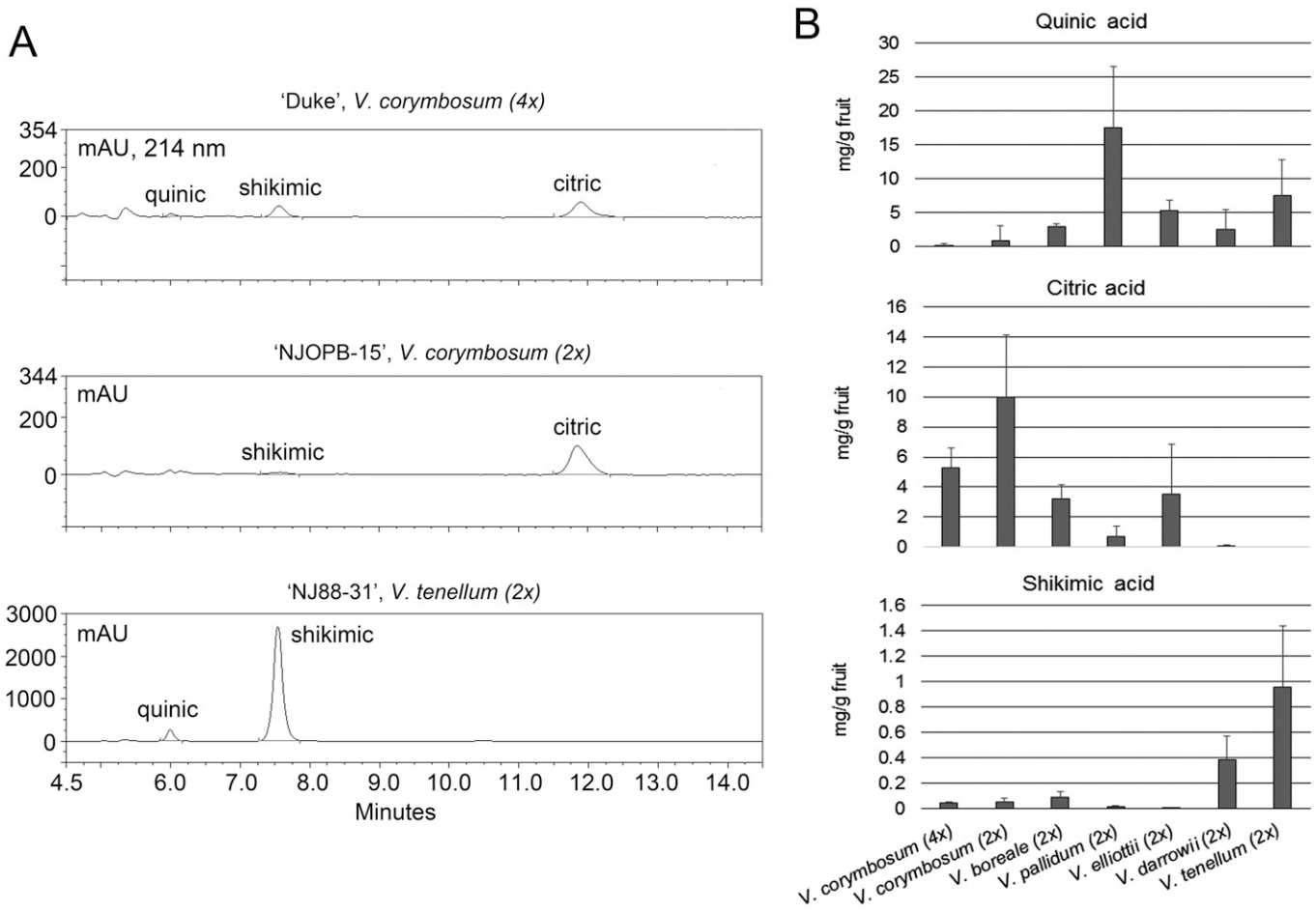

Fig. 7. Blueberry organic acid profiles. (**A**) High-performance liquid chromatography chromatograph of blueberry organic acids in three species. (**B**) Average concentrations of quinic, citric, and shikimic acids in blueberry species.

blueberry (*V. angustifolium*) (Vander Kloet, 1977). Their lack of succinic acid in the current study (Supplemental Table 4) is consistent with the absence of succinic acid in lowbush blueberry (Kalt and McDonald, 1996).

All studies reported citric acid as the primary organic acid in cultivated highbush blueberries, suggesting its contribution to blueberry flavor. Different organic acid compositions were observed not only between diploid (wild) and tetraploid (cultivated) blueberry species, but also among different diploid species (Fig. 7B). Most notably is the relative abundance of citric vs. quinic acid in different species. Although citric acid was the primary organic acid in tetraploid cultivars, accumulation of quinic acid was elevated in most of the diploid species (except *V. corymbosum*), and it was the primary organic acid in certain species such as *V. pallidum* and *V. tenellum*. The fact that quinic acid was absent in certain *V. corymbosum* clones and citric acid was not produced in *V. tenellum* and most *V. darrowii* clones (Supplemental Table 4) suggests the potential value of those blueberry materials in the study of plant organic acid biosynthesis regulation.

The differential flavonoid and organic acid profiles of diploid blueberry species

compared with cultivated tetraploids also suggests their potential value in blueberry breeding for phytochemical improvement. For instance, *V. pallidum*, *V. tenellum,* and *V. boreale* clones contain about double amounts of PACs compared with the tetraploid *V. corymbosum* clones (Supplemental Table 2). *V. pallidum*, *V. tenellum,* and *V. darrowii* clones produce high levels of quinic acid, which is extremely low in tetraploid clones (Supplemental Table 4). The diploid species have been an important source of alleles for blueberry breeding (Galletta, 1975; Hancock, 1988; Lyrene and Sherman, 1983; Sharpe and Sherman, 1971).

In summary, the flavonoid and organic acid profile of six different cultivated or wild blueberry species were comprehensively evaluated in the present study. Eighteen anthocyanins, seven PAC polymers/fractions, 23 flavonol glycosides, and three organic acids were characterized and quantified. Different blueberry clones exhibited both inter- and intraspecies variation on the composition and concentration of analyzed compounds. Flavonoids and organic acids are important phytochemicals in blueberry and other fruits, and have a great impact on fruit quality, taste, and potential human health benefits. The differential flavonoid and organic acid composition in cultivated tetraploid vs. wild diploid blueberry species revealed in this report offers future breeding efforts the prospect of breeding for specific flavonoid/organic acid profiles. The unique presence of certain flavonoids or organic acids in specific blueberry species also can be used to elucidate their biosynthesis pathways.

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Supplemental Table 1. Concentrations (mg/100 g fruit) of anthocyanins in different blueberry species.

Species	Clone	Del-gal	Del-glu	Cy-gal	Del-ara	Cy-glu	Pet-gal	Cy-ara	Pet-glu	Peo-gal	Pet-ara	Mal-gal	Mal-glu
V. corymbosum (4*x*)	Bluecrop	26.75	14.97	4.56	18.03	2.92	14.57	2.94	12.05	2.45	10.36	39.47	28.80
	Duke	105.01	3.21	19.34	47.68	0.00	70.28	10.06	3.30	10.99	27.17	164.00	10.50
	Cara's Choice	43.81	44.70	5.26	44.44	5.83	20.26	4.21	31.38	2.05	20.91	36.95	46.23
V. corymbosum (2*x*)	NJOPB-1	104.89	5.01	44.33	91.32	0.00	59.79	35.75	2.74	14.17	36.38	104.49	10.21
	NJOPB-8	103.18	3.36	23.09	59.87	0.00	52.36	13.45	1.88	4.94	22.82	49.49	3.07
	NJOPB-15	142.09	5.70	66.11	90.88	0.00	67.40	43.92	2.47	13.37	28.95	58.98	6.05
	NJ85-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	NJ17-9	102.71	1.78	36.84	55.96	3.23	69.25	22.29	1.37	14.11	27.41	112.92	6.26
V. boreale (2*x*)	NJ88-29	6.67	29.97	3.12	3.84	12.09	3.55	2.11	26.50	2.11	12.21	10.88	66.66
	NJ88-30	9.06	16.14	6.71	1.29	12.54	6.11	2.11	13.90	4.61	9.70	19.87	31.78
V. pallidum (2*x*)	NJ17-1	30.29	0.38	39.18	15.27	1.90	22.83	22.98	0.73	13.97	10.99	59.92	6.76
	NJ17-4	48.82	0.72	61.86	23.70	3.12	26.36	36.10	0.00	12.42	14.89	23.19	5.22
	NJ17-5	43.21	0.88	42.98	20.93	3.07	31.23	27.34	1.20	7.93	14.69	39.90	5.50
	NJ17-6	52.05	1.16	54.94	27.52	3.74	36.09	36.57	1.25	13.49	19.85	45.42	7.79
V. elliottii (2*x*)	NJ17-7	178.95	2.30	118.03	80.98	4.66	99.74	60.84	2.24	31.37	37.49	106.34	9.97
	NJ17-8	232.70	5.23	169.45	84.19	9.35	134.93	72.49	3.75	53.44	43.68	138.91	16.58
V. darrowii (2*x*)	NC84-6A	15.64	2.45	130.83	6.34	9.28	17.41	54.55	2.41	58.58	17.26	39.24	24.15
	NJ88-05	18.30	0.83	26.15	8.93	0.86	23.74	14.17	1.13	13.57	11.12	49.64	6.46
	NJ88-06	46.40	2.62	25.20	34.25	0.00	38.76	15.88	2.95	10.48	22.97	59.12	8.34
	NJ88-07	50.57	2.35	23.04	18.14	0.00	47.63	7.90	3.51	8.48	17.78	87.63	7.54
	NJ88-10	114.35	5.36	100.19	43.10	8.48	84.39	40.20	4.94	43.81	33.31	184.99	22.23
	NJ88-11	48.28	3.04	40.73	18.91	3.79	39.75	18.02	3.83	11.92	16.82	76.85	9.68
	NJ88-12	5.10	0.00	117.88	2.38	5.53	5.39	54.84	0.00	113.14	15.36	19.26	36.09
	NJ88-13	28.61	1.83	8.71	19.91	0.00	24.15	6.71	1.71	5.74	15.92	69.83	7.46
	NJ88-14	104.86	4.40	39.12	67.94	0.00	61.23	24.92	3.92	10.13	35.37	66.29	8.86
V. tenellum (2*x*)	NJ88-31	177.41	7.43	85.59	68.75	7.80	82.77	32.89	5.76	14.24	30.19	80.35	9.17
	NC78-8	128.70	6.99	25.47	58.41	0.00	84.59	14.14	6.99	11.36	34.94	158.71	17.31
	NC83-9	125.69	4.50	31.47	60.54	0.00	76.52	14.31	3.86	12.23	31.62	122.48	9.08

Species	Clone	Mal-ara	Del-(acet)gal	Mal-(acet)gal	Pet-(acet)glu	Peo-(acet)glu	Mal-(acet)glu	Total
V. corymbosum (4*x*)	Bluecrop	24.65	2.44	2.08	1.42	0.21	4.47	213.15
	Duke	59.94	0.76	0.51	0.00	0.06	0.17	532.99
	Cara's Choice	34.58	10.45	5.72	7.77	0.74	18.07	383.33
V. corymbosum (2*x*)	NJOPB-1	54.14	0.00	0.00	0.00	0.00	0.00	563.23
	NJOPB-8	22.65	0.00	0.00	0.00	0.00	0.00	360.14
	NJOPB-15	25.39	0.36	0.00	0.00	0.00	0.00	551.67
	NJ85-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	NJ17-9	39.75	0.00	0.00	0.00	0.00	0.00	493.89
V. boreale (2*x*)	NJ88-29	5.26	0.00	0.00	0.00	0.00	0.00	184.94
	NJ88-30	3.10	11.35	5.57	10.54	13.17	42.53	220.07
V. pallidum (2*x*)	NJ17-1	25.18	0.00	0.00	0.00	0.00	0.00	250.39
	NJ17-4	11.39	0.00	0.00	0.00	0.00	0.00	267.78
	NJ17-5	15.73	0.00	0.00	0.00	0.00	0.00	254.59
	NJ17-6	21.42	0.00	0.00	0.00	0.00	0.00	321.29
V. elliottii (2*x*)	NJ17-7	33.31	0.00	0.00	0.00	0.00	0.00	766.23
	NJ17-8	40.87	0.00	0.00	0.00	0.00	0.00	1005.57
V. darrowi (2*x*)	NC84-6A	18.81	0.64	0.00	0.00	0.00	0.00	397.58
	NJ88-05	20.15	0.19	0.00	0.00	0.00	0.00	195.25
	NJ88-06	32.78	1.56	0.00	0.25	0.13	0.00	301.70
	NJ88-07	39.97	0.00	0.00	0.00	0.00	0.00	314.53
	NJ88-10	76.20	0.00	0.00	0.00	0.00	0.00	761.55
	NJ88-11	29.06	0.52	0.00	0.00	0.00	0.00	321.18
	NJ88-12	11.43	0.00	0.00	0.00	0.32	0.00	386.70
	NJ88-13	54.87	1.60	0.00	0.00	0.00	0.00	247.05
	NJ88-14	41.13	0.70	0.00	0.00	0.00	0.00	468.88
V. tenellum (2*x*)	NJ88-31	26.96	1.37	0.09	0.36	0.09	0.00	631.21
	NC78-8	69.41	2.91	0.00	0.00	0.00	0.00	619.92
	NC83-9	55.54	2.38	0.00	0.37	0.00	0.00	550.59

Del = delphinidin; Cy = cyanidin; Pet = petunidin; Peo = peonidin; Mal = malvidin; gal = galactoside; glu = glucoside; ara = arabinoside.

Supplemental Table 2. Concentrations (mg/100 g fruit) of proanthocyanidins in different blueberry species.

Species	Clone	DP-2	DP-3	DP-4	DP-5	DP-6	DP7-10	DP-11+	Total
V. corymbosum (4*x*)	Bluecrop	3.61	6.44	5.17	3.59	3.07	7.68	11.81	41.39
	Duke	1.45	2.98	4.07	2.66	2.55	7.35	16.46	37.52
	Cara's Choice	3.81	6.45	6.66	4.39	4.29	12.26	24.47	62.33
V. corymbosum (2*x*)	NJOPB-1	1.21	1.87	2.65	2.17	2.02	5.68	17.49	33.09
	NJOPB-8	1.68	3.90	3.49	2.61	2.38	8.03	18.49	40.58
	NJOPB-15	1.50	3.02	3.16	2.81	2.82	10.24	33.90	57.46
	NJ85-1	1.09	2.08	1.97	1.61	1.34	5.14	14.53	27.74
	NJ17-9	5.41	6.86	5.85	4.79	3.96	11.89	19.05	57.82
V. boreale (2*x*)	NJ88-29	4.36	11.60	8.72	6.54	5.35	18.24	46.37	101.18
	NJ88-30	4.09	8.21	9.28	7.07	5.57	18.00	35.32	87.54
V. pallidum (2*x*)	NJ17-1	8.23	11.52	10.62	9.02	8.22	22.26	26.74	96.62
	NJ17-4	7.87	9.89	8.63	7.66	6.72	22.20	28.58	91.56
	NJ17-5	13.62	15.21	18.03	13.34	11.68	29.68	35.13	136.70
	NJ17-6	14.29	17.90	15.68	11.74	10.27	23.94	23.46	117.28
V. elliottii (2*x*)	NJ17-7	3.99	6.50	5.95	4.50	3.95	12.52	18.74	56.17
	NJ17-8	4.81	6.91	6.96	5.88	4.90	14.37	21.10	64.93
V. darrowii (2*x*)	NC84-6A	4.26	10.85	10.87	8.46	7.64	19.17	29.43	90.69
	NJ88-05	3.09	6.97	5.91	4.51	4.22	11.25	20.36	56.31
	NJ88-06	3.28	4.27	4.73	3.96	3.67	11.82	25.52	57.24
	NJ88-07	2.21	8.11	7.18	5.70	5.87	15.91	31.88	76.86
	NJ88-10	2.24	11.67	11.33	7.61	7.05	19.78	35.21	94.89
	NJ88-11	1.72	6.87	6.46	4.68	4.21	11.54	22.15	57.62
	NJ88-12	10.56	6.25	11.73	8.01	5.70	14.99	33.95	91.19
	NJ88-13	4.21	5.80	4.99	4.28	3.76	11.38	22.61	57.04
	NJ88-14	11.13	10.21	10.27	6.89	5.23	15.67	26.45	85.85
V. tenellum (2*x*)	NJ88-31	7.31	16.42	15.80	12.14	10.85	31.31	52.48	146.32
	NC78-8	8.41	10.80	16.28	6.92	5.30	14.26	32.74	94.71
	NC83-9	8.56	8.28	7.55	4.95	4.56	13.40	29.65	76.95

DP = degree-of-polymerization.

Supplemental Table 3. Concentrations (mg/100 g fruit) of flavonols in different blueberry species.

Species	Clone	M-gal	M-glu	M-pen	M-rha	Q-rut	Q-gal	Q-glu	Q-pen	Q-rha	Q-glu-ace	L-gal	L-glu
V. corymbosum (4*x*)	Bluecrop	2.29	0.21	0.19	0.00	3.98	5.47	2.47	0.54	0.02	3.09	0.32	0.06
	Duke	4.96	0.00	0.39	0.00	0.78	8.40	0.07	0.88	0.00	0.56	0.80	0.02
	Cara's Choice	3.90	0.42	0.37	0.00	11.95	9.00	1.85	0.79	0.00	0.55	0.36	0.08
V. corymbosum (2*x*)	NJOPB-1	5.26	0.20	0.63	0.00	0.00	1.30	0.08	0.30	0.00	0.01	0.79	0.04
	NJOPB-8	1.45	0.05	0.19	0.00	0.04	0.90	0.05	0.10	0.00	0.01	0.46	0.02
	NJOPB-15	2.31	0.08	0.21	0.00	0.02	1.16	0.11	0.17	0.00	0.00	0.33	0.01
	NJ85-1	0.01	0.00	0.00	0.00	0.01	0.40	0.03	0.05	0.00	0.00	0.00	0.00
	NJ17-9	5.01	0.07	0.40	0.01	0.49	5.44	0.26	0.75	0.01	0.04	0.56	0.02
V. boreale (2*x*)	NJ88-29	1.09	0.67	0.03	0.00	8.96	2.06	1.70	0.03	0.36	0.08	0.28	0.32
	NJ88-30	0.78	0.55	0.02	0.00	5.11	0.99	0.83	0.04	0.04	0.19	0.20	0.26
V. pallidum (2*x*)	NJ17-1	0.24	0.01	0.03	0.10	0.00	0.40	0.05	0.07	1.30	0.01	0.10	0.01
	NJ17-4	0.71	0.03	0.08	0.13	0.00	5.29	0.27	0.66	2.41	0.03	0.14	0.02
	NJ17-5	0.73	0.01	0.08	1.87	0.00	2.65	0.13	0.30	10.29	0.06	0.18	0.00
	NJ17-6	0.74	0.04	0.13	1.01	0.00	0.89	0.23	0.21	13.26	0.11	0.18	0.02
V. elliottii (2*x*)	NJ17-7	2.81	0.11	0.18	0.00	0.00	12.06	0.41	1.15	0.02	0.00	0.61	0.05
	NJ17-8	2.51	0.10	0.22	0.01	0.00	5.38	0.19	0.76	0.00	0.01	0.64	0.06
V. darrowii (2*x*)	NC84-6A	0.12	0.01	0.02	0.23	0.04	0.87	0.10	0.14	3.87	0.06	0.08	0.01
	NJ88-05	0.35	0.04	0.04	0.09	3.57	1.19	0.66	0.32	4.30	0.07	0.22	0.05
	NJ88-06	0.23	0.06	0.07	0.28	1.62	0.42	0.27	0.22	4.17	0.02	0.07	0.03
	NJ88-07	0.25	0.04	0.03	0.07	0.33	1.06	0.13	0.19	0.55	0.01	0.12	0.04
	NJ88-10	0.29	0.11	0.04	0.70	0.34	0.18	0.25	0.03	3.15	0.05	0.08	0.06
	NJ88-11	0.10	0.03	0.03	0.33	0.02	0.06	0.06	0.03	2.15	0.04	0.03	0.03
	NJ88-12	0.12	0.01	0.02	0.13	1.15	1.07	0.13	0.43	3.12	0.03	0.06	0.02
	NJ88-13	0.18	0.10	0.06	0.11	0.73	0.25	0.07	0.14	2.37	0.05	0.05	0.08
	NJ88-14	0.85	0.50	0.17	0.28	0.94	0.98	0.52	0.51	6.49	0.12	0.25	0.22
V. tenellum (2*x*)	NJ88-31	0.98	0.09	0.10	1.06	1.71	1.05	0.40	0.87	13.17	0.23	0.21	0.06
	NC78-8	0.48	0.06	0.52	0.21	1.03	0.44	0.20	0.32	2.88	0.05	0.15	0.04
	NC83-9	0.44	0.15	0.49	0.09	0.24	0.10	0.11	0.05	0.30	0.00	0.11	0.10

Species	Clone	L-pen	L-rha	K-rut	K-glu	I-gal	I-glu	S-gal	S-glu	S-penA	S-penB	S-rha	Total
V. corymbosum (4*x*)	Bluecrop	0.03	0.00	0.12	0.08	0.06	0.07	0.50	0.26	0.03	0.01	0.00	19.79
	Duke	0.08	0.00	0.00	0.02	0.21	0.01	1.99	0.21	0.10	0.00	0.01	19.50
	Cara's Choice	0.05	0.00	0.74	0.17	0.02	0.02	0.69	0.38	0.05	0.01	0.00	31.40
V. corymbosum (2*x*)	NJOPB-1	0.07	0.00	0.00	0.00	0.02	0.00	0.99	0.22	0.05	0.00	0.00	9.98
	NJOPB-8	0.02	0.00	0.00	0.00	0.01	0.00	0.45	0.16	0.02	0.00	0.00	3.94
	NJOPB-15	0.02	0.00	0.00	0.00	0.02	0.00	0.18	0.02	0.00	0.00	0.00	4.62
	NJ85-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50
	NJ17-9	0.05	0.00	0.00	0.01	0.02	0.00	0.55	0.00	0.03	0.00	0.00	13.70
V. boreale (2*x*)	NJ88-29	0.00	0.00	0.73	0.15	0.02	0.03	0.51	1.23	0.01	0.02	0.00	18.26
	NJ88-30	0.01	0.00	0.06	0.01	0.03	0.03	0.53	1.22	0.01	0.02	0.00	10.93
V. pallidum (2*x*)	NJ17-1	0.01	0.02	0.00	0.00	0.00	0.00	0.24	0.09	0.01	0.00	0.03	2.72
	NJ17-4	0.01	0.01	0.00	0.00	0.02	0.00	0.20	0.16	0.00	0.00	0.02	10.17
	NJ17-5	0.02	0.16	0.00	0.01	0.00	0.00	0.15	0.00	0.01	0.00	0.01	16.67
	NJ17-6	0.02	0.10	0.00	0.02	0.00	0.01	0.18	0.05	0.01	0.00	0.08	17.31
V. elliottii (2*x*)	NJ17-7	0.03	0.00	0.00	0.19	0.02	0.01	0.77	0.19	0.02	0.00	0.00	18.62
	NJ17-8	0.03	0.00	0.02	0.13	0.02	0.00	1.10	0.11	0.03	0.00	0.00	11.31
V. darrowi (2*x*)	NC84-6A	0.01	0.06	0.00	0.01	0.05	0.02	0.17	0.14	0.01	0.00	0.18	6.18
	NJ88-05	0.01	0.03	0.07	0.01	0.02	0.01	0.42	0.34	0.00	0.00	0.03	11.84
	NJ88-06	0.02	0.05	0.04	0.01	0.00	0.00	0.26	0.37	0.03	0.00	0.08	8.32
	NJ88-07	0.02	0.02	0.01	0.00	0.01	0.00	0.49	1.52	0.03	0.01	0.12	5.05
	NJ88-10	0.01	0.14	0.01	0.01	0.01	0.01	0.12	0.95	0.01	0.01	0.54	7.09
	NJ88-11	0.01	0.07	0.00	0.00	0.00	0.00	0.09	0.58	0.01	0.01	0.29	3.97
	NJ88-12	0.01	0.05	0.04	0.00	0.17	0.10	0.38	0.70	0.01	0.00	0.24	7.98
	NJ88-13	0.02	0.03	0.02	0.00	0.01	0.01	0.33	1.02	0.05	0.01	0.08	5.78
	NJ88-14	0.03	0.04	0.02	0.01	0.02	0.01	0.51	0.93	0.05	0.01	0.04	13.52
V. tenellum (2*x*)	NJ88-31	0.01	0.13	0.05	0.02	0.01	0.01	0.24	0.45	0.00	0.00	0.16	21.03
	NC78-8	0.10	0.04	0.04	0.01	0.00	0.00	0.39	0.67	0.02	0.02	0.13	7.79
	NC83-9	0.02	0.00	0.01	0.00	0.00	0.00	0.37	1.49	0.01	0.06	0.03	4.17

M = myricetin; Q = quercetin; L = laricitrin; K = kaempferol; I = isorhamnetin; S = syringetin; gal = galactoside; glu = glucoside; pen = pentoside; rha = rhamnoside; rut = rutinoside; glu-ace = glucoside acetate.

Supplemental Table 4. Concentrations (mg/g fruit) of organic acids in different blueberry species.

Species	Clone	Qunic acid	Shikimic acid	Citric acid	Total
V. corymbosum (4*x*)	Bluecrop	0.422	0.035	4.982	5.44
	Duke	0.225	0.059	4.089	4.37
	Cara's Choice	0.000	0.042	6.695	6.74
V. corymbosum (2*x*)	NJOPB-1	0.000	0.068	7.316	7.38
	NJOPB-8	0.000	0.061	12.688	12.75
	NJOPB-15	0.000	0.077	13.708	13.78
	NJ85-1	0.000	0.058	12.298	12.36
	NJ17-9	4.734	0.002	4.104	8.84
V. boreale (2*x*)	NJ88-29	3.292	0.120	3.883	7.30
	NJ88-30	2.573	0.057	2.559	5.19
V. pallidum (2*x*)	NJ17-1	12.825	0.006	0.276	13.11
	NJ17-4	28.537	0.013	0.392	28.94
	NJ17-5	8.150	0.026	1.765	9.94
	NJ17-6	20.835	0.026	0.354	21.22
V. elliottii (2*x*)	NJ17-7	6.412	0.003	1.176	7.59
	NJ17-8	4.150	0.003	5.887	10.04
V. darrowii (2*x*)	NC84-6A	1.160	0.579	0.240	1.98
	NJ88-05	0.353	0.264	0.208	0.82
	NJ88-06	5.960	0.533	0.000	6.49
	NJ88-07	0.000	0.134	0.000	0.13
	NJ88-10	2.275	0.345	0.000	2.62
	NJ88-11	1.287	0.354	0.000	1.64
	NJ88-12	0.809	0.160	0.000	0.97
	NJ88-13	3.409	0.550	0.000	3.96
	NJ88-14	8.334	0.595	0.000	8.93
V. tenellum (2*x*)	NJ88-31	13.013	1.384	0.000	14.40
	NC78-8	7.326	1.057	0.000	8.38
	NC83-9	2.450	0.433	0.000	2.88

Supplemental Table 5. One-way ANOVA with LSD mean separation analysis on the PC scores of blueberry flavonoids.

Anthocyanins

ANOVA: $P < 0.001$ (PC1); $P = 0.005$ (PC2)

LSD:

PC1, *P* value	*V. corymbosum* (4*x*)	*V. corymbosum* (2*x*)	*V. boreale*	*V. pallidum*	*V. elliottii*	*V. darrowii*
V. corymbosum (2*x*)	0.003					
V. boreale	0.016	<0.001				
V. pallidum	0.039	0.204	<0.001			
V. elliottii	<0.001	0.173	<0.001	0.022		
V. darrowii	0.011	0.234	<0.001	0.752	0.021	
V. tenellum	0.005	0.974	<0.001	0.251	0.186	0.299

PC2, *P* value	*V. corymbosum* (4*x*)	*V. corymbosum* (2*x*)	*V. boreale*	*V. pallidum*	*V. elliottii*	*V. darrowii*
V. corymbosum (2*x*)	0.445					
V. boreale	0.66	0.831				
V. pallidum	0.008	0.029	0.045			
V. elliottii	0.256	0.069	0.156	0.001		
V. darrowii	0.026	0.107	0.14	0.289	0.003	
V. tenellum	0.738	0.267	0.462	0.003	0.397	0.01

Proanthocyanidins

ANOVA: $P < 0.001$ (PC1); $P = 0.065$ (PC2)

LSD:

PC1, *P* value	*V. corymbosum* (4*x*)	*V. corymbosum* (2*x*)	*V. boreale*	*V. pallidum*	*V. elliottii*	*V. darrowii*
V. corymbosum (2*x*)	0.778					
V. boreale	0.044	0.02				
V. pallidum	<0.001	<0.001	0.074			
V. elliottii	0.427	0.281	0.236	0.004		
V. darrowii	0.064	0.02	0.41	0.001	0.479	
V. tenellum	0.002	0.001	0.347	0.334	0.032	0.032

PC2, *P* value	*V. corymbosum* (4*x*)	*V. corymbosum* (2*x*)	*V. boreale*	*V. pallidum*	*V. elliottii*	*V. darrowii*
V. corymbosum (2*x*)	0.358					
V. boreale	0.017	0.071				
V. pallidum	0.471	0.085	0.003			
V. elliottii	0.993	0.411	0.028	0.53		
V. darrowii	0.296	0.996	0.047	0.046	0.366	
V. tenellum	0.186	0.607	0.186	0.04	0.232	0.553

Flavonols

ANOVA: $P < 0.001$ (PC1); $P = 0.001$ (PC2)

LSD:

PC1, *P* value	*V. corymbosum* (4*x*)	*V. corymbosum* (2*x*)	*V. boreale*	*V. pallidum*	*V. elliottii*	*V. darrowii*
V. corymbosum (2*x*)	0.001					
V. boreale	0.004	0.897				
V. pallidum	<0.001	0.001	0.008			
V. elliottii	0.106	0.133	0.157	<0.001		
V. darrowii	<0.001	0.001	0.009	0.63	<0.001	
V. tenellum	<0.001	0.02	0.06	0.357	0.002	0.53

PC2, *P* value	*V. corymbosum* (4*x*)	*V. corymbosum* (2*x*)	*V. boreale*	*V. pallidum*	*V. elliottii*	*V. darrowii*
V. corymbosum (2*x*)	0.058					
V. boreale	0.001	<0.001				
V. pallidum	0.192	0.486	<0.001			
V. elliottii	0.186	0.746	<0.001	0.804		
V. darrowii	0.951	0.022	<0.001	0.115	0.138	
V. tenellum	0.762	0.108	0.001	0.319	0.287	0.756