Article

Evaluating Wild Germplasm Introgression into Autotetraploid Blueberry

Diego Cabezas [1,†], Ivone de Bem Oliveira [2,†], Mia Acker [1], Paul Lyrene [1] and Patricio R. Munoz [1,*]

[1] Blueberry Breeding and Genomics Lab, Horticultural Sciences Department, University of Florida, Gainesville, FL 32611, USA; dcabezas@ufl.edu (D.C.); miaacker@ufl.edu (M.A.); lyrene@ufl.edu (P.L.)

[2] Hortifrut North America, Inc., Estero, FL 33928, USA; ioliveira@hortifrut.com

[*] Correspondence: p.munoz@ufl.edu; Tel.: +1-352-273-4837

[†] These authors have contributed equally to this work.

Abstract: Wild germplasm can be classified as the raw material essential for crop improvement. Introgression of wild germplasm is normally used in breeding to increase crop quality or resilience to evolving biotic and abiotic threats. Here, we explore the potential of introgressing *Vaccinium elliottii* into commercial blueberry germplasm. *Vaccinium elliottii* is a wild diploid blueberry species endemic to the southeastern United States that possesses highly desirable and economically important traits for blueberry breeding such as: short bloom to ripe period, adaptation to upland sandy soils, disease resistance, firmness, and pleasant flavor. To examine the potential of hybridization, we evaluated populations of interspecific hybrids across multiple stages of breeding (i.e., F_1, F_2, and backcrosses) in two crop seasons. We used our extensive pedigree data to generate breeding values for pre-breeding blueberry hybrid populations. Hybrid performance was evaluated considering fitness (i.e., plant vigor and plant height) in addition to evaluating six fruit-quality and marketable-related traits (i.e., size, firmness, acidity, soluble solids, weight, and yield). Overall, F_2 and backcrosses rapidly achieved market thresholds, presenting values not significantly different from commercial blueberry germplasm. Our results confirmed the potential of exploiting the high genetic variability contained in *V. elliottii* for interspecific hybridization. Additionally, we developed germplasm resources that can be further evaluated and utilized in the breeding process, advancing selections for fruit quality and environmental adaptation.

Keywords: blueberry; pre-breeding; hybridization; *V. elliottii*; fruit quality

1. Introduction

Global blueberry production has more than tripled since 1985 [1,2]. Thus, the importance of blueberry as a crop has increased significantly [3], requiring continuous improvements to meet market needs. Breeding provides several possibilities to address market requirements [4,5]. One of these possibilities is the identification and development of potential germplasm resources such as native wild germplasm, which is of great importance for crop breeding and pre-breeding as well as for global food security [4,6,7].

A successful example of the use of wild species as genetic resources for blueberry breeding is the development of southern highbush blueberry (SHB) cultivars [3,4,8]. According to Sharpe [9], the three main species that were used to create the SHB germplasm were Northern highbush blueberry (*V. corymbosum*), Darrow's blueberry (*V. darrowii*), and Rabbiteye blueberry (*V. virgatum*). Collectively, the introgression of wild germplasm generated SHB hybrids with low chilling hours, early ripening in berries, increase in fruit quality and productivity, improved tolerance to higher soil pH, and added resistance to multiple diseases [4,10,11]. Current breeding efforts focus on introgressing further disease resistance, abiotic stress adaptation, earlier ripening, improved fruit quality traits such as flavor and texture, and traits conducive to machine harvesting [2,11].

When choosing wild species to introgress into a crop's commercial germplasm, breeding programs should prioritize species with a broad spectrum of desirable traits that will complement and improve the existing germplasm. Elliott's Blueberry (*Vaccinium elliottii* Chapm.) exhibits several beneficial fruit quality traits such as preferable flavor and fragrance, minimal scarring, small seeds, and juiciness [4,11,12]. Additionally, *V. elliottii* displays economically important traits for blueberry production, such as tolerance to drought and high pH, as well as presenting early ripening [11]. The species also presents high firmness [12], which is a valuable trait for developing machine harvestable varieties, and a critical breeding focus to decrease overall cost of production [13].

Moreover, as crop pest pressures continue to increase, research into wild species' resistance must continue to meet the escalating pressures that threaten global food production. Notably, wild species can allow for introgression of disease-resistance traits as previously seen in wheat [14], rice [15], and corn [16]. In addition to improved fruit quality and abiotic stress resistance, *V. elliottii* can also function as a genetic resource for biotic stress resistance. In this sense, *V. elliottii* introgression could enhance the SHB germplasm with fungal resistance to *Phytophthora* root rot, blueberry stem canker, and stem blight, as well as entomological resistance to the sharp-nosed leaf hopper [11].

Several attempts to cross *V. elliottii* wild genotypes into SHB germplasm have been made in the past [12,17,18]. The crosses' success led to the development of highly aromatic, flavorful, and commercially successful cultivars, such as "Carteret," "Snowchaser," and "Kestrel" [12,19]. Norden et al. [12] and Dweikat et al. [17] also obtained vigorous fertile hybrids when crossing *V. elliottii* into SHB germplasm. These studies generated valuable resources for the introgression of genetic diversity and beneficial traits into breeding populations. Undeniably, wild germplasm has proven to be a valuable historical and modern tool for blueberry breeding programs. More research is required, however, to fully explore the benefits of *V. elliottii* as a genetic resource for breeding programs, given *V. elliottii*'s intraspecific diversity [8,11,12], and its ability to produce quality hybrids [12,17,18]. Wild introgression from a species such as *V. elliottii* can allow breeding programs to maintain a dynamic and diverse germplasm to meet evolving environmental and economic demands of the blueberry industry.

Here, we evaluate the introgression of *V. elliottii* into SHB germplasm. We evaluated progeny's fruit quality and overall vigor traits through multiple crosses between *V. elliottii* and SHB over two crop seasons. In order to maximize the production of fertile progeny, tetraploid clones of *V. elliottii* were used. The interspecifically generated populations under investigation comprised different stages of the early breeding process, including the F_1, F_2, and backcrosses. The information generated here can help guide genotype selection toward the development of new improved blueberry genotypes.

2. Materials and Methods

2.1. Plant Material

Five breeding stages of the hybridization process between *V. elliottii* and *V. corymbosum* were evaluated in this study (i.e., F_1, F_2, BC_1, wild *V. elliottii*, and SHB). In this paper, pseudo-F_2 and pseudo-backcrossing schemes were designed to circumvent the high inbreeding depression observed for blueberry. In summary, F_1 families were obtained in crosses between tetraploid *V. elliottii* and selected SHB genotypes, pseudo-F_2 families were the product of crossing two different F_1 hybrid genotypes, and pseudo-BC_1 families were the product of backcrossing F_1 hybrids to a non-related SHB genotype (Figure 1A). For comparison with parental genotypes, we also evaluated wild *V. elliottii* families obtained from open-pollinated crosses between wild diploid *V. elliottii* genotypes and SHB commercial germplasm families. To facilitate understanding, we will omit the expression "pseudo" when talking about each breeding stage.

Two breeding sets were evaluated in this study. The seeds used to compose the first population, hereafter called 2017 nursery, were generated by Norden [12], encompassing two F_2 families and one BC_1 hybrid family. For comparison, data from two southern

highbush (SHB) families and one *V. elliottii* family established in the same nursery were collected. The seeds used to compose the second population, hereafter called 2018 nursery, were generated by Lyrene [20], which included individuals from eight F_1 families, three F_2 families, and two BC_1 families. Additionally, for comparison, data from four SHB families and two *V. elliottii* families established in the same nursery were collected. Individuals from *V. elliottii* used in this study were obtained from unreduced gametes and colchicine-treated plants (Supplemental Tables S1 and S2). Tetraploidy of these individuals was confirmed by flow cytometry and visual analysis of the pollen (as described by [12,20]).

As described by Norden [12] and Lyrene [20], these populations were generated through controlled crosses that were performed by emasculating the female recipient before their flowers open, followed by manual application of the pollen to the stigma. Pollen samples were manually collected from selected male parents. Seeds obtained from each cross were germinated in individual 2 L pots. Plants from the 2017 nursery families were transplanted into a high-density blueberry nursery in June 2017, while plants from the 2018 nursery were transplanted into a second high-density blueberry nursery in June 2018. Both nurseries were located at University of Florida Research Station in Citra, Florida (29°24′24.18″ N, 82° 8′29.53″ W). For both nurseries, each family contained approximately 100 seedlings. Genotypes were spaced at 15 cm between plants and 40 cm between rows. Standard cultivation procedures were applied, including irrigation at a flow of 10–17 L per minute, administered three times a week for 1.5 h with an overhead sprinkler irrigation system as needed, to prevent drought stress. Soil was tilled and amended with pine bark before planting. Slow-release fertilizer was applied approximately monthly from April to June (in mg/L): 15 N, 5 P_2O_5, 10 K_2O (Blueberry Mix, Growers Fertilizer Corporation, Lake Alfred, FL, USA). Manual weed control was performed. At the beginning of the second year, coarse pine bark was applied as a mulch in each nursery to retain moisture and control weeds. The experimental unit of this study consisted of breeding stages (i.e., F_1, F_2, BC_1, *V. elliottii*, and SHB) sampled within each nursery that were distributed in a complete randomized design.

2.2. Phenotypic Evaluations

During two crop seasons (2017 and 2018 nurseries), information was collected for nine traits relating to fruit quality and plant fitness. The traits evaluated were yield (g), fruit weight (g), fruit diameter (mm), fruit firmness (g mm^{-1} of compression force), soluble solids content (°Brix), total titratable acids (TTA), pH, plant height (cm), and plant vigor (rated using a 1–5 scale). The last two traits were only evaluated in the 2018 nursery.

For the evaluation of fruit quality and market-related traits (i.e., weight, diameter, firmness, °Brix, TTA, and pH), 15 fully mature berries from 20 individual plants were sampled from each family. Each plant was flagged and tagged with a sample ID number. Fruit weight (g) was determined using an analytical balance (CP2245, Sartorius Corp., Bohemia, NY, USA). The same 15 berries were then evaluated for firmness (g mm^{-1} of compression force) and fruit size diameter (mm) using FirmTech II equipment (BioWorks Inc., Wamego, KS, USA). Sensory quality traits were evaluated using the juice obtained from the same 15 berries, thus soluble solids (°Brix) measures were obtained from pipetting 1 mL of juice onto a digital pocket refractometer (Atago, Inc., Bellevue, WA, USA), and total titratable acids were determined using an automatic titrator (Mettler-Toledo, DL 115, Inc., Columbus, OH, USA). The pH was measured using a glass electrode in the remaining juice (Mettler-Toledo, DL 115, Inc., Columbus, OH, USA). Plant height was measured in the field with a ruler as the distance between the base of the plant to top of the highest branch. Vigor was evaluated using a 1–5 rating scale, where 1 represented low vegetative growth and 5 represented high vegetative growth.

Models

One-step single-trait Bayesian linear mixed models were used to obtain the breeding values for each evaluated individual, as follows

$$y = \mu + Xn + Z_1b + Z_2nxb + Z_3g + e, \tag{1}$$

where y is a vector of the phenotypic values of the trait being analyzed, μ is the population's overall mean, n is the fixed effect of nursery, b is the random effect of the ith breeding stage, nxb is the random effect of the nursery by breeding stage interaction $\sim N\left(0, I\sigma^2_{nxb}\right)$, g is the random effect of genotype $\sim N\left(0, A\sigma^2_a\right)$, where A is the population's pedigree-based relationship matrix, and e is the random residual effect $\sim N\left(0, I\sigma^2_e\right)$. Genotype effects were considered nested within nursery, since each nursery was constituted by different breeding populations containing individuals for each breeding stage. For traits measured in a single nursery, the same Equation (1) was used without considering the nursery terms. The variance components for each random variable were the additive $\left(\sigma^2_a\right)$, breeding stage $\left(\sigma^2_b\right)$, nursery-by-breeding stage interaction $\left(\sigma^2_{nxb}\right)$, and residual $\left(\sigma^2_e\right)$. X, Z_1, Z_2, and Z_3 were incidence matrices for nursery, breeding stage, nursery-by-breeding stage interaction, and genotype, respectively. A pseudo broad-sense heritability for each trait was obtained considering the ratio of the sum of the additive and breeding stage variances and the total phenotypic variance, and narrow-sense heritability was estimated considering the ratio between the additive variance component and the total phenotypic variance.

Pedigree information

A historical and extensive pedigree data set that contained information from 11,866 entries was used to obtain the relationship matrix for the individuals that composed this study. Pedigree information was obtained by combining data from NCGR–Corvallis Vaccinium Catalog [21], the Brooks and Olmo Register of Fruit and Nut Varieties [22,23], and internal pedigree records from the University of Florida blueberry breeding program, including the pedigree of the individuals used in this study. The R package AGHmatrix v. 1.0.2 [24] was used to obtain the pedigree-based relationship matrix (A) considering the autotetraploid model without double reduction as in Kerr et al. [25].

Model implementation

All models were fitted using the package BGLR v. 1.0.5. [26]. Predictions were based on 80,000 Gibbs sampler iterations, considering 40,000 for burn-in, and a thinning of five. Parameters' convergency was evaluated to define the final values used in the analysis. A single-step regression approach was applied to perform all pedigree-BLUP (P-BLUP) analysis. Default hyper-parameters were used, as previously described by Perez and de los Campos [27]. The packages lsmeans [28] and multcomp [29] were used to perform post hoc tests, using the Bonferroni multiple comparisons test to counteract the problem of multiple comparisons considering $\alpha = 0.05$. Graphical visualizations were obtained using ggplot2 [30]. All analyses were implemented on the R platform [31].

3. Results

3.1. Population Genetics

Large genetic variation was observed within the evaluated populations (Figure 1). The first two principal components of the pedigree-based matrix (A) used in this study explained approximately 65% of the variance. Clear clusters were observed for F_2 families and for the independent SHB families used as benchmarks in the study. Given that the SHB families evaluated in this study were obtained from multiple biparental crosses, some of those crosses shared a higher relationship than others, therefore, one can see the grouping in the PCA based on the relationship shared among SHB as well as their relationship share with the other breeding stages. The individuals representing *V. elliottii*, F_1, BC_1, and the remaining SHB (i.e., individuals used in the crosses) presented higher genetic similarity and clustered together (Figure 1).

Table 1 summarizes the posterior means of the genetic parameters. All traits displayed genetic variances significantly different than zero, showing that selection can be performed both within and between breeding stages. For firmness, TTA, and plant vigor, the additive variance (σ_a^2) represented, respectively, 50%, 38%, and 43% of the total variance observed. Conversely, for diameter, weight, and yield, more than 50% of the total variance was explained by breeding stage. Broad sense heritability results (h^2) were high (>0.70) for all traits, apart from plant height that presented $h^2 = 0.53$. Considering all traits, narrow-sense heritability values varied between 0.14 and 0.50, for yield and fruit firmness, respectively.
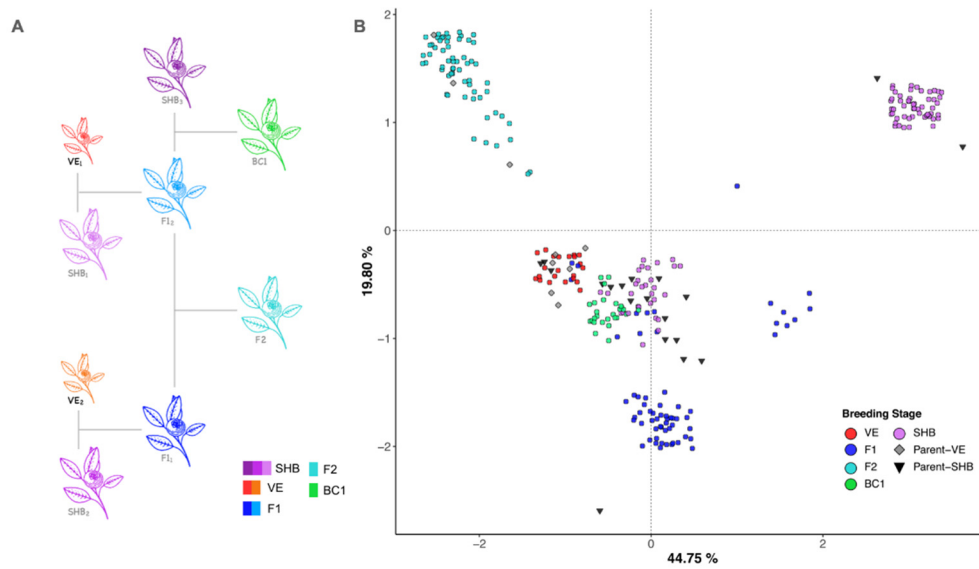

Figure 1. Description of the crosses and genetic variance observed for five stages of blueberry breeding. (**A**) Heredogram depicting how the families used in this study were obtained, *V. elliottii* introgressions are represented in smaller scale and (**B**) Percentage of the variance observed for the first and second principal components for the analysis of the relationship matrix considering the *V. elliottii* (VE), F_1, F_2, BC_1, and Southern highbush (SHB) families. The Pedigree-based matrix was computed using the AGHmatrix package [24] considering Kerr et al. [25] methodology.

Table 1. Genetic parameters and standard deviations estimated for nine fruit quality- and plant fitness-related traits.

Trait	σ_a^2	σ_b^2	σ_e^2	h^2	h_a^2*
Fruit firmness (g mm^{-1})	1118.55 (±345.90)	357.22 (±209.13)	768.34 (±202.15)	0.66	0.50
Fruit diameter (mm)	1.86 (±0.44)	9.91 (±6.01)	1.77 (±0.28)	0.87	0.14
Fruit weight (g)	0.14 (±0.05)	0.33 (±0.22)	0.17 (±0.03)	0.74	0.22
Total yield (g)	7080.91 (±2816.68)	30494.15 (±19,257.27)	14211.86 (±2282.682)	0.73	0.14
°Brix	2.03 (±0.69)	2.98 (±1.90)	1.47 (±0.40)	0.77	0.31
pH	0.05 (±0.01)	0.04 (±0.03)	0.03 (±0.01)	0.77	0.41
TTA	0.05 (±0.01)	0.04 (±0.02)	0.04 (±0.01)	0.70	0.38
Plant height (cm)	122.30 (±38.86)	143.15 (±95.22)	235.30 (±22.27)	0.53	0.24
Plant vigor (1–5 scale)	1.04 (±0.27)	0.69 (±0.46)	0.67 (±0.14)	0.72	0.43

*σ_a^2: additive variance; σ_b^2: breeding stages variance; σ_e^2: residual variance; h^2: broad-sense heritability; and h_a^2: narrow-sense heritability.

3.2. Hybrid Performance

To assess the performance of hybrid families, nine plant fitness-, fruit quality-, and marketable-related traits were evaluated and compared with the results obtained for commercial germplasm grown in the same conditions as the hybrids (i.e., SHB). Results were also compared with the performance of the wild relative, to confirm improvement towards commercial requirements. Commercial thresholds used by the University of Florida Blueberry breeding program were shown in the plots to facilitate understanding, when existing.

Hybrid performance was above commercial requirements for fruit diameter, fruit weight, and fruit firmness (Figure 2). Backcrossed families did not significantly differ from SHB germplasm regarding fruit diameter (Figure 2A,B) and fruit weight (Figure 2C). No significant differences were observed for fruit firmness between any of the breeding stages compared (Figure 2C), showing that requirements for size, weight, and firmness can be rapidly achieved after hybridization.
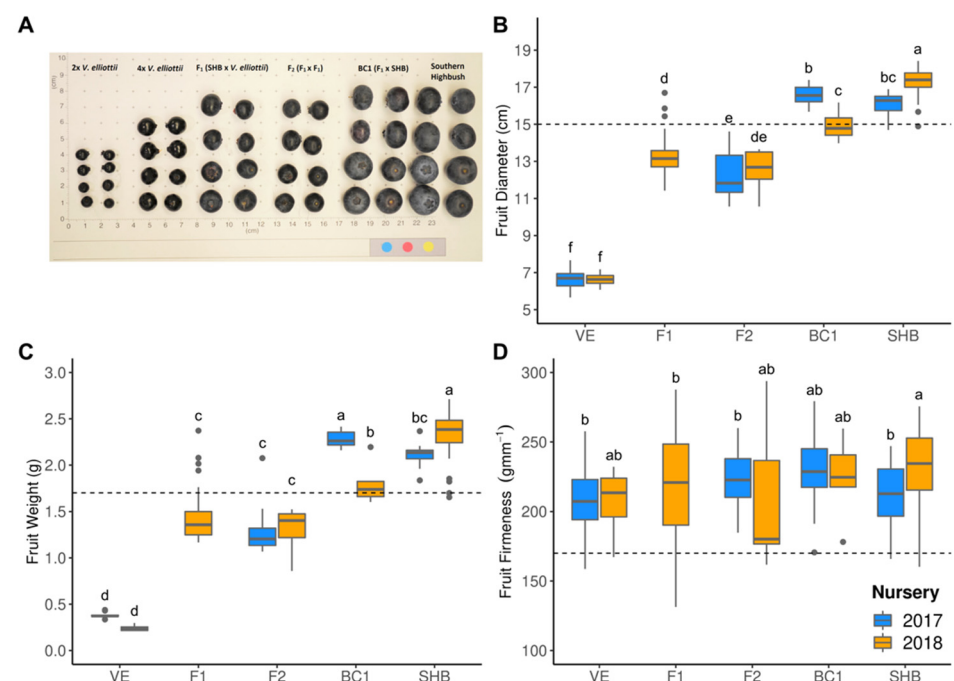

Figure 2. Fruit size, fruit weight, and fruit firmness observed for five stages of blueberry breeding. Fruit quality-related traits evaluated in five stages of blueberry breeding: *V. elliottii* (VE), F_1, F_2, BC_1, and Southern highbush (SHB) families. (**A**) Berry size and color, (**B**) Breeding values obtained for fruit diameter, (**C**) Breeding values obtained for fruit weight, and (**D**) Breeding values obtained for fruit firmness. Treatments with the same letters are not statistically different at alpha = 0.05.

V. elliottii presented a significantly higher amount of sugar (i.e., higher Brix) than both hybrids and SHB germplasm. Nonetheless, there was no significant difference for Brix between most of the hybrids and SHB families (Figure 3A), showing that commercial sugar requirements can be promptly obtained through hybridization. For *V. elliottii*, however, pH was significantly lower and TTA values were significantly higher when compared with the hybrids and SHB (Figure 3B,C). Moreover, some hybrid families (F_2 and BC_1) performed as well as or slightly better than SHB germplasm when considering pH and TTA values (Figure 3B,C).

Plant fitness was evaluated by measuring total yield, plant height, and plant vigor. With the exception of F_2, hybrids presented height values as good as or better than the ones observed for SHB germplasm (Figure S1). The same pattern was also observed for vigor (Figure S1), showing that interspecific hybridization between SHB and *V. elliottii* can produce healthy and robust progeny. However, plant yield can still be improved upon, and

promising results are expected, given BC_1 families presented yield measures significantly higher than the other hybrids for nursery 2017 and not significantly different from SHB families for the 2018 nursery (Figure 3D). Nevertheless, only one round of backcrossing was performed.
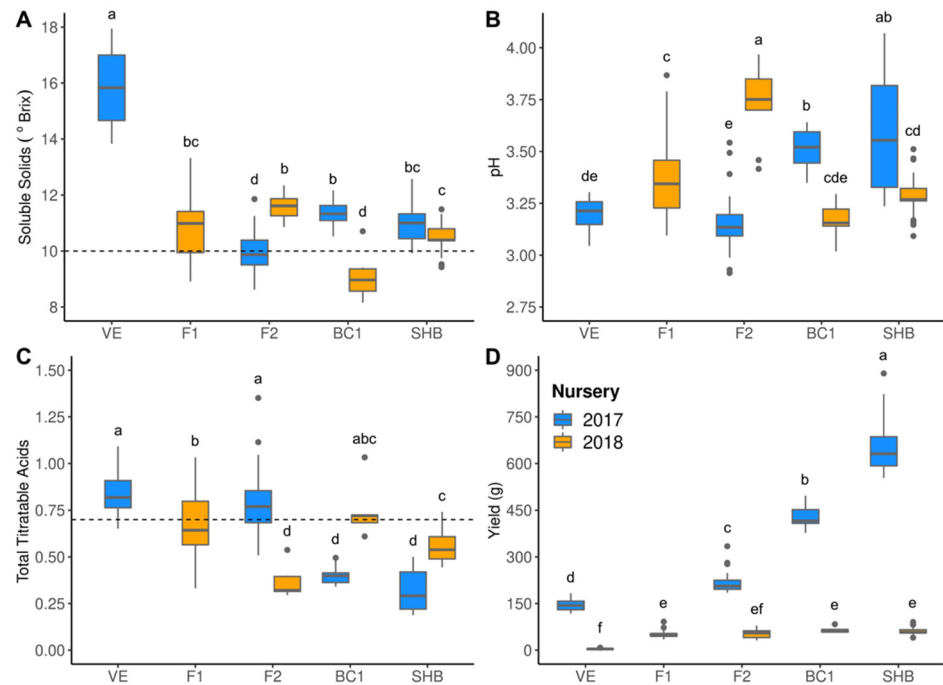

Figure 3. Soluble solids, pH, total titratable acids, and yield observed for five stages of blueberry breeding. Fruit quality-related traits evaluated in five stages of blueberry breeding: *V. elliottii* (VE), F_1, F_2, BC_1, and Southern highbush (SHB) families. (**A**) Breeding values obtained for soluble solids, (**B**) breeding values obtained for pH, (**C**) breeding values obtained for fruit total titratable acids (TTA), and (**D**) breeding values obtained for yield. Note the difference of other traits, with TTA being below the target dotted line. Treatments with the same letters are not statistically different at alpha = 0.05.

Figure 4 summarizes the performance of hybrids, wild germplasm, and SHB germplasm across crop seasons. Our results show that commercial requirements for fruit quality and marketable traits can be quickly achieved, given the BC_1 families performed as well as SHB families or slightly better for most of the traits evaluated. Hybrid individuals that presented satisfactory performance were visually evaluated for fruit color, wax coating, scar, and stem detachment. Individual genotypes that reached the quality standards of the blueberry breeding program were flagged and kept in the nurseries for future evaluations, factoring in the possibility of evaluating resistance to biotic and abiotic factors and usage in next stages of breeding.

Figure 4. Average values obtained by breeding stage for nine fruit quality- and plant fitness-related traits. Breeding values obtained across crop seasons for nine traits for five blueberry breeding stages: *V. elliottii* (VE), F_1, F_2, BC_1, and Southern highbush (SHB) families. Darker colors (either blue or orange) correspond to higher values. Treatments with the same letters are not statistically different at alpha = 0.05.

4. Discussion

4.1. Interspecific Hybridization in Blueberry

Interspecific hybridization is undeniably beneficial for modern agricultural advancements [7]. The use of wild species as raw material for breeding, both through classical and biotechnological techniques, can provide the genetic variability required to respond to environmental and demographical changes [6]. This process has contributed to the creation of commercial crops, such as sugarcane [32], banana [33], cotton [34], and bread wheat [35], as well as improving resistance and tolerance to biotic and abiotic factors. Prominent examples include bacterial blight resistance in rice [36], verticillium wilt resistance in canola [37], resistance to *Phytophthora infestans* and *Globodera pallida* in potatoes [38], and drought tolerance in wheat [39]. Introgression of traits from wild relatives has also improved fruit quality and marketable traits, such as fruit flavor, fruit size, and fruit texture in strawberry, tomatoes, and peppers [40,41].

Given blueberry's recent breeding and domestication history, beginning with Frederick Coville and Elizabeth White (early 1900s), blueberry can be considered a "modern crop" [2]. Since the beginning of domestication, hybridization has played a major role in blueberry improvement [42,43], starting with cross-pollination studies between highbush blueberry (*V. corymbosum* L.) and lowbush blueberry *(V. angustifolium* Ait.), generating a substantial amount of genetic and phenotypic variability [42]. Historical records show that blueberry breeding is extensively rooted in wild species introgression and interspecific hybridization [19,42–44]. The primary source of genetic variability for breeding is derived from three species: Highbush Blueberry (HB; i.e., *V. corymbosum*, 2n = 4x = 48), Lowbush Blueberry (LB; i.e., *V. angustifolium*, 2n = 4x = 48), and Rabbiteye Blueberry (i.e., *V. virgatum*, 2n = 6x = 72). However, wild species from the Cyanococcus section, such as *V. elliottii*, are considered a viable secondary source of variability [19,45] and have also been highly exploited in breeding [44,46,47].

Improvements obtained from interspecific hybridization in blueberry are not limited to the incorporation of novel traits, but also relate to the geographic expansion of the crop [3,19]. In 1948, Ralph H. Sharpe at the University of Florida developed the groundbreaking Southern Highbush Blueberry (SHB) that allowed for this expansion of blueberries into warmer climates [2]. The SHB blueberry encompasses cultivated hybrids that exhibit low cold hour requirements (i.e., chilling hours and hours of cold below 7 °C, e.g., ~300 h). This achievement resulted from the interspecific hybridization between HB, LB, Rabbiteye, and wild native diploid species [48,49], resulting in the development of hybrids with high vigor, resistance to diseases and abiotic stressors (i.e., heat and humidity), early maturation, vertical architecture, better fruit quality (e.g., firmness, color, and flavor), and traits consistent with commercial requirements [8,50]. Nevertheless, the global market is constantly requiring improvements and innovation in fruit quality, yield, and resources to

meet the escalating pressures that threaten production such as global warming and biotic and abiotic resistance/tolerance. Breeding provides several perspectives to address these demands. Most notably, introgression of germplasm from wild species has demonstrated the ability to provide the genetic variability for valuable trait improvements in blueberries.

4.2. The Use of Vaccinium elliottii as a Genetical Resource

Vaccinium elliottii is continually recognized as an important source of genetic variability to improve favorable traits for blueberry. Its ability to endure extreme environmental conditions, produce highly aromatic berries with a short bloom to ripe period, and maintain an upright vegetative architecture make this wild species a strong candidate for interspecific hybridization with SHB [12,44]. Successful released varieties such as 'Snowchaser', 'Kestrel', and 'Carteret' were produced by introgressing *V. elliottii* germplasm into SHB [12,19]. However, given the high variability shown within the species [12], more studies are required to verify *V. elliottii*'s potential as a genetic resource for blueberry breeding. Here, using a large breeding population composed of multiple hybrid stages, we show that promising results are expected when introgressing *V. elliottii* germplasm into SHB. Specifically, our study corroborated results from previous analysis, showing that market thresholds can be rapidly obtained in the hybrids [12], in addition to generating genetic variability using a wild species as genetic resource for breeding. Different from previous studies, such as Norden et al. [12] and Lyrene [20], we implemented a pedigree-based analysis using cross records and historic pedigrees for each hybrid, thus improving predictions for the families analyzed. To our knowledge, this is the first study characterizing such a large population of hybrids involving *V. elliottii*, as well as the first time that extensive pedigree data has been applied to generate breeding values to pre-breeding hybrid populations for blueberry.

We show that high genetic variability can be obtained when using *V. elliottii* as breeding resource. This is an important development for blueberry breeding, given previous studies illustrated declining heterozygosity of cultivated blueberry resulting from F. Coville's selective breeding founding event [19]. Both global food security and production are intrinsically connected to the wise use, conservation, and identification of biodiversity and wild germplasm genetic resources. These genetic resources can be classified as the raw material containing the genetic diversity necessary to build crops' resilience to evolving environmental and anthropogenic threats [6,7]. A broad-based foundation population, such as the one generated here, could be a new genetic resource for breeding [44]. This development broadens not only the genetic base of breeding populations but can also assist in developing successful commercial cultivars that can face escalating pressures threatening crop production. Given tetraploid intersectional hybrids between SHB and *V. elliottii* can be easily intercrossed with SHB genotypes [12], the segregating populations generated here can be further evaluated and used in different stages of breeding to capture positive and valuable traits.

One of the primary goals of breeding programs is the identification of superior genotypes when considering fruit quality. Current breeding efforts for blueberry focus on improving fruit firmness, fruit size, and flavor (i.e., sugar content, acidity, and volatiles) [2]. Fruit firmness is a key trait for blueberry breeding as it influences production/marketability from harvesting to transportability, shelf life, and consumer preference. Hand harvesting is normally associated with specialty crops, such as blueberry, which are destined for fresh market given the fragility of the fruit. Nevertheless, high costs and shortage of labor are directing the market to favor machine harvestable varieties [51]. Fruit firmness is a primary factor that allows for machine harvesting of blueberries, as firmer fruits better resist physical impacts [2,13,52]. Regarding postharvest behavior, firm fruits can withstand harvest handling and subsequent transportation better than soft fruits [53]. Even small differences in fruit firmness can affect shelf life [54]. High berry firmness is also associated with consumer liking. Gilbert et al. [55] has shown that firm fruits and crispness were generally preferred by consumers. Our results show that no significant differences for fruit

firmness were observed between hybrids and SHB genotypes. Given that *V. elliottii* presents high firmness, commercial thresholds were quickly obtained through hybridization, with F_1 individuals even displaying high values for firmness (Figure 2). Our outcomes corroborate results obtained in previous studies, showing that firmness values over 200 g mm^{-1} of compression force can be obtained in the first stages of hybridization [12].

Specific disadvantages can be observed when crossing SHB and *V. elliottii.* Among the major challenges is increasing fruit size and achieving commercial yield requirements [18]. For consumers' first purchases, appearance dictates preference. Gilbert et al. [55] illustrated that blueberry fruit size can additionally affect consumer preferences. The same is observed for other crop species, such as kiwi [56]. Lyrene and Sherman [18] and Lyrene [8] reported that berry size of BC_1 genotypes have the tendency to average below the normal commercial standards. Given the small berry size for *V. elliottii*, fruit size may be a difficult trait to recover when using the species for interspecific hybridization. Here, we show promising results in recovering fruit size when interbreeding *V. elliottii* and SHB. In our study, with only one round of backcrossing, genotypes presented diameter similar to the ones observed for SHB families across crop seasons (Figure 4). Our results probably differed from previous studies due to the selected *V. elliottii* stock. Given *V. elliottii*'s high diversity, one can expect resulting hybrids to display high phenotypic and genetic variability depending on the stock chosen. Similar results were observed, as expected, for fruit weight (Figures 2C and 4), since fruit size and fruit weight are positively and significantly correlated [5,57]. The effect of fruit weight and size as components of total yield for blueberry [58] can also be seen in the promising yield values observed for BC_1 and F_2 stages across crop season (Figure 4), considering that only one round of backcrossing was performed.

Beyond fruit weight, size, and yield, flavor is a fundamental component of fruit quality that affects consumer preference and marketability. Consumers are willing to pay more for better tasting varieties, justifying breeding for traits involved with flavor perception [59]. Flavor is a complex trait that is expensive to routinely evaluate in breeding programs [60]. However, sugar content and acidity greatly contribute to overall consumer liking and are easier and less expensive to phenotype than volatile profiles. Ferrão et al. [60] verified that overall liking of blueberry is positively correlated with high sugar content and negatively correlated with high acidity. Here, we show that we can achieve commercial requirements for total soluble solids and TTA in the first stage of hybridization (i.e., F_1). Our results show that some BC_1 and F_2 families presented sugar content and acidity equivalent to or better than the values presented by SHB individuals. Additionally, *V. elliottii* presented a significantly higher quantity of total sugars than SHB and all hybrid stages evaluated. However, *V. elliottii* also presented a significantly higher acidity and a lower pH than SHB and the hybrids. Similar results were also obtained by Norden et al. [12]. These results show that even though *V. elliottii* fruits presented more sugars, the perception of sweetness can be altered by the higher quantity of acids. *V. elliottii* germplasm could be further examined to screen for genotypes low in acidity (i.e., higher pH and lower TTA) with greater positive volatile and sugar contents, generating information for better stocks. Meeting these requirements could facilitate generation of hybrids with a better flavor perception and could further improve values for these traits.

5. Conclusions

By analyzing a large population comprising five different breeding stages across two seasons, here, we illustrate that the introgression of wild germplasm for blueberry breeding can generate a large amount of diversity and can generate promising and quick results towards fruit quality improvement. The use of *V. elliottii* through interspecific hybridization resulted in hybrids that quickly attained commercial benchmarks for most of the traits analyzed (e.g., fruit size, fruit weight, sugars, and acidity), and displayed height and vigor comparable to SHB germplasm. The segregating populations generated here can be used in different stages of breeding to introgress positive and valuable traits into commercial SHB germplasm. These findings also present encouraging implications for

the blueberry breeding industry, as future research can be performed to evaluate hybrid environmental adaptations and disease resistance, with the potential to corroborate the success of introgression of these traits from *V. elliottii* into SHB material.

References

1. FAOSTAT. Available online: http://www.fao.org/faostat/en/#data/QC (accessed on 18 May 2020).
2. Finn, C.E.; Hancock, J.F.; Olmstead, J.W.; Brazelton, D.M. Welcome to the party! Blueberry breeding mixes private and public with traditional and molecular to create a vibrant new cocktail. *Acta Hortic.* **2014**, *1017*, 51–62. [CrossRef]
3. Fang, Y.; Nunez, G.H.; da Silva, M.N.; Phillips, D.A.; Munoz, P.R. A Review for Southern Highbush Blueberry Alternative Production Systems. *Agronomy* **2020**, *10*, 1531. [CrossRef]
4. Lyrene, P.M. Low-chill highbush blueberries. *Fruit Var. J.* **1990**, *44*, 82–86.
5. Cellon, C.; Amadeu, R.R.; Olmstead, J.W.; Mattia, M.R.; Ferrao, L.F.V.; Munoz, P.R. Estimation of genetic parameters and prediction of breeding values in an autotetraploid blueberry breeding population with extensive pedigree data. *Euphytica* **2018**, *214*, 1–13. [CrossRef]
6. Esquinas-Alcázar, J. Protecting crop genetic diversity for food security: Political, ethical and technical challenges. *Nat. Rev. Genet.* **2005**, *6*, 946–953. [CrossRef] [PubMed]
7. Hajjar, R.; Hodgkin, T. The use of wild relatives in crop improvement: A survey of developments over the last 20 years. *Euphytica* **2007**, *156*, 1–13. [CrossRef]
8. Lyrene, P.M. Value of various taxa in breeding tetraploid blueberries in Florida. *Euphytica* **1997**, *94*, 15–22. [CrossRef]
9. Sharpe, R. Horticultural Development of Florida Blueberries. *Proc. Florida State Hort. Soc.* **1954**, *66*, 188–190.
10. Galletta, G.J. Blueberries and Cranberries. In *Advances in Fruit Breeding*; Purdue University Press: West Lafayette, IN, USA, 1975; pp. 154–196.
11. Retamales, J.B.; Hancock, J.F. *Blueberries—Jorge B Retamales, James F Hancock—Google Livros*, 2nd ed.; CABI: Boston, MA, USA, 2018; ISBN 9781780647265.
12. Norden, E.H.; Lyrene, P.M.; Chaparro, J.X. Ploidy, Fertility, and Phenotypes of F1 Hybrids between Tetraploid Highbush Blueberry Cultivars and Diploid *Vaccinium elliottii*. *HortScience* **2020**, *55*, 281–286. [CrossRef]
13. Cappai, F.; Benevenuto, J.; Ferrão, L.; Munoz, P. Molecular and Genetic Bases of Fruit Firmness Variation in Blueberry—A Review. *Agronomy* **2018**, *8*, 174. [CrossRef]
14. Fellers, J.P.; Matthews, A.; Fritz, A.K.; Rouse, M.N.; Grewal, S.; Hubbart-Edwards, S.; King, I.P.; King, J. Resistance to wheat rusts identified in wheat/*Amblyopyrum muticum* chromosome introgressions. *Crop Sci.* **2020**, 1957–1964. [CrossRef]
15. Brar, D.S.; Khush, G.S. Alien introgression in rice. *Plant Mol. Biol.* **1997**, *35*, 35–47. [CrossRef]
16. Wang, L.; Yang, A.; He, C.; Qu, M.; Zhang, J. Creation of new maize germplasm using alien introgression from *Zea mays* ssp. *mexicana*. *Euphytica* **2008**, *164*, 789–801. [CrossRef]
17. Dweikat, I.M.; Lyrene, P.M. Induced Tetraploidy in a *Vaccinium elliottii* Facilitates Crossing with Cultivated Highbush Blueberry. *J. Am. Soc. Hortic. Sci.* **1991**, *116*, 1063–1066. [CrossRef]

18. Lyrene, P.M.; Sherman, W.B. Mitotic Instability and 2n Gamete Production in *Vaccinium corymbosum* X *Vaccinium elliottii* Hybrids. *J. Am. Soc. Hortic. Sci.* **1983**, *108*, 339–342.

19. Brevis, P.A.; Bassil, N.V.; Ballington, J.R.; Hancock, J.F. Impact of Wide Hybridization on Highbush Blueberry Breeding. *J. Am. Soc. Hortic. Sci.* **2008**, *133*, 427–437. [CrossRef]

20. Lyrene, P. The Use of Vaccinium elliottii Chapmn. in Breeding Highbush Blueberry. In *Proceedings of the North American Blueberry Research and Extension Workers Conference*; Rutgers University: New Brunswick, NJ, USA, 2014.

21. NCGR-Corvallis Vaccinium Catalog: Blueberry Cultivars and Selections. Available online: http://www.xn--arsgrin-706c.gov/cor/catalogs/vactc.html (accessed on 1 March 2021).

22. Brooks, R.M.; Olmo, H.P. *Register of New Fruit and Nut Varieties*; University of California Press: Berkeley, CA, USA; Los Angeles, CA, USA, 1952.

23. Brooks, R.M.; Olmo, H.P. Register of New Fruit and Nut Varieties. *HortScience* **1997**, *32*, 785–805. [CrossRef]

24. Amadeu, R.R.; Cellon, C.; Olmstead, J.W.; Garcia, A.A.F.; Resende, M.F.R.; Muñoz, P.R. AGHmatrix: R Package to Construct Relationship Matrices for Autotetraploid and Diploid Species: A Blueberry Example. *Plant Genome* **2016**, *9*, 1–10. [CrossRef]

25. Kerr, R.J.; Li, L.; Tier, B.; Dutkowski, G.W.; McRae, T.A. Use of the numerator relationship matrix in genetic analysis of autopolyploid species. *Theor. Appl. Genet.* **2012**, *124*, 1271–1282. [CrossRef] [PubMed]

26. De los Campos, G.; Rodriguez, P.P. The Comprehensive R Archive Network. BGLR: Bayesian Generalized Linear Regression 2016. *Software User Manual*. Available online: https://cran.r-project.org/web/packages/BGLR/BGLR.pdf (accessed on 22 May 2020).

27. Pérez, P.; De Los Campos, G. Genome-wide regression and prediction with the BGLR statistical package. *Genetics* **2014**, *198*, 483–495. [CrossRef] [PubMed]

28. Lenth, R.V. Least-Squares Means: The {R} Package {lsmeans}. *J. Stat. Softw.* **2016**, *69*, 1–33. [CrossRef]

29. Hothorn, T.; Bretz, F.; Ag, N.P.; Westfall, P. Simultaneous Inference in General Parametric Models. *Biom. J.* **2008**, *50*, 346–363. [CrossRef]

30. Wickham, H. *ggplot2: Elegant Graphics for Data Analysis—Hadley Wickham—Google Books*; Springer: Berlin/Heidelberg, Germany, 2016; ISBN 9783319242774.

31. R: The R Project for Statistical Computing. Available online: https://www.r-project.org/index.html (accessed on 2 December 2020).

32. D'Hont, A. Unraveling the genome structure of polyploids using FISH and GISH; examples of sugarcane and banana. *Cytogenet. Genome Res.* **2005**, *109*, 27–33. [CrossRef] [PubMed]

33. D'hont, A.; Denoeud, F.; Aury, J.M.; Baurens, F.C.; Carreel, F.; Garsmeur, O.; Noel, B.; Bocs, S.; Droc, G.; Rouard, M.; et al. The banana (*Musa acuminata*) genome and the evolution of monocotyledonous plants. *Nature* **2012**, *488*, 213–217. [CrossRef] [PubMed]

34. Hu, Y.; Chen, J.; Fang, L.; Zhang, Z.; Ma, W.; Niu, Y.; Ju, L.; Deng, J.; Zhao, T.; Lian, J.; et al. *Gossypium barbadense* and *Gossypium hirsutum* genomes provide insights into the origin and evolution of allotetraploid cotton. *Nat. Genet.* **2019**, *51*, 739–748. [CrossRef] [PubMed]

35. Jiang, Y.; Yuan, Z.; Hu, H.; Ye, X.; Zheng, Z.; Wei, Y.; Zheng, Y.; Wang, Y.; Liu, C. Differentiating homoploid hybridization from ancestral subdivision in evaluating the origin of the D lineage in wheat. *New Phytol.* **2020**, *228*, 409–414. [CrossRef] [PubMed]

36. Yan, C.Q.; Qian, K.X.; Xue, G.P.; Wu, Z.C.; Chen, Y.L.; Yan, Q.S.; Zhang, X.Q.; Wu, P. Production of bacterial blight resistant lines from somatic hybridization between *Oryza saliva* L. and *Oryza meyeriana* L. *J. Zhejiang Univ. Sci.* **2004**, *5*, 1199–1205. [CrossRef]

37. Snowdon, R.J. Cytogenetics and genome analysis in *Brassica* crops. *Chromosom. Res.* **2007**, *15*, 85–95. [CrossRef]

38. Louwes, K.M.; Hoekstra, R.; Mattheij, W.M. Interspecific hybridization between the cultivated potato *Solanum tuberosum* subspecies *tuberosum* L. and the wild species *S. circaeifolium* subsp. circaeifolium Bitter exhibiting resistance to Phytophthora infestans (Mont.) de Bary and Globodera pallida (Stone) Behrens—2. Sexual hybrids. *Theor. Appl. Genet.* **1992**, *84*, 362–370. [CrossRef]

39. Mwadzingeni, L.; Figlan, S.; Shimelis, H.; Mondal, S.; Tsilo, T.J. Genetic resources and breeding methodologies for improving drought tolerance in wheat. *J. Crop Improv.* **2017**, *31*, 648–672. [CrossRef]

40. Gaston, A.; Osorio, S.; Denoyes, B.; Rothan, C. Applying the Solanaceae Strategies to Strawberry Crop Improvement. *Trends Plant Sci.* **2020**, *25*, 130–140. [CrossRef] [PubMed]

41. Moreno-Peris, E.; Cortés-Olmos, C.; Díez-Díaz, M.; González-Mas, M.C.; de Luis-Margarit, A.; Fita, A.; Rodríguez-Burruezo, A. Hybridization in Peppers (*Capsicum* spp.) to Improve the Volatile Composition in Fully Ripe Fruits: The Effects of Parent Combinations and Fruit Tissues. *Agronomy* **2020**, *10*, 751. [CrossRef]

42. Coville, F.V. Improving the wild blueberry. *Improv. Wild Blueberry* **1937**, 559–574.

43. Ballington, J.R. The deerberry [*Vaccinium starnineurn* L. *Vaccinium* Section *Polycodium* (Raf.) Sleumer]: A potential new small fruit crop. *J. Small Fruit Vitic.* **1996**, *3*, 21–28. [CrossRef]

44. Lyrene, P.M.; Olmstead, J.W. The Use of Inter-Sectional Hybrids in Blueberry Breeding. *Int. J. Fruit Sci.* **2012**, *12*, 269–275. [CrossRef]

45. Lyrene, P.M.; Ballington, J.R. Wide hybridization in vaccinium. *HortScience* **1986**, *21*, 52–57.

46. Hancock, J.F.; Lyrene, P.; Finn, C.E.; Vorsa, N.; Lobos, G.A. Blueberries and Cranberries. In *Temperate Fruit Crop Breeding*; Springer: Cham, Switzerland, 2008; p. 149.

47. Retamales, J.B.; Hancock, J.F. *Blueberries*; (Crop Production Science in Horticulture Book 21); Atherton, J., Ed.; CABI: Cambridge, UK, 2012; ISBN 1845938267.

48. Sharpe, R.H.; Darrow, G.M. Breeding blueberries for the Florida climate. *Proc. Florida State Hortic. Soc.* **1960**, *72*, 308–311.

49. Ballington, J.R. Germplasm resources available to meet future needs for blueberry cultivar improvement. *Fruit Var. J.* **1990**, *44*, 54–62.

50. Lyrene, P.M. Breeding Southern Highbush Blueberries in Florida. *Acta Hortic.* **2002**, 149–152. [CrossRef]

51. Gallardo, R.K.; Stafne, E.T.; DeVetter, L.W.; Zhang, Q.; Li, C.; Takeda, F.; Williamson, J.; Yang, W.Q.; Cline, W.O.; Beaudry, R.; et al. Blueberry Producers' Attitudes toward Harvest Mechanization for Fresh Market. *Horttechnology* **2018**, *28*, 10–16. [CrossRef]

52. Moggia, C.; Graell, J.; Lara, I.; González, G.; Lobos, G.A. Firmness at Harvest Impacts Postharvest Fruit Softening and Internal Browning Development in Mechanically Damaged and Non-damaged Highbush Blueberries (*Vaccinium corymbosum* L.). *Front. Plant Sci.* **2017**, *8*, 535. [CrossRef]

53. Hanson, E.J.; Beggs, J.L.; Beaudry, R.M. Applying Calcium Chloride Postharvest to Improve Highbush Blueberry Firmness. *HortScience* **1993**, *28*, 1033–1034. [CrossRef]

54. Beaudry, R.M.; Moggia, C.E.; Retamales, J.B.; Hancock, J.F. Quality of Ivanhoe and Bluecrop blueberry fruit transported by air and sea from Chile to North America. *HortScience* **1998**, *32*, 313–317.

55. Gilbert, J.L.; Guthart, M.J.; Gezan, S.A.; de Carvalho, M.P.; Schwieterman, M.L.; Colquhoun, T.A.; Bartoshuk, L.M.; Sims, C.A.; Clark, D.G.; Olmstead, J.W. Identifying Breeding Priorities for Blueberry Flavor Using Biochemical, Sensory, and Genotype by Environment Analyses. *PLoS ONE* **2015**, *10*, e0138494. [CrossRef] [PubMed]

56. Jaeger, S.R.; Harker, R.; Triggs, C.M.; Gunson, A.; Campbell, R.L.; Jackman, R.; Requejo-Jackman, C. Determining Consumer Purchase Intentions: The Importance of Dry Matter, Size, and Price of Kiwifruit. *J. Food Sci.* **2011**, *76*, S177–S184. [CrossRef]

57. Ferrão, L.F.V.; Benevenuto, J.; Oliveira, I.d.B.; Cellon, C.; Olmstead, J.; Kirst, M.; Resende, M.F.R.; Munoz, P. Insights Into the Genetic Basis of Blueberry Fruit-Related Traits Using Diploid and Polyploid Models in a GWAS Context. *Front. Ecol. Evol.* **2018**, *6*, 107. [CrossRef]

58. Milić, B.; Tarlanović, J.; Keserović, Z.; Magazin, N.; Miodragović, M.; Popara, G. Bioregulators can improve fruit size, yield and plant growth of northern highbush blueberry (*Vaccinium corymbosum* L.). *Sci. Hortic.* **2018**, *235*, 214–220. [CrossRef]

59. Sater, H.M.; Bizzio, L.N.; Tieman, D.M.; Muñoz, P.D. A Review of the Fruit Volatiles Found in Blueberry and Other *Vaccinium* Species. *J. Agric. Food Chem.* **2020**, *68*, 5777–5786. [CrossRef] [PubMed]

60. Ferrão, L.F.V.; Johnson, T.S.; Benevenuto, J.; Edger, P.P.; Colquhoun, T.A.; Munoz, P.R. Genome-wide association of volatiles reveals candidate loci for blueberry flavor. *New Phytol.* **2020**, *226*, 1725–1737. [CrossRef] [PubMed]