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Admixture Analysis Using Genotyping-by-Sequencing Reveals Genetic Relatedness and Parental Lineage Distribution in Highbush Blueberry Genotypes and Cross Derivatives

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Abstract

Blueberries (*Vaccinium* section Cyanococcus) are perennial shrubs widely cultivated for their edible fruits. In this study, we used admixture and genetic relatedness analysis of northern highbush (NHB, *V. corymbosum*) and southern highbush (SHB, *V. darrowii*) blueberry genotypes and F_2 progenies of the *V. corymbosum* × *V. darrowii* cross. Using genotyping-by-sequencing (GBS), we generated ~3.34 billion reads (75 bp). The GBS reads were aligned to the *Vaccinium corymbosum* cv. Draper v1.0 reference genome sequence, and ~2.8 million reads were successfully mapped. From the alignments, we identified 2,244,039 single nucleotide polymorphisms (SNPs), which were used for principal component, haplotype, and admixture analysis. PCA formed three main groups: 1) NHB cultivars, 2) SHB cultivars, and 3) BNJ16-5 progenies. The overall fixation index (F_{ST}) and nucleotide diversity for NHB and SHB, indicated wide genetic differentiation, and haplotype analysis revealed that SHB cultivars are more genetically diverse than NHB cultivars. The admixture analysis identified a mix of various lineages of parental genomic introgression. This study demonstrated the effectiveness of GBS-derived SNP markers in genetic and admixture analyses to reveal genetic relatedness and to examine parental lineages in blueberry, which may be useful for future breeding plans.

Keywords: *Vaccinium*, admixture analysis, genotyping-by-sequencing, genetic relatedness.

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1. Introduction

Blueberries (*Vaccinium corymbossum* section Cyanococcus) are perennial shrubs native to eastern North America but are widely cultivated for their edible fruit in several countries including Canada, Europe, Australia, New Zealand, Chile, and Argentina [1]. The *Vaccinium* genus includes important cultivated species such as blueberry and cranberry. The United States is the world's largest producer of blueberries [2].

Blueberry is a very high-value crop [3] that can thrive on acidic soils. Consumer demand for blueberries is at an all-time high, and hence its production around the world has quickly risen (http://www.fao.org/faostat, accessed November, 2020) primarily due to its health benefits [4]. Blueberries contain a large amount of antioxidant phenolic compounds, anthocyanins, flavonols, and phenolic acids. Several epidemiological studies associated regular small to moderate intake of blueberries with reduced risk of cardiovascular disease, cancer, obesity, and type 2 diabetes [5].

Blueberries were recently domesticated in the twentieth century [6], and breeding and genetic improvement started in 1909 [7] with the selection of clones from wild populations and cross-pollination leading to breeding and selection cycles. Initial blueberry improvement efforts mainly focused on developing cultivars adaptable to the broader climatic conditions, improving winter hardiness, fruit quality, and mechanical harvesting [1].

The *Vaccinium* genus includes approximately 450 species [8] and the blueberry germplasm include diploid (2n = 2x = 24), tetraploid (2n = 4x = 48) and hexaploid (2n = 2x = 72) species [9-13]. All of the species in *Vaccinium* sect. Cyanococcus have been shown to be highly self-sterile and are essentially, obligatory outcrossing [14-16]. Breeding largely at the tetraploid level has led to cultivars with earlier ripening berries, increased berry size, and higher fruit set [17]. Interspecific hybridization has played a crucial role in the development of cultivars with improved trait performance but has also led to complex relationships among blueberry species.

V. augustifolium is thought to be one of the first blueberry species used for fruit production in North America [18]. The highbush blueberry (*V. corymbosum* L.) is the major cultivated blueberry type in North America and the world [19]. Commercially grown cultivars of highbush contribute about two-thirds of total production, with the remaining one-third from the lowbush (*V. augustifolium*) species. *V. corymbosum* cultivars with high chilling requirements (> 800 chilling hours measured as accumulated hours of temperature <7°C) for the initiation of flowering are called northern highbush (NHB) cultivars [20], whereas those with lower chilling requirements are called southern highbush (SHB) cultivars. The commercial SHB cultivars were developed from NHB cultivars by the introgression of genes from *V. darrowii*, *V. virgatum*, and *V. tenellum*, of which *V. darrowii* being the largest contributor of genetic material [19]. There is evidence that *V. darrowii* had ancient evolutionary divergence from the taxon ancestral to section *Cyanococcus* [21] and *V. darrowii* may have played a greater role in evolution of this section as a sole survivor to the extant taxa [14]. In this process, today's cultivars represent a mixture of alleles from four different species, and such admixed populations complicate mapping endeavors of various loci governing complex traits. Hence, analyzing admixture of genetic makeup of individuals is extremely important for association mapping and population genetic analyses [22]. Presence of wide-ranging contribution of *V. corymbosum* genome in combination with lineages of *V. darrowii* make extant highbush cultivars. Admixture analysis using a high density SNPset that is distributed across all the chromosomes would help to reveal lineage sorting among cultivar germplasm.

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The use of high-density SNPs for genetic analysis research in blueberry has been limited until recent advancements in next-generation sequencing (NGS) technologies. Several studies have used old generation types of molecular markers in highbush blueberry for population structure analysis: random amplification of polymorphic DNA (RAPD), simple sequence repeats [6,19,23,24], expressed sequence tag (EST)-PCR markers [19,25,26], and retrotransposon-based sequence-specific amplification polymorphism markers [27]. However, such marker systems have several limitations and are not amenable for high throughput screening of larger populations.

Genotyping-by-sequencing (GBS) is a reduced representation method that utilizes NGS and can be used to resolve population structure for use in genome-wide association studies (GWAS). Furthermore, increased marker density across the chromosomes facilitates linkage disequilibrium (LD) analysis and haplotype calling [28]. SNPs can be valuable in marker-assisted selection (MAS) to facilitate the introgression of traits into domesticated genetic backgrounds, such as traits including aphid resistance from a diploid species *V. darrowii* [29] and unique fruit chemistry traits including fruit volatiles, organic acids, and flavonoids [30,31].

The objective of the present study was (1) to identify a large number of SNPs anchored to genome sequence, and (2) to utilize chromosome-specific SNP markers for admixture analysis and haplotype identification of the 99 blueberry accessions. The set included NHB and SHB cultivars, and F_1 parents and the F_2 progeny derived from an interspecific diploid cross of the NHB genotype *V. corymbosum* adapted to a temperate climate with the evergreen blueberry genotype *V. darrowii* adapted to a subtropical climate. We also sought to characterize the LD patterns and perform haplotype block analysis. The findings in this study will be useful in future GWAS, MAS, and genetic characterization of blueberry species.

2. Results

GBS with 99 Blueberry collection generated approximately 3.34 billion reads (334,600,452) of 75 bp in length (Supplementary Table S1). The average number of reads with tags per sample was 3.3 million, with a median of 3.4 million reads. Good barcoded tags with at least 3 read counts were used for SNP calling. The GBS reads were aligned to the *Vaccinium corymbosum* cv. Draper v1.0 reference genome sequence [32]. Details of the SNPs mapped to the longest 12 scaffold sequences of the Draper v1.0 genome are given in Supplementary Table S2. An average of 2.8 million reads with a tag per sample were successfully mapped to the reference genome, which corresponds to an overall mapping rate of 83% to the genome. From the alignments, we identified 2,244,039 SNPs with 99 selected accessions. The SNPs were filtered by using 1) read depth, DP<3, 2) minor allele frequency, MAF<0.05, and 3) call rate<0.9. After stringent SNP filtering, we obtained 92,048 SNPs distributed across the *V. corymbosum* reference genome, with an average of 5 SNPs per 1-kb genome length. The number of filtered SNPs mapped to the 12 scaffolds ranged from 6,191 SNPs for VACCDSCAFF12, to 8,994 for VACCDSCAFF2 (Table 1).

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Table 1. Scaffold-wise summary of the single nucleotide polymorphism (SNP) statistics from the genotyping-by-sequencing analysis across 99 accessions.

Chromosome	Chromosome length (bp)	Raw SNPs	Filtered SNPs (MAF<0.05; call rate <0.9; DP >3)	Average number of filtered SNPs per kb
Total Number of taxa		**99**	**99**	
VACCDSCAFF1	46,295,995	223,567	8,719	5
VACCDSCAFF2	44,818,276	188,522	8,994	5
VACCDSCAFF4	42,981,373	162,229	7,758	6
VACCDSCAFF6	42,795,824	190,595	7,090	6
VACCDSCAFF7	41,705,179	175,039	7,783	5
VACCDSCAFF11	40,122,599	211,211	8,194	5
VACCDSCAFF12	39,741,682	170,459	6,191	6
VACCDSCAFF13	39,652,356	177,072	7,654	5
VACCDSCAFF17	38,874,919	173,797	7,901	5
VACCDSCAFF20	37,996,905	187,631	7,116	5
VACCDSCAFF21	37,975,728	184,224	7,395	5
VACCDSCAFF22	37,315,645	199,693	7,253	5
Total Number of SNPs		**2,244,039**	**92,048**	

2.1. PCA

We used PCA to distinguish closely related individuals into groups and to understand the genetic relatedness of blueberry cultivars used in the present study. The PCA with first and second eigenvectors explained 10.8% of the total variance (Figure 1). From the results, three main groups were identified, including 1) NHB cultivars, 2) SHB cultivars, and 3) F_1 and F_2 progenies of the cross BNJ16-5. The F_1, F_2 plants, and parents were distinguished and all progenies were placed in between the parental lines. This suggested that the population can be explored for admixture analysis. The eigenvalues of the first 2 principal components for all blueberry accessions used in this analysis are given in Supplementary Table S3. The NHB group comprised 25 blueberry varieties: 2 accessions (NJOPB-8, and NJOPB-15) were diploid *V. corymbosum*. Most of the NHB types grouped closely in PCA, except 5 cultivars were diverse, along with the 2 diploid and wild *V. corymbosum* accessions at a distance from the main cluster (Figure 1). The NHB cultivars 'G751', 'Sweetheart', and 'Pink Lemonade' grouped close to the SHB group, thereby suggesting admixture. We do not know the pedigree backgrounds of 'G-751' and 'Sweetheart'. 'Pink Lemonade' is a hybrid derivative of *V. corymbosum* and rabbiteye blueberry (cross of NJ89-158-1 x Delite (*V. ashei*)).

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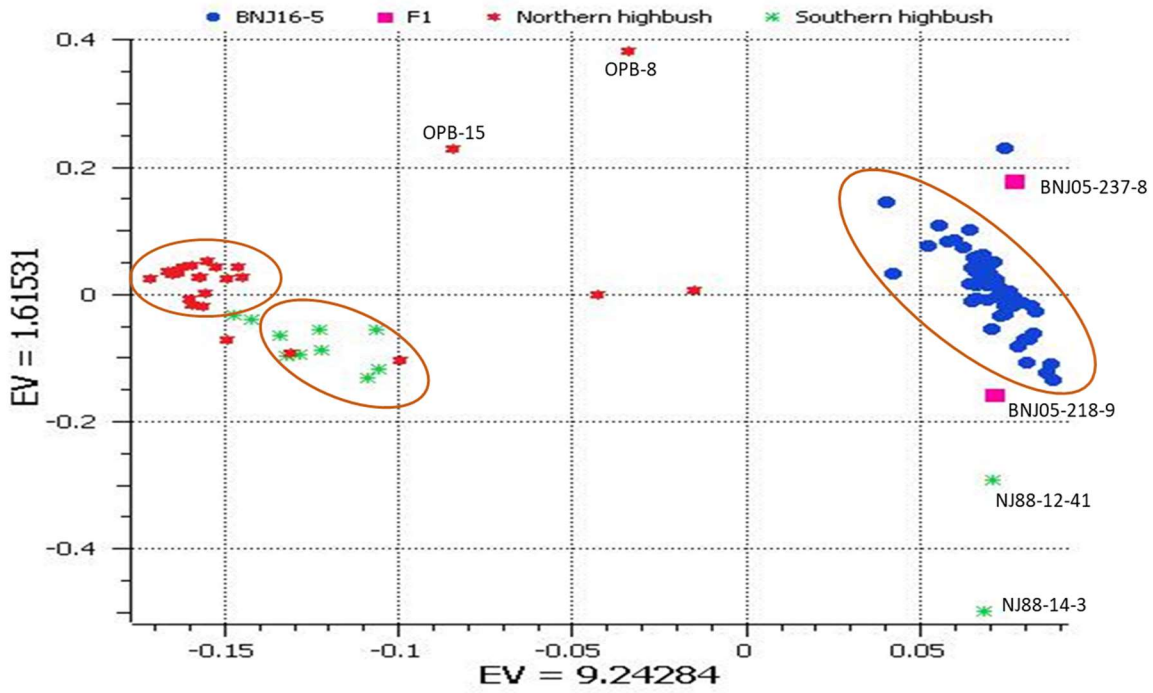

Figure 1. The first and second components of principal component analysis (PCA) for 99 blueberry accessions. See Supplementary Table S3 for eigenvalues for the respective positions of individual plants in this figure.

2.2 Admixture analysis

To resolve lineage sorting of tetraploid cultivated genomes, we used admixture analysis with the Landscape and Ecological Association model [33], which chooses cross-entropy criterion (prediction of a fraction of masked genotypes [matrix completion]). We iterated 6 runs (K) and chose K=3 because the value of K-3 showed a plateau of cross-entropy curve indicating a statistically significant lineage pattern (Figure 2A). From this analysis, the cultivars were admixed with 3 lineages (Figure 2B). Of note, 10 of 25 NHB cultivars had no admixture. 'Honey Creek', 'Blueray', 'Bonus', 'Aurora', 'Pioneer', 'Darrow', 'Patriot', 'Bluecrop', 'Duke', and 'Rancocas' had a single lineage and also were closely grouped (shown in orange color) in PCA. In contrast, SHB cultivars were highly admixed. The genetically diverse SHB group in this study consisted of 12 tetraploids and diploids of *V. elliotii* and *V. darrowii* (NJ88-12-41 and NJ88-14-3). NJ88-12-41 and NJ88-14-3 were the genotypes used as parents for the development of BNJ16-5 progenies. The admixture coefficients for SHB cultivars were in the range of 0.02 to 0.26, but those for the diploid *V. darrowii* species NJ88-14-3 and NJ88-12-41 were 0.93 and 0.95.

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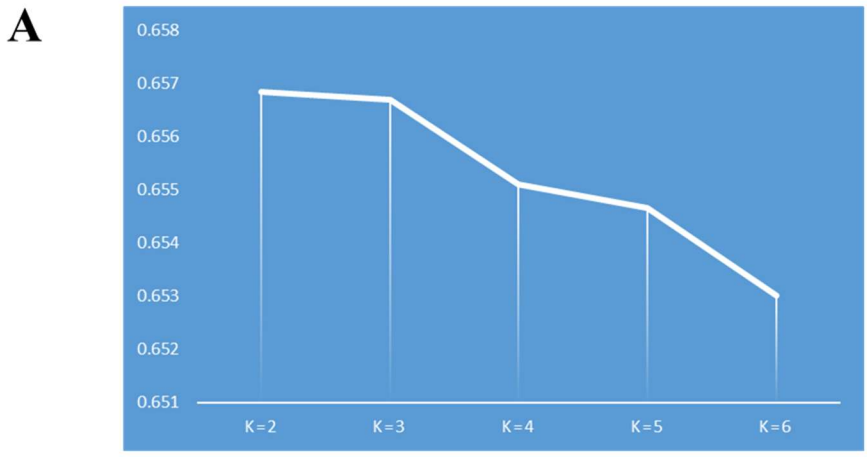

	K=2	K=3	K=4	K=5	K=6
min	0.654987	0.654943	0.652866	0.652187	0.65086
mean	0.656832	0.656693	0.655095	0.654664	0.653013
max	0.658459	0.658618	0.656798	0.658304	0.657414

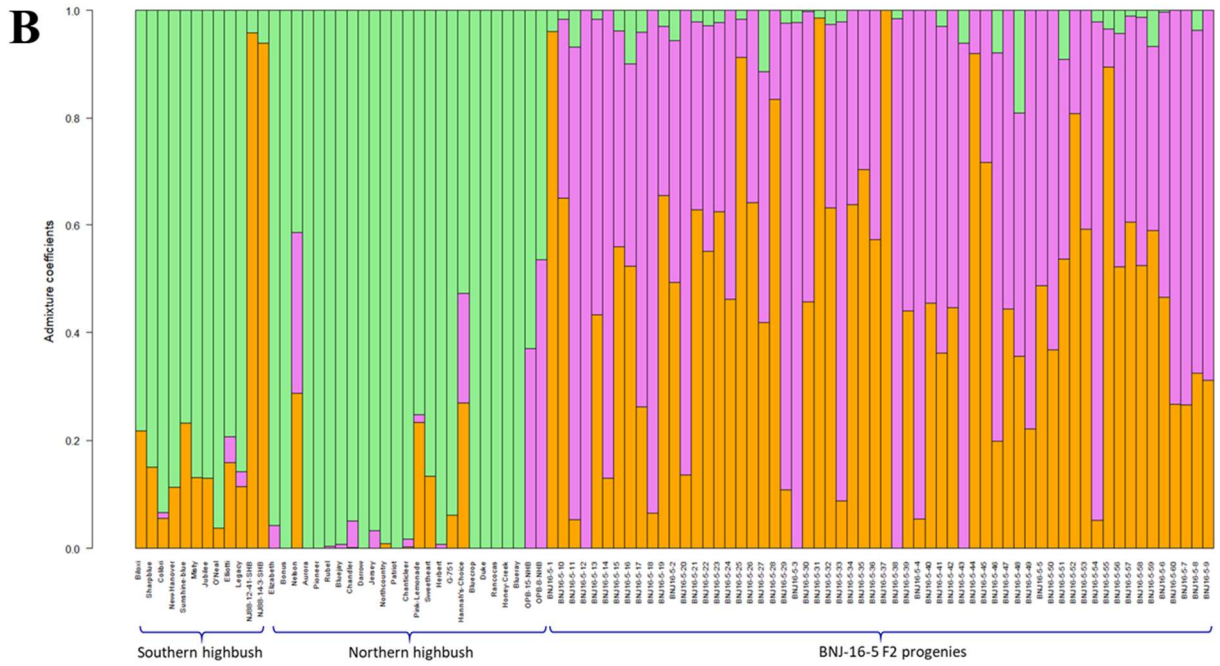

Figure 2. A. Detection of the number of clusters (K) based on the cross-entropy criterion by LEA software. The selected value in this analysis was K=2, for which the cross-entropy curve exhibits a plateau. B. Admixture analysis of the 97 blueberry accessions comprising northern and southern highbush types and F2 progenies. Each individual is represented by a vertical line depicting its membership into two clusters: orange bars are with a diploid *V. darrowii* background and magenta bars are with a diploid *V. corymbosum* background.

Admixture analysis to reveal the parental lineage is also significant in progenies of an interspecific cross in a breeding program and can be very helpful in the individual selection process. Hence, we

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explored progenies of an interspecific cross of *V. corymbosum* (NJOPB-8, and NJOPB-15) and *V. darrowii* (NJ88-14-3, and NJ88-12-41) to help understand the parental lineage distribution in the F_2 generation. The distribution of genomic proportions based on admixture coefficients of *V. darrowii* (represented by orange) and *V. corymbosum* (represented by magenta) across the progenies are shown in Figure 2B. Genetic lineage distribution by admixture analysis of the 60 F_2 progeny derived from a cross of two species *V. corymbosum* and *V. darrowii* was overall 50%, with wide variation for the admixture coefficients (from 0 to 1). For 8 of these progenies, the admixture coefficients were <0.2 lineage from *V. darrowii* and could be promising to select for lines with little introgression from wild materials. Of the progenies, BNJ16-5-4, BNJ16-5-11, BNJ16-5-18, and BNJ16-5-33 had < 10% lineage from *V. darrowii*. In contrast, BNJ16-5-25, BNJ16-5-44, and BNJ16-5-55 had < 10% lineage from *V. corymbosum*. Most of the cultivated NHB species are preferred for commercial production for desirable fruit and horticultural traits but due to high chilling requirements, are confined to colder environments. However, southern species require fewer chilling hours than do northern species, and SHB cultivars may have acquired heat tolerance from *V. darrowii*. Admixture analysis in this study identified progenies with <10% parental lineage from either species, which can be of great importance in selecting breeding blueberries with desirable traits, including chilling hour requirements, and fruit composition.

2.3 F_{ST} for characterizing selection footprints

We used F_{ST} analysis, estimating with 95% confidence intervals (CIs), to identify chromosomal regions that genetically distinguish NHB and SHB cultivars used in the analysis. The overall F_{ST} value between the NHB and SHB cultivars was 0.0473, which indicates wide genetic differentiation between these 2 highbush type cultivars. The F_{ST} distribution is shown in a Manhattan plot for all scaffolds showing regions with high F_{ST} markers (Figure 3). Pairwise F_{ST} values across all scaffolds are in Supplementary Table S4. A wide sweep on scaffold 11 was noted and was also characterized by decreased nucleotide diversity. From high pairwise F_{ST} indices, regions that underwent positive selection are noted in the Manhattan plot; they contributed to divergence of NHB from SHB cultivar groups (Figure 3) and could be important for genetic improvement.

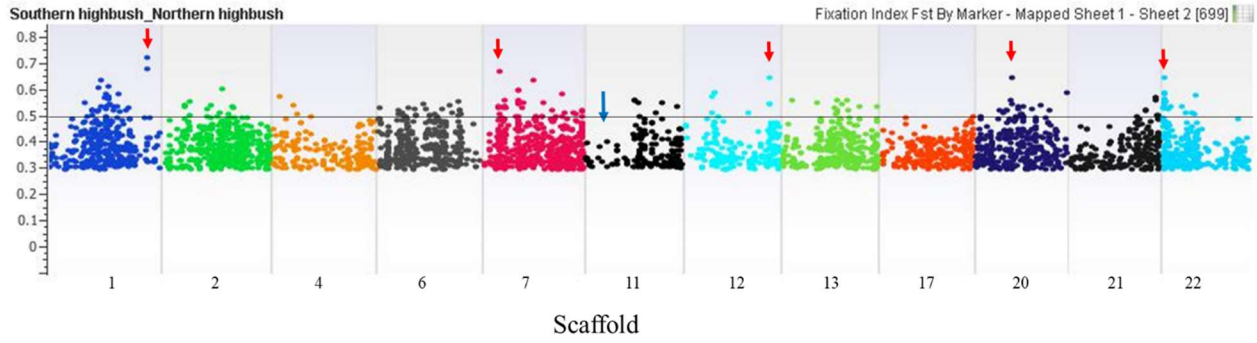

Figure 3. Genome-wide window-based pairwise fixation index F_{ST} values for northern highbush and southern highbush blueberry accessions across the 12 scaffolds. Red arrows indicate markers with high F_{ST} values. Blue arrow on part of scaffold 11 showed a distinct sweep. See Supplementary Table S4 for F_{ST} values.

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2.4 Nucleotide diversity

We estimated nucleotide diversity (π) across the 12 scaffolds to assess the patterns of diversity among all the 97 blueberry accessions used in this study (Figure 4). Scaffold-wise, nucleotide diversity of NHB cultivars ranged from 0.29 to 0.31 but from 0.34 to 0.35 for SHB cultivars (Supplementary Table S5).

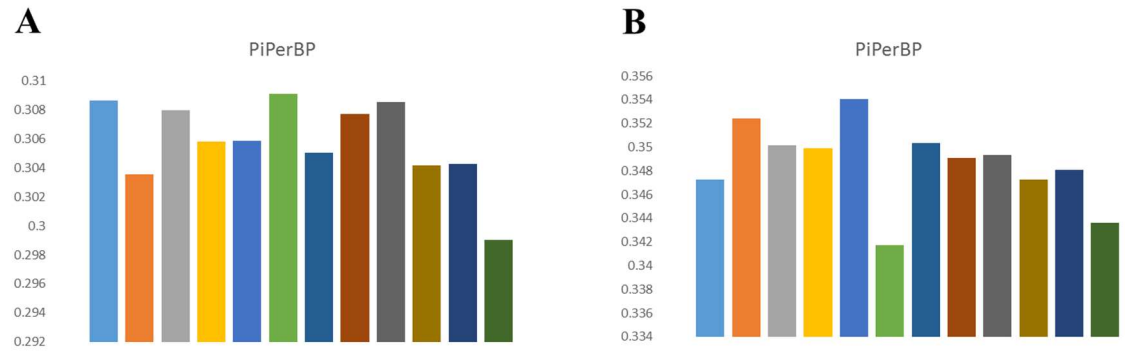

Figure 4. Scaffold-wise frequency spectrum for nucleotide diversity (π) in northern (A) and southern (B) highbush blueberry accessions.

2.5 LD and haplotype analysis

LD is the non-random association of alleles between different loci and is influenced by several factors including recombination rate, population structure, and genetic linkage. To understand the extent of genetic variation patterns, we performed an extensive LD block analysis of NHB and SHB cultivars. The largest LD blocks across the scaffolds were estimated (Table 2). In NHB genotypes, the largest LD block was 671.5 kb on scaffold 13, whereas the largest LD block in SHB genotypes was 425.4 kb. The size and number of haplotypes and their distribution are presented in Manhattan plots (Figure 5). For NHB genotypes, we identified 416 haplotype blocks with 993 SNPs in a range of 4 to 10 SNPs per haplotype but 209 haplotype blocks with 578 SNPs (in a range of 2 to 12 SNPs per haplotype) in SHB genotypes (Supplementary Table S6, S7). The average number of SNPs in the haplotype blocks was higher in SHB than NHB cultivars (2.73 vs 2.39). LD was estimated by using adjacent SNPs within a scaffold to reduce spurious associations. Highly significant LD blocks in the NHB and SHB genotypes and the BNJ16-5 population are shown in Figure 6 for comparison. Cross derivatives of the BNJ16-5 population showed a range of LD decays among the scaffolds, indicating variable recombination rates across scaffolds.

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Table 2. Scaffold-wise distribution of linkage disequilibrium (LD) blocks for northern and southern highbush cultivars used in this analysis

Chromosome	Largest LD block (kb)	
	Northern highbush blueberry	Southern highbush blueberry
VACCDSCAFF1	285	285
VACCDSCAFF2	425	425
VACCDSCAFF4	425	425
VACCDSCAFF6	518	418
VACCDSCAFF7	154	251
VACCDSCAFF11	255	329
VACCDSCAFF12	454	382
VACCDSCAFF13	672	261
VACCDSCAFF17	545	243
VACCDSCAFF20	247	255
VACCDSCAFF21	231	366
VACCDSCAFF22	189	194

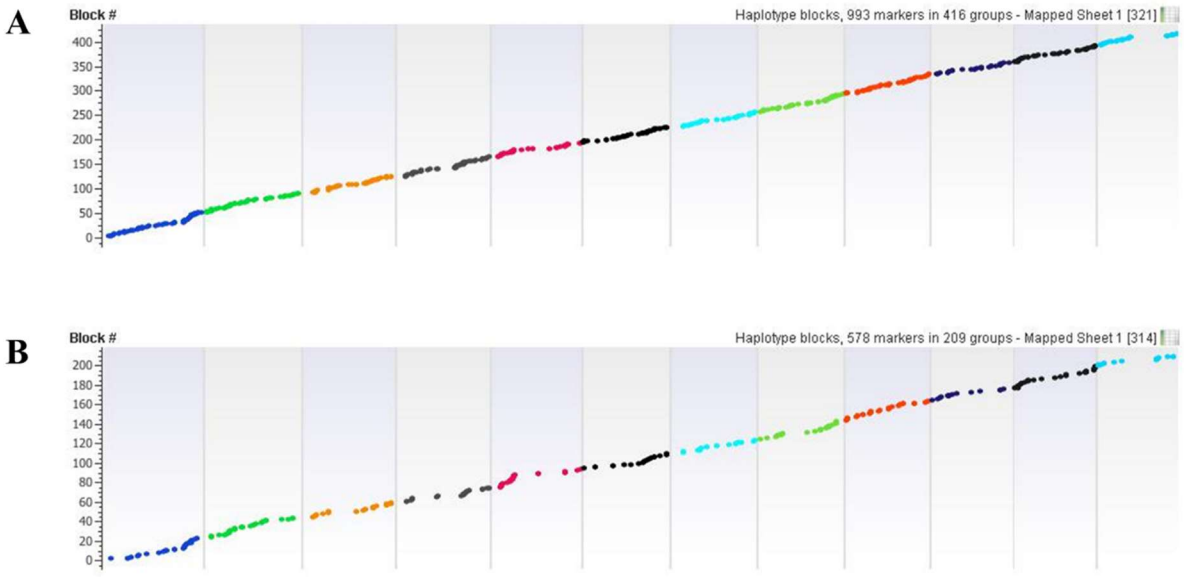

Figure 5. Haplotype distribution across the 12 scaffolds for (A) northern and (B) southern highbush blueberry accessions. See Supplementary Tables– S6 and S7 for detailed haplotype information.

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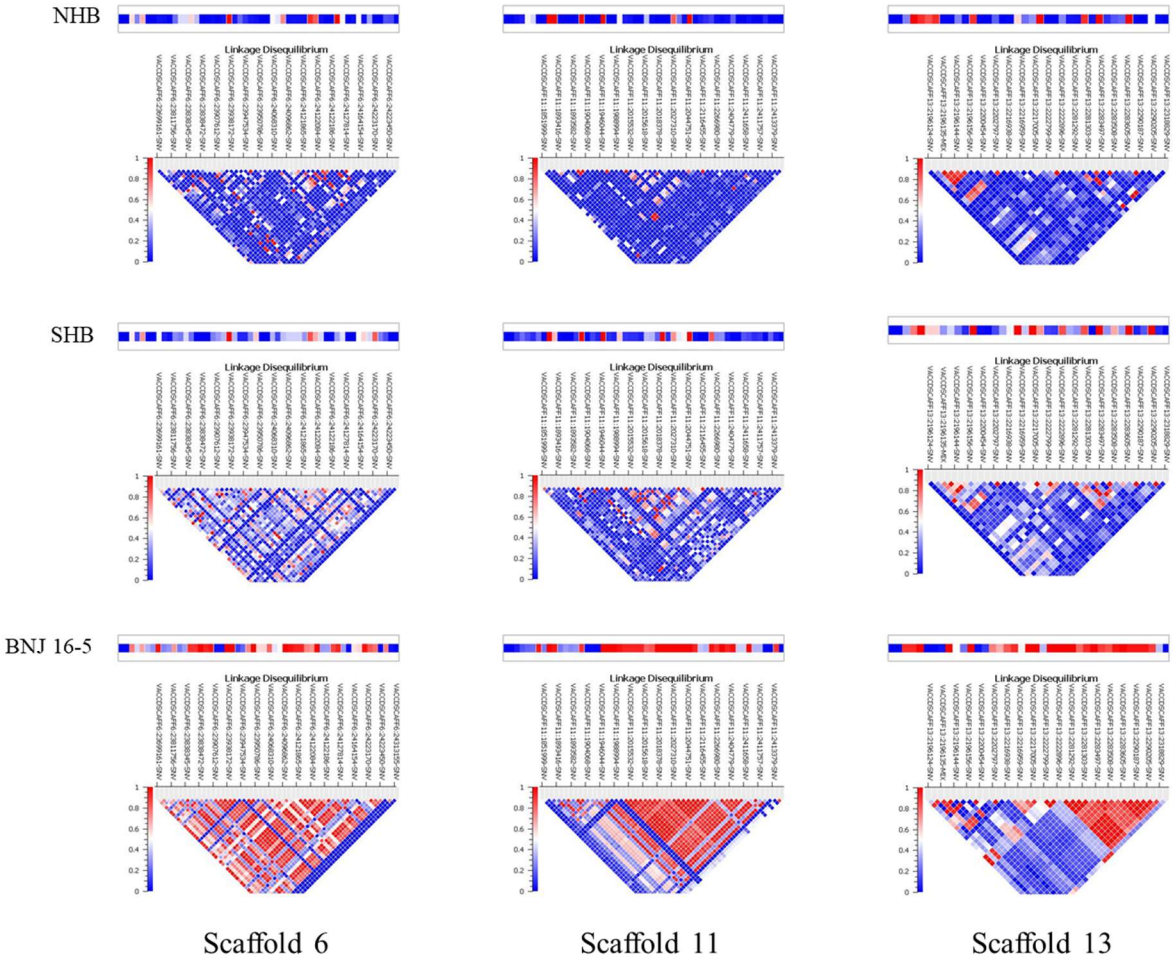

Figure 6. Comparative analysis of the linkage disequilibrium (LD) blocks across three scaffold regions in northern highbush (NHB), southern highbush (SHB) and BNJ 16-5 populations. Red-colored block indicates the highest LD and blue-colored blocks indicate the lowest LD values.

3. Discussion

Blueberry cultivars are derivatives of complex interspecific crosses involving four different species [25]. This situation warrants performing admixture analyses of progenies to understand lineage sorting primarily to classify them into NHB and SHB breeding material. This analysis will significantly reduce the time required in identifying progenies based on phenotype selections, the main selection criteria in traditional development programs. The PCA and admixture analysis in this study revealed a wide distribution of parental lineages with complex genetic makeup, which can be helpful for improvement.

To our knowledge, this is the first study in which the SNPs identified by using GBS were aligned to the tetraploid *Vaccinium corymbosum* cv. Draper v1.0 reference genome sequence [32], and made available for public use. In this study, we mapped 2.8 million reads to the reference genome, which corresponded to an overall 83% mapping to the genome. A stringent filtering with MAF =

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0.05 and 90% call rate yielded 92,048 SNPs. Furthermore, genome-wide LD blocks and haplotypes were characterized for comparing NHB and SHB cultivars.

The NHB blueberries are the most frequently cultivated species because of their high fruit quality and resistance to low temperatures [34]. NHB cultivars are reported to have significantly greater levels of anthocyanidins as compared with the other varieties. SHB cultivars were developed by further introgression of *V. darrowii* and other southern species. They combine nutritional benefits from the northern blueberries and low chilling requirements of the southern blueberries, which are adaptive to southern growing regions. From our PCA analysis, it was observed that three NHB cultivars were positioned close to the SHB group. Previous studies by Boches et al. [19] and Zong et al. [27] observed similar overlapping of the NHB and SHB cultivars, owing to the resemblance in the genetic backgrounds. Such shared germplasm sources of NHB and SHB can be of immense use for introgression of nutraceutically important traits as well as stress tolerance.

Blueberry domestication is relatively a recent event, initiated by Elizabeth White, a horticulturist for a private company in New Jersey (United States), and Frederick Coville, who was the chief botanist of the United States Department of Agriculture [35]. The germplasm selections from this project laid the foundation for modern plant breeding programs to develop improved varieties for commercial cultivation [36]. The useful characteristics in diploid section Cyanococcus species and the existence of key commercial cultivars at both the tetraploid and hexaploid levels helped blueberry breeders perform successful gene introgressions in tetraploid and hexaploid blueberries [37]. Such multiple interspecific crosses within *Vaccinium* species helped select for increased fruit size and yield and also expanded the geographic limits of highbush blueberry production. Highbush blueberries were domesticated because the domesticated plants produced bigger, higher quantity, and more uniform fruit than their wild ancestors [38-40]. These programs significantly widened the genetic diversity between the domesticated cultivars and diploid wild progenitors with a *V. corymbosum* background [23] and increased the genetic distance between diploid and polyploidy.

In this study, we resolved the genome-wide differences between cultivars and wild blueberry diploid *V. corymbosum* and *V. darrowii* accessions and their cross derivatives. In PCA, SHB and NHB cultivar groups were positioned adjacent to each other because the *V. corymbosum* genome was a common background. However, diploid wild accessions of *V. corymbosum* and *V. darrowii* were positioned further away from SHB and NHB cultivar groups. Our results indicated a wide genetic divergence between the blueberry cultivar groups and wild diploid accessions. Because the origin of 4x NHB from the progenitor diploid *V. corymbosum* is further supported by fruit chemistry [31], the observed variation may be sympatric, involving complex genetic processes underlying ploidy. Along similar lines, Wang et al. [31] found tetraploid (NHB) versus diploid *V. corymbosum* to be divergent for flavonol aglycone and glycosylation composition. Similar results were also described by Mengist et al. [41]. A significant proportion of genetic variation among the clones was documented in earlier studies [42].

Our analysis showed that 10 of 25 NHB cultivars had no admixture. Many of these cultivars are known to have 3.1% to 28% *V. augustifolium* background [25]. ‘Bluecrop’ and ‘Blueray’ were released around the 1950s and were known to have derived from the same parentage. The common background of these 2 cultivars was previously confirmed by Zong et al. [27]. The remaining NHB cultivars (‘Nelson’, ‘Hannah's Choice’, ‘Pink Lemonade’, ‘Sweetheart’, ‘G-751’, and the 2 diploid wild accessions NJOPB-8, and NJOPB-15) had lesser admixture coefficients in this study. Two

NHB cultivars, 'Nelson' and 'Hannah's Choice', did not cluster with the NHB cluster. 'Pink Lemonade', 'Sweetheart', and G 751 were clustered close to the SHB group on PCA. Current admixture analysis confirms that 'Bluecrop' is the parent of 'Nelson', as reported earlier [19,25]. Except for a few minor variations, clustering in the PCA was largely corroborated by the results of the admixture analysis.

Introgression of genes from undomesticated materials such as *V. darrowii* and *V. tenellum* into the SHB cultivars caused higher genetic diversity and expanded the geographic limits of the production of highbush blueberry (*V. ·corymbosum* L.) [43]. However, such introgressions also carried linkage drag, which could be very high in some SHB cultivars and affect agriculturally important traits. The hybrids between 4x *V. corymbosum* (CCCC) and diploid *V. darrowii* (DD) were most likely derived from 2*n* gametes from *V. darrowii* [37] giving a CCDD genomic composition in the hybrid. Preferential pairing in the polyploid of more homologous genomes would lead to linkage drag. Unfortunately, the unavailability of high-throughput screening methods may have restricted progeny selections. Admixture analyses using bi-allelic markers such as SNPs, which are spread all over the genome and are ubiquitous, can help reveal the proportion of lineages from the parental lines used in various development programs.

Fruit quality, tolerance to high soil pH and mineral soils, chilling requirement, and cold-hardiness have been identified as important traits in blueberry. Of these, low chilling hour requirements, and better fruit composition can be the 2 crucial traits in ideotype selection. Introgressions from wild blueberry species have been used to transfer desired traits into a *V. corymbosum* background. Thus, interspecific hybridizations between *V. corymbosum* and *V. darrowii* have produced blueberry cultivars with improved fruit quality, low chilling hours, and resistance to stress (biotic as well as abiotic) [44]. However, we must understand the parental lineage distribution in the interspecific hybridizations to minimize the unwanted linkage drag. From the F_2 progenies used in this study, BNJ16-5-4, BNJ16-5-11, BNJ16-5-18, and BNJ16-5-33 have < 10% parental lineage from *V. darrowii*. Similarly, BNJ16-5-25, BNJ16-5-44, and BNJ16-5-55 have < 10% parental lineage from *V. corymbosum*. These plants can be tested for their adaptability in climates with varying chilling hour conditions and may be promising lines for use in cultivar development. In this way, admixture analysis helps identify the exact proportions of the introgressions from each parental lineage, thereby helping in the selection process. It can further help improve the specific trait performance as desired in blueberry ideotype breeding.

4. Materials and methods

4.1 Plant materials

In this study, we used 99 blueberry accessions, including 25 NHB and 12 SHB cultivars. Of the highbush cultivars, 27 were from the inventory maintained at Delaware State University, and 6 were provided by Dr. Nicholi Vorsa (the Philip E. Marucci Center for Blueberry and Cranberry Research, Chatsworth, NJ, USA). Undomesticated diploid germplasm included *V. darrowii* grandparents (NJ88-14-3, NJ88-12-41), *V. corymbosum* grandparents (NJOPB-8, 15), F_1 progeny (BNJ05-237-8 [NJOPB-8 x NJ88-12-41], BNJ05-218-9 [NJ88-14-3 x NJOPB-15]), and 60 F_2 BNJ1-5 progeny (BNJ05-237-8 x BNJ05-218-9) of the cross *V. corymbosum* × *V. darrowii.* (Table 3). Note: BNJ05-237-8 was in *V. corymbosum* cytoplasm and BNJ05-218-9 in *V. darrowii* cytoplasm.

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Table 3. List of northern and southern blueberry cultivars used in the present work.

Category	Name of the variety/germplasm	PI number	Ploidy	Taxon	Pedigree information	Improvement status
Tetraploid northern highbush (23)	Elizabeth		4X	*Vc*	(Katharine x Jersey) x Scammel	
	Bonus	PI 666839	4X	*Vc*		
	Nelson	PI 618100	4X	*Vc*	Bluecrop × G 107	Cultivar
	Aurora		4X	*Vc*	Brigitta Blue × Elliott	
	Pioneer	PI 554815	4X	*Vc*	Brooks x Sooy	Cultivar
	Rubel	PI 554817	4X	*Vc*	Selection from wild *V. corymbosum* in NJ selected from the pine barrens of NJ	Cultivar
	Bluejay	PI 554846	4X	*Vc*	Berkeley x Michigan Highbush Sel. 241 (Pioneer x Taylor)	Cultivar
	Chandler	PI 657260	4X	*Vc*	Darrow x M-23	Cultivar
	Darrow	PI 618035	4X	*Vc*	F 72 × Bluecrop	Cultivar
	Jersey	PI 554897	4X	*Vc*	Rubel x Grover	Cultivar
	Northcountry	PI 554953	4X	*Vc x Va*	B6 (G65 x 'Ashworth' *V. corymbosum*) x R2P4 (open pollinated *V. corymbosum* x *V. angustifolium* hybrid)	Cultivar
	Patriot	PI 554843	4X	*Vc*	US 3 (Dixi x Mich LB-1) x Earliblue	Cultivar
	Chanticleer	PI 638765	4X	*Vc*	G-180 x MEUS 6620	Cultivar
	Pink Lemonade	PI 641330	4X	*Vc*	NJ89-158-1 x Delite (*V. ashei*)	Cultivar
	Sweetheart		4X	*Vc*	Need Pedigree	Cultivar
	Herbert	PI 554805	4X	*Vc*	Stanley (Katharine x Rubel) x GS-149 (Jersey x Pioneer)	Cultivar
	G 751		4X	*Vc*	Need Pedigree	Wild material
	Hannah's Choice	PI 657259	4X	*Vc*	G-136 x G-358	Cultivar
	Bluecrop	PI 554885	4X	*Vc*	GM-37 (Jersey x Pioneer) x CU-5 (Stanley x June)	Cultivar
	Duke	PI 554872	4X	*Vc*	G 100 (Ivanhoe x Earliblue) x 192-8 (E-30 x E-11)	Cultivar
	Rancocas	PI 554816	4X	*Vc*	394Y (Brooks x Russell) x Rubel	Cultivar
	Honey Creek		4X	*Vc*		

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	Blueray	PI 554887	4X	*Vc*	(Jersey x Pioneer) x (Stanley x June)	
Tetraploid southern highbush (9)	Biloxi	PI 618193	4X	*Vc*	Sharpblue x US 329 [US210 (US67 x US132) x FL 4-76 (Bluecrop x13-236)]	Cultivar
	Sharpblue	PI 554948	4X	*Vc*	*V. corymbosum* x *V. asheii* & *V. darrowi* (Fla 61-5 x Fla 62-4)tetraploid	Cultivar
	Colibri		4X	*Vc*		
	New Hanover		4X	*Vc*		
	Sunshine blue	PI 555316		*Vc x Va*	Avonblue OP	
	Misty	PI 555317	4X	*Vc*	Florida 67-I x Avonblue	
	Jubilee	PI 618195	4X	*Vc*	Sharpblue x MS60 [(Ashworth x Earliblue] x Bluecrop) x US-75]	Cultivar
	O'Neal	PI 554944	4X	*Vc*	Wolcott x Fla. 4-15 mainly *corymbosum*, some *angustifolium*, *ashei*, *darrowi*	Cultivar
	Legacy	PI 618164	4X	*Vc*	Elizabeth x (Fla. 4B x Bluecrop)	Cultivar
Vaccinium ellioti (1)	Elliotti	PI 657176	2X	*Ve*	Collected from the wild in Florida.	Wild material
Vaccinium darrowii (2)	NJ88-12-41		2X	*Vd*		Wild material
	NJ88-14-3		2X	*Vd*		Wild material
Vaccinium corymbosum (2)	NJOPB 8		2X	*Vc*		Wild material
	NJOPB 15		2X	*Vc*		Wild material
F_1 (2)	BNJ05-237-8			*Vc x Vd*		
	BNJ05-218-9			*Vd x Vc*		
F_2 (60)	BNJ16-5 population					

Vc, Vaccinium corymbosum; Vd, Vaccinium darrowii; Va, Vaccinium augustifolium; Ve, Vaccinium ellioti
*The number in the parenthesis indicates the total number of genotypes in the respective category/species

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4.2 DNA isolation

Leaf samples from young actively growing blueberry plants were collected in dry ice and stored at -80. About 100 mg leaf tissue was placed in a 2-ml round-bottom tube containing a single 5-mm stainless steel bead. The tubes containing the leaf samples were frozen in liquid nitrogen for 5 min and homogenized by using TissueLyser II (Qiagen, Germantown, MD, USA) at a frequency of 25 cycles/s, and two 3-min bursts. The homogenized leaf samples were stored at -80. The DNA extraction was performed with a commercially available Plant DNA extraction kit (DNeasy mini plant kit, Qiagen, Germantown, MD, USA) following the manufacturer's protocol with slight modifications. DNA was initially quantified by measuring absorbance at 260 nm by using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). To prepare the samples for GBS, DNA samples with better quality were quantified by using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and measured by using the Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and used for GBS library preparation. Sequencing was carried out at the Department of Biology, West Virginia State University, Institute, WA, USA.

4.3 GBS analysis

We genotyped 99 blueberry samples by using the GBS technology for variant identification and GWAS analysis. GBS was performed as described [45]. Genomic DNA was digested by using the *Ape*K1 restriction enzyme and ligated with barcoded adapters. The adapter-ligated library from each sample was pooled and amplified with Illumina sequencing primers. The quality and quantity of the GBS library was assessed by using Bioanalyzer 2100 (Thermo Fisher Scientific, Waltham, MA, USA) and Qubit 4 fluorimeter (Thermo Fisher Scientific, Waltham, MA, USA). The library was sequenced by using the NextSeq500 platform with paired-end sequencing chemistry. The resulting image files in bcl format were converted to FASTQ with 2x75 bp reads by using bcl2fastq (Illumina, San Diego, CA, USA). The GBS reads were de-multiplexed and variants were called by using a new workflow with GB-eaSy (https://github.com/dpwickland/GB-easy), which has an advantage of using paired-end reads from GBS data to call variants [46]. The resulting variant call file (vcf) was used for further downstream analysis.

4.4 Sequence alignment and SNP identification

Sequencing reads were aligned to the *Vaccinium corymbosum* cv. Draper (tetraploid) v1.0 genome sequence [32]. The assembled genome data were downloaded from GigaDB (http://gigadb.org/dataset/100537). The longest 12 scaffold sequences were used to align the sequencing reads. The mapped GBS reads were used to call SNPs by using GB-eaSy.

4.5 Principal component analysis (PCA)

The SNPs with minor allele frequency (MAF) ≥1% and missing data (call) rate ≤90% were used for analyses. For analyzing population structure, we used principal components, or eigenvectors, of PCA, and corresponding eigenvalues were estimated by using the EIGENSTRAT algorithm [47] with the SNP & Variation Suite (SVS v8.8.5; Golden Helix, Inc., Bozeman, MT, USA, *www.goldenhelix.com*).

4.6 Haplotype block analysis

For GBS data, we considered only SNPs successfully mapped to the whole-genome sequence draft, because knowing the physical location of SNPs helps prevent spurious LD and thereby calling

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unreliable haplotype blocks. Mapped SNPs were further filtered by call rate > 90%. Before studying LD decay, haplotype blocks were calculated for all markers by using the default settings in SVS v8.8.5. (Golden Helix, Inc., Bozeman, MT, USA, *www.goldenhelix.com*). Adjacent and pairwise measurements of LD for GBS data were calculated separately for SNPs in each scaffold. All LD plots and LD measurements and haplotype frequency calculations involved using SVS v8.8.5 (Golden Helix, Inc., Bozeman, MT, USA, *www.goldenhelix.com*)..

4.7 Admixture analysis

Admixture was analyzed using a least-squares optimization approach implemented in the sNMF function of the R package LEA [33,48]. This approach is based on estimating admixture coefficients based on sparse non-negative matrix factorization. The number of K populations was assessed from 1 to 6 clusters, and 10 replications were performed for each K value. The best K value was selected based on the minimum value of the cross-entropy criterion [48].

4.8 Nucleotide diversity analysis

Expected nucleotide diversity (ND) and Tajima's D for various chromosomes were estimated with sliding-window analysis by using TASSEL v5.0 as described [49]. Estimation of fixation index (F_{ST}) was based on Wright's F statistic [50] with use of SVS v8.8.5 (Golden Helix, Inc., Bozeman, MT, USA, *www.goldenhelix.com*).

5. Conclusions

In this study, we have shown the efficiency of GBS with a single restriction enzyme *Ape*K1 in generating high-density genotype data for genetic diversity and admixture analyses in blueberry. We successfully mapped the GBS-obtained sequence reads to the genome sequence of the tetraploid variety Draper, and the identified SNPs were used in PCA, haplotype, and admixture analysis to understand genetic relatedness in blueberry accessions. With goals to improve the adaptability of blueberries to wider geographies and warmer climates, interspecific hybridizations within *Vaccinium* species are set to increase greatly. In such a scenario, it will be highly crucial to resolve the genomic contribution of the 2 parental species in hybrid progenies. Admixture analysis of progenies using high-throughput SNP markers distributed across chromosomes will be useful to reveal genetic lineages. Our study showed how genetic admixture analysis is accurate for selecting progenies with desired parental lineage in intercross populations.

Supplementary Materials

Supplementary Table S1. GBS summary of all the blueberry genotypes and BNJ16-5 population

Supplementary Table S2. Details of the SNPs mapped to the longest 12 scaffold sequences of Draper v1.0 genome

Supplementary Table S3. Eigenvalues for the first three principle components estimated for NHB, SHB, and 16-5 populations

Supplementary Table S4. Pairwise FST values of NHB and SHB cultivars across all the scaffolds

Supplementary Table S5. Nucleotide diversity indices for NHB and SHB cultivars used in this study

Supplementary Table S6. Details of the haplotype block information of NHB cultivars across all the scaffolds

Supplementary Table S7. Details of the haplotype block information of SHB cultivars across all the scaffolds

Author Contributions: K.P.K implemented the project and wrote the manuscript; K.M and U.K.R conceived the project; K.P.K and P.N performed GBS analysis. N.V provided plant material and reviewed the manuscript; M.L provided blueberry genome sequence and reviewed the manuscript. K.M, S.E and U.K.R revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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