



OPEN Generation and characterization of novel transcriptome-derived SSR markers for genetic applications in blueberry

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Blueberries (*Vaccinium* spp.) are small fruit crops native to North America but grown commercially in several countries. There has been a steady increase in consumer demand for blueberries, owing to their numerous health benefits attributable to the high content of antioxidant compounds. Despite its importance, limited genomic resources are available, hindering genetic research and improvement in blueberry. In this study, we developed novel transcriptome-derived simple sequence repeat (SSR) markers from two divergent blueberry species. We identified 64,013 SSRs from 208,286 unigenes *de novo* assembled from 135 million high-quality sequence reads. SSRs with mononucleotide repeat motifs were most abundant, followed by di-, tri-, tetra-, penta-, and hexanucleotide repeat motifs. The repeat motif AG/CT was most abundant (84%) compared to the other three dinucleotide repeat motifs, AC/TG, AT/TA, and CG/GC. Similarly, AAG/CTT and AAAT/ATTT were the most abundant tri- and tetra-nucleotide repeat motifs, respectively. We designed 33,179 primer pairs from 64,013 SSR motifs. Genomic coordinates of these primers were identified using a chromosome-scale reference genome sequence of diploid blueberry clone W85-20. After discarding those markers mapped with MapQ < 1, genomic positions for 19,310 markers were identified. Thirty-six SSRs differing in nucleotide repeat motifs were randomly selected for PCR optimization, characterization, and amplification in 45 diverse *Vaccinium* species. Thirty-four primers generated 158 alleles with an average of 4.6 alleles per locus. Genetic structure analyses revealed a high genetic differentiation among the selected species, and divided 45 accessions into two to five clusters. Furthermore, the selected SSR markers showed 88.2–97% cross-species transferability, suggesting the possibility of using these markers for various genetics and comparative genomics applications in blueberry and cranberry species. Thus, the transcriptome-derived SSR markers developed in this study could serve as a valuable resource for further genetic and evolutionary studies in *Vaccinium* species.

Keywords *Vaccinium*, Blueberry, Cranberry, Transcriptome, RNAseq, EST-SSRs, Genetic relationships, Cross-species transferability

Blueberries (*Vaccinium* L. section *Cyanococcus* spp.) are small fruit crops native to North America but commercially grown in several countries worldwide¹. Blueberries have received much attention recently due to their health benefits attributable to the high content of anthocyanins, flavonols, procyanidins, and other types of phenolic compounds, which can protect against cancer, cardiovascular and neurodegenerative diseases, and aging². Antioxidant phenolic compounds are associated with numerous health benefits such as eye strain, prevention of macular degeneration, anticancer activity, and reduced risk of cardiovascular heart disease^{3,4}. Due to these types of benefits, there is a steady increase in demand for blueberries.

In the *Vaccinium* genus, the section *Cyanococcus* includes multiple species⁵ and species hybrids of various ploidy levels⁶. They are widespread throughout eastern North America, thriving in diverse surroundings and

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widening the genetic divergence among the species. Intraspecific hybridization is widespread in blueberry species and has been widely used in cultivar development since the early twentieth century⁷ incorporating novel traits from wild germplasm⁶. Natural and directed hybridization has led to complex genetic relationships among blueberry species⁸.

Several recent studies have used single nucleotide polymorphism (SNP) markers to elucidate the population structure and genetic divergence among the selected blueberry species^{6,9,10} providing essential insights into the evolutionary trajectories important for domestication and adaptation¹¹. However, many species did not have precise species boundaries, suggesting that some accessions may have a highly homogenous genetic background. SNPs are generally bi-allelic markers; hence, more loci are needed to obtain sufficient statistical power in population genetic analyses¹². SNPs occur in genomes at a much higher frequency than SSRs and can be genotyped in high-throughput systems with a high multiplex ratio. The comparison of SNP and SSR markers for population genetic differentiation and phylogenetic analyses have shown coherent results to each other^{13–15} although some studies implied that SSRs were shown to be more efficient for genetic diversity analysis in comparative studies¹⁶. Higher per-locus information content of SSRs makes them the marker of choice for parentage and assignment studies¹⁷ and an efficient genetic tool for assessing genetic diversity and subpopulation structure¹⁸. Thus, SSRs may help resolve the unspecified boundaries to provide evolutionary insights.

SSRs are codominant, multi-allelic, and reproducible markers¹⁹ characterized by high polymorphism and, hence, widely considered very powerful and informative across the plant species. They have wide genome coverage, are transferable to other species in the genus, and are easy to use²⁰. Due to their hypervariable nature, SSRs produce high allelic variations, even among very closely related varieties²¹. Genic microsatellite markers are derived from the coding regions or the 5' and 3' regions of mRNA. They often show a lower genetic variation than the genomic SSRs but provide higher transferability among the related species²². The genic SSRs offer valuable information on functional sequences and may help identify adaptive genetic variations²³. For these reasons, genic SSRs have found broad applications in genetic analyses, such as genetic and QTL mapping, phylogenetic and population structure, marker-assisted breeding, molecular characterization of core collections, and management of germplasm collections²⁴. Compared to other species, limited resources are available for genetic research in blueberry²⁵.

Dominant as well as codominant markers, including random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP), SSR, and expressed-sequence tag-polymerase chain reaction (EST-PCR) markers have been developed and used for analyzing genetic relationships in *Vaccinium* species^{5,26–34}. These studies have demonstrated that multiallelic SSRs facilitate the genetic and evolutionary analyses of blueberry.

Before the availability of reference genomes, SSR markers were developed from the genomic DNA by constructing the genomic library and sequencing the clones, which was time-consuming, costly, and labour-intensive^{35,36}. However, with the rapid development of next-generation sequencing (NGS) technologies and the dramatic reduction of sequencing costs, it has become feasible to use whole genome sequence data for SSR marker development³⁷. The SSR markers developed from genomic databases are categorized as genomic simple sequence repeat (gSSR) markers. In contrast, those derived from the transcriptome databases are termed as expression sequence tag-simple sequence repeat (EST-SSR) markers. Compared to gSSRs, the cost of developing EST-SSRs is lower; therefore, many EST-SSRs can be developed from transcriptome sequencing data.

EST-SSRs help to identify variation among the transcribed sequences, thereby facilitating the identification of functional candidate genes, and marker-assisted selection³⁸. Many EST-SSRs from conserved genic regions can be amplified in other related species and thus have high cross-species transferability^{39,40}. Such markers also act as anchors to develop high-resolution genetic maps using biparental mapping populations. Therefore, EST-SSRs have become attractive and valuable resources for the genetic breeding of important plant species⁴¹. However, few studies have reported the large-scale development of transcriptome-derived SSRs in blueberry. For instance, Wang et al.⁴² carried out large-scale transcriptome sequencing of 54 blueberry cultivars and developed > 26,974 SSR markers. Similarly, Rowland et al.⁴³ developed 7,705 SSRs from the transcriptome of the commercial highbush blueberry (*V. corymbosum*) cultivar Bluecrop. Li et al.⁴⁴ identified 8,796 SSRs from 15,457 unigenes from transcripts involved in various stages of fruit and color development in the blueberry cultivar 'Sierra' (*V. corymbosum*).

In this study, we developed SSR markers from the transcriptome data of two divergent blueberry species, *V. corymbosum* and *V. darrowii*⁴⁵. We *de novo* assembled the short reads from the RNAseq experiment and designed an extensive collection of SSR markers, which were validated using a set of 45 diverse blueberry accessions.

Results

SSR discovery, frequency, and distribution

RNAseq of *V. corymbosum* and *V. darrowii* samples obtained more than 135 million high-quality sequence reads (> 64.9 million from *V. corymbosum* and > 70.3 million from *V. darrowii*) using the Illumina NextSeq500 system⁴⁵. The summary of the RNA sequencing data is given in Table 1. These reads were *de novo* assembled into 208,286 unigene sequences after clustering. The average contig length ranged from 1,041 bp to 1,139 bp (Table 2). MISA software was used to identify potential microsatellites from all the 208,286 unigene sequences. A total of 95,034 (*V. corymbosum*) and 104,912 (*V. darrowii*) sequences were examined to identify SSRs. The summary of the SSRs identified using MISA software is given in Table 2.

A total of 29,634 and 34,379 SSRs were identified in *V. corymbosum* and *V. darrowii*, respectively (Table 2). Of these identified SSRs, 5,707 and 6,621 unigene sequences contained more than 1 SSR, and 2,553 and 2,941 SSRs were present in compound form in *V. corymbosum* and *V. darrowii*, respectively. Six types of repeat motifs, with significant variation in the number of each type of repeat, were found in these SSRs (Fig. 1A). Among 29,634 SSRs in *V. corymbosum*, SSRs with mononucleotide repeat motifs were most abundant (13,817; 47%), followed

Particulars	<i>V. corymbosum</i>	<i>V. darrowii</i>
Total number of assembled genes	99,315	109,431
Total number of assembled transcripts	162,307	189,613
Percent GC	41.6	41.7
Contig N50 (bp)	1,832	2,007
Average contig length (bp)	1,075	1,165
Total assembled bases	174,558,273	220,941,571

Table 1. Summary of *de novo* transcriptome assembly.

Parameters	<i>V. corymbosum</i>	<i>V. darrowii</i>
Total number of sequences examined	95,034	104,912
Total size of examined sequences (bp)	70,019,775	79,652,233
Total number of identified SSRs	29,634	34,379
Number of SSR-containing sequences	21,790	25,312
Number of sequences containing more than 1 SSR	5,707	6,621
Number of SSRs present in compound formation	2,553	2,941

Table 2. Summary of the SSRs identified using MISA tool.

by di- (12,862; 43%), tri- (2,712, 9%), tetra (139; <1%), penta (90; <1%), and hexanucleotide (14; <1%) repeat motifs (Fig. 1A). Similarly, among 34,379 SSRs in *V. darrowii*, SSRs with mononucleotide repeat motifs were most abundant (16,448; 48%), followed by di- (14,682; 43%), tri- (2,949, 9%), tetra (181; <1%), penta (103; <1%), and hexanucleotide (16; <1%) repeat motifs (Fig. 1A, Supplementary Tables S1 and S2).

The frequency distribution of classified SSR motif with distinct tandem repeats is illustrated in Fig. 2. In the mononucleotide repeats in *V. corymbosum*, the dominant repeat motif was T (7,305, 53%), followed by A (6,402, 46%), C (60, <1%) and G (50, <1%). Similarly, in *V. darrowii*, the dominant repeat motif was T (8,653), followed by A (7,635), C (84, <1%), and G (76, <1%) (Fig. 2A). The next most abundant classified repeat motif was dinucleotide, comprising 43% of the total SSRs. The dinucleotide repeat motif AG/CT (10,863 and 12,346) was most abundant (84%) compared to the other three dinucleotide repeat motifs, AC/TG (1,213 and 1,522), AT/TA (734 and 764) and CG/GC (52 and 50) in *V. corymbosum* and *V. darrowii*, respectively (Fig. 2B).

Among trinucleotide repeat motifs, AAG/CTT was the most abundant, comprising 35% in *V. corymbosum* and 36% in *V. darrowii*. The other two following dominant trinucleotide repeat motifs were AGG/CCT (14% in *V. corymbosum* and 13% in *V. darrowii*) and ACC/GGT (13.6% in *V. corymbosum* and 13.1% in *V. darrowii*) (Fig. 2C). Similarly, in tetra-nucleotide repeat motifs, AAAT/ATTT was most abundant (30%) in both species (Fig. 2D). In contrast, in pentanucleotide repeat motifs, AAAAG/CTTTT was most abundant (24.3% in *V. corymbosum* and 29.7% in *V. darrowii*) compared to the other repeat motifs (Fig. 2E).

Primer design and identification of genomic coordinates

Primers were designed for 15,414 out of 29,634 SSR loci identified in *V. corymbosum* and 17,765 out of 34,379 SSR loci in *V. darrowii* using Primer 3 software with selected parameters. These primers were designated as Vc_SSR_1 to Vc_SSR_15414 (Vc, *V. corymbosum*) and Vd_SSR_1 to Vd_SSR_17765 (Vd, *V. darrowii*) to distinguish the markers from both the species. Primers could not be designed for the remaining SSR loci, which could be either because they did not fit the criteria considered for the primer design using Primer 3 software or due to the lack of adequate DNA sequences flanking the SSR loci in the 5’ or 3’ unigene ends.

To identify the genomic coordinates, SSRs were aligned against the chromosome-scale reference genome sequence of diploid blueberry clone W85-20⁴⁶. After discarding those markers mapped with MapQ < 1, genomic positions for 9,211 SSRs from *V. corymbosum* and 10,099 SSRs from *V. darrowii* were identified. The highest number of markers were identified for chromosome 2 (2,102), whereas the lowest number of markers were identified for chromosome 5 (1,298) (Fig. 3). The chromosomal distribution of the SSRs from *V. darrowii* and *V. corymbosum* is given in Fig. 3. The SSRs from both species showed great coverage across all the chromosomes. Especially at both ends of chromosomes, SSR density was higher (red regions) than the central regions (green regions). These SSRs greatly varied in terms of the number of repeats and SSR length, as shown in Fig. 1B,C. Overall, 6–8 and 10–12 ranged repeats were more abundant. Detailed information of the markers (such as chromosomal position, repeat motif, lengths, product size, and forward and reverse primer sequences) are given in Supplementary Tables S1 and S2.

PCR validation and assessment of genetic diversity

PCR validation of selected 36 markers was performed using 45 accessions comprising cultivars and wild relatives of blueberry and cranberry from the *Vaccinium* genus. Of 36 markers, 30 were amplified at 55 annealing temperatures using Promega GoTaq (Promega, USA), providing clear band patterns on agarose gels. Further optimization of the PCR program with Promega HotStart enzyme (Promega, USA) amplified another four

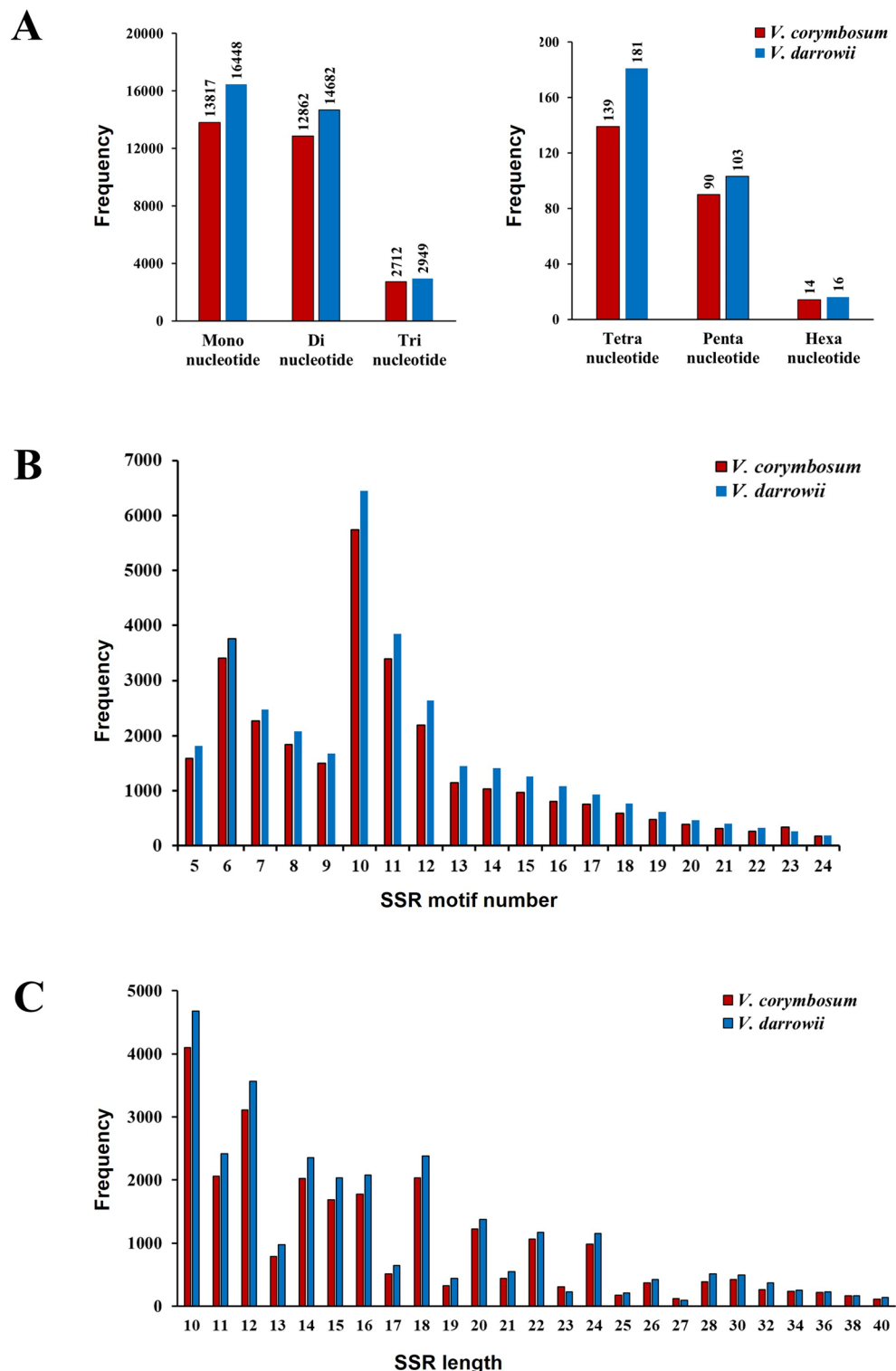


Fig. 1. Frequency distribution of the transcriptome-derived SSRs in *Vaccinium corymbosum* and *Vaccinium darrowii*. (A) Unit length; (B) Number of repeats; (C) SSR length.

markers. Thus, all the blueberry accessions were genotyped using 34 SSR markers, and amplicons were scored for the presence or absence of bands. The markers showed alleles in the range of 3 to 9, with a mean of 4.6 alleles/marker. The marker Vc_SSR_05524 yielded the highest number of alleles (9), and the lowest numbers of alleles (2) were obtained from markers Vd_SSR_00885, Vd_SSR_01750, Vc_SSR_01896, and Vc_SSR_01294. The average PIC values of these markers ranged from 0.15 (Vd_SSR_04972) to 0.36 (Vd_SSR_00885), with a mean of 0.24 (Table 3). The average Shannon's information index (I^*) ranged from a low of 0.33 to a high of 0.69,

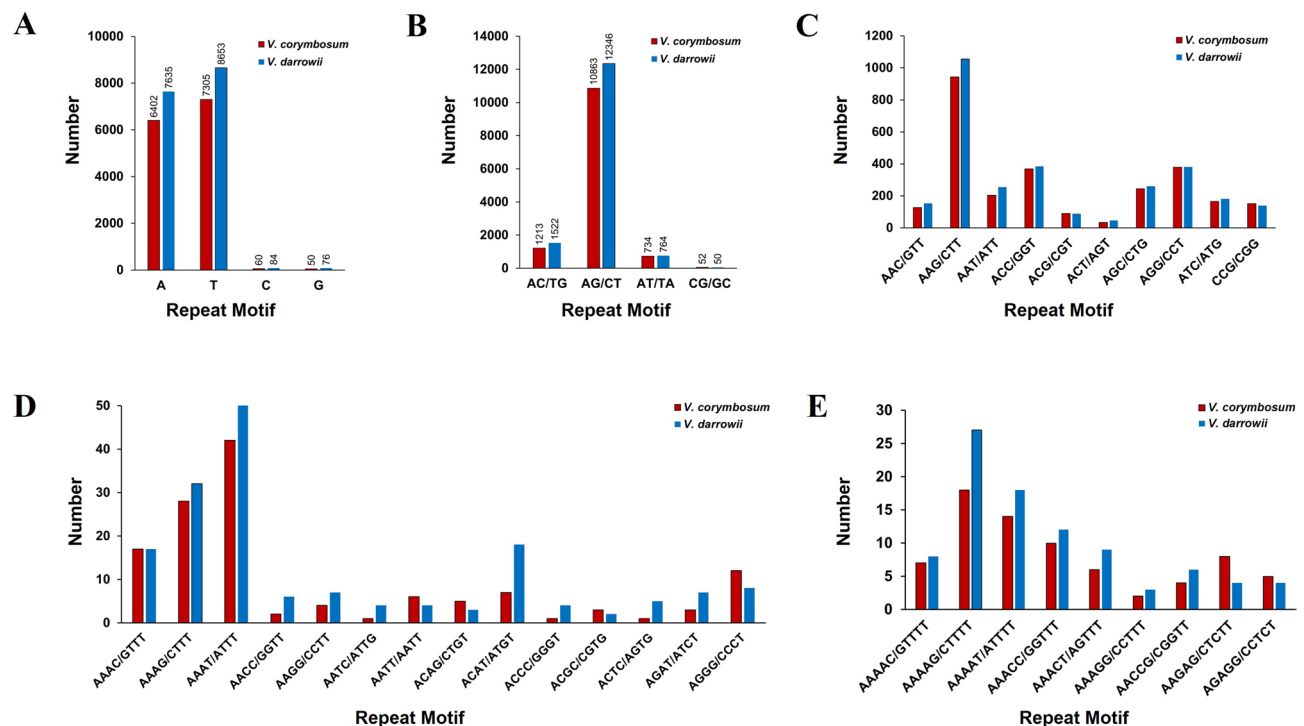


Fig. 2. Frequency of classified repeat types in transcriptome derived SSRs in *Vaccinium corymbosum* and *Vaccinium darrowii*. (A) Mononucleotide repeat motif. (B) Dinucleotide repeat motif. (C) Trinucleotide repeat motif. (D) Tetranucleotide repeat motif. (E) Pentanucleotide repeat motif.

with a mean value of 0.50. The average Nei's gene diversity (h^2) ranged from a low of 0.18 to a high of 0.49, with a mean value of 0.34 (Table 3).

Genetic relationship and phylogenetic analysis

The allelic information of 158 loci was used for PCA and phylogenetic analysis to reveal genetic relatedness among the selected accessions. The first two principal components (PC) accounted for 24.9% and 13.5% of total variability, respectively (Fig. 4A). The population can be divided into two clusters: the first comprising all the *V. macrocarpon* accessions and cultivars, and the second containing all the blueberry accessions. To further differentiate the blueberry accessions in the second cluster, we performed focused PCA, which included marker data only for blueberry accessions. The first two components of the PCA accounted for 26.5% (axis X = 14.8%, and axis Y = 11.7%). All the blueberry accessions formed three clusters on the PCA plot (Fig. 4B, Supplementary Table S3). Cluster 1 included all 5 *V. boreale* accessions, whereas Cluster 2 included all the southern highbush and northern highbush cultivars (*V. corymbosum*), except Pink Lemonade. The plurispecific cluster 3 included *V. angustifolium*, *V. darrowii*, *V. tenellum*, *V. myrsinites*, *V. ovatum*, *V. reticulatum*, and one *V. corymbosum* cultivar Pink Lemonade.

The genetic relationships of the selected 45 blueberry accessions are given in the UPGMA tree Fig. 5. The accessions were largely grouped into five clusters: (1) for *V. angustifolium*, (2) for *V. boreale*, (3) for *V. darrowii*, *V. tenellum*, *V. myrsinites*, *V. ovatum*, and *V. reticulatum*, (4) for northern and southern highbush cultivars (mainly comprising *V. corymbosum*), and (5) *V. macrocarpon* (cranberry accessions). The phylogenetic analysis further indicated that *V. darrowii*, *V. tenellum*, *V. myrsinites*, *V. ovatum*, and *V. reticulatum* did not have clear species boundaries. These results were highly consistent with those of PCA (Fig. 4).

AMOVA

Analysis of molecular variation (AMOVA) with 34 SSR markers revealed 25% variation among the populations and 75% variation within the populations (Table 4). The extent of the estimated variance of components was 6.4% among the populations and 19.6% within the populations, making 26% of the variance in all the selected *Vaccinium* accessions.

Cross-species transferability of the SSRs

To assess the cross-transferability, 36 SSR primers were screened in 45 accessions belonging to eight different *Vaccinium* species. The SSR markers showed variable extent of cross-transferability in the analyzed species. Of the 34 primer pairs, 27 markers successfully amplified in at least one accession from each selected species of the *Vaccinium* genus. These markers showed 100% transferability between *V. corymbosum* and *V. darrowii* and showed high polymorphism. Further, the percentage of transferability was 88.2, 85.2, and 91.2 for *V. ovatum*, *V. reticulatum*, and *V. angustifolium*, respectively. The markers showed 97% transferability for *V. boreale*, *V.*

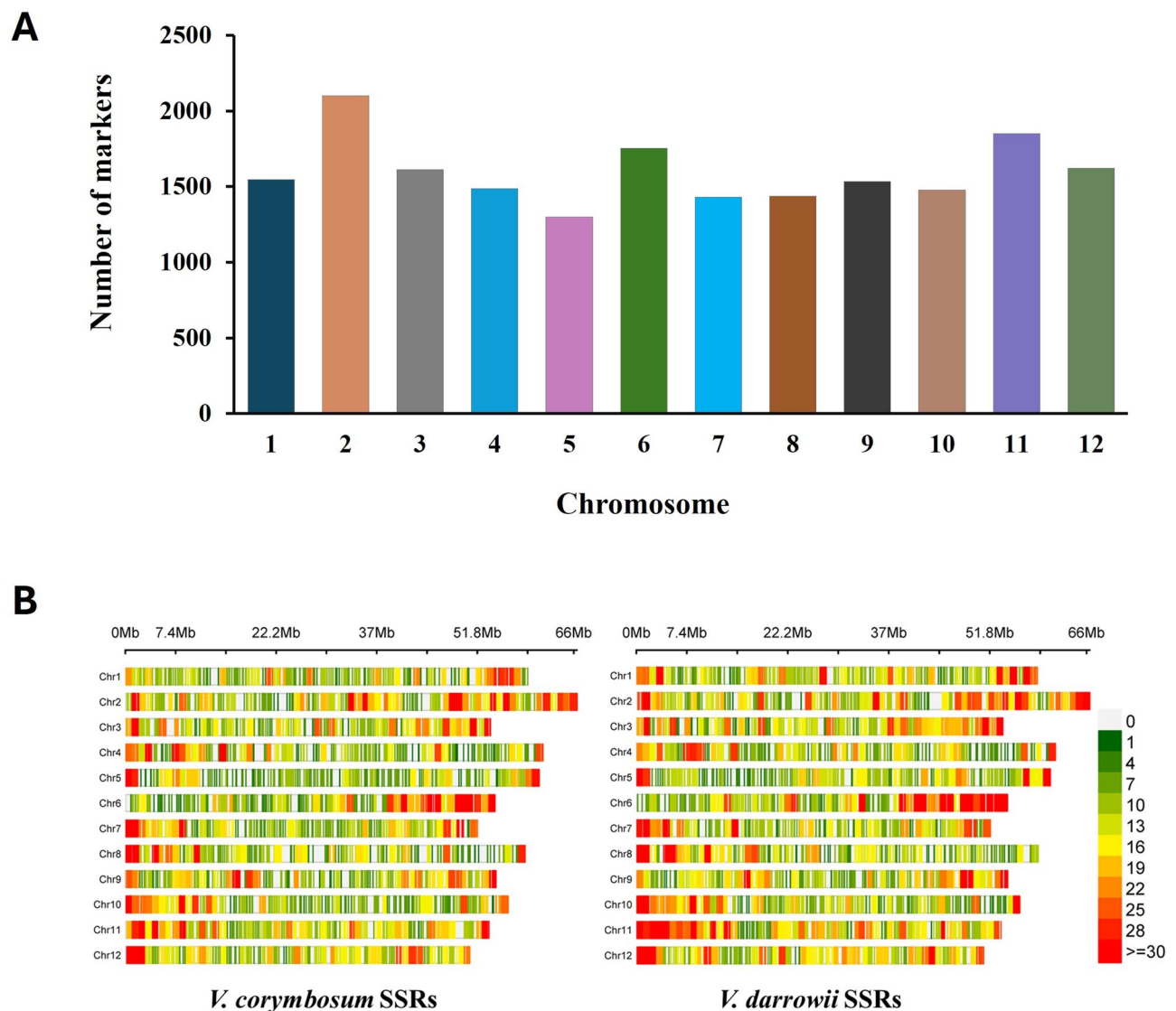


Fig. 3. Chromosome-wide number (A) and distribution and density (B) of the identified SSR markers from transcriptome data.

tenellum, and *V. myrsinites*. For cranberry accessions, 94% transferability was observed. The percentage of cross-transferability in different species is depicted in Fig. 6. Therefore, the transferability of SSR markers was higher in all the selected *Vaccinium* species.

Discussion

Blueberries have a very recent history (100 years) of domestication compared to many other fruit crops, such as apple, peach, and grape, which have been domesticated for hundreds or thousands of years. The cultivar development since then has been through phenotypic selection for large-sized fruits from wild populations. Since interspecific hybridization is widespread in blueberries, several breeding efforts have been taken to improve the blueberry cultivars through repetitive breeding and selection cycles for desired traits, mainly fruit size and quality. However, genetic improvements of blueberry for horticulturally important characteristics have been lagging due to a lack of genetic and genomic resources. Recent advancements in NGS technologies make it possible to develop transcriptome-derived SSR markers for selection and breeding purposes. Transcriptome-derived SSR markers offer many advantages, including rapid and inexpensive development and high levels of cross-taxon portability, and have the potential to facilitate evolutionary analyses in a wide variety of taxa²². Here, we report on developing large-scale transcriptome-derived nuclear SSR markers in blueberry. This study examined unigene sequences assembled from the short-read Illumina sequence read data from an RNAseq experiment⁴⁵ for SSR repeat motifs, and developed a large set of transcriptome-derived SSR markers, which were validated using diverse *Vaccinium* species.

Using the Trinity *de novo* assembly program, we assembled the short read sequences from *V. corymbosum* and *V. darrowii* samples into 162,307 and 189,613 transcripts, which were then clustered into 99,315, and

Marker	Chr	Repeat motif	N_a	PIC	Gene diversity**	I^*
Vd_SSR_11666	1	(GA)21	4	0.22	0.36	0.54
Vc_SSR_06705	1	(AG)11	4	0.27	0.28	0.44
Vc_SSR_05524	2	(GA)22	9	0.25	0.25	0.41
Vc_SSR_04327	2	(GAA)19	3	0.16	0.19	0.33
Vc_SSR_02111	2	(TC)8	5	0.21	0.37	0.56
Vc_SSR_02719	3	(TC)8	4	0.29	0.26	0.40
Vd_SSR_00885	3	(GA)24	2	0.36	0.49	0.69
Vc_SSR_10297	3	c*	4	0.31	0.38	0.56
Vc_SSR_02210	4	(CT)22	3	0.26	0.40	0.59
Vc_SSR_12082	4	(AAG)15	3	0.27	0.36	0.52
Vc_SSR_05228	4	(CA)6	4	0.25	0.41	0.59
Vd_SSR_01750	5	(AG)13	2	0.22	0.48	0.67
Vc_SSR_01907	5	(TAC)16	8	0.23	0.24	0.39
Vc_SSR_04056	5	(GA)9	3	0.20	0.18	0.33
Vd_SSR_02071	6	c*	8	0.23	0.34	0.51
Vc_SSR_08332	6	(CT)15	3	0.17	0.31	0.48
Vc_SSR_11947	6	(GAA)6(AAAAG)5	4	0.21	0.37	0.53
Vd_SSR_01601	7	(GA)23	6	0.24	0.33	0.50
Vd_SSR_04609	7	c*	7	0.25	0.37	0.47
Vc_SSR_12358	7	(AGAAA)5	3	0.31	0.32	0.47
Vd_SSR_10879	8	(TTC)14	5	0.22	0.34	0.51
Vd_SSR_04972	8	(AG)24	4	0.15	0.37	0.53
Vd_SSR_10393	8	(TC)22	5	0.25	0.28	0.44
Vc_SSR_01896	9	(T)11gaactg(T)13	2	0.26	0.40	0.59
Vd_SSR_01952	9	c*	8	0.25	0.26	0.40
Vc_SSR_06298	9	(TC)8	6	0.24	0.26	0.40
Vc_SSR_03536	10	(CT)15	4	0.22	0.46	0.65
Vd_SSR_08786	10	(TC)23	8	0.25	0.28	0.43
Vc_SSR_00789	10	(GA)21	5	0.22	0.33	0.49
Vc_SSR_10853	11	(GA)8	3	0.23	0.36	0.54
Vc_SSR_01294	11	(A)12	2	0.23	0.36	0.54
Vc_SSR_07732	11	(TC)21	6	0.23	0.38	0.38
Vd_SSR_13458	12	(TG)8(AG)9 g(GA)7	7	0.22	0.31	0.48
Vd_SSR_06280	12	c*	4	0.23	0.40	0.58
Mean			158	0.24	0.34	0.50
Range			2–9	0.15–0.36	0.18–0.49	0.33–0.69

Table 3. Characteristics of the polymorphic transcriptome-derived SSR markers used in genotyping of selected blueberry and cranberry DNA samples. c*, complex repeat motif** Average [Nei's (1973) gene diversity] I^* , Shannon's Information index N_a , number of alleles PIC polymorphism information content

109,431 unigenes, respectively. The unigenes lengths (1075 bp and 1165 bp in *V. corymbosum* and *V. darrowii*, respectively) we observed in this study were higher than the lengths previously reported by Zifkin et al.⁴⁷, Li et al.⁴⁴, Qi et al.⁴⁸ and An et al.⁴⁹ in blueberry. Moreover, our unigenes lengths were higher than those reported in previous studies on EST-SSR development using transcriptome sequence data in other crop species such as mung bean⁵⁰ (*Vigna radiata* L.), *Brassica napus*⁵¹, rice bean⁵² (*Vigna umbellata* L.), coriander⁵³ (*Coriandrum sativum* L.). The large unigene lengths provided a higher number of repeat motifs (64,013), which is higher than identified by Rowland et al.⁴³, Li et al.⁴⁴ and Wang et al.⁴².

Characteristics of SSRs may vary in different species and may be associated with their adaptation and evolution⁵⁴. SSRs within genes are subjected to stronger selective pressure than other genomic regions because of their functional importance⁵⁵. The difference in SSR motif length and frequency and its distribution is the result of selection pressure during the evolutionary process, and hence, understanding the distribution and variability of microsatellite sequences in the genome of a given species might bring different insight into genome evolution^{56,57}. In the present study, AG/CT dinucleotide repeat motif was found to be the predominant motif (84%), which was higher than reported previously by Boches et al.²⁷ (69%), Rowland et al.⁴³ (38%), Bian et al.⁵ and Liu et al.²³ in blueberry. Such predominant nature of AG/CT repeat motif is evident in other plant species such as *Triticeae* species (87%), pearl millet (81%), Chinese bayberry (57%, *Myrica rubra*), pineapple (*Ananas comosus* (L.) Merr.), Citrus (26%), and sweet potato (26%, *Ipomoea batatas*)^{58–62}.

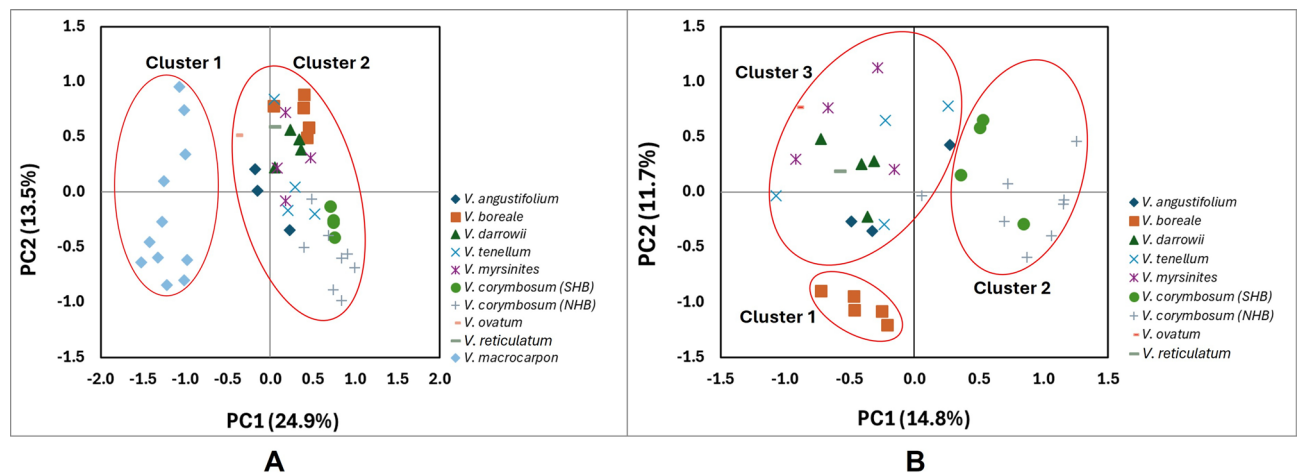


Fig. 4. Principal component analysis (PCA) of 45 blueberry and cranberry accessions using SSR markers. PCA figure (A) includes both blueberry and cranberry accessions, whereas figure (B) includes only blueberry accessions.

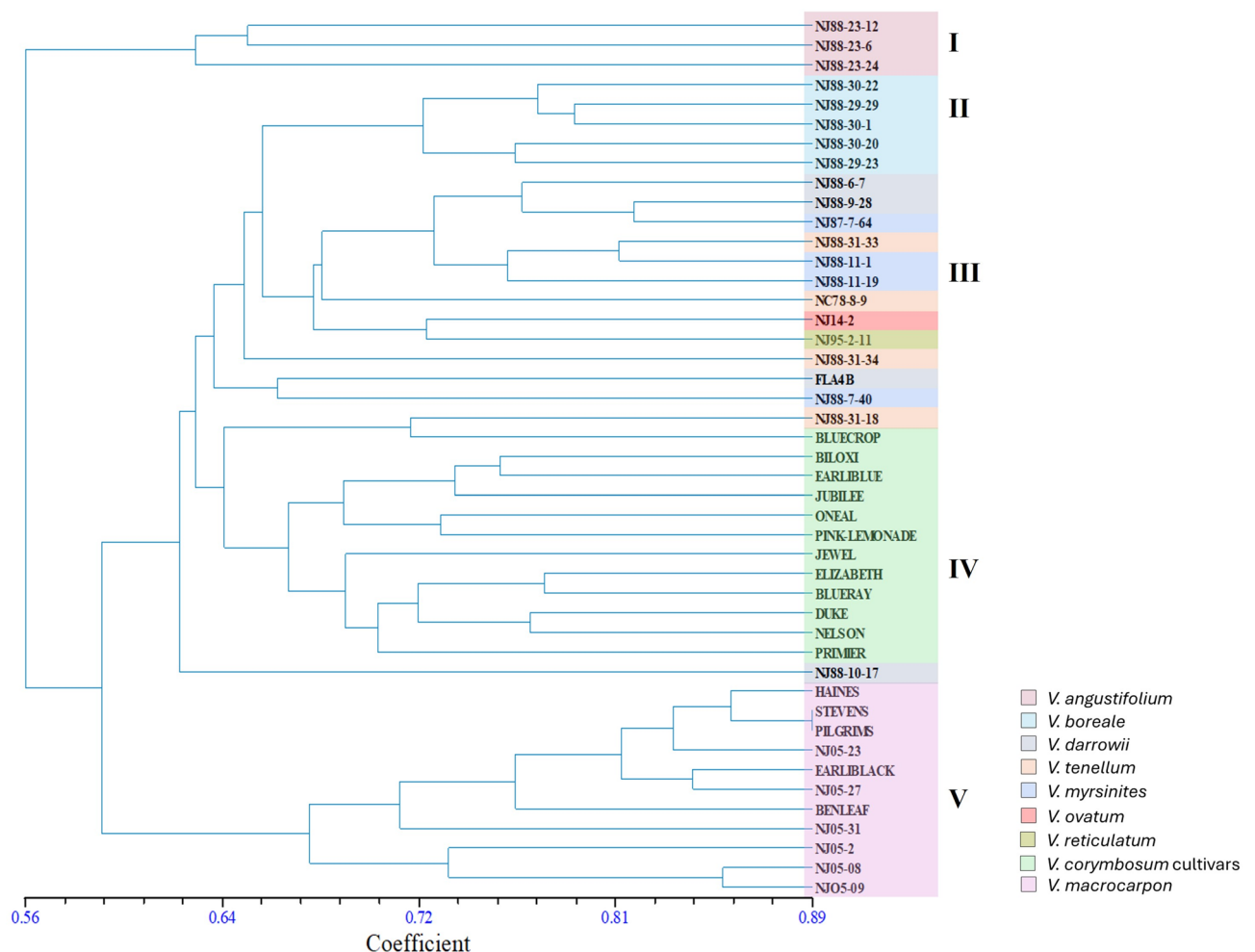


Fig. 5. Dendrogram showing genetic relationship between blueberry and cranberry species based on 34 SSR markers.

Source of variation	d.f.	Sum of squares	MS	Estimated variance of components	Percentage of variation (%)	P value
Among Pops	7	370.834	52.976	6.413	25	< 0.001
Within Pops	35	686.329	19.609	19.609	75	< 0.001
Total	42	1057.163		26.023	100	

Table 4. Analysis of molecular variance (AMOVA) for 43 *Vaccinium* species clustered in 8 populations.

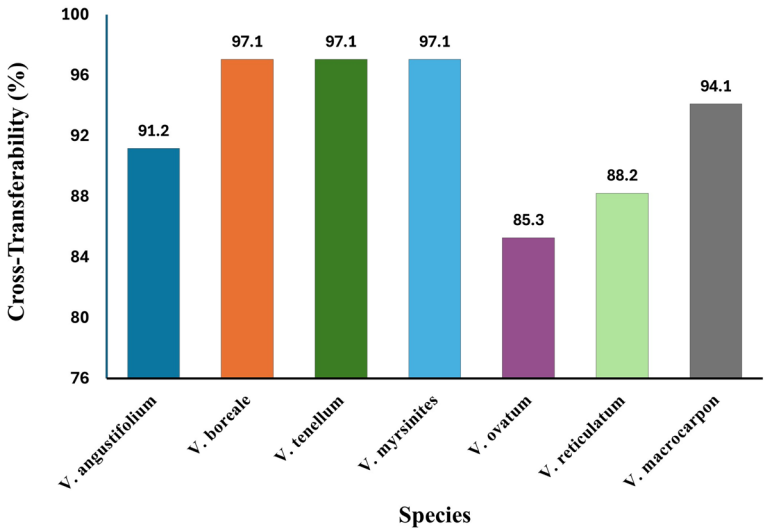


Fig. 6. Percentage of cross transferability observed in the selected *Vaccinium* species.

We designed primers for 52% of the SSR loci identified from the transcriptome data of both the species, *V. corymbosum* and *V. darrowii*. Lack of adequate flanking sequences could be the most common reason for not designing primers for the remaining SSR loci. Although the unigene lengths we obtained in this study were higher than some previously reported unigene sizes, the repeat loci at the 5' or 3' ends might fall short of flanking sequences on either side. Nevertheless, the number of primers we designed is higher than those reported in previous SSR development studies in blueberry^{42–44}. We identified chromosomal locations of 19,310 markers using a chromosome-scale reference genome of diploid blueberry W85-20. The chromosome-wide distribution and frequency of the physically mapped SSRs revealed the highest frequency on chromosome 2, whereas the lowest frequency was observed on chromosome 5. The SSRs are densely distributed across all the genomic regions of all the 12 chromosomes. The detailed information on these markers will be helpful for the selection of suitable genome-wide SSR markers across the 12 chromosomes for various genetic applications.

Genotyping of 45 diverse *Vaccinium* species, including blueberry and cranberry accessions and cultivars, with 45 SSR primers yielded 185 alleles. The average PIC values (0.15 to 0.36) and the number of alleles (4.6) were low to moderate compared to previous studies in blueberry^{23,27} in which as high as ten alleles per cultivar were observed. Further, 22 primers amplified four or more alleles (67% of the total) in this study, which is over 4 times higher than reported in Boches et al.²⁷. AMOVA detected significant variation among and within populations, and the proportion of estimated variance was higher than reported by Tailor et al.⁶³. The high variation and allelic polymorphism we observed could be due to the diversity of accessions that differed in sections, species, ploidy level, and type of material. The high levels of polymorphisms detected with the EST-PCR markers in blueberry are anticipated, considering that blueberry is a highly heterozygous, primarily outcrossing, and polyploid crop⁶⁴. Thus, the Transcriptome-derived SSR markers developed in this study could be valuable for all species within the *Cyanococcus* section and *Oxycoccus*, including the cranberry species^{26,64,65}. Using sensitive detection technologies such as gene analyzers or ABI sequencers may increase the number of alleles, as seen in Bian et al.⁵.

The overall species-wise genetic differentiation among the selected *Vaccinium* accessions was seen in the UPGMA tree, which is broadly consistent with PCA. In both analyses, *V. macrocarpon* accessions clustered separately from the blueberry species. Furthermore, the northern and southern highbush cultivars grouped together. The initial phases of blueberry domestication and breeding involved selection-based cultivar development by planting individuals with superior traits, mainly large fruit sizes selected from wild lowbush and highbush blueberry populations⁸. Later, southern highbush cultivars were developed through the introgression of southern species in northern *V. corymbosum* cultivars. Thus, *V. corymbosum* is involved in the selection process of southern highbush and northern highbush blueberry cultivars⁸ and a significant proportion of *V. corymbosum* background remains in southern highbush blueberry cultivars. Hence, the mixture of southern and northern highbush blueberry cultivars in the phylogenetic tree and PCA is expected and is consistent with our earlier

reports^{9–11}. Thus, it is apparent that the shared background in blueberry species is driving high heterozygosity in blueberry species.

Lowbush blueberry (*V. angustifolium*) is proposed to be an autotetraploid of *V. boreale*⁶⁶ or an allotetraploid of *V. boreale* x *V. pallidum* or *V. boreale* x *V. myrtilloides*^{67,68}. Several recent studies have investigated the genetic heterogeneity and relatedness of *V. angustifolium* within and among populations^{6,29,30,34} and documented genetic diversity within confined regions of majorly grown areas. Nevertheless, the founder effect on genetic diversity and selection pressure could cause a decrease in the genetic diversity among the existing lowbush accessions³⁴. It is noted that *V. boreale* and *V. angustifolium* cohabitate across Canada's Allegheny range, which may be the center of diversity¹¹. In our study, *V. angustifolium* has lower similarity coefficients than *V. boreale*, which suggests that *V. angustifolium* had higher genetic variation than *V. boreale*. Previously, we observed that *V. boreale* (collected from Nova Scotia, Canada) is a genetically distinct outgroup compared to the other blueberry accessions¹¹. Thus, further research involving more *V. angustifolium* and *V. boreale* accessions is needed to understand the genetic differentiation among these two species.

EST-SSR markers originate from the transcribed regions of DNA, which are the most conserved regions in the plant genomes⁶⁹. They are evolutionarily conserved and have higher transferability in closely related species than the genomic SSRs⁷⁰. The percentage of cross-transferability may vary depending on the number and type of accessions, and it decreases with the inclusion of more diverse accessions in the set. We found a 100% transfer rate of SSR amplification between *V. darrowii* and *V. corymbosum*, which indicates a very high rate of cross-transferance, consistent with the previous reports of Boches et al.²⁷ and Rowland et al.⁴³. Furthermore, more than 80% of the SSRs were amplified in at least one plant accession in each species, including *V. ovatum* and *V. reticulatum*, which had only one genotype in the selected 45 accessions. The cross-transferability rate we observed in this study was higher than some of the previous reports in *Vaccinium* species and other plants. For instance, Boches et al.²⁷ reported 83% cross-transferability, whereas Liu et al.²³ reported 68.5–88.90% cross-transferability in 8 *Vaccinium* species. Therefore, the SSRs developed in this study may have potential applications in comparative genomics and evolutionary studies, such as population genetic analyses²². Since these markers originate from the heat-responsive genes, they may help identify genetic variations associated with a plant's ability to tolerate high temperatures and potentially lead to the development of heat-resistant cultivars.

Conclusion

In this study, we developed 19,310 transcriptome-derived SSR markers from the RNA-seq data of two divergent blueberry species. The genomic positions of these markers were identified using a chromosome-scale genome sequence of blueberry clone W85-20. Thirty-four out of 36 primers showed amplification and revealed 158 alleles with a mean of 4.6 alleles per locus in 45 diverse *Vaccinium* species, indicating high levels of polymorphism. These alleles effectively uncovered the genetic differentiation among the selected *Vaccinium* species through PCA and phylogenetic analysis. Furthermore, the markers demonstrated high cross-species transferability, which suggested that the developed SSRs may have applications in related species, thereby facilitating genetic diversity and genetic relationship studies across different *Vaccinium* species. Thus, the novel transcriptome-derived SSR markers developed in this study are a rich, publicly available genomic resource that will aid genetic research in blueberry and related species.

Materials and methods

Plant materials

A total of 45 accessions belonging to the *Vaccinium* species (Table 5) were used to analyze the polymorphism of SSR markers in this study. These individuals represented the following species: *V. angustifolium* Ait ($N=3$), *V. boreale* ($N=5$), *V. darrowii* ($N=4$), *V. tenellum* ($N=4$), *V. myrsinites* ($N=4$), *V. ovatum* ($N=1$), *V. reticulatum* ($N=1$), and *V. corymbosum* ($N=12$). *V. myrsinites* and *V. corymbosum* accessions are tetraploid ($2n=4x=48$), and *V. angustifolium*, *V. boreale*, *V. darrowii*, *V. tenellum*, *V. ovatum*, and *V. reticulatum* accessions are diploid ($2n=2x=24$). The set also included 11 cranberry (*V. macrocarpon*) species differing in ploidy levels ($2X$ to $6X$). All the plants comprising the wild relatives of the selected species were collected across North America in the 1980s and 1990s, propagated, and maintained under greenhouse conditions at Philip E. Marucci Center for Blueberry & Cranberry Research and Extension managed by Rutgers, the State University of New Jersey. We confirm that the IUCN Policy Statement includes all methods as per the Convention on the Trade in Endangered Species of Wild Fauna and Flora. Since these are perennial plants, all the above accessions remain at Philip E. Marucci Center for Blueberry & Cranberry Research and Extension. For genomic DNA extractions, young leaves (~1–2 g) were collected in 15 mL Nunc™ conical sterile polypropylene centrifuge tubes, transferred to the Delaware State University in dry ice, and stored at -80°C . The northern and southern highbush cultivars (predominantly comprising *V. corymbosum* species) were procured from various plant nurseries and maintained in the greenhouse at the Delaware State University.

Sample preparation and DNA extraction

Leaf tissue ranging from 100 to 120 mg was placed in a 2.0 mL Eppendorf safe-lock tube (US Scientific, USA) containing a metallic bead. The tissue samples were homogenized to fine powder using TissueLyzer-II (Qiagen, USA), and tubes were stored at -80°C . Genomic DNA was extracted using a modified cetyl trimethyl ammonium bromide (CTAB) protocol. The DNA quality and concentration were checked using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The quality of each DNA sample was also verified on 1% agarose gels, stained with the SYBR™ Green I Nucleic Acid Gel Stain and visualized under UV light. Samples were stored at -20°C . The working concentration of DNA was adjusted to 50 ng/ml for use in marker evaluations.

Taxon	Genotype	Ploidy	Improvement status
<i>V. corymbosum</i>	Elizabeth	4X	Cultivar
	Nelson	4X	Cultivar
	Pink Lemonade	4X	Cultivar
	Bluecrop	4X	Cultivar
	Duke	4X	Cultivar
	Blueray	4X	Cultivar
	Earliblue	4X	Cultivar
	Primier	4X	Cultivar
	Biloxi	4X	Cultivar
	Jubilee	4X	Cultivar
	O'Neal	4X	Cultivar
	Jewel	4X	Cultivar
<i>V. angustifolium</i>	NJ88-23-12	2X	Wild material
	NJ88-23-24	2X	Wild material
	NJ88-23-6	2X	Wild material
<i>V. boreale</i>	NJ88-30-22	2X	Wild material
	NJ88-30-20	2X	Wild material
	NJ88-29-29	2X	Wild material
	NJ88-29-23	2X	Wild material
	NJ88-30-1	2X	Wild material
<i>V. darrowii</i>	NJ88-6-7	2X	Wild material
	NJ88-10-17	2X	Wild material
	FLA4B	2X	Wild material
	NJ88-9-28	2X	Wild material
<i>V. tenellum</i>	NC78-8-9	2X	Wild material
	NJ88-31-18	2X	Wild material
	NJ88-31-34	2X	Wild material
	NJ88-31-33	2X	Wild material
<i>V. myrsinites</i>	NJ88-11-1	4X	Wild material
	NJ88-11-19	4X	Wild material
	NJ88-7-40	4X	Wild material
	NJ87-7-64	4X	Wild material
<i>V. ovatum</i>	NJ14-2	2X	Wild material
<i>V. reticulatum</i>	NJ95-2-11		Wild material
<i>V. macrocarpon</i>	Haines	2X	Cultivar
	Earliblack	2X	Cultivar
	Stevens	2X	Cultivar
	Benleaf	2X	Cultivar
	Pilgrims	2X	Cultivar
	NJ05-2	4X	Selection
	NJ05-08	6X	Selection
	NJO5-09	6X	Selection
	NJ05-23	6X	Selection
	NJ05-27	6X	Selection
	NJ05-31	6X	Selection

Table 5. List of blueberry and cranberry accessions used in this study.

SSR marker identification and primer design

Unigenes identified from the transcriptome data obtained from the RNAseq experiment involving *V. darrowii* and *V. corymbosum* plants subjected to heat stress at 45 °C were used⁴⁵. The experiment details, including RNA extraction, library preparation, and RNAseq by Illumina sequencing and *de novo* transcriptome assembly were given in Callwood et al.⁴⁵. The assembled unigenes were used to predict simple sequence repeats using the MicroSatellite (MISA) search engine (<https://webblast.ipk-gatersleben.de/misa/>⁷¹). The minimum number of repeats used for selecting the EST-SSRs was ten for mononucleotide-based loci, six for dinucleotide loci, five for tri-nucleotide loci, and three for all the larger repeat types (tetra- to hexanucleotide motifs). The maximum

length of sequence between two SSRs was set for 100 bp to register as a compound SSR. The primer pairs for SSR markers were designed based on sequences flanking the selected microsatellite loci using the Primer3 software⁷².

Identification of genomic coordinates

To identify the genomic positions, the SSR primer sequences from *V. corymbosum* and *V. darrowii* were aligned versus the chromosome-scale genome sequence of diploid blueberry clone W85-20⁴⁶ using Burrows-Wheeler Aligner (BWA, <http://bio-bwa.sourceforge.net/bwa.shtml>) considering first the ALN and then the SAMSE options for each set, independently. The resulting SAM file was then filtered using SAMTOOLS⁷³ for the mapping quality of \geq one, and the unique mapping SSR sequences were retained. The mapping result for each set was used for the downstream analysis.

PCR optimization and validation of SSR markers

From the designed markers, 36 SSRs (three markers per chromosome) were selected for primer synthesis and validation using 45 accessions from the *Vaccinium* genus. Based on the physical position of the markers, three markers for each chromosome were chosen in the following way: One marker near the end of the short arm, one marker in the center, and one marker at the end of the long arm. The marker names, forward and reverse primer sequences, repeat motifs, and expected amplicon size for all the selected SSR markers are given in Supplementary Table S4.

PCR amplification was performed in 10 μ l volume reactions containing 0.5 μ M of Forward and Reverse primer, 1X GoTaq[®] Green Master Mix, which contains optimal concentrations of GoTaq[®] DNA Polymerase, dNTPs, MgCl₂ and reaction buffer (Promega, USA), and 50 ng of genomic DNA. For some of the primers, a temperature gradient PCR using GoTaq[®] Hot Start Master Mixes (containing GoTaq[®] Hot Start Polymerase, magnesium, dNTPs, and buffer) was performed. The PCR was performed in DNA thermocycler (Eppendorf, Germany). The PCR program was: initial denaturation at 95 °C for 3 min, 34 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s. This was followed by a final extension at 72 °C for 3 min. The PCR products were stored at 4 °C before loading. The PCR samples were loaded on 3% agarose gels run in TBE buffer. The SYBR[™] Green I Nucleic Acid Gel Stain (Thermo Fisher Scientific, Waltham, MA, USA) was used to stain the amplified DNA products, and images were taken using the Gel documentation system (iBlot, Thermo Fisher Scientific, Waltham, MA, USA). The gel images were used to score the allele patterns of the SSRs for selected primers. The amplicon sizes were estimated based on a 100 bp GeneRuler as a DNA ladder (Thermo Fisher Scientific, Waltham, MA, USA).

Polymorphism estimation and data analysis

The genotype profiles produced by the SSR markers were scored manually. Each allele was scored as present (1) or absent (0) of each of the SSR loci. Principal component analysis (PCA) and analysis of molecular variance (AMOVA) were performed using GenAlEx 6.5⁷⁴. For AMOVA, the blueberry genotypes were divided into seven groups, of which five groups comprised wild clones (*V. angustifolium*, *V. boreale*, *V. darrowii*, *V. tenellum*, *V. myrsinites*). The southern highbush and northern highbush *V. corymbosum* cultivars were placed in the sixth and seventh groups, respectively, whereas cranberry accessions were categorized into the eighth group.

Clustering analysis

Phylogenetic analysis was conducted using Jaccard's similarity coefficient through NTSYS-pc software Version 2.11⁷⁵. The similarity matrix was used in cluster analysis using the unweighted pair-group method with arithmetic averages (UPGMA) and sequential, agglomerative, hierarchical, and nested (SAHN) clustering algorithm to obtain dendrogram. Nei's⁷⁶ genetic diversity (h^2) and Shannon index (I^*)⁷⁷ were calculated with POPGENE Ver. 1.32⁷⁸. Additional genetic diversity parameters such as polymorphic information content (PIC) and average number of alleles (NA) were calculated using the PowerMarker version 3.25⁷⁹.

Data availability

The RNAseq datasets from our previous study were used to extract SSR information. The datasets, in the form of raw paired-end Illumina RNA sequencing reads, are available in the Sequence Read Archive (SRA) at NCBI under the Bio project accession nos. PRJNA691351 and PRJNA691352. The SSR marker data is available in the article's supplementary information files.

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Author contributions

K.P.K. performed and conceived the experiments, analysed the data, and wrote the manuscript; RKA, helped in genotyping; JJC, NV, provided the plant material for the research; UKR, provided SSR repeat information; SE reviewed the manuscript; HB, and MI, provided genomic coordinates for the markers; KM, supervised the project. All authors reviewed and approved this submission.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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