forests

MDPI

Article

Comparative Analysis of Complete Chloroplast Genome Sequences and Insertion-Deletion (Indel) Polymorphisms to Distinguish Five *Vaccinium* Species

Yonguk Kim [1], Jawon Shin [1], Dool-Ri Oh [1], Ah-Young Kim [2] and Chulyung Choi [1,*]

[1] Jeonnam Institute of Natural Resources Research, Jeollanam-do 59338, Korea; kyu9801@hanmail.net (Y.K.); sjo8127@naver.com (J.S.); pp0806@nate.com (D.-R.O.)
[2] School of Biological Sciences and Technology, Chonnam National University, Gwangju 61186, Korea; kimay1243@hanmail.net
* Correspondence: blockstar@hanmail.net; Tel.: +82-61-860-2620

Received: 3 August 2020; Accepted: 24 August 2020; Published: 25 August 2020

Abstract: We report the identification of interspecific barcoding InDel regions in *Vaccinium* species. We compared five complete *Vaccinium* chloroplast (cp) genomes (*V. bracteatum*, *V. vitis-idaea*, *V. uliginosum*, *V. macrocarpon*, and *V. oldhamii*) to identify regions that can be used to distinguish them. Comparative analysis of nucleotide diversity from five cp genomes revealed 25 hotspot coding and noncoding regions, occurring in 65 of a total of 505 sliding windows, that exhibited nucleotide diversity (*Pi*) > 0.02. PCR validation of 12 hypervariable InDel regions identified seven candidate barcodes with high discriminatory powers: *accD-trnT-GGU*, *rpoB-rpoA*, *ycf2-trnL-GAA*, *rps12-ycf15*, *trnV-GAC*, and *ndhE-ndhF*. Among them, the *rpoB-rpoA(2)* and *ycf2-trnL-CAA* sequences clearly showed the intraspecific and interspecific distance among five *Vaccinium* species by using a K2P technique. In phylogenetic analysis, included five *Vaccinium* species (n = 19) in the Bayesian and Neighbor-Joining (NJ) analysis revered all species in two major clades and resolved taxonomic position within species groups. These two locus provide comprehensive information that aids the phylogenetics of this genus and increased discriminatory capacity during species authentication.

Keywords: chloroplast genome; InDel; nucleotide diversity; species authentication; *Vaccinium*

1. Introduction

Genus *Vaccinium* (Ericaceae) includes more than 450 species across Europe, North America, Central America, Central and South East Africa, Madagascar, South East Asia and Malaysia, Korea, China, and Japan [1,2]. Blueberry (*Vaccinium ashei* J.M. Reade, *Vaccinium corymbosum* L., *Vaccinium angustifolium* Aiton), bilberry (*Vaccinium myrtillus* L.), cranberry (*Vaccinium macrocarpon* Aiton, *Vaccinium oxycoccos* L., *Vaccinium myrtilloides* Michx.), and lingonberry (*Vaccinium vitis-idaea* L.) are representative of this genus.

Blueberry is generally divided into three types: The highbush blueberry (*V. corymbosum*), the lowbush blueberry (*V. angustifolium*), and the rabbiteye blueberry (*V. ashei*) [3]. It is commercially cultivated worldwide, particularly in North America, Europe, and Asia [4]. Bilberry, also known as European blueberry, is native to northern and central Europe as well as North America. It is commercially cultivated mainly in North America, while France, Netherlands, Germany, Poland, and Spain are the leading European producers [5]. Cranberry is commonly separated into two types: The American cranberry (*Vaccinium macrocarpon*) and the European cranberry (*Vaccinium oxycoccos*). The American cranberry is native to Eastern North America and is commercially cultivated there and throughout Canada [6,7]. The European cranberry, commonly known as the small cranberry,

is cultivated in Central and Northeastern Europe [8]. Lingonberry is native to the alpine regions of North America, Northern Europe, Iceland, Greenland, and Northern Asia [9]. These edible fruits are popular worldwide as a "superfood" due to their bioactive properties, and high levels of antioxidant compounds (phenolics, flavonoids, and tannins), fruit colorants (anthocyanins and carotenoids), vitamins (ascorbic acid), and minerals [1]. Phenolic compounds are exceptionally strong antioxidants which prevent chronic and degenerative diseases, such as cancer and cardiovascular disorders [10].

The southern and northern alpine regions of Korea are native to five *Vaccinium* species: *V. bracteatum* Thunb., *V. oldhamii* Miq. *V. uliginosum* L., *V. Koreanum* Nakai., and *V. vitis-idaea* L. [11]. Although these wild berries have long been used as an edible fruit or traditional medicinal herb, breeding for cultivar improvement is still in the early stages. The commercial value of wild berries has reduced due to the dependence on imported blueberries. Also, most Korean blueberry farms grow the highbush blueberry cultivars, which is a major blueberry crop [12].

In August 2018, Korea enacted and enforced the Nagoya Protocol—an international agreement that aims to share the benefits arising from the use of genetic resources in a fair and equitable way [13]. Native wild berries have therefore attracted the attention of Korean researchers: They are commercially valuable, with potential bio-industrial and medicinal applications, and are also a good alternative to cultivated blueberries. Furthermore, with increasing demand for healthy foods worldwide, studies have shifted their focus to discovering wild relatives with higher concentrations of bioactive compounds [14–16].

In addition, we may be required to provide supporting the phylogenetic and taxonomical research for genetic diversity between *Vaccinium* species.

The multi-copy organellar genomes, including chloroplast (cp) and mitochondrion (mt) genomes, are valuable resources in molecular phylogenetic analysis [17]. The cp genome data can authenticate evolutionary relationships and confirm phylogenetic classifications for plants at the family and genus level. Higher plants contain cp genomes that range between 120 and 180 kb. They contain a pair of inverted repeat (IR) regions separated by a large single copy (LSC) and a small single copy (SSC) region [18]. This quadripartite structure is highly conserved in gene content and genome organization relative to the nuclear and mitochondrial genomes [19]. Comparative analyses between cp genomes of plant species reveal structural variations, such as IR or gene loss, as a result of environmental adaptation [17,18]. The cp genome contains informative genetic markers for phylogenetic and taxonomic analysis at the genus and species levels, due to its mostly uniparental inheritance, dense gene content, and slower evolutionary rate [17].

Recent studies have demonstrated the increased effectiveness of using complete chloroplast genomes over partial cpDNA sequences in land plant phylogenetics [20,21]. However, many phylogenetic studies continue to use DNA barcodes in chloroplast sequences, such as *trnH-psbA*, *rbcL*, and *matK*, which are limited in sequence divergence in several genera.

In this study, we sequenced the complete cp genomes of three *Vaccinium* species using the paired-end sequencing method on an Illumina HiSeq X Ten platform. We compared the gene content of five *Vaccinium* cp genomes and identified hyper-variable insertion-deletion (InDel) regions. We further confirmed that candidate DNA barcodes can be used to authenticate these species. These results provide valuable sequence information for molecular phylogenetics and aid the development of molecular markers in *Vaccinium* species.

2. Materials and Methods

2.1. Comparison of Cp Genomes and Identification of InDel Loci

All five cp genome sequences of the *Vaccinium* genus with complete genome sequence information were downloaded from Genbank (*V. bracteatum*; LC521967, *V. uliginosum*; LC521968, *V. vitis-idaea*; LC521969, *V. macrocarpon*; NC_019616, and *V. oldhamii*; NC_042713). The sequences were aligned using the Clustal W algorithm from MEGA 7.0 [22]. The mVISTA program (http://genome.lbl.gov/vista/

mvista/submit.shtml) in Shuffle-LAGAN mode was used to compare the four *Vaccinium* cp genomes, using the *V. macrocarpon* cp genome as a reference. DNaSP version 6.0 [23] was used to calculate nucleotide diversity (*Pi*) among the five *Vaccinium* cp genomes. Only regions with a nucleotide diversity (*Pi*) value of >0.02 were considered. CPGAVAS2 [24] was used to annotate the cp genomes and predict the rRNA/tRNA sequences of *V. macrocarpon* and *V. oldhamii*. The comparison of the LSC/IRB/SSC/IRA junctions among these related species was visualized by IRscope (http://irscope.shinapps.io/irapp/), based on the annotations of their available cp genomes in Genbank.

2.2. Development and Validation of the Candidate DNA Barcodes

To validate interspecies polymorphisms within the chloroplast genomes, specific primers were designed using Primer 3Plus, based on hotspot regions with high nucleotide diversity identified in these *Vaccinium* cp genomes [25]. All DNA and fresh leaf samples were showed in Table 1 and identified based on our previous study [26]. For the extraction of total genomic DNA, fresh leaf samples of all species (80 mg wet weight) were added to a tube filled with stainless steel beads (2.38 mm in diameter) from a PowerPlantPro DNA Isolation Kit (Qiagen, Valencia, CA, USA), and the mixture was homogenized in a Precellys®Evolution homogenizer (Bertin Technologies, Montigny-le-Breonneux, France). Genomic DNA was extracted using the PowerPlantPro DNA Isolation Kit according to the manufacturer's instructions. PCR amplifications were performed in a reaction volume of 50 µL containing 5 µL 10x Ex Taq buffer (with $MgCl_2$), 4 µL dNTP mixture (each 2.5 mM), Ex Taq (5 U/µL), (Takara, Japan), 10 ng genomic DNA, and 1 µL (10 pM) forward and reverse primers. The mixtures were denatured at 95 °C for 5 min and amplified for 40 cycles at 95 °C for 30 s, 55 °C for 20 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min. The target DNA was extracted and purified using a MinElute PCR Purification Kit (Qiagen). PCR products were visualized on 1.5% agarose gels with ethidium bromide. Purified PCR products were sequences by CosmoGenetech (Seoul Korea) using forward and reverse primers. The sequencing results were analyzed by BLAST searches of the GenBank database. Sequence alignment and data visualization were carried out using the CLC sequence viewer 8.0 [27].

Table 1. *Vaccinium* samples used in this study.

No.	Scientific Name (n)	Common Name	Collection Site	Specimen Code
1	*Vaccinium bracteatum* Thunb. (n = 3)	*Sea blueberry*	33°31′03.5″ N 126°43′00.1″ E	NIBRGR0000424873
2			34°22′17.0″ N 126°17′11.5″ E	NIBRGR0000424891
3			34°20′55.1″ N 126°41′30.5″ E	NIBRGR0000067902
4	*Vaccinium vitis-iaea* L. (n = 3)	Cowberry	38°10′14.5″ N 128°27′39.3″ E	JINR000002321
5			38°10′14.5″ N 128°27′39.3″ E	JINR000002322
6			38°10′14.5″ N 128°27′39.3″ E	JINR000002323
7	*Vaccinium uliginosum* L. (n = 3)	Bog bilberry, Moonberry	33°19′37.6″ N 126°34′09.3″ E	NIBRGR0000424897
8			38°10′14.5″ N 128°27′39.3″ E	JINR000002324
9			38°10′14.5″ N 128°27′39.3″ E	JINR000002325
10	*Vaccinium macrocarpon* Ait. (n = 3)	Cranberry	34°40′54.3″ N 126°54′38.1″ E	JINR000002326
11			34°40′54.3″ N 126°54′38.1″ E	JINR000002327
12			34°40′54.3″ N 126°54′38.1″ E	JINR000002328
13	*Vaccinium oldhamii* Miq. (n = 7)	Oldham blueberry	36°20′45.2″N 128°01′54.5″ E	NIBRGR0000424923
14			35°28′18.7″N 126°39′10.6″ E	NIBRGR0000424929
15			33°24′47.3″ N 126°24′41.9″ E	NIBRGR0000424965
16			36°33′33.7″ N 126°20′20.6″ E	NIBRGR0000424969
17			34°45′16.8″ N 127°58′43.8″ E	NIBRGR0000424971
18			34°22′36.6″ N 126°17′26.6″ E	NIBRGR0000424895
19			34°48′49.7′ N 126°20′10.2″ E	NIBRGR0000374901

2.3. Phylogenetic Analysis

N = 19 samples (3–7 samples per species) from each five species of genus *Vaccinium*, were collected from various localities in South Korea (Table 1). The specimens were deposited at the National Institute of Biological Resources (NIBR) and Jeollanamdo Institute of Natural Resources Research (JINR Korea). The seven loci sequences of each *Vaccinium* species obtained during this study were compared with *Vaccinium* chloroplast genomes in Genbank (accession numbers LC521967, LC521968, LC521969, NC_019616, and NC_042713) using the Basic Local Alignment Search Tool (BLAST, available at http://blast.ncbi.nlm.nih.gov/Blast.cgi). *Rhododendron delavayi* (MN711645) and *Rhododendron pulchrum* (MN182619) was used as the outgroup taxon. The sequences were aligned using the Clustal W algorithm implemented in MEGA ver. 7.0. The phylogenetic tree was constructed using the neighbor-joining (NJ) method in MEGA software. The Komura 2-parameter (K2P) model and bootstrap analysis with 1000 replicates were included. Genetic distances were calculated using the K2P model. Bayesian analysis was conducted with MrBayes ver. 3.2 using two replicates of 1 million generations with the nucleotide evolutionary model. The best-fit model GTR + I + G was implemented using the Akaike Information Criterion (AIG) in MrModeltest ver. 2.3.

3. Results

3.1. Comparative Analyses of the Chloroplast Genome of Five Vaccinium Species

A previous study reported that the three *Vaccinium* cp genome sequences were deposited in NCBI Genbank and published [26]. We compared the features of the newly sequenced cp genomes with those of *V. macrocarpon* (NC_019616) and *V. oldhamii* (NC_042713), already available in NCBI Genbank. The five *Vaccinium* cp genomes contained a pair of inverted repeat regions (IRs: 30,637–34,242 bp) which were separated by a small single copy region (SSC: 2979–3518 bp) and a large single copy region (LSC: 104,552–106,565 bp). All five varied in the number of genes; total gene number ranged from 117 to 147, protein–coding genes from 75 to 85, and tRNA genes from 30 to 38. All five cp genomes contained 8 rRNA genes. The overall GC content in each cp genome was approximately 37.1% (Table 2).

Table 2. Summary of complete chloroplast genomes for five *Vaccinium* species.

Name of Taxon	*V. bracteatum*	*V. uliginosum*	*V. vitis-idaea*	*V. macrocarpon*	*V. oldhamii*
Accession number	LC521967	LC521968	LC521969	NC_019616	NC_042713
Genome length	174,404	173,356	173,967	176,045	173,245
LSC length	106,565	105,856	106,013	104,552	108,904
SSC length	2979	3114	3518	3009	3067
IR length	32,430	32,193	32,218	34,242	30,637
Total gene number	117	125	125	147	130
No. of protein coding genes	79	79	79	75	85
No. of tRNA genes	30	38	38	36	37
No. of rRNA genes	8	8	8	8	8
GC content in genome (%)	36.8	36.8	36.7	37.1	37.2

These genes can be classified into five categories based on their different roles in the chloroplast (Table 3). The *rpoA*, *rps7*, *rps12*, *rps16*, *petB*, and *petD* genes were present in *V. macrocarpon* and *V. oldhamii*, but not in the other three. The *trnG-GCC* gene was absent from *V. macrocarpon* and *V. oldhamii*. The *trnD-GUC*, *trnfM-CAU*, *trnK-UUU*, *trnR-UCU*, *trnV-UAC*, *trnY-GUA*, and *rpl2* genes were absent from *V. bracteatum*. The *rpl20* and *psbZ* genes were absent from *V. macrocarpon*. The *lhbA*, *infA*, *ycf2*, *ycf15b*, and *ycf68* genes were present only in *V. macrocarpon*.

Table 3. List of genes encoded by the five *Vaccinium chloroplast* genomes. [a] Gene with two copies; * Gene with one intron; ** Gene with two intron; The symbol • indicate the presence of the gene; -gene loss; Vb, *Vaccinium bracteatum*; Vu, *Vaccinium uliginosum*; Vv, *Vaccinium vitis-idaea*; Vm, *Vaccinium macrocarpon*; Vo, *Vaccinium oldhamii*.

Gene Category	Gene Group	Gene Names	Vb	Vu	Vv	Vm	Vo
Self-replication	RNA polymerase	*rpoA*	-	-	-	• [a]	•
		rpoB	•	•	•	•	•
		rpoC1 *	•	•	•	•	•
		rpoC2	•	•	•	•	•
	rRNA genes	*rrn16* [a]	•	•	•	•	•
		rrn23 [a]	•	•	•	•	•
		rrn4.5 [a]	•	•	•	•	•
		rrn5 [a]	•	•	•	•	•
	tRNA genes	*trnA-UGC* [a,*]	•	•	•	•	•
		trnC-GCA	•	•	•	•	•
		trnD-GUC	-	•	•	•	•
		trnE-UUC	•	•	•	•	•
		trnfM-CAU	-	•	•	• [a]	• [a]
		trnG-GCC	•	•	•	-	-
		trnG-UCC	• *	• *	• *	•	•*
		trnH-GUG [a]	•	•	•	•	•
		trnI-CAU	•	•	•	•	•
		trnI-GAU *	•	• [a]	• [a]	• [a]	• [a]
		trnK-UUU	-	• *	• *	-	• *
		trnL-CAA	•	•	•	•	•
		trnL-UAG [a]	•	•	•	•	•
		trnM-CAU	•	•	•	•	•
		trnN-GUU [a]	•	•	•	•	•
		trnP-GGG	-	-	-	•	-
		trnQ-UUG	•	•	•	•	•
		trnR-ACG [a]	•	•	•	•	•
		trnR-UCU	-	•	•	•	•
		trnS-GCU	•	•	•	•	•
		trnS-GGA	•	•	•	•	•
		trnS-UGA	•	•	•	•	•
		trnT-UGU	•	•	•	•	•
		trnT-GGU	•	•	•	•	•
		trnV-GAC	•	•	•	•	•
		trnV-UAC	-	• *	• [*]	• *	• [*]
		trnW-CCA	•	•	•	•	•
		trnY-GUA	-	•	•	•	•
	Small subunit of ribosome	*rps2*	•	•	•	•	•
		rps3	• *	• *	• *	• [a]	•
		rps4	•	•	•	•	•
		rps7	-	-	-	•	•
		rps8	•	•	•	•	•
		rps11	•	•	•	•	•
		rps12	-	-	-	• [a]	• [a]
		rps14	•	•	•	•	•
		rps15 [a]	•	•	•	•	•
		rps16 [a]	• *	• *	• *	-	• *
		rps18	•	•	-	• [a]	•
		rps19	•	•	•	•	•

Table 3. *Cont.*

Gene Category	Gene Group	Gene Names	Vb	Vu	Vv	Vm	Vo
Photosynthesis	Large subunit of ribosome	*rpl2*	-	●	●	●	●
		rpl14	●	●	●	●	●
		rpl16	-	-	-	●	●
		rpl20	●	●	●	-	●
		rpl22	●	●	●	● [a]	●
		rpl23	●	●	●	●	●
		rpl32 [a]	●	●	●	●	●
		rpl33	●	●	●	●	●
		rpl36	●	●	●	●	●
	Photosystem I	*psaA*	●	●	●	●	●
		psaB	●	●	●	●	●
		psaC [a]	●	●	●	●	●
		psaI	●	●	●	●	●
		psaJ	●	●	●	●	●
		ycf3 **	●	●	●	●	●
	Photosystem II	*lhbA* [a]	-	-	-	●	-
		psbA	-	-	● [a]	● *	-
		psbB	●	●	●	●	●
		psbC	●	●	●	●	●
		psbD	●	●	●	●	●
		psbE	●	●	●	●	●
		psbF	●	●	●	●	●
		psbH	●	●	●	●	●
		psbI	●	●	●	●	●
		psbJ	●	●	●	●	●
		psbK	●	●	●	●	●
		psbL	●	●	●	●	●
		psbM	●	●	●	●	●
		psbN	●	●	●	●	●
		psbT	●	●	●	●	●
		psbZ	●	●	●	-	●
	ATP synthase	*atpA*	●	●	●	●	●
		atpB	●	●	●	●	●
		atpE	●	●	●	●	●
		atpF *	●	●	●	●	●
		atpH	●	●	●	●	●
		atpI	●	●	●	●	●
	NADH dehydrogenase	*ndhA* [a,*]	●	●	●	●	●
		ndhB *	●	●	●	●	●
		ndhC	●	●	●	●	●
		ndhD [a]	●	●	●	●	●
		ndhE [a]	●	●	●	●	●
		ndhF	●	●	●	●	●
		ndhG [a]	●	●	●	●	●
		ndhH [a]	●	●	●	●	●
		ndhI [a]	●	●	●	●	●
		ndhK	●	●	●	●	●
		ndhJ	●	●	●	●	●
	Cytochrome b/f complex	*petA*	●	●	●	●	●
		petB	-	-	-	●	● *
		petD	-	-	-	● *	● *
		petG	●	●	●	●	●
		petL	●	●	●	●	●
		petN	●	●	●	●	●
		rbcL	●	●	●	●	●
	Rubisco	*accD*	●	●	●	●	●
Other genes	Subunit of acetyl-CoA-carboxylase	*ccsA*	●	●	●	●	●
	C-type cytochrome synthesis gene	*infA*	-	-	-	●	-
	Translational initiation factor	*cemA*	●	●	●	●	●
	Envelop membrane protein	*matK*	●	●	●	●	●

Table 3. *Cont.*

Gene Category	Gene Group	Gene Names	Vb	Vu	Vv	Vm	Vo
Gene of unknown function	Open reading frame	*ycf4*	•	•	•	•	•
Putative pseudogenes		*ycf2*	-	-	-	•	-
		ycf15b	-	-	-	•	-
		ycf68	-	-	-	• [a]	-

In addition, we observed variation in the copy numbers and intron numbers of several genes. Six protein-coding genes, four rRNA genes, and two tRNA genes were present in two copies. Furthermore, *rpoA*, *rps3*, *rps18*, and *rpl22* had two copies only in *V. macrocarpon*. Moreover, two copies of the *rps12* gene were identified in both *V. macrocarpon* and *V. oldhamii*. Fourteen genes contained introns: These included the *rpoC1* RNA polymerase gene, seven tRNA genes, and five protein-coding genes. *rpoC1*, *trnA-UGC*, *trnI-GAU*, *ndhA*, and *ndhB* genes contained one intron in all five cp genomes. *trnG-UCC* and *rps16* genes with one intron were identified in the four cp genomes other than *V. macrocarpon*. The *trnK-UUU* gene in *V. bracteatum* and *V. macrocarpon*, and the *trnV-UAC* gene in *V. bracteatum*, contained no introns, while *rps3* had one intron in the three cp genomes other than *V. macrocarpon* and *V. oldhamii*. The *psbA*, *petB*, and *petD* genes in *V. macrocarpon* and *V. oldhamii* contained one intron. Only the *ycf3* genes had two introns in each of the five cp genomes. All of the above divergences are shown in Table 3.

We compared the border structure of the five cp genomes in detail (Figure 1). IR regions contained *rpl32* and the IRA/LSC border contained a part of the *psbA* gene. *V. vitis-idaea* contained two copies of the *psbA* gene: One in the IRA/LSC border and the other in the IRB region. The *ndhF* gene was located in the SSC region, between 87 and 203 bp away from the borders. *rpl32* resided in IRB, 616-669 bp away from the SSC/IRA border. In *V. uliginosum* and *V. vitis-idaea*, the 38 bp *trnV-UAC* gene was located in the LSC region, while *V. macrocarpon* had a 661 bp variant, and *V. bracteatum* lacked this gene.

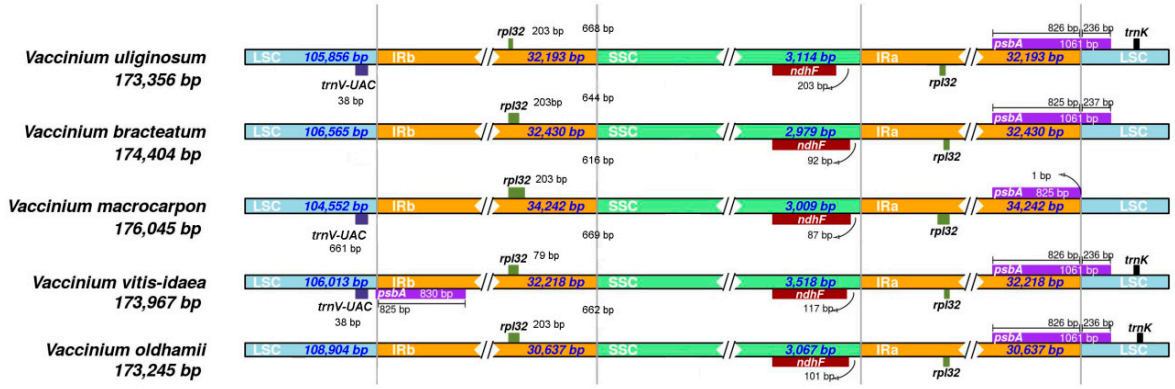

Figure 1. Distance between adjacent genes and junctions of the large single copy (LSC), small single copy (SSC), and two inverted repeats (IRs) among cp genomes from five *Vaccinium* species.

3.2. Divergence Hotspots of Five Vaccinium Cp Genomes

We compared the sequence divergence among the five *Vaccinium* cp genomes using mVISTA, with *V. macrocarpon* annotation as the reference (Figure 2). In general, non-coding regions were more divergent than coding regions. Seventeen non-coding regions—*rps4-ndhJ*, *ndhC-rbcL*, *atpE-psaI*, *petA-psbM*, *petN-rps18*, *psbL-psbE*, *petD-psbD*, *lhbA-rps14*, *psaA-psbK*, *rpoB-rpoA*, *rpl16-rps3*, *rpl23-ndhB*, *rps7-rps15*, *ndhI-ndhE*, *rpl32-ndhF*, and *ndhF-rpl32*—were highly variable among the five cp genomes. Coding regions were more conserved, with the exception of *ndhF*. To determine the level of sequence divergence, we calculated *Pi* value for regions spanning 300 bp on either end of coding regions in five

Vaccinium cp genomes with DnaSP 6.0 software. The average value of *Pi* for InDel diversity for all cp genome sites was 0.01032. Among the 505 windows, 65 windows showed much higher *Pi* values than the cp genome average (>0.02); this included 17 noncoding and their associated intergenic space regions (*trnM-CAU-psaI*, 0.028; *psbM-petN*, 0.02; *trnC-GCA-rps12*, 0.02; *psbJ-psbB*, 0.047; *accD-trnT-GGU*, 0.027; *psaA-trnQ-UUG*, 0.021; *rpoB-rpoA*, 0.055; *rpl16-rps18*, 0.027; *ycf2-trnL-CAA*, 0.02; *trnN-GUU-rps15*, 0.104; *psbA-ndhI*, 0.03; *rps15-rpl32*, 0.055; *ndhI-psbA*, 0.133; *ndhG-ndhI*, 0.032; *ndhI-psbA*, 0.029; *rps32-rps15*, 0.0515; *ndhI-psbA*, 0.133), and 8 coding and their associated intergenic space regions (*ycf3-trnS-GGA*, 0.022; *trnT-UGU*, 0.021; *rps12-ycf15*, 0.025; *trnV-GAC*, 0.028; *ndhE-ndhF*, 0.138; *ndhF*, 0.0312; *ndhF-ndhG*, 0.032; *psbA*, 0.037; (Figure 3). Of the 25 candidates, 13 lie in the LSC region, 4 in both LSC and SSC regions, 4 in the SSC region, 2 in both SSC and IR regions, and 2 in the IR region. The regions containing *ycf3-trnS-GGA* and *ndhF* had slightly less polymorphism, and *trnM-CAU-psaI* contained species-nonspecific polymorphisms and multicopy regions. Furthermore, *psbJ-psbB*, *rpl16-rps18*, *trnN-GUU-rps15*, *psbA-ndhI*, *rps15-rpl32*, *ndhI-psbA* (3), *ndhF-ndhG*, *ndhG-ndhI*, *rpl32*,*-rps15*, and *psbA* had multicopy sequences. Therefore, we did not consider these regions for further analyses.

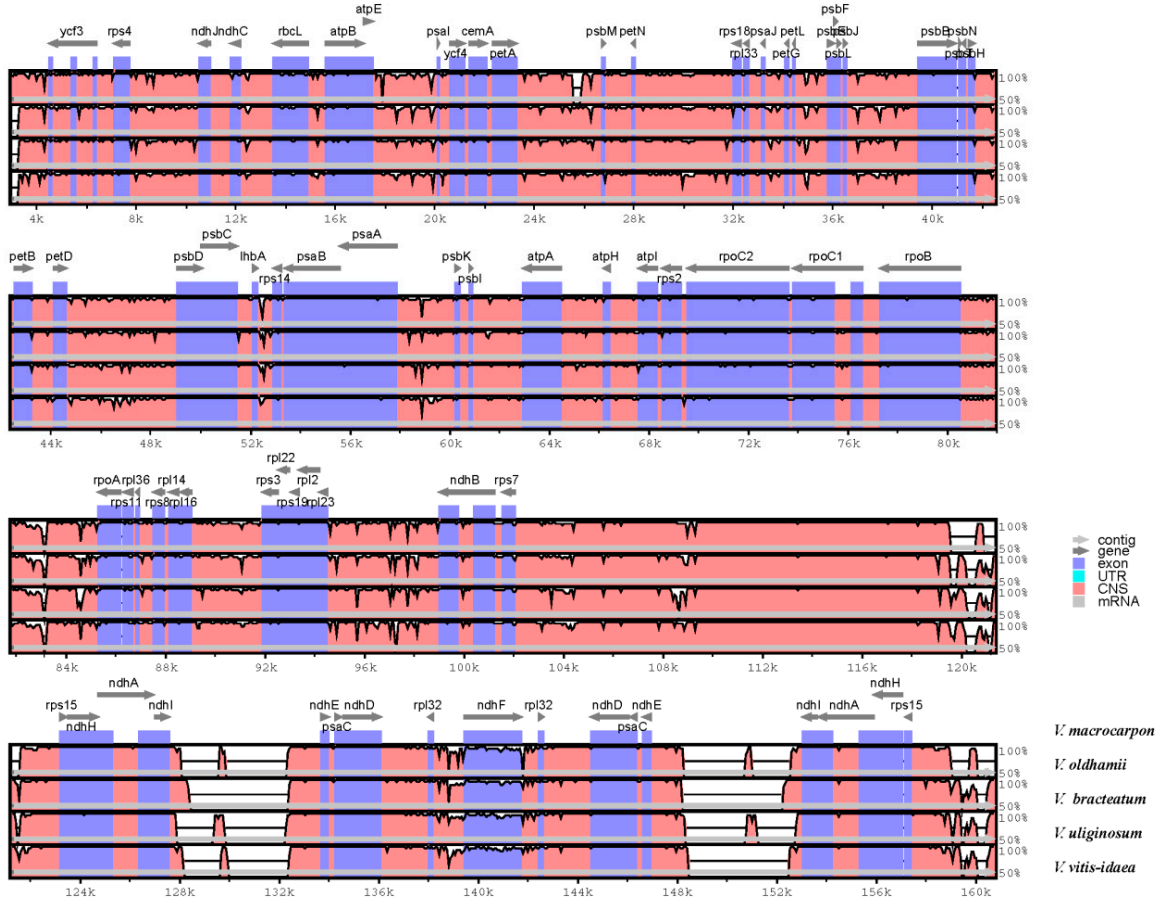

Figure 2. Comparison of five *Vaccinium* chloroplast genome using mVISTA. The cp genomes of four *Vaccinium* species were compared with that of *V. macrocarpon*. Blue blocks: Conserved genes, red blocks: Conserved non-coding sequences (CNS). White represents regions with sequence variation among the five *Vaccinium* species.

We validated the remaining 12 InDel-variable loci by PCR in the *Vaccinium* species samples (n = 19), to test their suitability as DNA barcodes (Table S1). The eight specific primer sets for six loci (*accD-trnT-GGU*, *rpoB-rpoA*, *ycf2-trnL-CAA*, *rps12-ycf15*, *trnV-GAC*, and *ndhE-ndhF*) successfully amplified their targets in all five species. For the other loci, we failed to amplify the following: *trnT-UGU* of *V. bracteatum*, *V. uliginosum*, and *V. oldhamii*; *psbM-petN* of *V. uliginosum* and *V. vitis-idaea*;

trnC-GCA–rps12 of *V. vitis-idaea*; and *psaA-trnQ-UUG* of *V. bracteatum* and *V. oldhamii* (Table 4). The *accD-trnT-GGU* primers specific to *V. uliginosum*, *V. vitis-idaea*, and *V. macrocarpon*, were derived from 8 indels with PCR products of 639, 636, and 630 bp, respectively, whereas the amplicons of *V. bracteatum* and *V. oldhamii* were identical in size (634bp). The *rpoB-rpoA* (2) and (3) primers had unique amplicon sizes that were specific to each species. *V. bracteatum*, *V. uliginosum*, *V. vitis-idaea*, *V. macrocarpon*, and *V. oldhamii* yielded band sizes of: 611, 603, 605, 706, and 656 bp spanning 5 indels; 552, 556, 558, 659, and 597 bp spanning 13 indels; and 770, 729, 725, 762, and 733 bp spanning 8 indels, respectively. The *ycf2-trnL-CAA* primers were specific to five species and spanned 11 indels that yielded amplicons of 908, 924, 905, 643, and 911 bp, respectively. The *rps12-ycf15* and *trnV-GAC* primers spanned 6 and 11 indels with amplicons of 704, 698, 705, 701, and 709 bp, and 625, 647, 640, 637, and 646 bp, respectively. The *ndhE-ndhF* primers were specific to *V. uliginosum*, *V. vitis-idaea*, and *V. macrocarpon* and spanned 7 indels with PCR products of 627, 601, and 643 bp, respectively, while the amplicons of *V. bracteatum* and *V. oldhamii* were identical in size (587 bp). To evaluate the sequence divergence in the seven hypervariable InDel regions with successful PCR amplification, we calculated the average pairwise distance for each marker using MEGA 7.0. The *rpoB-rpoA (2)* locus was the most divergent with a maximum pairwise distance of 0.05182, followed by *ndhE-ndhF* (0.04406). Although the lowest genetic distance was observed at *ycf2-trnL-CAA* (0.01768), there are still differences between the five species at that locus. The phylogenetic tree generated from *rpoB-rpoA (2)* and *ycf2-trnL-CAA* sequences datasets by NJ approach tree were identical except for *ndhE-ndhF* sequences. In tree topology of *rpoB-rpoA (2)* and *ycf2-trnL-CAA*, all nineteen *Vaccinium* species recovered in two major clades (A and B) and was placed in the basal position as the sister to the rest of the clades of the five *Vaccinium* species. In clade A, three species (*V. oldhamii*, *V. bracteatum*, and *V. uliginosum*) were present with bootstrap support (76–82%), while clade B covered two species (*V. macrocarpon and V. vitis-idaea*) with bootstrap support (77–87%). For *ndhE-ndhF*, in clade A, four species (*V. oldhamii*, *V. vitis-idaea*, *V. bracteatum*, and *V. uliginosum*) were present with a bootstrap support of 85%, while clade B covered only one species (*V. macrocarpon*) with a boostrap support of 79% (Figure S1). K2P genetic distances within and between the different species of *Vaccinium* for each marker are given in Table S2. Among them, the obtained sequences from *rpoB-rpoA* (2) and *ycf2-trnL-CAA* locus clearly showed the intraspecific and interspecific distance among five *Vaccinium* species. Intraspecific variation of the *rpoB-rpoA (2)* and *ycf2-trnL-CAA* sequences in the *V. oldhamii* was as high as 1.0% and 0.9%, respectively, whereas in *V. bracteatum*, *V. uliginosum*, *V. vitis-idaea*, and *V. macrocarpon* the variation was lower (maximum 0.7%). The *rpoB-rpoA (2)* and *ycf2-trnL-CAA* sequences of *V. macrocarpon* are clearly distinct from those of *V. bracteatum, V. uliginosum*, and *V. oldhamii* (K2P genetic distances 3.3–5.8% and 3.7–5.8%, respectively). Furthermore, the *rpoB-rpoA (2)* and *ycf2-trnL-CAA* of *V. vitis-idaea* are clearly distinct from those of *V. bracteatum*, *V. uliginosum*, and *V. oldhamii* (K2P genetic distances 4.4–5.6% and 3.2–5.2%, respectively). However, the *rpoB-rpoA (2)* and *ycf2-trnL-CAA* sequences of *V. oldhamii*, with intraspecific variation of 0.0–2.0% and 0.0–1.9%, respectively, are not clearly distinct from the sequences of *V. bracteatum* (K2P genetic distances 1.2–2.0% and 1.6–2.7%, respectively) and *V. uliginosum* (K2P genetic distances 2.2–3.4% and 1.4–2.8%, respectively). In addition, the *rpoB-rpoA (2)* and *ycf2-trnL-CAA* sequences of *V. macrocarpon* are not clearly distinct from the sequences of *V. vitis-idaea* (K2p genetic distance 1.9–2.6% and 1.1–2.2%, respectively).

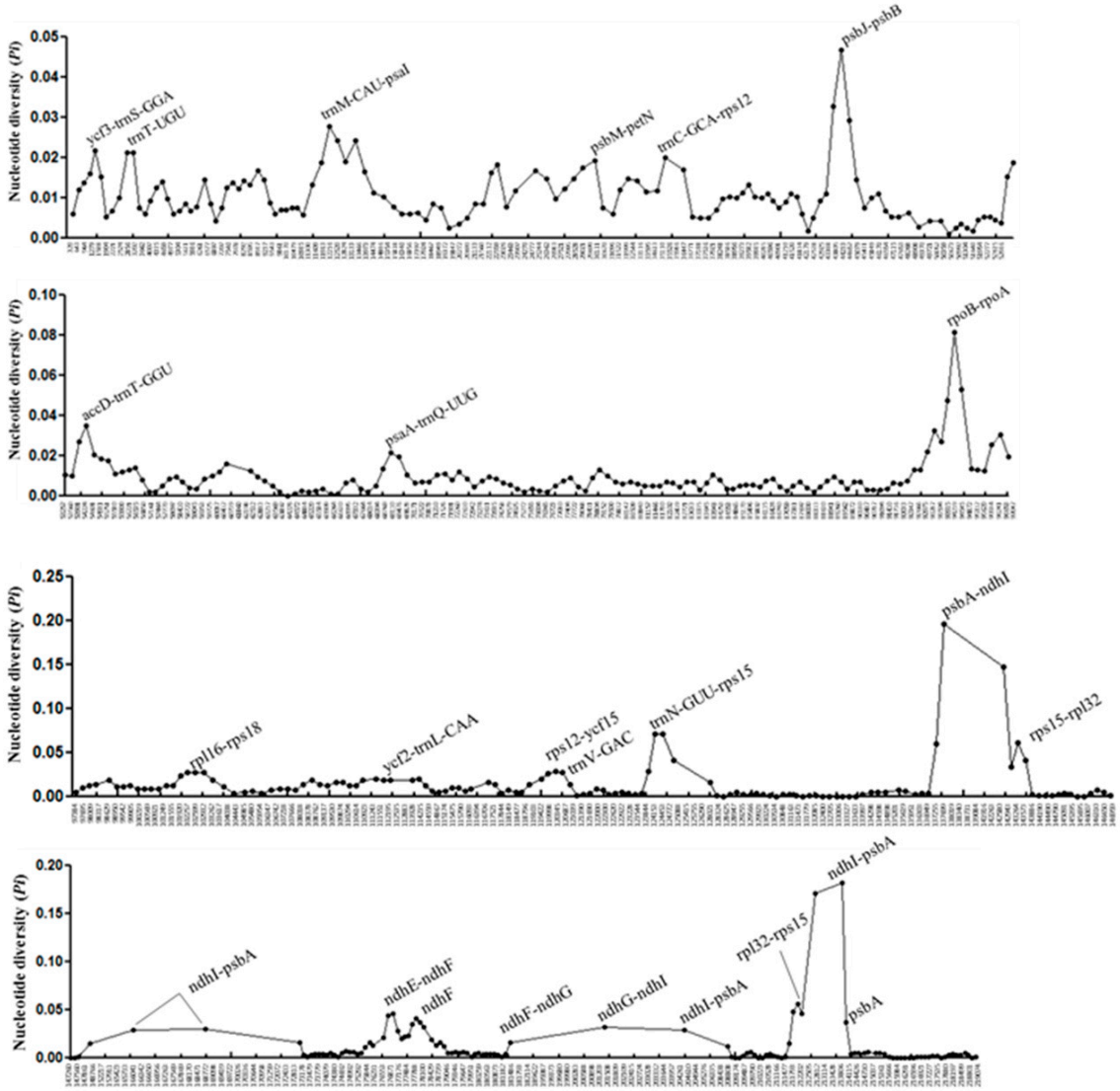

Figure 3. Comparison of nucleotide diversity values among the five *Vaccinium* cp genomes.

Table 4. Validation of 12 molecular markers derived from InDel regions of five *Vaccinium* cp genomes.

No.	Locus	Forward Primer (Sequence 5′ to 3′)	Reverse Primer (Sequence 5′ to 3′)	Product Range (bp)	AS	*No.* of InDels	Mean Pairwise Distance
1	*trnT-UGU*	CAGTAATCTTTGCA AAAGGAAAAAC	TTCGTCGTAACTTA CACCTTTATGA	617–653	40	4	0.01683
2	*psbM-petN*	ATGAGAGCTTCT TCGAATAATTTTG	CATTTTCTCTTTCA CTCGTAGTATGG	650–665	60	2	0.01467
3	*trnC-GCA-rps12*	AATTCGATTG AATAAAATGGAGGA	GGAAATTGCC AACGTCAA	611–630	80	3	0.02496
4	*accD-trnT-GGU*	GGATCTAAAT TAGGCCTCGTGTG	ATGATAGAGTCG ACTTGACAATGC	630–639	100	8	0.02504
5	*psaA-trnQ-UUG*	ATCCCCCGGT ATCTTATCTACATT	TTGCTGAATAT CAAGTCAAACAGAA	682–718	60	6	0.01364

Table 4. *Cont.*

No.	Locus	Forward Primer (Sequence 5′ to 3′)	Reverse Primer (Sequence 5′ to 3′)	Product Range (bp)	AS	*No.* of InDels	Mean Pairwise Distance
6	*rpoB-rpoA (1)*	AAAAAGCCAAT TACAAGCCAAATA	ATCCAACGGAAA TGACATTCTTAT	603–706	20	5	0.02594
7	*rpoB-rpoA (2)*	GCACTGAGATC TGCCACTTTATT	GTCATCGACGAG ATTTTTGTAGC	552–659	100	14	0.05182
8	*rpoB-rpoA (3)*	CTTTCTTCGCT TTGATCCTCATA	TCCCCTCTTATGTA TGTTTTTGC	725–770	100	7	0.02646
9	*ycf2-trnL-CAA*	ATTCTTTCGAC TCATTTTCCTGAC	CTAGGAGCCAA AACTATGTGATTG	643–924	100	11	0.01768
10	*rps12-ycf15*	CTTACACTCGGT CCCAAAGAAC	CTTTTCTCATGGG ACAATGCTCT	698–709	100	6	0.01991
11	*trnV-GAC*	GAGCTCTTAAATG GAAATGGAAAA	GCCATTGTATAAC CATTCATCAAC	625-647	100	11	0.03506
12	*ndhE-ndhF*	AATTCTATGAGG CACTGTTTCGAT	GAAGATTTTTCG TTGCTCTTGG	587–643	100	7	0.04406

AS: Amplified Success rate (%).

4. Discussion

In our previous work, we sequenced the cp genomes of *V. bracteatum*, *V. vitis-idaea*, and *V. uliginosum* using Illumina Hiseq platform, which provided resources for evolutionary and genetic studies of *Vaccinium* [26]. Although a recent study submitted sequences of a few *Vaccinium* species, such as *V. macrocarpon* and *V. oldhamii*, to the NCBI Genbank database, most research has been limited to "core" DNA barcodes, with resolution limited to the species level. By comparing the gene structure, content, and arrangement of five *Vaccinium* cp genomes, we have detected valuable variations in intergenic spacer lengths, which could serve as interspecific DNA barcodes.

Of these five species, *V. macrocarpon* had the largest cp genome and IR length; other species exhibited minor differences in genome and IR length, whereas *V. vitis-idaea* and *V. oldhamii* had the largest SSC and LSC length, respectively. All five cp genomes contained variation in protein coding and tRNA genes, with the exception of *V. uliginosum* and *V. vitis-idaea*, which were identical to the reference. All cp genomes had identical rRNA genes. The *ycf15* and *ycf68* genes were lost in three of the cp genomes, and was inferred as a pseudogene in *V. macrocarpon*. Their function as hypothetical genes is ambiguous in various land plants [28]. The *infA* gene, which codes for a translation initiation factor, was missing in all species other than *V. macrocarpon.* Millen et al. 2001 [29] demonstrated at least 24 independent losses of *infA* in angiosperms, with a transfer into the nucleus in four lineages.

DNA barcodes are universal DNA sequences, such as *rbcL*, *trnH-psbA*, and *matK*, that have a high mutation rate. Their use allows researchers to distinguish a species within a given taxon, and to reliably identify plant species. Because the core DNA barcodes lack sufficient variation between closely related taxa, none of them work across all plant species [17]. With advances in NGS technologies, recent barcoding studies have focused on the use of whole-chloroplast genome-based barcodes. Because they are more efficient at detecting gene loss and determining gene order than the established DNA barcoding, they are better able to distinguish between closely related taxa [30]. The continuing advances in NGS technology may make these the method of choice for plant identification. In contrast to SNPs and SSRs, INDELs have received more attention recently; they are relatively abundant, spread throughout the genome, contribute to both intra- and inter-specific variation, and are suitable for fast and cost-effective genotyping.

In our assessment of nucleotide diversity among five *Vaccinium* cp genomes, we observed sequence divergence in noncoding regions. Among 12 hot spot regions derived from cp genome sequences, we validated seven regions in terms of amplification success and a large number of interspecific indels (*accD-trnT-GGU*, *rpoB-rpoA(2),(3)*, *ycf2-trnL-CAA*, *rps12-ycf15*, *trnV-GAC*, and *ndhE-ndhF*).

Our phylogenetic analysis of *rpoB-rpoA(2)* and *ycf2-trnL-CAA* gene sequences, the two major clade (A and B) were confirmed, where the clade A represent the species with the fruit color of red type (a closely related species to cranberry) and the clade B represent species with the fruit color of dark blue type (a closely related species to blueberry). Many researchers reported that *V. macrocarpon* is closely related to *V. vitis-idaea* based on a phylogenetic analysis of nrITS sequence data [31,32]. We suggest that these intergenic spacers are suitable for use as DNA barcodes; they have good priming sites, and exhibit length variation and interspecific variation. This study analyzed a limited number of *Vaccinium* species based on available cp genomes; more complete cp genome sequences are needed to resolve the comprehensive phylogenies and genetic divergence within the *Vaccinium* genus.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/11/9/927/s1, Table S1: Hypervariable InDels of 12 intergenic spacer regions among five *Vaccinium* cp genomes. Vb, *Vaccinium bracteatum* cp genome; Vu, *Vaccinium uliginosum* cp genome; Vv, *Vaccinium vitis-idaea* cp genome; Vm, *Vaccinium macrocarpon* cp genome; Vo, *Vaccinium oldhamii* cp genome. Table S2. Intra- and interspecific variation of K2P genetic distances of *rpoB-rpoA (2)*, *ycf2-trnL-CAA*, and *ndhE-ndhF* sequences for five *Vaccinium* species; Vb, *Vaccinium bracteatum*; Vu, *Vaccinium uliginosum*; Vv, *Vaccinium vitis-idaea*; Vm, *Vaccinium macrocarpon*; Vo, *Vaccinium oldhamii*. Figure S1. Phylogenetic tree derived from (A) *rpoB-rpoA* (2), (B) *ycf2-trnL-CAA*, and (C) *ndhE-ndhF* sequences of specimens of the *V. bracteatum*, *V. uliginosum*, *V. vitis-idaea*, *V. macrocarpon*, *V. oldhamii* and outgroup Rh. delavayi and Rh. pulchrum. See details in Table 1.

Author Contributions: Conceptualization, Y.K. and C.C.; methodology, Y.K. and A.-Y.K.; investigation, Y.K. and J.S.; resources, D.-R.O. and J.S.; validation, J.S. and A.-Y.K.; software, Y.K.; writing—original draft preparation, Y.K.; writing—review and editing, Y.K. and C.C.; project administration, C.C.; funding acquisition, D.-R.O. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Bio & Medical Technology Development Program of the National Research Foundation (NRF) and funded by the Korean government (MSIT) [No. 2019M3A913084518].

Acknowledgments: This paper is dedicated to the memory of Heungsu Kim and Jaeman Kim for inspiring and mentoring our work and teaching us as a curious, inquisitive inventor and scientist with a passion for plant biology.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Luby, J.J.; Ballington, J.R.; Draper, A.D.; Pliszka, K.; Austin, M.E. Blueberries and cranberries (*Vaccinium*). *Genet. Resour. Temp. Fruit Nut Crops* **1991**, *290*, 393–458. [CrossRef]
2. Ballington, J.R. Collection, utilization, and preservation of genetic resources in *Vaccinium*. *HortScience* **2001**, *36*, 206–213. [CrossRef]
3. Abreu, O.A.; Barreto, G.; Prieto, S. *Vaccinium* (Ericaceae): Ethnobotany and pharmacological potentials. *Emir. J. Food Agric.* **2014**, *26*, 577. [CrossRef]
4. Strik, B.C. A review of optimal systems for organic production of blueberry and blackberry for fresh and processed markets in the northwestern United States. *Sci. Horticult.* **2016**, *208*, 92–103. [CrossRef]
5. Primetta, A.K.; Jaakola, L.; Ayaz, F.A.; Inceer, H.; Riihinen, K.R. Anthocyanin fingerprinting for authenticity studies of bilberry (*Vaccinium myrtillus* L.). *Food Control* **2013**, *30*, 662–667. [CrossRef]
6. Zhu, H.; Senalik, D.; McCown, B.H.; Zeldin, E.L.; Speers, J.; Hyman, J.; Zalapa, J.E. Mining and validation of pyrosequenced simple sequence repeats (SSRs) from American cranberry (*Vaccinium macrocarpon* Ait.). *Theor. Appl. Genet.* **2012**, *124*, 87–96. [CrossRef]
7. Jothi, G.A.; Sircom, J. Native pollinators alone provide full pollination on small-scale commercial cranberry (Ericaceae) farms. *Can. Entomol.* **2019**, *151*, 745–756. [CrossRef]
8. Jurikova, T.; Skrovankova, S.; Mlcek, J.; Balla, S.; Snopek, L. Bioactive compounds, antioxidant activity, and biological effects of European cranberry (*Vaccinium oxycoccos*). *Molecules* **2019**, *24*, 24. [CrossRef]
9. Gustavsson, B.A. Genetic variation in horticulturally important traits of fifteen wild lingonberry *Vaccinium vitis-idaea* L. populations. *Euphytica* **2001**, *120*, 173–182. [CrossRef]
10. Hokkanen, J.; Mattila, S.; Jaakola, L.; Pirttilä, A.M.; Tolonen, A. Identification of phenolic compounds from lingonberry (*Vaccinium vitis-idaea* L.), bilberry (*Vaccinium myrtillus* L.) and hybrid bilberry (*Vaccinium x intermedium* Ruthe L.) leaves. *J. Agric. Food Chem.* **2009**, *57*, 9437–9447. [CrossRef]
11. Lee, T.B. *Coloured Flora of Korea*; Hyangmunsa: Seoul, Korea, 2003; Volume 2, pp. 27–29. (In Korean)

12. Kim, C.Y.; Kim, Y.H.; Han, S.H.; Ko, H.C. Current Situations and Prospects on the Cultivation Program of Tropical and Subtropical Crops in Korea. *Korean J. Plant Res.* **2019**, *32*, 45–52.
13. Sherman, B.; Henry, R.J. The Nagoya Protocol and historical collections of plants. *Nat. Plants* **2020**, *6*, 430–432. [CrossRef] [PubMed]
14. Lee, B.L.; Kang, J.H.; Kim, H.M.; Jeong, S.H.; Jang, D.S.; Jang, Y.P.; Choung, S.Y. Polyphenol-enriched *Vaccinium uliginosum* L. fractions reduce retinal damage induced by blue light in A2E-laden ARPE19 cell cultures and mice. *Nutr. Res.* **2016**, *36*, 1402–1414. [CrossRef] [PubMed]
15. Oh, B.T.; Jeong, S.Y.; Velmurugan, P.; Park, J.H.; Jeong, D.Y. Probiotic-mediated blueberry (*Vaccinium corymbosum* L.) fruit fermentation to yield functionalized products for augmented antibacterial and antioxidant activity. *J. Biosci. Bioeng.* **2017**, *124*, 542–550. [CrossRef]
16. Oh, D.R.; Yoo, J.S.; Kim, Y.; Kang, H.; Lee, H.; Lm, S.J.; Hong, J. *Vaccinium bracteatum* leaf extract reverses chronic restraint stress-induced depression-like behavior in mice: Regulation of hypothalamic-pituitary-adrenal axis, serotonin turnover systems, and ERK/Akt phosphorylation. *Front. Pharmacol.* **2018**, *9*, 604. [CrossRef]
17. Li, X.; Yang, Y.; Henry, R.J.; Rossetto, M.; Wang, Y.; Chen, S. Plant DNA barcoding: From gene to genome. *Biolog. Rev.* **2015**, *90*, 157–166. [CrossRef]
18. Ravi, V.; Khurana, J.P.; Tyagi, A.K.; Khurana, P. An update on chloroplast genomes. *Plant Syst. Evol.* **2008**, *271*, 101–122. [CrossRef]
19. Gualberto, J.M.; Mileshina, D.; Wallet, C.; Niazi, A.K.; Weber-Lotfi, F.; Dietrich, A. The plant mitochondrial genome: Dynamics and maintenance. *Biochimie* **2014**, *100*, 107–120. [CrossRef]
20. Bi, Y.; Zhang, M.F.; Xue, J.; Dong, R.; Du, Y.P.; Zhang, X.H. Chloroplast genomic resources for phylogeny and DNA barcoding: A case study on Fritillaria. *Sci. Rep.* **2018**, *8*, 1–12. [CrossRef]
21. Chen, X.; Zhou, J.; Cui, Y.; Wang, Y.; Duan, B.; Yao, H. Identification of Ligularia herbs using the complete chloroplast genome as a super-barcode. *Front. Pharmacol.* **2018**, *9*, 695. [CrossRef]
22. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870–1874. [CrossRef] [PubMed]
23. Rozas, J.; Ferrer-Mata, A.; Sánchez-DelBarrio, J.C.; Guirao-Rico, S.; Librado, P.; Ramos-Onsins, S.E.; Sánchez-Gracia, A. DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Mol. Biol. Evol.* **2017**, *34*, 3299–3302. [CrossRef]
24. Shi, L.; Chen, H.; Jiang, M.; Wang, L.; Wu, X.; Huang, L.; Liu, C. CPGAVAS2, an integrated plastome sequence annotator and analyzer. *Nucleic Acids Res.* **2019**, *47*, W65–W73. [CrossRef] [PubMed]
25. Untergasser, A.; Nijveen, H.; Rao, X.; Bisseling, T.; Geurts, R.; Leunissen, J.A. Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Res.* **2007**, *35* (Suppl. 2), W71–W74. [CrossRef] [PubMed]
26. Kim, Y.; Shin, J.; Oh, D.R.; Kim, D.W.; Lee, H.S.; Choi, C. Complete chloroplast genome sequences of *Vaccinium bracteatum* Thunb. *V. vitis-idaea* L. and *V. uliginosum* L. (Ericaceae). *Mitochondrial DNA B* **2020**, *5*, 1843–1844.
27. Knudsen, B.; Knudsen, T.; Flensborg, M.; Sandmann, H.; Heltzen, M.; Andersen, A.; Mønsted, S. *Bio-Qiagen, C.L.C.*; CLC Sequence Viewer: Aarhus, Denmark, 2011; Volume 6.
28. Raubeson, L.A.; Peery, R.; Chumley, T.W.; Dziubek, C.; Fourcade, H.M.; Boore, J.L.; Jansen, R.K. Comparative chloroplast genomics: Analyses including new sequences from the angiosperms *Nuphar advena* and *Ranunculus macranthus*. *BMC Genom.* **2007**, *8*, 174. [CrossRef]
29. Millen, R.S.; Olmstead, R.G.; Adams, K.L.; Palmer, J.D.; Lao, N.T.; Heggie, L.; Calie, P.J. Many parallel losses of infA from chloroplast DNA during angiosperm evolution with multiple independent transfers to the nucleus. *Plant Cell* **2001**, *13*, 645–658. [CrossRef]
30. Nock, C.J.; Waters, D.L.; Edwards, M.A.; Bowen, S.G.; Rice, N.; Cordeiro, G.M.; Henry, R.J. Chloroplast genome sequences from total DNA for plant identification. *Plant Biotechnol. J.* **2011**, *9*, 328–333. [CrossRef]
31. Kron, K.A.; Powell, E.A.; Luteyn, J.L. Phylogenetic relationships within the blueberry tribe (*Vaccinieae*, Ericaceae) based on sequence data from matK and nuclear ribosomal ITS regions, with comments on the placement of Satyria. *Am. J. Bot.* **2002**, *89*, 327–336. [CrossRef]
32. Sultana, N.; Pascual-Díaz, J.P.; Gers, A.; Ilga, K.; Serçe, S.; Vitales, D.; Garcia, S. Contribution to the knowledge of genome size evolution in edible blueberries (genus *Vaccinium*). *J. Berry Res.* **2019**, *10*, 243–257. [CrossRef]