

Article

Genetic Diversity of Lowbush Blueberry throughout the United States in Managed and Non-Managed Populations

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Abstract: Expressed sequenced tagged-polymerase chain reaction (EST-PCR) molecular markers were used to evaluate the genetic diversity of lowbush blueberry across its geographic range and to compare diversity among four paired managed/non-managed populations. Seventeen populations were sampled in a north–south transect throughout the eastern United States with 27 km to 1600 km separating populations. The majority of genetic variation was found within populations (75%) with each population genetically unique ($p \leq 0.0001$) with the exception of the Jonesboro, ME, and Lubec, ME, populations. The effects of management for commercial fruit harvesting on genetic diversity were investigated in four locations in Maine with paired managed and non-managed populations. Significant differences were found between the populations indicating that commercial management for fruit production influences the diversity of lowbush blueberries in the landscape, even though planting does not occur. Forests are harvested and the existing understory blueberry plants become established.

Keywords: EST-PCR; *Vaccinium angustifolium*; geographic range; domestication

1. Introduction

Lowbush blueberry (*Vaccinium angustifolium* Aiton) is an outcrossing, rhizomatous, tetraploid ($2n = 4 \times = 48$) woody perennial in the family Ericaceae. It is native to eastern North America from Newfoundland, Canada, south to North Carolina and west to Manitoba (United States Department of Agriculture (USDA) Plants Database; [1]). Plants are commonly found in disturbed acidic soils, especially glacial outwash plains, but also in forests, bogs, or exposed rocks [2,3]. Multiple cold hardiness zones are present throughout its geographic range. Lowbush blueberries are valued primarily for their fruit, but also for their leaves for tea, and utility as a horticultural plant in the landscaping industry. Management for commercial fruit production is primarily located in Maine, Quebec, and the Canadian Maritimes with the largest acreage in the vast barrens along the Atlantic coast [4]. Lowbush blueberry is unique among fruit crops, as fields are not planted with cultivars of known traits, or genetics. Rather, an area of forest is cleared and managed with fire, mowing, and herbicides to promote the growth and spread of existing understory plants through rhizomes and the colonization of bare ground by new plants via seeds [5]. The resulting field is a mosaic of genetically unique plants, referred to as “clones”, that are visually distinct with varying pollination, yields, cold hardiness, fertility response and susceptibility to pests [6–9].

The underlying genetic diversity of lowbush blueberry is relatively unknown compared to other fruit crops [8]. Recently, a variety of techniques and molecular markers have been used to investigate the genetic heterogeneity and relatedness of *V. angustifolium* populations [1,8,10–15]. These studies have shown *V. angustifolium* to be highly diverse in Maine and the Canadian Maritimes. Restricted geographic sampling may not detect diversity influenced by latitudinal clines and geographic factors [16]. Latitudinal clines also drive the development of ecotypes in regards to photoperiod sensitivity and cold hardiness within a species [17,18]. Air temperatures are expected to increase by 1.1–1.7 °C in the primary lowbush blueberry producing regions of North America by 2050 due to climate change [19]. Already, growers in Maine are experiencing a growing season that has increased by one month since 1950 resulting in an increase in pests and pathogens [20], fall bloom [21] and 50% more days with precipitation during bloom [22].

Vaccinium angustifolium is believed to be one of the first blueberry species to be managed in North America. Clearing and burning of forests by Native Americans for harvesting occurred before the arrival of Europeans [23]. Although attempts have been made to improve or develop cultivars, cultivation has remained unchanged for hundreds of years, relying on wild plant populations pre-existing in fields [24,25]. It is assumed that plants managed for commercial harvesting retain the same genetic diversity as plants growing in a mature forest, however there has been no investigation to evaluate these assumptions. Loss of diversity has been documented in many domesticated crops as they have been selected by humans [26,27]. Despite its relatively recent domestication (1939), commercially important rabbiteye blueberry (*Vaccinium virgatum* Aiton) cultivars can be traced to four individual plants and as such inbreeding depression and high genetic load in cultivars have been an issue for breeders [28]. Existing lowbush genotypes in the landscape could have a founder effect on the genetic diversity of lowbush fields. A large stochastic event of removing forest cover may result in a population of plants ultimately descended from relatively few parents [29].

The objective of this study was to utilize expressed sequenced tagged-polymerase chain reaction (EST-PCR) molecular markers developed for use in *Vaccinium* species to evaluate the genetic diversity of lowbush blueberry across the eastern United States and to compare diversity between managed and non-managed populations [30]. Increasing the geographic assessment of population genetic composition will provide a landscape-level quantification of the genetic diversity in lowbush blueberry throughout a large portion of its native range. Sampling populations representing climates warmer than those found in Maine and the Canadian Maritimes may provide insight into how diversity may change in northern populations with rising annual temperatures, fewer chilling hours, and an increased growing season due to climate change. Additionally, incorporating managed and non-managed populations will help determine whether commercial management changes the genetics of the species.

2. Materials and Methods

2.1. Plant Material

Lowbush blueberry stems and leaves were collected from seven locations in Maine (Lubec, Jonesboro, Old Town, Winterport, Hope, Salem, and Sebago) representing areas of primary fruit production within the state. These locations were geographically separated by at least 27 km. Populations outside of Maine ($n = 6$) were selected based upon prior knowledge of existing populations along a general north to south transect that would represent the geographic range in the eastern United States. These sites included Fitchburg, MA, Groton, VT, Shokan, NY, Milton, PA, Dolly Sods, WV, and New Castle, VA (Figure 1). Several USDA cold hardiness zones were represented in the populations from 4b (−31.7 to −28.9 °C) in Salem, ME to 7a (−17.8 to −15 °C) in New Castle, VA, USA (<http://planthardiness.ars.usda.gov/PHZMWeb/>).

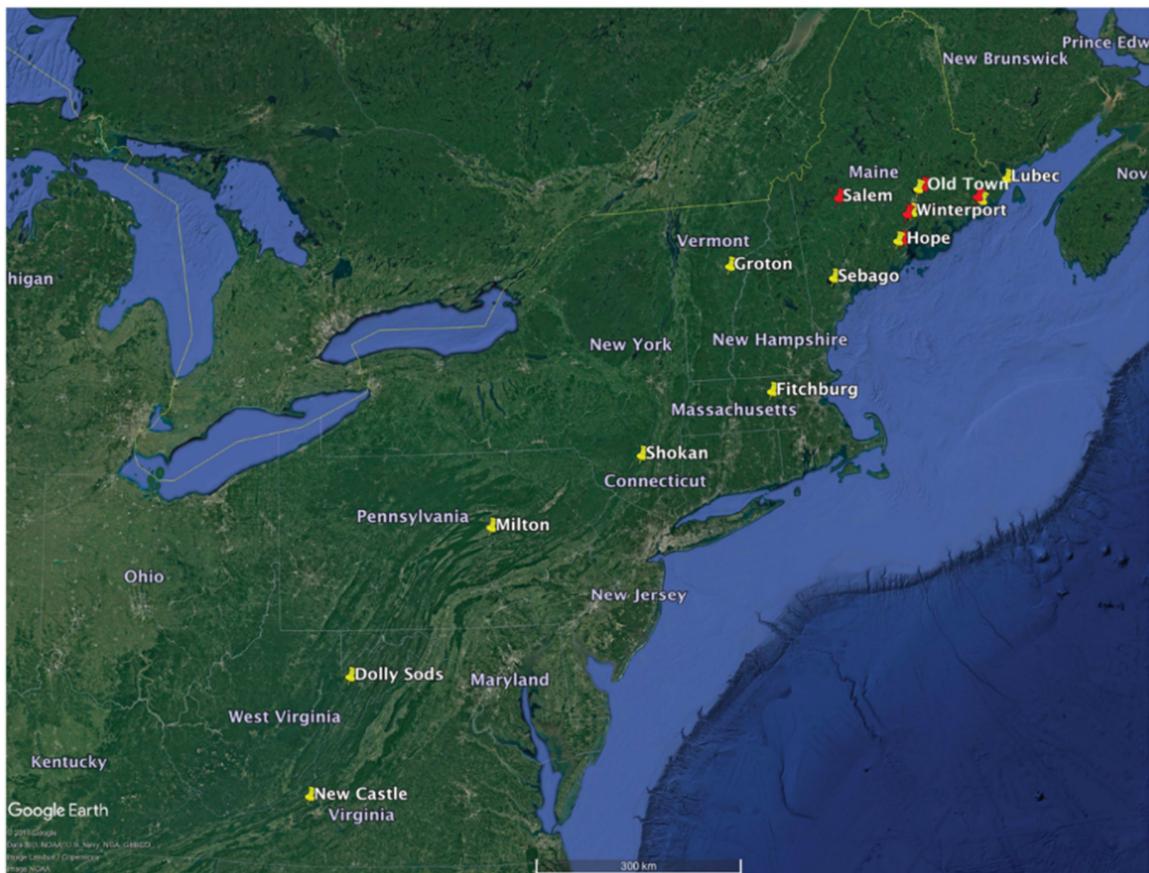


Figure 1. Sampled lowbush blueberry populations. Populations were collected along a 1600 km transect and represented a range of cold hardiness zones and a general north to south gradient which includes a large portion of the native growing range in the eastern United States (Gleason and Cronquist, 1991). Populations in Maine include plants commercially managed (red pins) for fruit production (Jonesboro, Old Town, Winterport, Hope, Salem) and plants from non-managed (yellow pins) natural forested landscape sites (Lubec, Jonesboro, Old Town, Winterport, Hope, Sebago). All locations outside of Maine were sampled from a forested landscape with no known history of commercial management.

To assess genetic diversity among managed commercial fields and wild unmanaged landscapes, we collected plants in a paired design in four regions in Maine making a total of 11 populations sampled in Maine (four paired and three not paired). The four “paired” populations were sampled by collecting 20 individual plants from a field managed for fruit production and 20 individual plants from an adjacent (>500 m) forest with no historical record or evidence (large mature tree stands) of management. These paired populations were sampled in Hope, Winterport, Old Town, and Jonesboro, ME. The buffer of at least 500 m between managed fields and natural areas in each pair was chosen to minimize any management “overflow” in commercial fields that could have impacted the plants in the natural areas over time, but at the same time be within the average bee-foraging distance [31] and the hypothesized fruit/seed dispersal range by birds [12].

Approximately one gram of leaf material was collected from 12–20 genetically distinct individuals in each population along transects. Since lowbush blueberry is rhizomatous, care was taken to collect from samples that were visually distinct, at least 6 m from the nearest sampled neighbor, and large enough to survive leaf removal. Plants were taxonomically verified to ensure only *V. angustifolium* was sampled and not visually similar (*V. myrtilloides*, *V. boreale*, and *V. pallidum*) which are commonly found in the same landscapes [32].

2.2. DNA Isolation and Amplification

Genomic DNA was isolated from approximately 2 mg of young, still expanding, leaf material using Qiagen DNeasy Plant Mini Kit (Valencia, CA, USA) or a CTAB (cetyl trimethylammonium bromide) extraction protocol developed by Doyle and Doyle [33]. DNA concentration and purity was measured with a Thermo Scientific NanoDrop 2000 spectrophotometer (Wilmington, DE, USA). Primer pairs previously reported in lowbush blueberry diversity studies were used, and additional EST-PCR molecular marker primers were developed from *V. corymbosum* EST sequences available in the blueberry genomic database (BBGD454) representing known general housekeeping and stress response genes [11,12,34]. Primer sequences were designed to amplify as much of the target gene as possible by choosing primer sequences at extreme 5' and 3' ends of an EST sequence using the Primer3 interface [35]. Eleven stress response [36,37], and 10 general housekeeping genes [38] were screened for polymorphisms and suitability for genetic diversity analysis. A total of 45 primer pairs were screened for this study, of which 16 were used for analysis (Table 1). Amplification protocols were performed as previously described by Rowland et al. [39]. An AdvanCE FS96 systemTM (Advanced Analytical Technologies; Ames, IA, USA) was used for separation and digital visualization of polymorphic bands via capillary electrophoresis.

Table 1. Primer pairs used for analyses that yielded reproducible polymorphic bands throughout the dataset.

Primer Name	Sequence (5' to 3')	Tm (°C)	Annealing Temperature (°C)
02675	AAGGAAGGGGGAGGGTTTAT	58	49
	AAAAAGGGGCACAAAGAAGAA	54	
00125	AGTAGGGGACACAGCCACAC	62	55
	TGGCAGAGGGTAGAACTTGC	60	
00064	CACAGTTTGTACGGTGATGG	56	51
	TGATTGCTGCACCAAGACTC	58	
CO	AAACTACCCGATGTCGATGC	57	49
	TCACAAAAACGATGGAACGA	54	
00313	CAGCACAAATTGCAGAGCAT	56	50
	GCATGGGAAAGGAATTCTGA	55	
NA799	TTTACCTCCCTTGTCCACAC	57	52
	GGAAATCCCACAGCTCAGAA	57	
CA448F	GTGGGCAGAGTGAGGAAGAG	60	53
	ACACAAACCAGGGAGAAACG	58	
CA15F	ACCAAAGCTGAGCAACCAAT	57	52
	GTCTGCCATGAAAACCCAAC	57	
CA65F	GTCGAATCCGAAGCCTTCTC	58	48
	AAACAGCAAATCCCAATCG	53	
CA1463	GAAGATGTCGTGGAGGTGGT	59	51
	TAATGCGGGTTGATGTAGCA	56	
CA1423	TCATAGCCAATACACTCGAACC	56	51
	GCCCCACCTTAGCAAATC	56	
CA1785L	CACCACCACTGCGTACACC	62	50
	GCATGAGCCGAACATAATCA	55	
EST133	AAACAATCCACCAATCAACTTGT	54	49
	CCTCTCCACAGTCCGATCAA	59	
EST193	GAGGGATTGAGCACGAAGAG	58	50
	CAACATCATCAACCCCAACA	55	
EST248	TGGAGACTGGAGTGATGCAA	58	49
	AAGTGCATTAAGCATCCGAAA	54	
EST1029	GAAGTTTCCGTCTCTGCAA	55	50
	CTGCAGCTAGGACCGAAGAG	60	

2.3. Molecular Marker Analysis

Clearly defined, reproducibly amplified fragments between 200 and 1500 base pairs were scored as dominant markers (present or absent) with PROsize™ software (Advanced Analytical Technologies; Ames, IA, USA). Primer pairs that failed to amplify in all individuals or that did not produce consistently reproducible bands were not used for further analysis. Binary scoring matrices for each EST-PCR primer pair were exported and combined in Microsoft Excel™ for analysis with the GenAlEx™ 6.5 statistical package add-in [40]. Distance measures (genetic and geographic) were calculated for single and combined populations to be used as the basis for analysis of molecular variance (AMOVA), spatial autocorrelation, and principal coordinate analysis (PCoA). Analysis of variance for band richness between populations was calculated with JMP™ software (SAS Institute 2017; Cary, NC, USA). Comparisons between managed and non-managed populations were made using non-parametric analysis of variance (PerMANOVA) measures in PC-ORD 6 with a 2-way factorial design, 4999 permutations, using Sorensen distance measures (MJM Software, Gleneden Beach, OR, USA). Bonferoni correction of p -values to maintain an overall experiment-wise error rate was conducted [41].

2.4. Sequencing of Amplified Fragments

Amplified fragments were sequenced to determine if polymorphic bands produced by EST-PCR molecular markers were different alleles in the tetraploid lowbush blueberry. DNA from 20 clones (10 managed and 10 non-managed) was amplified with a subset of 5 EST-PCR primer pairs that reliably produced 2–7 polymorphic bands. Each amplification was repeated three times per individual and then separated by electrophoresis on a 1.5% agarose gel. Bands were excised from the gel and pooled for extraction with the Qiagen QIAquick Gel Extraction Kit (Valencia, CA, USA). Sequencing of bands was performed at the University of Maine's DNA Sequencing Facility (Orono, ME, USA). Sequences were aligned and assembled using Geneious R7 software (Biomatters, Auckland, New Zealand) before being compared with the original template EST sequence from which the primer was designed and similar sequences available in GenBank using a nucleotide BLAST (program to search a nucleotide database).

3. Results

3.1. Managed vs. Non-Managed Populations

The genetics of 175 clones were assessed with EST-PCR molecular markers. Of the 45 primer pairs tested, only 16 produced reliable bands with no missing data for further evaluation. Although GenAlEx can handle missing data (entered as -1), markers missing (not amplified) from a few locations were removed from all sites for comparing diversity among populations, as further analysis in PC-ORD 6 could not incorporate missing data. In all, a total of 142 polymorphic bands were used for comparing commercial fields and natural areas. AMOVA analysis with 9999 permutations estimated 75% of the total genetic variance within sampled populations and the remaining 25% was estimated to be among populations. Pairwise comparisons between populations provided evidence for significant differences ($p < 0.0001$) between all but one managed field and non-managed natural site in neighboring forest and also between location sites of the pairs, when Bonferoni corrected for the number of comparisons made (Table 2). Similarities among managed populations were observed based on PhiPT values (an F_{ST} analogue measure allowing intra-individual variation) compared to their non-managed natural site counterparts. Further analysis via PerMANOVA also provided evidence of differences between locations, management, and the interaction between location and management ($p = 0.0002$) (Table 3). While there is a trend for fewer polymorphic bands in the populations from managed fields when compared to non-managed natural site populations, this trend is not as large for the Hope location ($p = 0.077$, Figure 2). The contribution to overall diversity by stress-related primer pairs within and among managed and non-managed natural sites was also assessed with AMOVA. Separate AMOVA

analyses were conducted for managed and non-managed populations using only stress-related primer pairs or only “neutral” primer pairs. Results of these analyses showed no increase or decrease in diversity between managed and non-managed populations based upon origin of primer pairs ($p < 0.001$ and $p < 0.001$, respectively) (data not shown).

Table 2. Pairwise analysis of molecular variance (AMOVA) comparison between populations currently managed for commercial blueberry production and those from a mature forest not managed for fruit production. p -values are shown (random > data) based on 9999 permutations in GenAlEx 6.5. All populations are significantly different from all others, except Jonesboro managed from Winterport managed when the p -values are Bonferoni corrected. The Old Town managed is different at $p \leq 0.10$ (Bonferoni corrected) from the Jonesboro managed. Managed populations are significantly different from their associated paired non-managed population (bold p -values) found in close proximity (≤ 500 m).

		Managed				Non-Managed			
		Hope	Jonesboro	Old Town	Winterport	Hope	Jonesboro	Old Town	Winterport
Non-Managed	Winterport	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	
	Old Town	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001		
	Jonesboro	0.0001	0.0001	0.0001	0.0001	0.0001			
	Hope	0.0001	0.0001	0.0001	0.0001				
Managed	Winterport	0.0001	0.0056 (ns)	0.0001					
	Old Town	0.0001	0.0023 †						
	Jonesboro	0.0001							
	Hope								

† p -value ≤ 0.10 when Bonferoni corrected to adjust for the number of comparisons.

Table 3. Non-parametric analysis of variance (PerMANOVA) for paired (location) managed and non-managed population comparisons. Significant differences are found between location, management, and the interaction ($p = 0.0002$), where df = degrees of freedom, SS = sums of squares, MS = mean square, F = F statistic, and p = probability of significance.

Source	df	SS	MS	F	p
Location	3	7.1233	2.3744	16.299	0.0002
Management	1	3.7856	3.7856	25.986	0.0002
Interaction	3	3.1031	1.0344	7.1004	0.0002
Residual	152	22.143	0.14568		
Total	159	36.155			

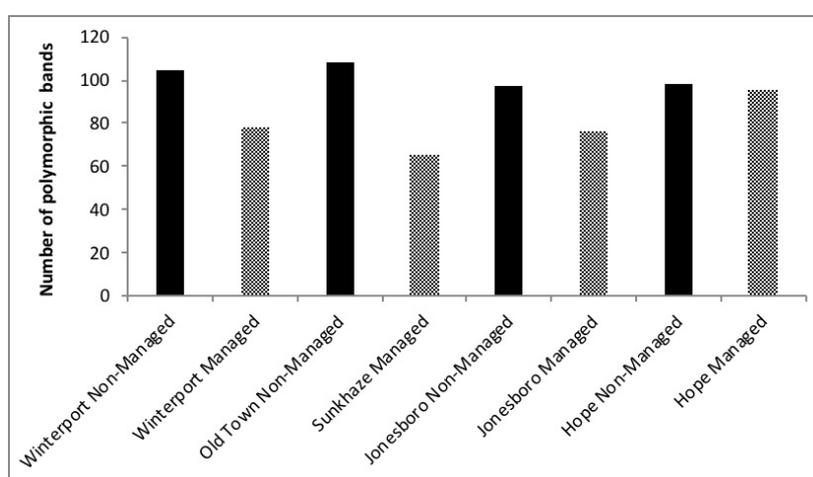


Figure 2. Number of polymorphic bands in lowbush blueberry populations managed for commercial production and those from non-managed populations in mature forests without a known history of commercial management.

3.2. Genetic Diversity throughout the Eastern United States

Of the 45 primer pairs screened, 24 EST-PCR markers were polymorphic and reliably amplified in greater than 90% of the sampled clones. Those individual plants with poor marker amplification were assigned -1 for analysis in GenAlEx [42]. A total of 202 polymorphic bands were used for analysis of 338 individuals in 17 populations. Prior results showed significant differences between managed and non-managed populations so paired populations were split and treated as individual populations. Seventy five percent of the variance associated within the populations with the remaining 25% found among populations with a total differentiation between populations of 0.252 (PhiPT, $p \leq 0.001$) (Table 4). Pairwise comparisons suggest significant differences between almost all populations ($p = 0.0001$) with the exception of the non-managed Lubec, ME and managed Jonesboro, ME sites ($p = 0.228$) (Table 5). With the entire data set, populations tended to be more similar, although statistically different, among managed locations when compared to non-managed populations based on PhiPt values (PhiPt 0.037, 0.049, 0.101, 0.405, $p < 0.100$).

Table 4. AMOVA for all sampled populations with 9999 permutations. The majority of the variance associated with our data (75%) is found within the populations with the remainder (25%) found among the populations. There are significant differences between populations ($p \leq 0.0001$) indicating a high level of genetic diversity. This is confirmed with a relatively high PhiPT (0.252) for all populations showing little similarity among populations.

Source	df	SS	MS	Est. Var.	%
Among Pops	16	1482.821	92.676	4.063	25%
Within Pops	321	3878.995	12.084	12.084	75%
Total	337	5361.817		16.147	100%
Stat	Value	<i>p</i>			
PhiPT	0.252	0.0001			

3.3. Spatial Analysis and Population Structure

Spatial autocorrelation was performed on all 17 populations with variable distance classes representing intra-field distance classes (50 m, 100 m, 250 m) and inter-field distance classes (500 m, 1000 m, 2.5 km, 10 km, 25 km, 100 km, and 1000 km) (Figure 3). Distance influenced genetic heterogeneity at the 50 m and 1000 m class as the calculated r fell above the upper confidence limit (95%). Principal coordinate analysis (PCoA) showed no clearly defined populations based on geographic and genetic distance (Figure 4). Spatial autocorrelation in conjunction with PCoA show limited spatial structure in lowbush blueberry, primarily within field distances. We attempted to cluster similar individuals with STRUCTURE software using Bayesian algorithms [43]. This was ultimately unsuccessful (data not shown), as an increasingly high number (100+) of K populations were needed for successful clustering providing little information about population structure over a large geographic range [44].

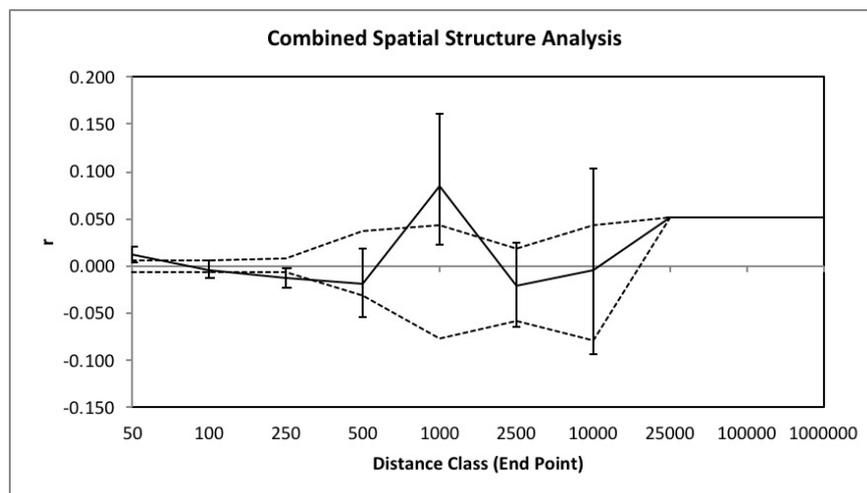


Figure 3. Spatial autocorrelation analysis. Distance classes (meters) were assigned to represent the distances between clones in a population (≤ 50 m) and the distances between populations at different sites (≥ 1000 m). Dashed lines indicate the upper and lower 95% confidence intervals while the solid line indicates the calculated r . Positive spatial structure appears at 50 m within a population and then again at 1 km among sites.

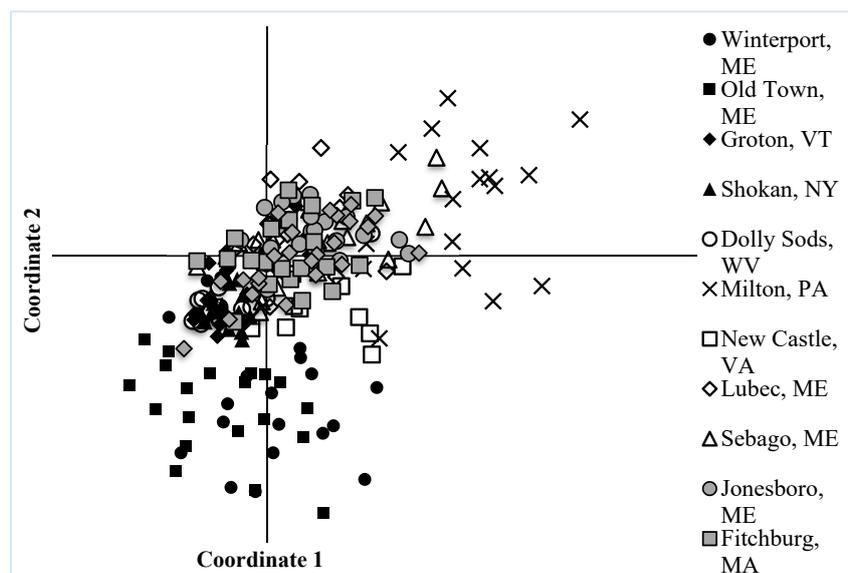


Figure 4. Principal coordinates (PCoA) analysis of genetic composition of non-managed lowbush blueberry populations ($n = 11$).

3.4. Evaluation of Stress-Related Expressed Sequenced Tagged-Polymerase Chain Reaction (EST-PCR) Molecular Markers

EST-PCR primers were developed from EST sequences of housekeeping and stress-related genes with the hypothesis that EST-PCR molecular markers would produce a higher rate of polymorphic bands due to the relaxed selective pressure compared to housekeeping genes [45]. Our results found this not to be the case as the average number of bands per primer pair designed from stress genes and housekeeping genes did not differ significantly, 7.0 and 7.16 respectively ($p = 0.863$). We also observed no difference in the frequency of the number of bands between management. EST-PCR markers developed from stress-related genes proved useful for genetic diversity analysis, but they did not have an advantage over the existing EST-PCR markers in terms of increased number of bands or frequency between populations. The effect of latitude and cold hardiness zone on the number of stress-related bands were evaluated with linear regression analysis. Neither latitude ($r^2 = 0.244$, $p = 0.126$) nor cold hardiness zone ($r^2 = 0.452$, $p = 0.494$) had a significant effect on the number of stress-related bands present in any of the sampled populations (data not shown).

3.5. Sequencing of Selected Polymorphic Bands

All sequenced bands were homologous to primer sequences at the ends, but BLAST results varied in regards to similarities with the original EST sequence from which they were designed. Generally, sequences of the same size and primer pair were highly homologous (>90%) between all 20 lowbush blueberry clones with most variation between sequences associated with gaps in the refined sequence resulting from background noise in the raw sequence contigs. At least one polymorphic band from each primer pair was homologous to the original EST sequence with other bands having little or no homology. Multiple polymorphic bands of differing size with homology to the original sequence, such as the case with primer 00064 bands of 210 bp and 250 bp, were highly similar (>98%) but the shorter sequence aligned to the middle of the longer sequence. Thus, we conclude that polymorphic bands for a particular EST marker do not generally represent different alleles.

4. Discussion

We found that lowbush blueberries managed for commercial production do not retain the same genetic diversity as found in an established forest. Although fields of lowbush blueberries are not planted with selected cultivars, management removes some genotypes from the landscape. The application of herbicides, such as hexazinone, for weed control can remove sensitive genotypes, which has been well documented in weedy species, but herbicide application can also have phytotoxic or lethal effects on lowbush blueberry plants [46–48]. Lowbush blueberry is managed on a two-year cycle, with a cropping year followed by a pruning year where the fields are mowed or burned [49]. Burn pruning for lowbush blueberry fields is an effective disease control measure as it removes pathogens in the stems and on the soil surface, but the intensity (heat and duration) can have a negative effect on the plants and the organic layer of the soil [49–51]. Although the negative effects of burn pruning have been contested, the increase in price of oil used for most burn applications has provided incentive for many growers to adopt flail mowing as an alternative pruning method with lower risk to the plants but again may negatively impact some genotypes [52].

If the Hope site populations are taken out for an analysis, there is significantly less genetic diversity in the managed populations in Old Town, Winterport, and Jonesboro when compared to the non-managed populations ($p = 0.044$). We are unable to draw conclusions about functional diversity in managed populations since polymorphic bands did not generally appear to represent alleles, as many of the sequenced polymorphic bands did not have homology to the original EST sequence from which the primer was designed, except at the ends. Other studies have found that the selection process under stress conditions results in a more tightly refined stress response pathway [45].

Domestication of wild crops (maize, wheat, apple, etc.) by the selection and planting of individuals with superior traits has resulted in a genetic bottleneck in many of our current traditionally-bred

crops [26,53]. In contrast, intentional rogueing of plants is not a current practice among lowbush blueberry growers, so plants with less desirable traits remain in the gene pool retaining a higher-level diversity relative to domesticated plants.

Overall genetic diversity for lowbush blueberry was retained for all sampled populations reflecting the results of previous studies in Maine and the Canadian Maritimes [10,12,54]. Bell et al. [12] described differences among four managed fields in the Down East region of Maine. Similar to our findings, Bell described variance (91.6%) associated within populations relative to among populations (8.4%). Positive spatial autocorrelation (SA) within fields at the 50 m-distance class found in our study was also reported by Bell et al. [12]. Differences between lowbush blueberry populations has been attributed to the last receding ice sheet that covered much of Maine [12]. Our results would not entirely support this hypothesis, as we found no correlation between diversity and latitude as would be expected with receding ice sheets [55]. Genetic differentiation as a result of geographic isolation has been found in bilberry (*Vaccinium myrtillus* L.) throughout Scandinavia, and the same may be true for lowbush blueberry here in North America but greater geographical separation than is reported here is likely to be a necessity [56]. The large geographic range, outcrossing, and animal seed dispersal traits associated with lowbush blueberry likely contributes to genetic diversity [57].

Our sampled populations from 8 states spanning 1600 km represent the native growing range of lowbush blueberry in the eastern United States. Spanning multiple cold hardiness zones, the populations are a good representation of climatic zones that could provide insight into how an increased average temperature may influence the lowbush blueberry industry in Maine. Wild plants can be found growing in a variety of cold hardiness zone from zone 0 (Quebec) to zone 7a (North Carolina). While plants may tolerate the extremes of the range, larger populations can be found in zones 4–6 which encompasses most of the commercial harvesting regions. Climate change has increased the growing season for lowbush blueberry by approximately one month in the past 50 years in Maine [20]. The revised USDA cold hardiness map in 2012 reflected warmer average minimum temperatures compared to the last map update in 1990 with the majority of Maine's blueberry growing region shifting from predominantly zone 5a (−28.9 to −26.1 °C) to predominantly zone 5b (−26.1 to −23.3 °C) and to a lesser extent zone 6a (−23.3 to −20.6 °C). Populations sampled from warmer locations (New Castle, VA, Dolly Sods, WV, Milton, PA) maintained a high level of diversity similar to cooler, northern populations. Pairwise comparisons between each site and also comparisons between northern (ME, VT, MA, NY) and southern (VA, WV, PA) populations did not reveal any population structure based on latitude or prevailing hardiness zones. The numbers of stress-related marker bands also were not correlated with latitude or hardiness zone. Other studies have found population structure in lowbush blueberry based on geographic distances in the Canadian Maritimes and Quebec [58]. Our results did not show any population structure based on location, cold hardiness zone, and management. It is possible plants sampled for our study lack genetic isolation and inbreeding depression associated with genetic similarities [59].

5. Conclusions

These findings suggest that while growers in Maine may need to adapt management practices to a warmer and longer growing season, the overall genetic diversity of the plants would remain high, but might not provide the genetic ability to adapt rapidly to extreme climate change.

Author Contributions: L.B. collected and analyzed the data and wrote the first draft of the manuscript. L.J.R. provided expertise and technical assistance in conducting the genetic diversity assessment and she contributed to editing the manuscript. F.D. was involved in the conceptual design of the study, sampling protocol, assisted in the interpretation of statistical analyses, and contributed to the editing of the manuscript.

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References

1. Bell, D.J.; Rowland, L.J.; Smagula, J.; Drummond, F.A. Recent Advances in the Biology and Genetics of Lowbush Blueberry. *Tech. Bull.* **2009**, *203*, 1–28.
2. Vander Kloet, S.P. *The Genus Vaccinium in North America*; Research Branch, Agriculture Canada: Ottawa, ON, Canada, 1988; 201p.
3. Drummond, F.; Smagula, J.; Annis, S.; Yarborough, D. Organic Wild Blueberry Production. *Tech. Bull.* **2009**, *852*, 43.
4. Yarborough, D. Factors contributing to the increase in productivity in the wild blueberry industry. *Small Fruits Rev.* **2004**, *3*, 33–43. [[CrossRef](#)]
5. Barker, W.; Hall, I.; Aalders, L.; Wood, G. The lowbush blueberry industry in Eastern Canada. *Econ. Bot.* **1964**, *18*, 357–365. [[CrossRef](#)]
6. Hepler, P.; Yarborough, D. Natural variability in yield of lowbush blueberries. *HortScience* **1991**, *26*, 245–246. [[CrossRef](#)]
7. Cappiello, P.; Dunham, S. Seasonal variation in low-temperature tolerance of *Vaccinium angustifolium* Ait. *HortScience* **1994**, *29*, 302–304. [[CrossRef](#)]
8. Bell, D.J.; Stommel, J.; Rowland, L.; Drummond, F. Yield variation among clones of lowbush blueberry as a function of kinship and self-compatibility. *J. Am. Soc. Hort. Sci.* **2010**, *135*, 259–270. [[CrossRef](#)]
9. Penman, L.N.; Annis, S.L. Leaf and flower blight caused by *Monilinia vaccinii-corymbosi* on lowbush blueberry: Effects on yield and relationship to bud phenology. *Phytopathology* **2005**, *95*, 1174–1182. [[CrossRef](#)] [[PubMed](#)]
10. Burgher, K.L.; Jamieson, A.R.; Lu, X. Genetic relationships among lowbush blueberry genotypes as determined by randomly amplified polymorphic DNA analysis. *J. Am. Soc. Hort. Sci.* **2002**, *127*, 98–103. [[CrossRef](#)]
11. Bell, D.J.; Rowland, L.; Drummond, F.; Polashock, J. Suitability of EST-PCR markers developed in highbush blueberry (*Vaccinium corymbosum* L.) for genetic fingerprinting and relationship studies in lowbush blueberry and related species. *J. Am. Soc. Hort. Sci.* **2008**, *133*, 701–707. [[CrossRef](#)]
12. Bell, D.J.; Rowland, L.; Zhang, D.; Drummond, F. Spatial genetic structure of lowbush blueberry, *Vaccinium angustifolium*, in four fields in Maine. *Botany* **2009**, *87*, 932–946. [[CrossRef](#)]
13. Bell, D.J.; Rowland, L.; Drummond, F. Does pollen ‘neighborhood’ affect berry yield in lowbush blueberry (*Vaccinium angustifolium* Ait)? *Int. J. Fruit Sci.* **2012**, *12*, 65–74. [[CrossRef](#)]
14. Bell, D.J.; Drummond, F.; Rowland, L. Evidence of functional gender polymorphisms in a population of the hermaphroditic lowbush blueberry (*Vaccinium angustifolium*). *Botany* **2012**, *90*, 393–399. [[CrossRef](#)]
15. Rowland, L.J.; Bell, D.J.; Alkharouf, N.; Bassil, N.V.; Drummond, F.; Beers, L.; Buck, E.; Finn, C.E.; Graham, J.; McCallum, S.; et al. Generating genomic tools for blueberry improvement. *Int. J. Fruit Sci.* **2012**, *12*, 276–287. [[CrossRef](#)]
16. Eckert, C.G.; Samis, K.E.; Lougheed, S.C. Genetic variation across species’ geographical ranges: The central–marginal hypothesis and beyond. *Mol. Ecol.* **2008**, *17*, 1170–1188. [[CrossRef](#)] [[PubMed](#)]
17. Howe, G.T.; Hackett, W.P.; Furnier, G.R.; Klevorn, R.E. Photoperiodic responses of a northern and southern ecotype of black cottonwood. *Physiol. Plant* **1995**, *93*, 695–708. [[CrossRef](#)]
18. Friedman, J.; Roelle, J.; Gaskin, J.; Pepper, A.; Manhart, J. Latitudinal variation in cold hardiness in introduced *Tamarix* and native *Populus*. *Evol. Appl.* **2008**, *1*, 598–607.
19. Fernandez, I.J.; Schmitt, C.; Stancioff, E.; Birkel, S.D. *Maine’s Climate Future: 2015 Update*; University of Maine Climate Change Institute: Orono, ME, USA, 2015; 20p.
20. Drummond, F.A.; Yarborough, D.E. Growing season effects on wild blueberry (*Vaccinium angustifolium*) in Maine and implications for management. *Acta Hort.* **2012**, *1017*, 101–107. [[CrossRef](#)]

21. Collins, J.A.; Drummond, F. Fertilizer and fungicides: Effects on wild blueberry growth, insect attack, and leaf spot disease incidence. In Proceedings of the North American Blueberry Research and Extension Workers Conference, Orono, ME, USA, 12–15 August 2018.
22. Drummond, F.A.; Dibble, A.C.; Stubbs, C.; Bushmann, S.; Ascher, J.S.; Ryan, J. A Natural History of Change in Native Bees Associated with Lowbush Blueberry in Maine. *Northeast. Nat.* **2017**, *24*, 49–68. [[CrossRef](#)]
23. Eck, P.; Childers, N.F. *Blueberry Culture*; Rutgers University Press: New Brunswick, NJ, USA, 1966; 400p.
24. Hall, I.; Aalders, L. Blomidon Lowbush Blueberry. *Can. J. Plant Sci.* **1992**, *62*, 519–521. [[CrossRef](#)]
25. Hall, I. Genetic improvement of the lowbush blueberry, *Vaccinium angustifolium*. *Can. J. Plant Sci.* **1983**, *63*, 1091–1092. [[CrossRef](#)]
26. Doebley, J.F.; Gaut, B.S.; Smith, B.D. The molecular genetics of crop domestication. *Cell* **2006**, *127*, 1309–1321. [[CrossRef](#)]
27. van de Wouw, M.; Kik, C.; van Hintum, T.; van Treuren, R.; Visser, B. Genetic erosion in crops: Concept, research results and challenges. *Plant Gen. Resour.* **2009**, *8*, 1. [[CrossRef](#)]
28. Aruna, M.; Ozias-Akins, P.; Austin, M.; Kochert, G. Genetic relatedness among rabbiteye blueberry (*Vaccinium ashei*) cultivars determined by DNA amplification using single primers of arbitrary sequence. *Genome* **1993**, *36*, 971–977. [[CrossRef](#)] [[PubMed](#)]
29. Loveless, M.D.; Hamrick, J.L. Ecological determinants of genetic structure in plant populations. *Ann. Rev. Ecol. Syst.* **1984**, *15*, 65–95. [[CrossRef](#)]
30. Rowland, L.J.; Dhanaraj, A.L.; Polashock, J.J.; Arora, R. Utility of blueberry-derived EST-PCR primers in related Ericaceae species. *HortScience* **2003**, *38*, 1428–1432. [[CrossRef](#)]
31. Groff, S.C.; Loftin, C.S.; Drummond, F.A.; Bushmann, S.; McGill, B. Spatial prediction of lowbush blueberry native bee pollinators in Maine, USA. *Environ. Model. Softw.* **2016**, *79*, 1–9. [[CrossRef](#)]
32. Gleason, H.A.; Cronquist, A. *Manual of Vascular Plants of Northeastern United States and Adjacent Canada*, 2nd ed.; Botanical Garden: New York, NY, USA, 1991; 910p.
33. Doyle, J.J.; Doyle, J.L. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* **1987**, *19*, 11–15.
34. Darwish, O.; Rowland, L.J.; Alkharouf, N.W. BBGD454: A database for transcriptome analysis of blueberry using 454 sequences. *Bioinformatics* **2013**, *9*, 883–886. [[CrossRef](#)]
35. Untergasser, A.; Cutcutache, I.; Koressaar, T.; Ye, J.; Faircloth, B.C.; Remm, M.; Rozen, S.G. Primer3—New capabilities and interfaces. *Nucleic Acids Res.* **2012**, *40*, e115. [[CrossRef](#)]
36. Thomashow, M.F. Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1999**, *50*, 571–599. [[CrossRef](#)] [[PubMed](#)]
37. Shinozaki, K.; Yamaguchi-Shinozaki, K. Gene networks involved in drought stress response and tolerance. *J. Exp. Bot.* **2007**, *58*, 221–227. [[CrossRef](#)]
38. Coker, J.S.; Davies, E. Selection of candidate housekeeping controls in tomato plants using EST data. *BioTechniques* **2003**, *35*, 740–748. [[CrossRef](#)] [[PubMed](#)]
39. Rowland, L.J.; Mehra, S.; Dhanaraj, A.; Ogden, E.L.; Slovin, J.P.; Ehlenfeld, M.K. Development of EST-PCR markers for DNA fingerprinting and genetic relationship studies in blueberry (*Vaccinium*, section *Cyanococcus*). *J. Am. Soc. Hort. Sci.* **2003**, *128*, 682–690. [[CrossRef](#)]
40. Peakall, R.; Smouse, P. GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Mol. Ecol. Notes.* **2006**, *6*, 288–295. [[CrossRef](#)]
41. Simes, R.J. An improved Bonferroni procedure for multiple tests of significance. *Biometrika* **1986**, *73*, 751–754. [[CrossRef](#)]
42. Peakall, R.; Smouse, P. GenA1Ex 6.5: Genetic analysis in Excel. Population genetic software for teaching and research—An update. *Bioinformatics* **2012**, *28*, 2537–2539. [[CrossRef](#)]
43. Pritchard, J.K.; Stephens, M.; Donnelly, P. Inference of population structure using multilocus genotype data. *Genetics* **2000**, *155*, 945–959.
44. Evanno, G.; Regnaut, S.; Goudet, J. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Mol. Ecol.* **2005**, *14*, 2611–2620. [[CrossRef](#)]
45. Zhen, Y.; Ungerer, M. Relaxed selection on the CBF/DREB1 regulatory genes and reduced freezing tolerance in the southern range of *Arabidopsis thaliana*. *Mol. Biol. Evol.* **2008**, *25*, 2547. [[CrossRef](#)]
46. Holt, J.S.; Powles, S.B.; Holtum, J. Mechanisms and agronomic aspects of herbicide resistance. *Ann. Rev. Plant Biol.* **1993**, *44*, 203–229. [[CrossRef](#)]

47. Yarborough, D.; Cote, J. Pre-and post-emergence applications of herbicides for control of resistant fine leaf sheep fescue in wild blueberry fields in Maine. In Proceedings of the North American Blueberry Research and Extension Workers Conference, Atlantic City, NJ, USA, 23–26 June 2014.
48. Jensen, K.I.N.; Yarborough, D.E. An Overview of Weed Management in the Wild Lowbush Blueberry—Past and Present. *Small Fruits Rev.* **2004**, *3*, 229–255. [[CrossRef](#)]
49. Yarborough, D. Wild blueberry culture in Maine. *Univ. Maine Coop. Ext. Fact. Sheet.* **2015**. No. 220. Available online: <https://extension.umaine.edu/blueberries/factsheets/production/wild-blueberry-culture-in-maine/> (accessed on 28 May 2019).
50. DeGomez, T. Pruning lowbush blueberry fields. *Univ. of Maine Coop. Ext. Wild Blueberry Fact. Sheet.* **1988**. No. 229. Available online: <https://extension.umaine.edu/blueberries/factsheets/production/pruning-lowbush-blueberry-fields/> (accessed on 28 May 2019).
51. Smith, D.W.; Hilton, R.J. The Comparative Effects of Pruning by Burning or Clipping on Lowbush Blueberries in North-Eastern Ontario. *J. Appl. Ecol.* **1971**, *8*, 781. [[CrossRef](#)]
52. Hanson, E.J.; Ismail, A.A.; Metzger, H.B. *A Cost Analysis of Pruning Procedures in Lowbush Blueberry Production*; Life Sciences and Agriculture Experiment Station: Ithaca, NY, USA, 1982; Bulletin 780.
53. Rao, V.R.; Hodgkin, T. Genetic diversity and conservation and utilization of plant genetic resources. *Plant Cell Tissue Organ. Culture* **2002**, *68*, 1–19.
54. Debnath, S.C. Development of ISSR markers for genetic diversity studies in *Vaccinium angustifolium*. *Nordic J. Botany* **2009**, *27*, 141–148. [[CrossRef](#)]
55. Hewitt, G. The genetic legacy of the Quaternary ice ages. *Nature* **2000**, *405*, 907–913. [[CrossRef](#)] [[PubMed](#)]
56. Zoratti, L.; Palmieri, L.; Jaakola, L.; Häggman, H. Genetic diversity and population structure of an important wild berry crop. *AoB Plants* **2015**, *7*, plv117. [[CrossRef](#)]
57. Hamrick, J.L.; Godt, M.J.W.; Sherman-Broyles, S.L. Factors influencing levels of genetic diversity in woody plant species. *New For.* **1992**, *6*, 95–124. [[CrossRef](#)]
58. Tailor, S.; Bykova, N.V.; Igamberdiev, A.U.; Debnath, S.C. Structural pattern and genetic diversity in blueberry (*Vaccinium*) clones and cultivars using EST-PCR and microsatellite markers. *Genet. Resour. Crop. Evol.* **2017**, *64*, 2071–2081. [[CrossRef](#)]
59. Willi, Y.; Van Buskirk, J. Genomic compatibility occurs over a wide range of parental genetic similarity in an outcrossing Plant. *Proc. R. Soc.* **2005**, *272*, 1533–1538.



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