Toward Marker-assisted Breeding for Root Architecture Traits in Southern Highbush Blueberry

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ABSTRACT. Root growth and root system architecture (RSA) are affected by edaphic and genetic factors and they can impact plant growth and farm profitability. Southern highbush blueberries [SHBs (*Vaccinium corymbosum* hybrids)] develop shallow, fibrous root systems, and exhibit a preference for acidic soils where water and ammonium are readily available. The amendments used to create these soil conditions negatively affect the profitability of SHB plantations. Hence, breeding for RSA traits has been suggested as an alternative to soil amendments. *Vaccinium arboreum* is a wild species that is used in SHB breeding. *V. arboreum* exhibits greater drought tolerance and broader soil pH adaptation than SHB, and—according to anecdotal evidence—it develops deep, taproot-like root systems. The present study constitutes the first in-depth study of the RSA of *Vaccinium* species with the intention of facilitating breeding for RSA traits. Root systems were studied in rhizotron-grown seedling families. In separate experiments, we tested the effect that growth substrate and family pedigree can have on root growth and RSA. Subsequently, a genotyping by sequence approach was used to develop single nucleotide polymorphism (SNP) markers that could be used along with the phenotyping method to investigate the heritability of RSA traits and look for marker-trait associations. We found that RSA is affected by growth substrate and family pedigree. In addition, we found that *V. arboreum* exhibited greater maximum root depth and a lower percentage of roots in the top 8 cm of soil than SHB, and interspecific hybrids generally exhibited intermediate phenotypes. Also, we found that RSA traits exhibit moderate to low heritability and genetic correlations among them. Finally, we found 59 marker-trait associations. Among these markers, 37 were found to be located in exons, and 16 of them were annotated based on protein homology with entries in National Center for Biotechnology Information (NCBI) GenBank. Altogether, the present study provides tools that can be used to breed for root architecture traits in SHB.

Root growth and RSA are affected by edaphic and genetic factors. Soil characteristics such as mechanical impedance, moisture content, and nutrient availability can affect the size of the root system (Gioia et al., 2015; Hund et al., 2009; Jin et al., 2015), but their effects go beyond biomass accumulation. Edaphic and genetic factors also affect the root growth angle, average root diameter, and maximum root depth (Hund et al., 2009; Jin et al., 2015; Nagel et al., 2015) of various plant species. Similarly, the availability of nitrogen, phosphorus, and other elements can affect root branching, secondary root length, and maximum root depth (Gioia et al., 2015; Lambers et al., 2015; York et al., 2015). Although there is a growing body of literature documenting the edaphic factors that affect root growth and RSA of agronomic crops like wheat [*Triticum* sp. (Gioia et al., 2015; Jin et al., 2015; Nagel et al., 2015)] and maize [*Zea mays* (Hund et al., 2009; York et al., 2015)], research about the edaphic factors that affect root growth and RSA of horticultural crops, particularly the SHB, is lacking.

A cultivated specialty crop, SHB is derived from interspecific crosses between *V. corymbosum* and other *Vaccinium* species, including *Vaccinium darrowii* and *Vaccinium virgatum* (Brevis et al., 2008). SHB plants develop shallow, fibrous root systems (Bryla and Strik, 2007), and exhibit a preference

for growth on acidic soils (pH <5.5) (Coville, 1910; Finn et al., 1993) where ammonium is the predominant form of nitrogen (Pritts and Hancock, 1992). These characteristics are relatively rare in agricultural soils. Consequently, SHB growers use costly soil amendments to provide favorable conditions for SHB cultivation (Williamson et al., 2015).

A related species with different soil adaptation and RSA is *V. arboreum*. This species can withstand growth in higher pH (up to pH 6.4) and mineral, drought-prone soils (Stockton, 1976). In addition, *V. arboreum* plants exhibit greater capacity for nitrate uptake than SHB plants (Poonnachit and Darnell, 2004), and—according to field-level observations—*V. arboreum* plants develop deep, taproot-like root systems (Lyrene and Brooks, 1995). Although there is a lack of quantitative data about the roots of SHB and *V. arboreum* plants grown under the same conditions, deeper root systems in other species have been shown to confer an adaptive advantage in soils where water (Padilla and Pugnaire, 2007; Pinheiro et al., 2005) and nitrate (Gastal and Lemaire, 2002; King et al., 2003) percolate to deeper horizons. Moreover, deeper root systems encounter increasing soil acidity at greater soil depths (Lynch and Wojciechowski, 2015), which would be an adaptive advantage for acid-loving plants like *Vaccinium* species. Hence, the RSA of *V. arboreum* might be related to its soil adaptation. These RSA traits and the fact that *V. arboreum* has been previously used in SHB breeding (Lyrene and Olmstead, 2012; Olmstead et al., 2013) make *V. arboreum* a potential source of RSA traits that could be used to expand the soil adaptation of SHB.

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Breeding for RSA traits is underway in other crop species. Plant breeders have identified genetic sources of desirable root growth and RSA traits in domestic (Fita et al., 2008; Henry et al., 2012; Khan et al., 2012; Steele et al., 2006) and wild (Dias et al., 2004; Fita et al., 2006; Johnson et al., 2000) populations of many species. Since phenotypic selection for root traits remains largely unfeasible due to the challenge of large-scale phenotyping below ground (Kuijken et al., 2015), breeding for root growth and RSA traits has focused on the development of tools for marker-assisted selection (MAS). Accordingly, a multitude of quantitative trait loci (QTL) and molecular markers that are associated with root growth (Khan et al., 2012) and RSA traits such as root length (Ron et al., 2013; Steele et al., 2006), diameter (Li et al., 2015), growth angle (Hamada et al., 2012), branching (Fita et al., 2008), and depth (Johnson et al., 2000) have been identified. Although less frequent, MAS has been successfully used to create breeding lines with optimal RSA traits in maize and rice [*Oryza sativa* (Liu et al., 2012; Steele et al., 2006)]. However, to date, there has been limited application of root phenotyping and MAS in SHB.

The present study focuses on the edaphic and genetic factors affecting root growth and RSA in SHB, *V. arboreum*, and their interspecific hybrids. Considering that roots of *Vaccinium* species are understudied with respect to RSA, and that *V. arboreum* is a promising source of RSA traits for SHB breeding, we set out to identify a rooting substrate that allows practical phenotyping of *Vaccinium* species' roots. Then, we explored family-level differences between SHB and *V. arboreum* grown under similar conditions. Finally, we used an interspecific family with *V. arboreum* introgression to search for molecular markers associated with RSA traits. We hypothesized that: a) growth substrate affects the RSA of *Vaccinium* species, b) *V. arboreum* seedlings exhibit deeper root systems than SHB seedlings, and interspecific hybrids exhibit intermediate phenotypes, and c) deoxyribonucleic acid (DNA) markers could be associated with one or more RSA traits from *V. arboreum*.

Materials and Methods

Each of the previously mentioned hypotheses was tested in a separate experiment. We refer to seedlings from the same controlled cross or open pollination event as families, and pedigrees of the families used for each study are in Table 1. Although all SHB genotypes are interspecific hybrids, in the present study, a family was considered SHB if it did not have introgression from a pure *Vaccinium* species source for over three generations. The pollen sources for the open-pollinated *V. arboreum* families used were assumed to be from the *V. arboreum* due to the bloom timing (average bloom date of 1 Feb. through 1 Mar. for SHB, *V. virgatum*, and hybrids vs. 1 May onwards for *V. arboreum*) and ploidy level differences between *V. arboreum*, SHB, and *V. virgatum*.

Seeds were germinated in 3.8-L pots filled with sphagnum peatmoss (peat) in a temperature-controlled greenhouse under intermittent mist as previously described (Moore, 1965). When the average seedling reached 5 cm height from the soil line, seedlings were transplanted to bench-top rhizotrons. Bench-top rhizotrons were built with two 37.5 × 37.5-cm glass plates and three 37.5 × 3.0 × 3.5-cm plastic inserts. Plastic inserts were used to close off the space between three of the four sides of the glass plates, forming a container that fit ≈3100 cm³ of substrate

(Fig. 1A). In Expts. 1 and 2, there were three seedlings per rhizotron. In Expt. 3, there were two seedlings per rhizotron. After transplant, rhizotrons were placed as cassettes in racks holding 12 rhizotrons (Fig. 1B). Each rack was then wrapped with black polyurethane plastic to further exclude light from the root compartments, such that only the substrate surface and growing seedlings were exposed. All experiments were run in greenhouses where daily temperature ranged between 10.3 and 37.8 °C, with daylength ranging between 11 h 46 min and 14 h 2 min, and average photosynthetic photon flux of 521 μmol·m⁻²·s⁻¹. Rhizotrons were watered as needed and fertilized weekly with 6 g·L⁻¹ of 20N–8.7P–16.6K soluble fertilizer with micronutrients (Tracite; Helena Chemical Co., Collierville, TN) for the duration of the experiments. All plants were harvested at the same time; when the deepest roots of the largest plant in each experiment reached the bottom of the rhizotron.

To access the root systems, one of the glass plates in each rhizotron was removed and a nail board (four 5.72-cm nails per 6.5 cm²) was put in its place to minimize root movement and breakage. Calipers were used to determine the in situ maximum root depth of each seedling before flushing the substrate away with tap water. In Expt. 1, clean roots were spread in transparent trays filled with tap water for scanning. In Expts. 2 and 3, roots were stained with 5 g·L⁻¹ Neutral Red (Sigma-Aldrich, St. Louis, MO) for 24 h as per Bouma et al. (2000) before transfer to the trays for scanning. Longer staining periods and different dyes produced comparable results in a preliminary experiment (data not shown). Where necessary, root systems were split into more than one part before scanning to reduce root overlap in the images. This was accomplished by cutting at the highest order root available, and scanning this root with all its attached lower order roots in a separate image. Roots were scanned on a scanner (LX1100; Seiko Epson Corp., Tokyo, Japan) at a resolution of 600 dots per inch (dpi) in Expt. 1 and 800 dpi in Expts. 2 and 3. Scanning at resolutions up to 1000 dpi did not yield significantly different results than scanning at 800 dpi in a preliminary experiment (data not shown). The resulting images were grayscale TIFF files (8786 pixels wide × 12,251 pixels long) and they were analyzed using WinRhizo Pro 2013b (Regent Instruments, Quebec, QC, Canada). RSA variables measured were total root length, total root volume, total root surface area, specific root length [SRL (meters of root length per gram of root dry weight)], number of root branching events, number of root tips, and average root diameter. To ameliorate overestimation of root tips when root systems were split, the number of root tips was corrected by subtracting the number of split points from the number of root tips calculated by the software. Given that previous studies have found weak correlation between root diameter and root order (Valenzuela-Estrada et al., 2008), we used the count of root tips in a root system as a proxy to count first-order roots. Previously, root tips have been used as indirect measurement of root branching (Fita et al., 2008). After scanning, roots and shoots were dried at 72 °C to a constant weight, unless otherwise noted.

Expt. 1: substrate effect

To test the effect of growth substrate on root growth and RSA of *Vaccinium* species, bench-top rhizotrons were filled with one of two substrate treatments: peat or a 3:1 peat:perlite mixture. Nine uniform seedlings per family, from five different families (Table 1), were transplanted to rhizotrons filled with each substrate treatment and grown for 77 d before harvest.

Table 1. Pedigree of *Vaccinium* families used in three separate experiments to study the effect that substrate and pedigree have on root system architecture and to develop molecular markers to breed for root system architecture traits.

Expt.	Family name	Pedigree
1: Substrate effect	S1	Open pollinated diploid <i>Vaccinium arboreum</i>
	S2	FL92-255 ^z × Tifblue ^c
	S3	FL10-529 (Scintilla ^y × FL06-730 ^x) × FL08-496 (FL91-333MIK ^y × FL06-754 ^y)
	S4	FL10-633 (FL95-138 ^y × FL06-730 ^x) × Meadowlark ^y
	S5	FL11-46 (FL08-036 ^y × FL00-266 ^y) × FL07-338 (FL98-358 ^y × FL01-006 ^y)
2: Pedigree effect	P1	FL11-1000 ^w × FL11-1001 ^w
	P2	FL12-22 (FL09-006 ^y × FL01-050 ^y) × FL06-360 IV (FL03-027 ^y × Windsor ^y)
	P3	Open pollinated <i>V. arboreum</i> seed collected near Branford, FL
	P4	FL08-483 (NC3115 ^y × FL06-753 ^x) × FL12-604 (FL02-106 ^y × FL06-730 ^x)
	P5	FL10-678 (Bluecrisp ^y × FL06-730 ^x) × Flicker ^y
	P6	FL10-616 (Farthing ^y × FL08-447 F1) × Flicker ^y
3: Marker-trait association	G1	Southern Belle ^y × FL08-467 (Primadonna ^y × FL06-753 ^x)

^z*Vaccinium virgatum*.

^ySouthern highbush blueberry (SHB). Although all SHB genotypes are interspecific hybrids, in the present study, individuals or families were considered SHB if they did not have introgression from a pure *Vaccinium* species source for over three generations.

^xColchicine-induced tetraploid *V. arboreum*.

^wWild *Vaccinium corymbosum*.

Fig. 1. Bench-top rhizotron system used to study the root system architecture of *Vaccinium* species. (A) Open bench-top rhizotron showing two *Vaccinium* species seedlings 69 d after transplant. (B) Rack used to hold bench-top rhizotrons during experiments. Racks were wrapped with black polyurethane plastic to exclude light from the root compartments, such that only the substrate surface and growing seedlings were exposed during experiments (not shown).

Data for RSA traits were compared between substrate treatments across families (main effect) or between substrate treatments within each family (interaction). Shoots from four randomly selected seedlings per family × substrate combination were destructively harvested as above.

Expt. 2: pedigree effect

To test the effect of *V. arboreum* introgression on root growth and RSA of *Vaccinium* species, 21 uniform seedlings from each of six additional families (Table 1) were transplanted to bench-top rhizotrons filled with peat. Roots and shoots from all seedlings were harvested as indicated above after 67 d of growth. In addition to the previously described data collection, a visual estimate of the percentage of roots in the top 8 cm of soil was recorded for each plant at the time of harvest. This depth corresponded to about 1/4 of the depth of the rhizotron,

and was chosen because previous research found that the majority of *V. corymbosum* roots was in the top 1/4 of the soil profile (Bryla and Strik, 2007). Data were compared among families, making each seedling a pseudo-replication of the pedigree treatment.

STATISTICAL ANALYSIS. Expts. 1 and 2 followed a randomized complete block design where substrate treatment (Expt. 1) or family pedigree (Expt. 2) were independent variables and rhizotrons were the blocking variable. Dry weight data were analyzed with generalized linear models (proc GLM). RSA data were analyzed through a generalized linear mixed model (proc GLIMMIX), where substrate treatment, family, and/or their interaction were qualitative independent variables, and root dry weight was used as a covariate. Treatments were compared after normalizing for root dry weight. This was done since previous studies found that root system size (measured as fresh or dry weight) confounded size-independent variation in RSA traits (Fita et al., 2008; Johnson et al., 2000). Where significant interactions were found, interaction terms were sliced by family. Analyses were carried out in SAS (version 9.3; SAS Institute, Cary, NC).

Expt. 3: marker-trait association

To identify molecular markers that are associated with RSA traits, 267 seedlings from a pseudo-backcross family (Table 1) were transplanted to bench-top rhizotrons filled with peat. After 69 d of growth, seedlings were harvested as above. Since the pseudo-backcross family was of interest to the blueberry breeding program, individuals were not destructively harvested at the end of the experiment and RSA traits were not size-adjusted. Previously, others have successfully used unadjusted RSA data to study the genetic control of RSA traits (Ron et al., 2013), and similar QTL have been found where size-adjusted and unadjusted data were used simultaneously (Johnson et al., 2000; Khan et al., 2012).

Leaf tissue from each seedling was used to extract total genomic DNA using the E-Z 96[®] Plant DNA Kit[™] (Omega Bio-Tek, Norcross, GA). Then, DNA was quantified using Quanti-iT[™] PicoGreen[®] dsDNA Assay Kit[™] (Invitrogen, Grand Island, NY) and DNA libraries were built as previously described

(Bhakta et al., 2015). Briefly, 400 ng of DNA from each individual were digested for 2 h at 37 °C with 1 unit of PstI-HF® restriction enzyme (New England BioLabs, Ipswich, MA), in a mix containing 1× buffer and 18 nM adapter mix (final volume 20 µL). The adapter mix contained 30 nM of barcode and 30 nM of common adapter that were annealed separately in a thermocycler program consisting of 95 °C for 2 min, ramp down to 25 °C (rate 0.1 °C·s⁻¹), 25 °C for 30 min, and hold at 4 °C. Sequences of common adapters can be found in Supplemental Table 1. Following digestion, DNA was ligated to the adapters using 2.5 µL of T4 DNA ligase (400 CEU/µL; New England BioLabs), 5 µL of 10× T4 Ligase Buffer, and 22.5 µL of sterile H₂O. The ligation reaction was incubated at 25 °C for 1 h, followed by a 30-min incubation at 65 °C to inactivate the ligase. Then, samples were cleaned by filtration through a Millipore® MultiScreen™ PCR filter plate (EMD Millipore, Billerica, MA) that had been soaked with 100 µL of DNA grade H₂O (Sigma-Aldrich). Samples were diluted with 150 µL of DNA grade H₂O before applying a tension of 25 cm of Hg for 15 min in a vacuum manifold. DNA was eluted from the filter using 30 µL DNA grade H₂O as per manufacturer's instructions. Following this, a polymerase chain reaction (PCR) reaction was set up with 8 µL of cleaned DNA, 1× PCR buffer, 1.5 mM MgCl₂, 200 µM dNTPs, 0.5 µM of each primer, and 0.025 units of GoTaq polymerase (final volume 50 µL). Primers contained common forward and reverse sequences (Supplemental Table 2), as well as 4 to 8 bases-long barcodes on the forward primer that were used to multiplex the sequencing reaction (Supplemental Table 3). The PCR program consisted of 72 °C for 3 min, 98 °C for 30 s, 22 cycles of 98 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s, followed by a final elongation step at 72 °C for 5 min and hold at 4 °C. PCR products were cleaned using a MultiScreenHTS PCRu96 filter plate (EMD Millipore) and quantified as above before pooling 20 ng from each individual into a family pool. Then, fragments between 200 and 500 bp were selected from this pool using a BluePippin instrument (Sage Science, Beverly, MA). Finally, fragments were submitted for multiplexed, 150-bp, single-end sequencing using the NextSeq500 platform (Illumina, San Diego, CA) at the Interdisciplinary Center for Biotechnology Research at the University of Florida (Gainesville). Subsequently, sequencing data were processed with the TASSEL-UNEAK nonreference genotyping by sequencing SNP calling pipeline (Lu et al., 2012) to produce a haplotype map (hap map) of the individuals with SNP loci identified. Loci with fewer than five tags per locus or more than 25% missing data were discarded. A genetic linkage map for this population is currently under construction (H.P. Rodriguez-Armenta, personal communication). However, due to technical challenges in autotetraploid populations, it has not been completed. Although SHB is an autotetraploid [$2n = 4x = 48$ (Qu et al., 1998)] and *V. arboreum* used for intersectional crosses is a colchicine-induced tetraploid (Lyrene and Olmstead, 2012), a $2n$ ploidy level was used for the analyses due to unresolved technical challenges in autotetraploids (discussed in McCallum et al., 2012). The hapmap was used to estimate a kinship matrix based on genome-wide SNP data (Yang et al., 2010) in TASSEL version 5.2.13 (Bradbury et al., 2007). Then, the kinship matrix, hapmap, and phenotypic data were used to find marker-trait associations with RSA traits using a mixed linear model (Yu et al., 2006). Genetic (σ_G^2) and residual variances (σ_R^2) were used to compute the broad-sense heritability (H^2) of each trait.

The genetic covariance between two traits ($\sigma_{G(AB)}$) was used to estimate the genetic correlation between them as previously done (Fita et al., 2006, 2008; Hebert et al., 1994; Lynch and Walsh, 1998). Where genetic correlations fell outside the range -1 to $+1$ (Hebert et al., 1994; Lynch and Walsh, 1998), they were considered nonestimable. SNP loci that exhibited statistically significant association (defined as $P \leq 10^{-3}$ after adjusting for multiple comparisons through the Bonferroni method) with heritable RSA traits (defined as $H^2 \geq 0.10$) were termed candidate markers and used for in silico annotation.

The multiple sequence reads containing the SNP loci were aligned and consensus sequences (87 bp) were produced using STACKS (Catchen et al., 2013). These consensus sequences (Supplemental Table 4) were used as queries for homology search via Basic Local Alignment Search Tool [BLAST version 2.2.31 (Altschul et al., 1990)] in two custom-built databases created with root transcriptome contig sequences from SHB and *V. arboreum* from a previous study (J.W. Olmstead, unpublished data). Then, the sequence of the best BLAST match for each marker from the SHB transcriptome, or the *V. arboreum* transcriptome in absence of the former, was searched against the nonredundant protein database from NCBI (Pruitt

Table 2. Effects of family, substrate, and the interaction between family and substrate on dry weight of *Vaccinium* seedlings from five different families grown in bench-top rhizotrons for 77 d.

Family ^z	Substrate ^y	Root dry wt (mg) ^x	Shoot dry wt (mg) ^w	Whole plant dry wt (mg) ^w
S1		175 b	482	861
S2		143 b	462	853
S3		300 a	362	581
S4		200 ab	449	764
S5		158 b	479	1,186
	Peat	236 a	592	1,100
	Peat:perlite	155 b	302	600
S1	Peat	217 a	628	1,117
	Peat:perlite	134 a	337	606
S2	Peat	186 a	683	1,044
	Peat:perlite	101 a	241	663
S3	Peat	230 b	260	175
	Peat:perlite	370 a	463	987
S4	Peat	324 a	675	1,310
	Peat:perlite	77 b	223	219
S5	Peat	224 a	714	1,852
	Peat:perlite	93 b	243	519
<i>P</i> value				
Family		0.0024	0.5867	0.5917
Substrate		0.0025	0.2130	0.0583
Family × substrate		0.0003	0.1709	0.1359

^zFamilies' pedigree information can be found in Table 1.

^ySubstrates were 100% peat or 3:1 peat:perlite.

^xValues shown are means of $n \geq 7$ replications per family × substrate combination. Letters indicate significant differences ($P < 0.05$) among families, substrates, or substrates within a family as determined by post hoc Tukey honestly significant difference (HSD) tests.

^wValues shown are means of $n = 4$ replications per family × substrate combination. Means were not significantly different among families, substrates, or substrates within a family as determined by post hoc Tukey HSD tests.

et al., 2005) via BLASTX (Altschul et al., 1990) in Sept. 2015. A sequence was considered satisfactorily annotated if an alignment with $e\text{-value} \leq 10^{-4}$ and 30% or higher identity was produced over 30 or more amino acids. This percentage identity cutoff was selected bearing in mind that transcriptome-based query sequences—like the ones used here and in previous functional annotation efforts in blueberry (Gupta et al., 2015)—can contain multiple transcripts, which lowers the percentage identity in alignments. Alignments with uncharacterized proteins were not considered satisfactory. Where satisfactory annotation was achieved, the description string of the best BLASTX match was adopted for the sequence, the GenBank identifier of the best match was listed, and the function of the protein was investigated in UniProtKB (The UniProt Consortium, 2015).

Results

SUBSTRATE EFFECT. While neither family, substrate treatment, nor their interaction significantly affected whole plant or shoot dry weight (Table 2), all three of these factors significantly affected the root dry weight of *Vaccinium* species seedlings. On average, seedlings of family S3 had significantly larger root systems than seedlings in families S1, S2, and S5. The root systems of families S1, S2, S4, and S5 did not differ among themselves. Across families, peat-grown seedlings exhibited significantly greater root dry weight than peat:perlite-grown seedlings. The same effect was observed between peat-grown and peat:perlite-grown seedlings in families S4 and S5. The opposite effect was observed in family S3. Growth substrate did not affect root dry weight in families S1 and S2. Maximum root depth, SRL, root volume, average root diameter, total root length, total root surface area, and number of branching points were positively correlated with root dry weight ($P < 0.001$ in all cases). Substrate treatment significantly affected RSA traits (Table 3). Peat-grown seedlings exhibited significantly lower maximum root depth and SRL than peat:perlite-grown seedlings. Additionally, peat-grown seedlings exhibited greater root volume and average root diameter than peat:perlite-grown seedlings. Peat-grown seedlings exhibited fewer first-order roots than peat:perlite-grown seedlings. Total root length, total root surface area, and the number of branching points were not significantly affected by substrate treatment (data not shown). In this experiment, the average root system was 1.27 m long, had a surface area of 385 cm², and contained 7511 branching points.

FAMILY PEDIGREE EFFECT. Family pedigree significantly affected root, shoot, and whole plant dry weight (Table 4). Seedlings in family P2 exhibited significantly higher biomass

accumulation than seedlings in all other families. Families P1, P3, P4, P5, and P6 were not significantly different among themselves. Maximum root depth, percentage of roots in the top 8 cm of soil, root length, and number of branching points were positively correlated with root dry weight ($P < 0.001$ in all cases). Average root diameter and SRL were not correlated with root dry weight ($P > 0.145$ in all cases). Family pedigree significantly affected RSA traits (Table 5). Seedlings in families P1 and P2 exhibited significantly lower maximum root depth than all other families. Seedlings in families P1 and P2 had greater percentage of their root systems in the top 8 cm of soil than seedlings in families P3 and P4. Root architecture differences between families P2 and P3 can be observed in Fig. 2. Seedlings in family P4 exhibited significantly greater total root length than seedlings in all other families (Table 5). Seedlings in family P2 exhibited significantly lower root length than seedlings in families P1, P3, P4, and P6. Root surface area and volume followed the same statistical separation as root length (data not shown). Seedlings in family P2 exhibited significantly lower SRL than seedlings in families P3 and P4 (Table 5). Seedlings in family P4 exhibited significantly more branching points than seedlings in all other families except P6, while family P2 exhibited significantly fewer branching points than families P4 and P6. Seedlings in family P4 exhibited more first-order roots than seedlings in all families except P3, while family P2 exhibited significantly fewer first-order roots than families P3, P4, and P6. Seedlings in families P1 and P2 exhibited significantly greater average root diameter than seedlings in families P3, P4, and P5. The average root diameters of family P1 and P6 were not significantly different.

Table 3. Substrate effect on root system architecture traits of *Vaccinium* seedlings grown in bench-top rhizotrons for 77 d.

Substrate ^z	Maximum root depth (cm) ^y	Specific root length (m·g ⁻¹)	Root vol (cm ³)	Avg root diam (mm)	First order roots (×1,000)
Peat	22.13 b	71.20 b	9.83 a	0.98 a	2.44 b
Peat:perlite	24.37 a	84.31 a	9.00 b	0.93 b	3.24 a
<i>P</i> value					
Root dry weight	<0.0001	0.0005	<0.0001	0.0001	<0.0001
Substrate	0.0065	0.0382	0.0189	0.0420	0.0077
Family × substrate	0.0582	0.0992	0.6947	0.3883	0.0078

^zSubstrates were 100% peat or 3:1 peat:perlite.

^yValues shown are means for each substrate after normalizing for root dry weight. Letters indicate significant differences ($P < 0.05$) as determined by post hoc Tukey honestly significant difference (HSD) tests.

Table 4. Effects of family pedigree on the dry weight of six families of *Vaccinium* seedlings grown in bench-top rhizotrons for 67 d.

Family ^z	Root dry wt (mg) ^y	Shoot dry wt (mg)	Whole plant dry wt (mg)
P1	48 b	173 b	221 b
P2	121 a	466 a	587 a
P3	50 b	198 b	248 b
P4	71 b	277 b	348 b
P5	53 b	221 b	274 b
P6	66 b	245 b	311 b
<i>P</i> value			
Family pedigree	0.0002	<0.0001	<0.0001

^zFamilies' pedigree information can be found in Table 1.

^yValues shown are means of $n = 21$ replications per family. Letters indicate significant differences ($P < 0.05$) as determined by post-hoc Tukey honestly significant difference (HSD) tests.

Table 5. Effects of family pedigree on root system architecture traits of six families of *Vaccinium* seedlings grown in bench-top rhizotrons for 67 d.

Family ^z	Maximum root depth (cm) ^y	Roots in the top 8 cm of soil (%)	Root length (cm)	Specific root length (m·g ⁻¹)	Branching points (×1,000)	First order roots (×1,000)	Avg root diam (mm)
P1	14.59 b	58.97 ab	560.13 b	81.53 bc	3.93 bc	2.67 dc	0.34 ab
P2	13.92 b	71.06 a	309.49 c	62.38 c	2.58 c	1.76 d	0.35 a
P3	24.48 a	33.45 dc	663.73 b	120.65 ab	4.06 bc	4.22 ab	0.29 c
P4	26.30 a	25.94 d	899.10 a	139.43 a	5.87 a	5.44 a	0.29 c
P5	21.18 a	48.43 abc	427.37 bc	95.32 abc	3.16 bc	2.80 dc	0.29 c
P6	22.77 a	38.77 bcd	656.32 b	91.56 abc	4.34 ab	3.51 bc	0.30 bc
<i>P</i> value							
Root dry weight	<0.0001	<0.0001	<0.0001	0.1448	<0.0001	<0.0001	0.5796
Family	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

^zFamilies' pedigree information can be found in Table 1.

^yValues shown are means for each family after normalizing for root dry weight. Letters indicate significant differences ($P < 0.05$) as determined by post hoc Tukey honestly significant difference (HSD) tests.

Fig. 2. Root system architecture of southern highbush blueberry [SHB (family P2)] and *Vaccinium arboreum* (family P3) seedlings grown in bench-top rhizotrons for 67 d. Pedigree information for these families can be found in Table 1. (A) SHB root system: root dry weight = 67 mg, total root length = 620 cm. (B) *V. arboreum* root system: root dry weight = 69 mg, total root length = 640 cm. Root systems pictured to scale. Size bars represent 8 cm.

MARKER-TRAIT ASSOCIATION WITH RSA TRAITS. A total of 3447 SNPs and eight RSA traits measured in 227 individuals resulting from a cross between a SHB cultivar and an interspecific hybrid between SHB and *V. arboreum* were used for the association study. All RSA traits except root length exhibited H^2 greater than 0.10 (Table 6). The H^2 of root length was <0.01. Genetic correlations between RSA traits ranged between -0.11 and 0.47 (Table 7). Genetic correlations with average root diameter were nonestimable (data not shown). A total of 57 significant marker-trait associations were found between 6 RSA traits and 54 markers (Table 6; Supplemental Table 4). Marker TP30609 was significantly associated with root surface area, root volume, number of branching points, and number of first-order roots. All other marker-trait associations were one to one. The proportion of phenotypic variation explained by markers ranged between 6.72% and 14.45%. The sequences of 37 of the 54 markers exhibited significant

homology with contigs in the SHB or *V. arboreum* root transcriptome (Table 8). Markers with no homology were not analyzed further. Markers TP10972, TP20147, TP2069, and TP5008 were found in the same contig in the SHB and *V. arboreum* transcriptome. All other markers had one-to-one matches. Median contig length was 501 bp. In the BLASTX search, 18 markers did not have significant or satisfactory matches and were not annotated. The neighboring regions of the 16 successfully annotated markers contained genes that code for proteins involved in ubiquitination (TP16403, TP16736, and TP17162), auxin response (TP53 and TP30062), and translation (TP12923), as well as proteins with functions as transcription factors (TP17298, TP17930, TP29772, TP33561, and TP7074), transmembrane transporters (TP5555), and enzymes (TP18501, TP22590, and TP283).

Discussion

RSA traits of cultivated SHB are attractive targets for developing MAS assays, as phenotypic characterization of below-ground traits cannot be accomplished without significant resources and/or destructive sampling. In this study, we developed an assay for RSA traits by testing for family-level differences among *Vaccinium* species. In particular, we sought to determine if differences in RSA traits could be quantified among various levels of introgression from *V. arboreum* into the SHB genetic background. This introgression of favorable RSA traits from *V. arboreum* has long been a goal in SHB breeding (Lyrene, 1997; Olmstead et al., 2013).

The RSA of *Vaccinium* species was affected by the substrate in which seedlings were grown. The greater root biomass in peat-grown seedlings compared with peat:perlite-grown seedlings supports previous studies on lowbush blueberry (*Vaccinium angustifolium*) and other crops demonstrating substrate effects on root biomass accumulation (Gioia et al., 2015; Hicklenton et al., 2000; Hund et al., 2009; Jin et al., 2015). Although peat-grown seedlings exhibited a tendency to have higher shoot and total dry weight than peat:perlite-grown seedlings, differences were not significant likely due to the limited number of replications used in Expt. 1. Root dry weight was positively correlated with most traits, in agreement with previous studies (Fita et al., 2008; Johnson et al., 2000; Khan et al., 2012). However, substrate treatments affected RSA beyond the effect on biomass accumulation. The thicker roots of peat-grown compared with peat:perlite-grown plants led to

significant differences in the total root volume. In addition, peat-grown plants had fewer first-order roots than peat:perlite-grown ones. Considering that the SRL of first-order roots is higher than the SRL of higher order roots in *Vaccinium* species (Valenzuela-Estrada et al., 2008), the lower abundance of first-order roots in peat-grown plants compared with peat:perlite-grown ones explains the lower SRL of seedlings in the former treatment. Although high SRL has been associated with growth in low-fertility soils (Eissenstat, 1992; Ostonen et al., 2007), the hydraulic and nutritional characteristics of the substrates used in this experiment were not measured. Hence, conclusions cannot be drawn about specific substrate characteristics affecting SRL in *Vaccinium* species.

In light of our finding that roots of peat-grown seedlings had greater average diameter than peat:perlite-grown ones, assuming that thicker roots can withstand cleanup better than thin ones, and considering that flushing peat away from the root was easier than flushing peat:perlite, peat was selected as the substrate for subsequent RSA studies and scanning procedures and image acquisition parameters were adjusted (as noted in the Materials and Methods section).

Comparisons among families revealed significant differences in root, shoot, and whole plant dry weight. Seedlings in family P2 were the result of a cross between advanced selections from the University of Florida blueberry breeding program and they were bigger than seedlings in all other families in Expt. 2. This is consistent with plant vigor being one of the standards used for phenotypic selection the breeding program (Lyrene, 2007). Families also differed on their RSA. The shallower roots and greater percentage of roots close to the surface in SHB seedlings (family P2) compared with *V. arboreum* seedlings (family P3) are the first set of empirical evidence documenting RSA differences between plants in these two taxa grown under the same conditions. These results are in agreement with previous reports based on field observations (Lyrene, 1991; Lyrene and Brooks, 1995). Water acquisition from subsoil strata through a deeper root system is likely the reason for the reported

Table 6. Single nucleotide polymorphism markers with statistically significant association ($P \leq 10^{-3}$) with heritable root system architecture traits in an interspecific pseudo-backcross family between southern highbush blueberry and *Vaccinium arboreum*.

Trait	Mean	Range	H^{2z}	Marker	R^2	P value ^y
Roots in the top 8 cm of soil (%)	32.42	10–90	0.36	TP10972	0.0941	<0.0001
				TP12275	0.0851	<0.0001
				TP1255	0.0858	<0.0001
				TP12923	0.0853	<0.0001
				TP14807	0.0882	<0.0001
				TP15079	0.0878	<0.0001
				TP16401	0.0866	<0.0001
				TP16403	0.1007	<0.0001
				TP16736	0.0852	<0.0001
				TP171	0.0930	<0.0001
				TP17162	0.0970	0.0001
				TP17244	0.0876	<0.0001
				TP17298	0.0849	<0.0001
				TP17930	0.0858	<0.0001
				TP18062	0.0888	0.0001
				TP18501	0.0849	<0.0001
				TP18747	0.0849	<0.0001
				TP1952	0.0903	<0.0001
				TP20147	0.0855	<0.0001
				TP2069	0.1000	0.0002
				TP2117	0.0852	<0.0001
				TP2155	0.0864	<0.0001
				TP21868	0.1169	<0.0001
				TP22519	0.0852	<0.0001
				TP22590	0.0882	<0.0001
				TP23301	0.0851	0.0001
				TP23574	0.0920	<0.0001
				TP25121	0.0672	0.0009
				TP25656	0.1445	<0.0001
				TP26551	0.0929	<0.0001
				TP283	0.0867	<0.0001
				TP28439	0.0857	<0.0001
				TP29772	0.0677	0.0004
				TP30062	0.0896	<0.0001
				TP30258	0.0854	<0.0001
				TP33561	0.0875	<0.0001
				TP4589	0.0853	<0.0001
				TP4941	0.0854	0.0001
				TP5008	0.0885	<0.0001
				TP5072	0.0858	<0.0001
				TP5246	0.0852	<0.0001
				TP53	0.0935	0.0001
				TP5555	0.0849	<0.0001
				TP5627	0.0899	<0.0001
				TP5668	0.0867	<0.0001
				TP702	0.0867	<0.0001
				TP7074	0.0955	<0.0001
				TP8034	0.0855	<0.0001
				TP9464	0.0903	<0.0001
				TP9982	0.0849	0.0006
Maximum root depth (cm)	26.41	3.7–37.2	0.33	None found		
Root surface area (cm ²)	400.46	36.46–999.73	0.17	TP30609	0.085	<0.0001
Average root diameter (mm)	0.26	0.20–0.39	0.25	TP14326	0.083	<0.0001
				TP16027	0.084	<0.0001
				TP26072	0.074	<0.0001

Continued next page

Table 6. Continued.

Trait	Mean	Range	H^2z	Marker	R^2	P value ^y
Root volume (cm ³)	2.64	0.22–8.08	0.30	TP30609	0.071	<0.0001
First order roots (×1000)	24.17	3.23–44.16	0.25	TP30609	0.102	<0.0001

^zBroad-sense heritability.^yAdjusted for multiple comparisons with the Bonferroni method.Table 7. Genetic correlations between root system architecture traits in an interspecific pseudo-backcross family between southern highbush blueberry and *Vaccinium arboreum*.

Root system architecture traits	T1	T2	T3	T4	T5	T6	T7
Roots in the top 8 cm of soil [T1 (%)]	1.00	−0.03	−0.02	0.00	−0.11	−0.01	0.00
Maximum root depth [T2 (cm)]		1.00	−0.02	0.01	0.47	−0.01	0.00
Total root length [T3 (cm)]			1.00	0.11	−0.02	−0.01	0.00
Root surface area [T4 (cm ²)]				1.00	0.38	0.00	0.00
Root volume [T5 (cm ³)]					1.00	0.00	0.00
First-order roots [T6 (×1,000)]						1.00	0.00
Branching points [T7 (×1,000)]							1.00

drought-tolerance of *V. arboreum* plants (Stockton, 1976). Additionally, deeper roots would give *V. arboreum* access to nitrate that has percolated to deeper parts of the soil (Lynch and Wojciechowski, 2015), which combined with its greater nitrate reductase activity (Poonnachit and Darnell, 2004), could explain the soil nitrogen adaptation of this plant species. Finally, while soil acidity and high aluminum concentrations at greater soil depths are challenges to deep root production in other plant species (Lynch and Wojciechowski, 2015), *V. arboreum* and its offspring would be particularly well adapted for growth at greater depths due to their preference for acidic soils (Finn et al., 1993) and mycorrhizae-mediated aluminum toxicity tolerance (Yang and Goulart, 2000). Altogether, our results suggest that *V. arboreum* could be a source of greater root depth in SHB breeding programs.

Families with different levels of *V. arboreum* introgressions exhibited midrange phenotypes between the RSA of families P2 and P3, with the exception of family P4, which exhibited accentuated *V. arboreum*-like RSA. The finding that families with *V. arboreum* parentage in the three preceding generations exhibit *V. arboreum*-like root depth and SRL suggests that RSA traits are heritable. The root architecture of family P4 might have been caused by a combination of the root depth and high SRL alleles from its two different *V. arboreum* grandparents, as this family is the product of the cross of two interspecific F1 hybrids between SHB and *V. arboreum*. Altogether, these results provide evidence of differences in RSA traits among families of *Vaccinium* species and highlight the opportunity to use *V. arboreum* as a genetic source for SHB breeding.

Except for root length, all RSA traits studied in the pseudo-backcross family exhibited moderate to low broad-sense heritabilities that fall within the range found for RSA traits in seedlings of other species (Fita et al., 2008; Hamada et al., 2012; Khan et al., 2012). This supports the conclusion from Expt. 2 that both maximum root depth and root distribution in the top soil layer are heritable in *Vaccinium* species. However, future work will be necessary to extend these results beyond the growth conditions used in this study. RSA traits exhibited moderate to low genetic correlations among them, which fall within the range published for RSA traits in other species (Fita et al., 2006, 2008). In the present study and in previous research

(Hebert et al., 1994), some genetic correlations exhibited magnitudes outside of theoretical limits, likely due to the use of an indirect estimation method and a single family in a single environment as discussed in Lynch and Walsh (1998) and references therein.

All but one of the heritable RSA traits were significantly associated with at least one marker. While there were not any markers associated with maximum root depth, the majority of the associations were with the percentage of roots in the top 8 cm of soil. Hence, MAS against these alleles may lead to *Vaccinium* species plants with greater percentages of roots in deeper soil strata. Across all heritable RSA traits, marker effects were small, but some traits were asso-

ciated with more than one marker. This suggests that several small effects could be stacked to breed for an RSA ideotype, as previously done in other crops (Liu et al., 2012; Steele et al., 2006). Moreover, this further emphasizes the quantitative nature of RSA traits (Johnson et al., 2000). In previous studies, several QTL that affect RSA traits have been discovered in other plant species (Fita et al., 2008; Hamada et al., 2012; Johnson et al., 2000; Li et al., 2015; Ron et al., 2013; Steele et al., 2006), which suggests that QTL that affect RSA could exist in SHB. Nevertheless, in absence of a genetic map that includes the markers used here, we were unable to test this hypothesis.

Instead, we pursued a two-step annotation process analogous to the candidate gene mining efforts undertaken in model organisms (Liu et al., 2012; Monclus et al., 2012). First, due to the short sequences produced by the genotyping by sequencing pipeline for each polymorphism, we gathered the sequence of the regions neighboring significantly associated markers in the available *Vaccinium* species root transcriptomes. Then, we annotated these regions based on protein homology. Only 19 of the 54 markers were successfully annotated. The remaining markers were either not represented in the SHB or *V. arboreum* transcriptome or they did not have satisfactory matches in the NCBI database. Markers not present in the transcriptome are potentially intronic. Lack of significant homology with databases was likely caused by the short transcriptome sequences used as queries.

The annotation resulted in several genomic regions that affect RSA traits. Among these, sequence homologies to genes involved in root development in other species were identified. For instance, the peptides neighboring TP53 and TP30062 exhibited homology with proteins involved in auxin-dependent gene expression (Ellis et al., 2005) and polar auxin transport (Lin and Wang, 2005), respectively; which have been found to have an effect in root development in *Arabidopsis thaliana* (Liu et al., 2014). Similarly, the peptide neighboring TP29772 has homology with transcription factor STOP1, which controls root-level responses to proton and aluminum toxicity in *A. thaliana* (Iuchi et al., 2007). Although the functions of the peptides in the neighboring region of other significantly associated markers did not expose direct relationships to RSA traits, all the annotated genes constitute targets for future studies in *Vaccinium* species and beyond.

Table 8. In silico annotation based on protein homology with the NCBI GenBank database of the transcribed regions neighboring markers significantly associated with root system architecture traits in an interspecific pseudo-backcross family between southern highbush blueberry and *Vaccinium arboreum*.

Marker	Homologous sequence in transcriptome		Contig length (bp)	Annotation ^y	NCBI GenBank accession no. of best match
	SHB ^z	<i>V. arboreum</i>			
TP10972, TP20147, TP2069, TP5008	Yes	Yes	430	Putative major spike protein G-like	WP_047924110.1
TP12275	Yes	Yes	1,453	NS	
TP12923	Yes	Yes	287	Putative elongation factor 1 alpha- like protein	AAV34910.1
TP16027	Yes	No	2,998	NS	
TP16401	Yes	No	200	NA	
TP16403	Yes	Yes	2,523	Putative ubiquitin-protein ligase	XP_011084290.1
TP16736	Yes	Yes	305	Putative T-complex protein	XP_008362565.1
TP171	Yes	Yes	953	NA	
TP17162	Yes	Yes	644	Putative ubiquitin fusion degradation protein	KHG02024.1
TP17298	Yes	Yes	1,832	Putative deoxyribonucleic acid binding protein	XP_002521085.1
TP17930	Yes	Yes	360	Putative AP2 domain-containing transcription factor	XP_007014965.1
TP18062	Yes	Yes	282	NS	
TP18501	Yes	Yes	1,740	Putative aerogenate dehydratase 3	XP_002268701.1
TP2117	Yes	Yes	501	NS	
TP21868	Yes	Yes	561	NA	
TP22519	Yes	Yes	271	NS	
TP22590	Yes	Yes	1,021	Putative type I inositol 1,4,5- trisphosphate 5-phosphatase 12-like protein	XP_009758451.1
TP23574	Yes	No	247	NS	
TP26072	Yes	No	510	NS	
TP283	Yes	Yes	174	Putative nudix hydrolase 2-like protein	KHG07900.1
TP29772	Yes	Yes	329	Putative sensitive to proton rhizotoxicity 1-like transcription factor	XP_010062013.1
TP30062	Yes	Yes	133	Putative ABC transporter B family member	KHG09918.1
TP30258	Yes	Yes	710	NS	
TP30609	Yes	Yes	968	NS	
TP33561	Yes	Yes	1,320	Putative zinc finger domain- containing protein	XP_009601385.1
TP4589	No	Yes	337	NS	
TP4941	No	Yes	308	NA	
TP5072	Yes	Yes	1,959	NS	
TP5246	Yes	No	494	NA	
TP53	Yes	Yes	1,679	Putative auxin response factor 1	AFQ94050.1
TP5555	Yes	Yes	468	Putative V-type H ⁺ ATPase subunit a1	XP_006424665.1
TP5668	Yes	Yes	590	NS	
TP7074	Yes	No	1,206	Putative nucleotide-binding site leucine-rich repeat protein	XP_013464423.1
TP9464	Yes	Yes	2,474	NS	

NA = no significant similarity found; NS = alignment found with uncharacterized proteins.

^zSouthern highbush blueberry.

^yDescription string of the best BLASTX match (e-value $\leq 10^{-4}$ and 30% or higher identity) in NCBI GenBank.

An area that warrants further investigation is the relationship between the RSA of seedlings and adult plants, and seedlings and plants with adventitious roots. Previous studies have found

that RSA differences and genetic architecture are consistent between seedlings and mature plants in other species (Dias et al., 2004; Fita et al., 2006), but studies in *Vaccinium* species

are not available. Nevertheless, candidate genes associated with RSA in *Vaccinium* species seedlings were homologous to genes that impact root phenotypes of mature specimens of *A. thaliana*. This suggests that the plant breeding tools developed here are based on plant physiological processes that affect the RSA of plants across growth stages. RSA differences between seedlings and rooted cuttings have been described in other species, including lowbush blueberry (Morrison et al., 2000). Yet, to the best of our knowledge, there have not been studies focusing on the genetic elements controlling seminal and adventitious RSA in SHB or other *Vaccinium* species. Further investigation on this subject will be requisite considering that commercial propagation of SHB is done through rooted cuttings or in vitro methods (Lyrene, 2007).

Altogether, the present study identified a growth medium for root phenotyping of thin-rooted *Vaccinium* species. Additionally, root depth differences between SHB and *V. arboreum* grown under the same conditions were found. This trait is of interest for SHB breeding as it may impact drought tolerance, access to nutrients, and soil adaptation. The intermediate RSA of interspecific hybrid seedlings between SHB and *V. arboreum*, and the non-zero heritability estimates for RSA traits in an interspecific pseudo-backcross family suggest that breeding for RSA traits in SHB is feasible. SNP markers significantly associated with RSA traits highlight the possibility of employing MAS for this means. In silico annotation of the transcribed regions neighboring these markers identified biologically relevant genomic regions that affect RSA in *Vaccinium* species and other plants suggesting that these markers may be a robust tool for breeding for RSA traits.

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Supplemental Table 1. Sequences of common adapters used in library construction for genotyping by sequence of an interspecific pseudo-backcross family between southern highbush blueberry and *V. arboreum*.

Primer name	Sequence (5' to 3')
Com_F	AGA TCG GAA GAG CGG TTC AGC AGG AAT GCC GAG
Com_R	CTC GGC ATT CCT GCT GAA CCG CTC TTC CGA TCT TGC A

Supplemental Table 2. Sequences of common primers used in library construction for genotyping by sequence of an interspecific pseudo-backcross family between southern highbush blueberry and *V. arboreum*.

Primer name	Sequence (5' to 3')
PstI_F	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT CTC CAT GCA
PstI_R	TGG AGA GAT CGG AAG AGC GTC GTG TAG GGA AAG AGT GT

Supplemental Table 3. Barcode sequences used for multiplexed library construction for genotyping by sequence of an interspecific pseudo-backcross family between southern highbush blueberry and *V. arboreum*.

Barcode	Sequence (5' to 3')	Barcode	Sequence (5' to 3')
A01.F	CTCC	E01.F	CTATTA
A02.F	TGCA	E02.F	GCCAGT
A03.F	ACTA	E03.F	GGAAGA
A04.F	CAGA	E04.F	GTACTT
A05.F	AACT	E05.F	GTTGAA
A06.F	GCGT	E06.F	TAACGA
A07.F	CGAT	E07.F	TGGCTA
A08.F	GTAA	E08.F	TATTTTTT
A09.F	AGGC	E09.F	CTTGCTT
A10.F	GATC	E10.F	ATGAAAC
A11.F	TCAC	E11.F	AAAAGTT
A12.F	TGCGA	E12.F	GAATTCA
B01.F	CGCTT	F01.F	GAACTTC
B02.F	TCACC	F02.F	GGACCTA
B03.F	CTAGC	F03.F	GTCGATT
B04.F	ACAAA	F04.F	AACGCCT
B05.F	TTCTC	F05.F	AATTGCA
B06.F	AGCCC	F06.F	ACGTGTT
B07.F	GTATT	F07.F	ATTAATT
B08.F	CTGTA	F08.F	ATTGGAT
B09.F	ACCGT	F09.F	CATAAGT
B10.F	GCTTA	F10.F	CGCTGAT
B11.F	GGTGT	F11.F	CGGTAGA
B12.F	AGGAT	F12.F	CTACGGA
C01.F	ATTGA	G01.F	GCGGAAT
C02.F	CATCT	G02.F	TAGCGGA
C03.F	CCTAC	G03.F	TCGAAGA
C04.F	GAGGA	G04.F	TCTGTGA
C05.F	GGAAC	G05.F	TGCTGGA
C06.F	GTCAA	G06.F	ACGACTAC
C07.F	TAATA	G07.F	TAGCTGCA
C08.F	TACAT	G08.F	TAGGCCAT
C09.F	TCGTT	G09.F	TGCAAGGA
C10.F	GGTTGT	G10.F	TGGTACGT
C11.F	CCAGCT	G11.F	TCTCAGTC
C12.F	TTCAGA	G12.F	CCGGATAT
D01.F	TAGGAA	H01.F	CGCCTTAT
D02.F	GCTCTA	H02.F	AACCGAGA
D03.F	CCACAA	H03.F	ACAGGGAA
D04.F	CTTCCA	H04.F	ACGTGGTA
D05.F	GAGATA	H05.F	CCATGGGT
D06.F	ATGCCT	H06.F	CGCGGAGA
D07.F	AGTGGA	H07.F	CGTGTGGT
D08.F	ACCTAA	H08.F	GCTGTGGA
D09.F	ATATGT	H09.F	GGATTGGT
D10.F	ATCGTA	H10.F	GTGAGGGT
D11.F	CATCGT	H11.F	TATCGGGA
D12.F	CGCGGT	H12.F	TTCCTGGA

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Supplemental Table 4. Continued.

Marker name	Sequence (5' to 3')
TP23574	CGACGCTCTCCGATCTCTACGGAAATGCAGGGTTGGCAAAGTTCTCAAAATTGCCAGTAGTGTTTTCACATTAGGAACCTGCAAGATCGG
TP25121	GGTTGACTAGCTAAAATCAAAAAGAGATTTTGTGGGATTCAGAGAAATTAGGGCTTTGGTTCAGAGAAATAAGGCTTTTAAATCTA
TP25656	GTAAACAGACTCTATTAGCTCTTCTCAAAATTTTGA AATGGTAAGTGCAGGATCACTTCAAGTGTAGATCCCTGGACTCAACCAAGAGA
TP26072	GTACAAAGTGTTCTTAGCGTGATCCAAATCGTTTAAAAGTGTTTAAATAAACAGATTGTGTGAAAGCTTCTTATCGCAAAATCTAAA
TP26551	GTAGCAGGAACCTCGTACAGTGGTAGCCTCTATCATACCGGTGGTTTCAAGACAATCATCAACATCTGATATGAATATTCAAACC
TP28439	GTCTCAGTATCTAGGTAACAATTGAGTTTTTGAATTTGATACGACTTTTGATTGGACCTGTAGTGGATGGTGTGATTGGTC
TP29772	GTGGA AATCAATTGAACCTATAAATTGAAGTAAGATCAGCAGTGACAAGCTGTTGTTGCTGAGCTACAAGTTCAATCAGCCCGAGAGGGA
TP30062	GTGGGTCTGTGTATCCTCACTATCAGGATTATATACAACAGCAATGA AAGCTGCAAGATCGGAAGAGCGGTTTCAGCAGGAATGCC
TP30258	GTGTACTGTAGCAGAACTGTCTTCGAGGATGCAACTCCAGAGCTGGTCAGAGACTTCTTCTGGGATGATGAGTTCGTCTCTAAATGG
TP30609	GTTAAAATATCTCCAGTTAATTTGTGATTTTCAGCTGGAAAGATGGAATCGAAGCACAAGTTTGGAAATGGGCTTTTGAATCTTAC
TP33561	GTTTTTGGAGTACAAACATTTCTCTGTATCTTTTCTCTTGTATGGCTCTGCAAGATCGGAAGAGCGGTTTCAGCAGGAATGCCGAGA