

Construction of a genetic linkage map of an interspecific diploid blueberry population and identification of QTL for chilling requirement and cold hardiness

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Abstract A genetic linkage map has been constructed from an interspecific diploid blueberry population [(*Vaccinium darrowii* Fla4B × *Vaccinium corymbosum* W85-20) F₁#10 × *V. corymbosum* W85-23] designed to segregate for cold hardiness and chilling requirement. The map is comprised of 12 linkage groups (equivalent to the haploid chromosome number of diploid blueberry) and totals 1,740 cM. Included on the map are 265 markers based on simple sequence repeats, expressed sequence tag-polymerase chain reactions, single nucleotide polymorphisms, and

randomly amplified polymorphic DNAs. The estimated map coverage is 89.9 %, and the average distance between markers is 7.2 cM. The mapping population was evaluated for 2 years (2009 and 2010) for mid-winter bud cold hardiness and for 3 years (2011–2013) for chilling requirement under controlled conditions. Broad-sense heritability of both cold hardiness and chilling requirement was quite high under these conditions with values of 0.88 and 0.86, respectively. One quantitative trait locus/loci (QTL) for cold hardiness and one for chilling requirement were identified that were consistent over at least 2 years. A second weaker QTL for chilling requirement was detected in only one of the 3 years.

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Abbreviations

CH	Cold hardiness
CR	Chilling requirement
CR50	Chill units resulting in 50 % floral bud break
EST-PCR	Expressed sequence tag-polymerase chain reaction
<i>H</i>	Broad-sense heritability
HRM	High-resolution melting
IM	Interval mapping
LOD	Logarithm of the odds
LT50	Lethal temperature causing 50 % injury
PM	Permutation test
QTL	Quantitative trait locus/loci
RAPD	Randomly amplified polymorphic DNA
rMQM	Restricted multiple QTL mapping
SNP	Single nucleotide polymorphism
SRA	Sequence read archive
SSR	Simple sequence repeat

Introduction

Blueberry production has risen by about 50 % over the last 5 years, making it one of the most rapidly expanding fruit crops in the world today. Popularity among consumers has increased, too, no doubt propelled by research findings on their many health benefits. For example, blueberries have been shown to be an especially rich source of anthocyanins (Prior et al. 1998), which have been associated with many health benefits from reducing eyestrain, improving night vision, and helping prevent macular degeneration, to exhibiting anticancer activity (Cho et al. 2004; Kalt et al. 2007). A recent human study found that blueberries may even improve certain features of metabolic syndrome and related cardiovascular risk factors like high blood pressure at dietary achievable doses (Basu et al. 2010).

Blueberry is a native North American fruit crop. Several species of various ploidy levels within the *Cyanococcus* section of the genus *Vaccinium*, family *Ericaceae*, are grown commercially. The major commercial types in the USA are cultivars of *Vaccinium corymbosum* L. (tetraploid highbush blueberry) and, to

a lesser extent, *V. virgatum* Ait. (hexaploid rabbiteye blueberry; syn. *V. ashei* Reade), and native, managed stands of *V. angustifolium* Ait. (tetraploid lowbush blueberry). Highbush blueberry cultivars are classified into northern and southern types. Southern highbush cultivars, with low chilling requirements, have been developed through the introgression of the low-chilling southern diploid species *Vaccinium darrowii* Camp, through the production of unreduced gametes, into the tetraploid *V. corymbosum* background (Rowland et al. 2011). The southern highbush cultivars have the advantage of being earlier ripening than rabbiteye blueberries, which are also grown in the southern USA.

The highbush blueberry was domesticated in the USA in the early twentieth century. Breeding efforts have focused on the development of cultivars with broader climatic adaptation (increased freezing tolerance for northern regions and reduced chilling requirements for southern regions), season extension, disease and pest resistance, mechanical handling tolerance, and high fruit quality (Galletta and Ballington 1996). However, evaluating breeding populations in the field for traits related to climatic adaptation can be very difficult, as chilling hours and temperatures vary greatly from year to year and site to site. The availability of molecular markers for help in combining traits for climatic adaptation with other important traits like fruit and nutritional quality would be very useful. Woody perennials, like blueberry, are especially suitable for improvement via marker-assisted breeding because of their long generation times, high heterozygosity, self-infertility, inbreeding depression, and polyploidy of commercial types.

A study of winter injury in field-grown blueberry found heritability estimates over 3 years to be low although individual year estimates were higher. General combining ability variance components were consistently higher than specific combining ability, suggesting that additive variance is more important than nonadditive variance (Fear et al. 1985). However, because winter survival in the field is determined by multiple factors, the authors recommended screening for cold hardiness under controlled conditions to accurately ascertain additive, dominance, and epistatic interactions.

We have developed an interspecific diploid blueberry population suitable for mapping cold hardiness, chilling requirement, and multiple fruit quality traits (Rowland et al. 1999). This population is a testcross generated by

crossing an F₁ hybrid of a low-chilling, freezing-sensitive *V. darrowii* selection and a high-chilling, freezing tolerant diploid *V. corymbosum* selection, to another diploid *V. corymbosum* selection. A true F₂ or backcross population would be difficult, if not impossible, to generate for mapping due to self-sterility (Galletta and Ballington 1996) and inbreeding depression (Krebs and Hancock 1988) in diploid blueberry. The testcross population described here, along with other related populations, has been used previously in genetic analyses of cold hardiness (Arora et al. 2000; Rowland et al. 1999) and chilling requirement (Rowland et al. 1998, 1999). Degree of dominance and generation means analysis revealed that cold hardiness is a quantitative trait controlled largely by additive gene effects and, to a lesser extent, by dominance gene effects, with cold sensitivity appearing to be partially dominant. Cold tolerance could be explained adequately by a simple additive-dominance model of gene action, although two epistatic models involving additive-additive and dominance-dominance interactions also fit the data (Arora et al. 2000). Formal generation means analysis has not been carried out successfully for chilling requirement; however, segregation patterns suggest that it, too, is largely quantitatively inherited with low chilling requirement showing additive and perhaps some dominance gene action (Rowland et al. 1998, 1999, 2011).

Here, the construction of a genetic linkage map for the interspecific diploid blueberry population described above is described. The map is comprised of 265 molecular markers and includes simple sequence repeat (SSR), expressed sequence tag-polymerase chain reaction (EST-PCR), single nucleotide polymorphism (SNP), and randomly amplified polymorphic DNA (RAPD) markers. We also report the identification of quantitative trait loci (QTL) for chilling requirement and cold hardiness by evaluating the population for these traits over 5 years under controlled conditions.

Materials and methods

Plant material

A blueberry interspecific diploid testcross population, segregating for mid-winter cold tolerance, chilling requirement, and fruit quality traits, was generated for genetic linkage map construction and QTL identification. The population, developed by crossing an interspecific F₁ plant [*V. darrowii* selection Fla4B

(low chilling, cold sensitive, evergreen) × diploid *V. corymbosum* selection W85-20 (high chilling, cold hardy, deciduous)] to another diploid *V. corymbosum* W85-23 (high chilling, cold hardy, deciduous, like W85-20), was propagated to give approximately three to four clones of each individual. The plants from this cross (F₁#10 × W85-23) are maintained in a greenhouse in 4- to 12-l pots at the Beltsville Agricultural Research Center-West, Beltsville, MD. The selection Fla4B was originally collected from Florida, and W85-20 and W85-23 were collected from New Jersey (Arora et al. 2000). Fla4B has been used extensively in blueberry breeding programs and is still the primary source of low chilling requirement alleles in southern highbush cultivars (Rowland et al. 2011).

DNA extraction and general overview of marker scoring

DNA was extracted from young leaf tissue of 95 mature individuals of the mapping population (F₁#10 × W85-23), parents, and grandparents as described previously (Rowland et al. 2003b) using a modified CTAB procedure. A variety of markers, including those based on EST-PCRs (Rowland et al. 2003b), SSRs, SNPs, and RAPDs, were generated and screened first on the parents (F₁#10 and W85-23) and the grandparents (Fla4B and W85-20). Our intent was to develop a genetic map of the F₁#10, and not W85-23, because of concerns that W85-23 may be fixed for genes responsible for phenological traits like cold hardiness and chilling requirement, and thus, these genes may not segregate in the mapping population. The F₁#10, on the other hand, was specifically developed to be heterozygous for these genes. Therefore, only markers that met the following criteria were used for map construction: the marker must have been (1) polymorphic between Fla4B and W85-20, (2) present in the F₁#10, and (3) absent in W85-23. Markers were scored on an EXCEL spreadsheet as present or absent in each individual.

Marker design and application

For the EST-PCR markers, emphasis was initially placed on designing primers from blueberry ESTs representing cold-responsive genes, identified previously from microarrays (Dhanaraj et al. 2007) and subtracted/reverse subtracted libraries (Naik et al. 2007). These ESTs were derived from Sanger

sequencing and are present in the EST database of GenBank/NCBI (Dhanaraj et al. 2004, 2007). Later, PCR primers were designed from contigs assembled from transcriptome data obtained using 454 sequencing (Rowland et al. 2012). These 454 sequences are available on our Web site (<http://bioinformatics.towson.edu/BBGD454/>) and in the sequence read archive (SRA) of GenBank (under accessions SRX100856, SRX100859, and SRX100861-SRX100867). The EST-PCR marker names, forward and reverse primer sequences, origin of sequences, GenBank accession numbers, and the genes encoded by the sequences are given in Sheet 1 of the Online Resource 1 spreadsheet. Prefixes in marker names indicate the source of the ESTs from which the primers were designed. Methods for designing EST-PCR primers, amplifying products, and separating the products on agarose gels were those described in Rowland et al. (2003b).

SSR markers were developed from blueberry sequences originating from several different sources including: 454-sequenced ESTs provided at <http://bioinformatics.towson.edu/BBGD454/> and in the SRA of GenBank (VCB prefix in marker names stands for *V. corymbosum* from 'Bluecrop,' Rowland et al. 2012); Sanger-sequenced ESTs in the EST database of GenBank (GVC prefix for GenBank-mined *V. corymbosum*); private Sanger-sequenced ESTs developed by the New Zealand Institute for Plant and Food Research Ltd. (GenBank accession numbers used for prefix); genomic sequences from the largest scaffolds of the current whole genome assembly of diploid blueberry selection W85-20 (KAN prefix for Kannapolis, NC, where the genome sequence is being assembled); and from sequences near specific gene sequences of interest identified from the whole genome assembly (various prefixes for gene names). The SSR marker names, forward and reverse primer sequences, and origin of sequences are given in Sheet 2 of the Online Resource 1 spreadsheet. Sequences were mined for SSRs and primers designed using the online SSR tool (SSR Server) available at the Genome Database for *Vaccinium* (GDV, <http://www.vaccinium.org>) as described in Rowland et al. (2012). Sanger ESTs from GenBank were mined after eliminating sequences that contained previously developed SSRs (Boches et al. 2006).

To identify SSRs near genes of interest, such as anthocyanin biosynthesis genes (Jaakola et al. 2002) and CBF (C-repeat or CRT binding factor), a

transcriptional activator involved in cold acclimation (Jaglo-Ottosen et al. 1998; Liu et al. 1998), amino acid sequences of these genes were first identified from blueberry, grape, strawberry, or *Arabidopsis*, from the UniProt database. Sequences were then used in tBLASTn searches of the diploid *V. corymbosum* W85-20 genome assembly. Blueberry scaffolds with the best *E* values (threshold of e^{-10}) were mined using the online *Vaccinium* SSR tool. Primer pairs for the best three SSRs were synthesized for screening. Criteria for best SSRs included: presence of di- or trinucleotide motifs close to the alignment's starting location, large number of repeats, and expected PCR products of 100–700 bp.

PCR and capillary electrophoresis conditions for SSR genotyping varied somewhat among the laboratories that performed the work, depending on the equipment that was available and optimization protocols established for that equipment at each location. At the USDA-ARS, National Clonal Germplasm Repository laboratory (NCGR), PCR reactions contained 1 × reaction buffer, 2 mM MgCl₂, 0.2 mM dNTPs, 0.5 μM of fluorescent M13 primer (Schuelke 2000), 0.12 μM forward primer, 0.50 μM reverse primer, 0.075 units of GoTaq[®] DNA Polymerase (Promega Corporation, Madison, WI, USA), and 4.5 ng genomic DNA in a total volume of 15 μL. Amplification conditions consisted of 94 °C for 3 min; followed by 10 cycles of 94 °C for 40 s, 65 °C for 45 s (−1.0 °C per cycle), and 72 °C for 45 s; 20 cycles of 94 °C for 40 s, 52 °C for 45 s, and 72 °C for 45 s; eight cycles of 94 °C for 40 s, 53 °C for 45 s, and 72 °C for 45 s; and finally 72 °C for 30 min. Successful amplification was first assessed by 2 % agarose gel electrophoresis. PCR products were separated by capillary electrophoresis using a Beckman Coulter (Indianapolis, IN, USA) CEQ 8000 genetic analyzer and analyzed with software provided by the manufacturer.

At the New Zealand Institute for Plant and Food Research Ltd., PCR amplification contents were the same as those outlined for NCGR using the M13 fluorescently labeled approach of Schuelke (2000). Amplification conditions consisted of 94 °C for 2 min 45 s; then 10 cycles at 94 °C for 55 s, 60 °C for 55 s (−0.5 °C per cycle), and 72 °C for 1 min 30 s; 30 cycles at 94 °C for 55 s, 55 °C for 55 s, and 72 °C for 1 min 30 s; and finally 72 °C for 10 min. PCR products were analyzed on an ABI PRISM[®] 377 DNA sequence analyzer (Applied Biosystems, Foster

City, CA, USA) using dye-labeled GeneScan™ -350 ROX™ size standard (Applied Biosystems) on Long Ranger® Singel® packs (Lonza Rockland, ME, USA), and a 64-well casting comb (0.18 mm; single use; no. 4309455 Shark's Tooth Comb; Applied Biosystems) according to the manufacturer's protocol. Allele sizes were visualized using Peak Scanner™ Software v.1.0 (Applied Biosystems).

At the USDA-ARS, Genetic Improvement of Fruits and Vegetables laboratory, the PCR conditions were the same as those at NCGR except that no fluorescent M13 primer was used. Separation of PCR products was achieved using the Advanced Analytical FS96 system and software provided by Advanced Analytical (Ames, IA, USA).

At the James Hutton Institute, PCR amplifications consisted of 25 µl reactions containing 1× PCR buffer (10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl, pH 8.3), 0.2 mM dNTPs, 1.0 µM fluorescently labeled forward primer, 1.0 µM reverse primer, 0.1 units *Taq* polymerase (Roche Applied Science, Indianapolis, IN, USA), and 25 ng template DNA. Amplification conditions were as follows: 95 °C for 5 min; followed by 40 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 45 s; followed by a final extension of 72 °C for 10 min. PCR products were first resolved on 1.5 % agarose gels followed by analysis on an ABI 3730 DNA sequence analyzer (Applied Biosystems) using Rox 500 (Applied Biosystems) as an internal size standard. Allele sizes were visualized using GeneMapper software version 3.7 (Applied Biosystems).

Some of the monomorphic SSR markers were screened using the high-resolution melting (HRM) point analysis technique, in an attempt to detect SNPs and other small sequence and length variations between different PCR products (Reed and Wittwer 2004; Wittwer et al. 2003; Montgomery et al. 2007). HRM-PCRs and analyses were performed as described in Bushakra et al. (2012), with conditions as outlined in Chagne et al. (2008). Names of the mapped SNP markers (based on HRM), forward and reverse primer sequences, and origin of sequences are shown in Sheet 3 of the Online Resource 1 spreadsheet.

RAPDs were generated using 10-base RAPD primers from Operon Technologies, Inc. (Alameda, CA, USA) and from the Biotechnology Laboratory, University of British Columbia (Vancouver, British Columbia, Canada). DNA amplification and electrophoresis conditions were those described in Rowland

et al. (2003b) except that 0.2 µM of primer was used. Primers that generated mapped markers are listed in Sheet 4 of the Online Resource 1 spreadsheet.

Map construction

Markers that met mapping criteria described above were used for map construction. A linkage map for the parent F₁#10 was generated using the regression approach in JoinMap 4.0 (Van Ooijen 2006). Linkage groups (LG) were established with independence LOD of 4.0–5.0; and Haldane's mapping function was used to calculate map distances. Other default parameters of JoinMap were accepted. Several methods were used to minimize potential sources of error in the map including removing markers that had the most skewed segregation ratios ($P < 0.0001$), appeared repeatedly in genotypes with very low probabilities in the double recombinant table, were the least informative, and had suspicious linkages. The graphical presentation of the map was prepared with MapChart 2.2 (Voorrips 2002). Map coverage was calculated by method four of Chakravarti et al. (1991).

Cold hardiness determinations

To achieve cold acclimation, the mapping population, along with parents and grandparents, were moved from a heated to an unheated hoop house at the USDA-ARS in Beltsville, MD, in late December and early January of 2008–2009 and 2009–2010. Plants were evaluated for cold hardiness using a detached shoot assay as previously described (Arora et al. 2000, 2004; Rowland et al. 2005) when they had received approximately 500–550 chill units (hours of exposure to temperatures in the range of 0–7 °C, measured as described below under 'chilling requirement evaluations'). Hoop house temperatures were monitored with a biophenometer, Model TA51 (Omnidata International, Inc., Logan, UT, USA). Beginning in early February of 2009 and 2010, ~35 terminal shoots were collected from several plants (1–4) of each genotype. Sets of shoots, 5 to 6 cm long with at least three flower buds attached, were subjected to a freeze-thaw protocol, which included exposure to a range of temperature treatments. The three most distal buds on each of three shoots were evaluated for damage per treatment temperature.

The protocol consisted of placing three randomly sampled shoots/treatment temperature from each genotype in test tubes with 0.5 ml of water and freezing them in a glycol bath (model 2325; Forma Scientific, Marietta, OH, USA). Bud temperature was monitored by copper-constantan thermocouples (TT-T-30) attached to a thermometer (DP465, Omega Engineering, Stamford, CT, USA). Ice nucleation was initiated at $-1\text{ }^{\circ}\text{C}$. Samples were allowed to equilibrate for 45 min and then further cooled at $0.5\text{ }^{\circ}\text{C}/30\text{ min}$ down to $-4\text{ }^{\circ}\text{C}$, at $1\text{ }^{\circ}\text{C}/30\text{ min}$ down to $-8\text{ }^{\circ}\text{C}$, and at $2\text{ }^{\circ}\text{C}/30\text{ min}$ thereafter to respective final treatment temperatures. Treatment temperatures chosen for cold-acclimated buds spanned a range from -10 to $-28\text{ }^{\circ}\text{C}$ at $2\text{ }^{\circ}\text{C}$ increments, to represent 0–100 % injury for most, if not all, genotypes. Controls consisted of shoots held at $4\text{ }^{\circ}\text{C}$ without exposure to freezing. To assess injury, shoots were removed from the freezing bath at respective treatment temperatures and samples were allowed to thaw overnight at $4\text{ }^{\circ}\text{C}$ followed by a 24-h incubation at $20\text{ }^{\circ}\text{C}$. Subsequently, the three most distal buds were dissected and observed for injury (visual browning) of the ovaries in individual flowers (Arora et al. 2000; Flinn and Ashworth 1994), and a percent damage value was calculated for each bud.

Bud cold hardiness was measured as LT50 values, i.e., the temperature causing 50 % injury overall. For each genotype \times year combination, bootstrap estimates (Manly 1997) of LT50 values and their 95 % fiducial confidence intervals were calculated using the PROBIT procedure of the Statistical Analysis System (SAS) 9.2 package (SAS Institute Inc., Cary, NC, USA). For this, the nine observed data points (three distal buds on each of the three shoots) for each temperature were resampled ($n = 9$ with replacement) 30 times. For each genotype, year and replicate, the 30 LT50 estimates were used to fit a logistic regression model, from which an LT50 and a 95 % confidence interval were estimated.

Chilling requirement determinations

The mapping population, as well as parents and grandparents, were evaluated for chilling requirement at the USDA-ARS in Beltsville, MD, USA, in the winters of 2010–2011, 2011–2012, and 2012–2013. One to six plants of each genotype were held in a heated greenhouse until November of each year, specifically until 11-26-10, 11-25-11, and 11-9-12, at which point

plants had set a sufficient number of flower buds and were dormant. On those dates, plants were moved into an unheated hoop house covered with opaque winter-white plastic to minimize daytime temperature increases and were exposed to ambient light and temperature conditions. Chill units (hours from 0 to $7\text{ }^{\circ}\text{C}$) were measured with a Lascar EL-USB-2-LCD+ datalogger (Lascar Electronics, Erie, PA, USA). Sets of shoots (2–6), 5 to 6 cm long with three to eight flower buds attached, from multiple plants of each genotype, were collected every 200 chill units (200, 400, 600, 800, and 1,000). Shoots were then rated for floral bud development after 3 weeks at $24\text{ }^{\circ}\text{C}$ (14-h photoperiod) as described by Spiers (1978). Accordingly, each bud was examined for signs of budbreak and given a score from stage 1 through 7, with 1 representing the stage with no sign of visible swelling, 2 representing the stage of first visible swelling and separation of bud scales, etc., and finally 7 representing the stage after which corollas had completely expanded and dropped. Mean chilling requirements were determined from the percentage of breaking buds (growth stage >2) after plants had received the various chill units (200, 400, 600, 800, and 1,000) as described in Rowland et al. (1998). Mean chilling requirements were calculated by fitting a straight-line regression equation of the frequency of buds at growth stage >2 against the number of chill units. Slopes and y-intercepts of equations with r^2 values >0.75 were used to calculate the chill units required for 50 % of the buds to break (CR50).

Distribution, correlation, and heritability analyses

The normality of cold hardiness and chilling requirement distributions was assessed using the UNIVARIATE procedure of SAS v. 9.3. The CORR procedure was used to test for correlations between phenotypic data from different years for the same trait and between different traits. The Pearson correlation coefficient (r) was used, because both traits had a normal distribution pattern most years. Correlations were considered significant at $P < 0.001$. Correlations were classified as high if $r > 0.7$, moderate if $0.4 < r < 0.7$, and weak if $r < 0.4$.

Broad-sense heritability (H) was estimated for cold hardiness and chilling requirement as the ratio of genotypic variance to total phenotypic variance. SAS PROC MIXED (SAS v. 9.2) was used to determine variance components for

$$H = \sigma_G^2 / ((\sigma_{\text{error}}^2 / re) + (\sigma_{G \times E}^2 / e) + \sigma_G^2)$$

where r = number of replications and e = number of environments (years) (Holland et al. 2003). Mean squares (genotype and genotype \times environment) from PROC GLM (SAS v. 9.2) were used to construct the 95 % confidence intervals (Knapp et al. 1985).

QTL analyses

Phenotypic data for chilling requirement (CR50) and cold hardiness (LT50) were used in QTL analyses with MapQTL 5.0 (Van Ooijen 2004). Because a map was not developed for the parent W85-23, however, these analyses are limited to markers and QTL present in the F₁#10 that contribute to the observed phenotypic variation. The genome-wide (GW) thresholds for each trait and year were calculated with the permutation test (PM) set at 1,000 permutations. The QTL were considered significant in interval mapping (IM) and restricted multiple QTL mapping (rMQM) when the LOD score exceeded the GW 90th percentile (i.e., significant at 10 % error rate). Forward selection was performed by first identifying QTL by IM, selecting nearest markers to set as cofactors, re-analyzing with rMQM, and then repeating rounds of cofactor selection and rMQM until no additional QTL were identified.

Results

Plant population chosen for mapping

The generation of interspecific diploid testcross populations that segregate for mid-winter cold tolerance and chilling requirement has been described in detail previously (Rowland et al. 1999). Briefly, three *V. darrowii* selection Fla4B (low chilling, cold sensitive, and evergreen) \times *V. corymbosum* selection W85-20 (high chilling, cold hardy, and deciduous) hybrids were crossed to another *V. darrowii* NJ88-13-15 and another *V. corymbosum* W85-23. Fla4B has an average mid-winter cold tolerance LT50 of -14.5 °C and chilling requirement of 330 chill units. W85-20 has a cold tolerance LT50 of -25.5 °C and chilling requirement of 790 chill units. The various populations were evaluated for chilling requirement

and cold tolerance to identify the best one to use for the identification of QTL (data not shown). Based on the results, we decided to focus on the cross of F₁#10 \times W85-23, which had a wide distribution of both chilling requirement and cold hardiness levels (Fig. 1).

Phenotypic data

The distribution of cold hardiness in the mapping population for the 2 years tested, 2009 and 2010, was normal according to the Shapiro–Wilk and Kolmogorov–Smirnov tests for normality (Fig. 1a). The cold hardiness levels for the 2 years were moderately correlated ($r = 0.663$; $P < 0.0001$) (Table 1). The distribution of chilling requirement for the 3 years tested, 2011, 2012, and 2013, was also normal except for the 2013 data (according to the Shapiro–Wilk test), where the distribution was somewhat skewed toward a preponderance of genotypes with relatively low chilling requirements (Fig. 1b). The chilling requirements for the 3 years were moderately to highly correlated (for CR2011:CR2012, $r = 0.587$, $P < 0.0001$; for CR2012:CR2013, $r = 0.694$, $P < 0.0001$; for CR2011:CR2013, $r = 0.763$, $P < 0.0001$) (Table 1). Cold hardiness appeared weakly negatively correlated with chilling requirement but only for the CH2009:CR2012 comparison (the lower the cold hardiness LT50, the higher the chilling requirement). This was only significant at the $P < 0.01$ level (Table 1).

Broad-sense heritability (H) for cold hardiness was estimated to be 0.88, with a 95 % confidence interval of 0.70–0.88. For chilling requirement, H was calculated to be 0.86, with a 95 % confidence interval of 0.78–0.90. Because of the way chilling requirement was determined, however, years were treated as reps, and there were no within-year reps. Therefore, in the H calculation, there was no genotype \times environment component, and hence, H may be overestimated for chilling requirement if the genotype \times environment interaction is large.

Map construction

Blueberry progeny from the cross of F₁#10 \times W85-23 was genotyped for 340 markers. Of these, 265 markers were mapped (Fig. 2) and are described in detail in the Online Resource 1 spreadsheet. In total, 219 primer pairs generated the 265 mapped markers. A summary

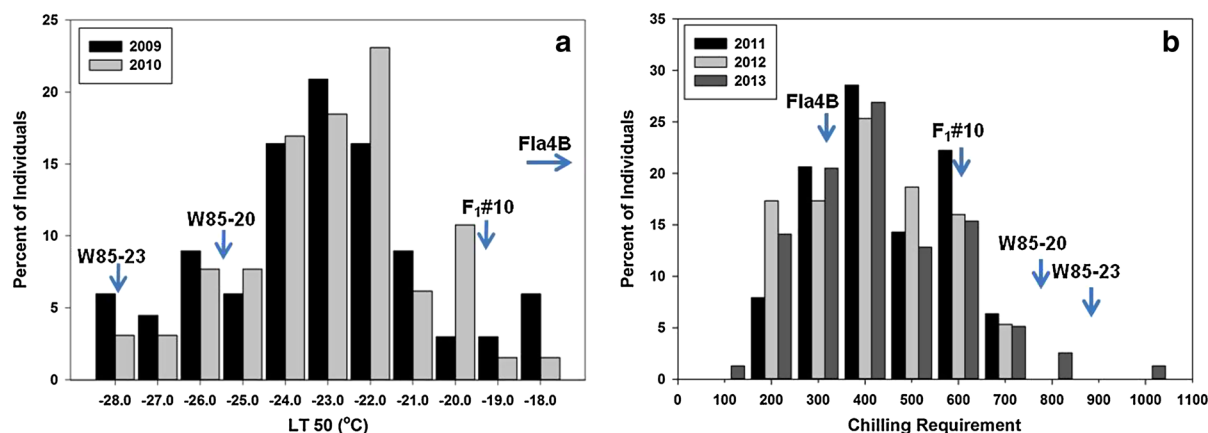


Fig. 1 Distribution of cold hardiness and chilling requirement in the diploid blueberry mapping population. **a** Distribution of cold hardiness (LT 50 in °C) in the diploid blueberry mapping population [(Fla4B × W85-20) F₁#10 × W85-23] in 2009 and 2010. Cold hardiness levels of parent plants are indicated by

arrows. **b** Distribution of chilling requirement (chill units required for 50 % bud break) in the diploid blueberry mapping population in 2011, 2012, and 2013. Chilling requirements of parent plants are indicated by arrows

Table 1 Pearson correlation coefficients of cold hardiness (CH) and chilling requirement (CR) and their probabilities in the blueberry mapping population in different years

Pearson correlation coefficients					
Prob > r under H ₀ : $\rho = 0$					
	CH2009	CH2010	CR2011	CR2012	CR2013
CH2009	1.000	0.663	-0.236	-0.358	-0.298
		<0.0001	0.1033	0.0062	0.0207
CH2010		1.000	-0.147	-0.315	-0.257
			0.2990	0.0143	0.0477
CR2011			1.000	0.587	0.763
				<0.0001	<0.0001
CR2012				1.000	0.694
					<0.0001
CR2013					1.000

of the types of markers mapped, the percentage showing distorted segregation ratios, the percentage that yielded multiple mapped fragments, and, of those, the percentage that were allelic, is shown in Table 2. Most of the 265 mapped markers segregated 1:1, as expected for dominant markers in a testcross population (Aa × aa). However, 74 of the markers still had significantly distorted ratios from the expected 1:1 ratio ($P < 0.05$) even after removal of those with the most skewed segregation ratios ($P < 0.0001$). The markers with significantly distorted ratios are

indicated by asterisks on Sheet 5 of the Online Resource 1 spreadsheet along with their positions on the map.

The 265 mapped markers were organized into 12 LG (Fig. 2), which is equal to the haploid chromosome number for diploid blueberry. The total map length was 1,740 cM, with an average distance between markers of 7.2 cM. The largest gap was 33 cM. LG ranged in size from 56 to 220 cM, and the number of markers per LG ranged from 5 to 50. The average number of markers per LG was 22.1. The estimated map coverage, calculated according to method four of Chakravarti et al. (1991), was 89.9 %.

QTL analyses

We identified one QTL for cold hardiness and two for chilling requirement from the F₁#10 genetic map (Fig. 2) and 5 years of data (2 years for cold hardiness and 3 years for chilling requirement evaluations). Table 3 provides detailed information (LOD scores, % trait variance explained, etc.) for the QTL and their associated markers each year.

In both 2009 and 2010, one QTL for cold hardiness was identified on LG 4 by IM followed by restricted multiple QTL mapping (rMQM). The nearest marker to the QTL peak was EST-PCR marker leaf-00248. This QTL was significant at the 0.05 level in 2009 (LOD 5.12, GW95 % 3.1) and in 2010 (LOD 3.14,

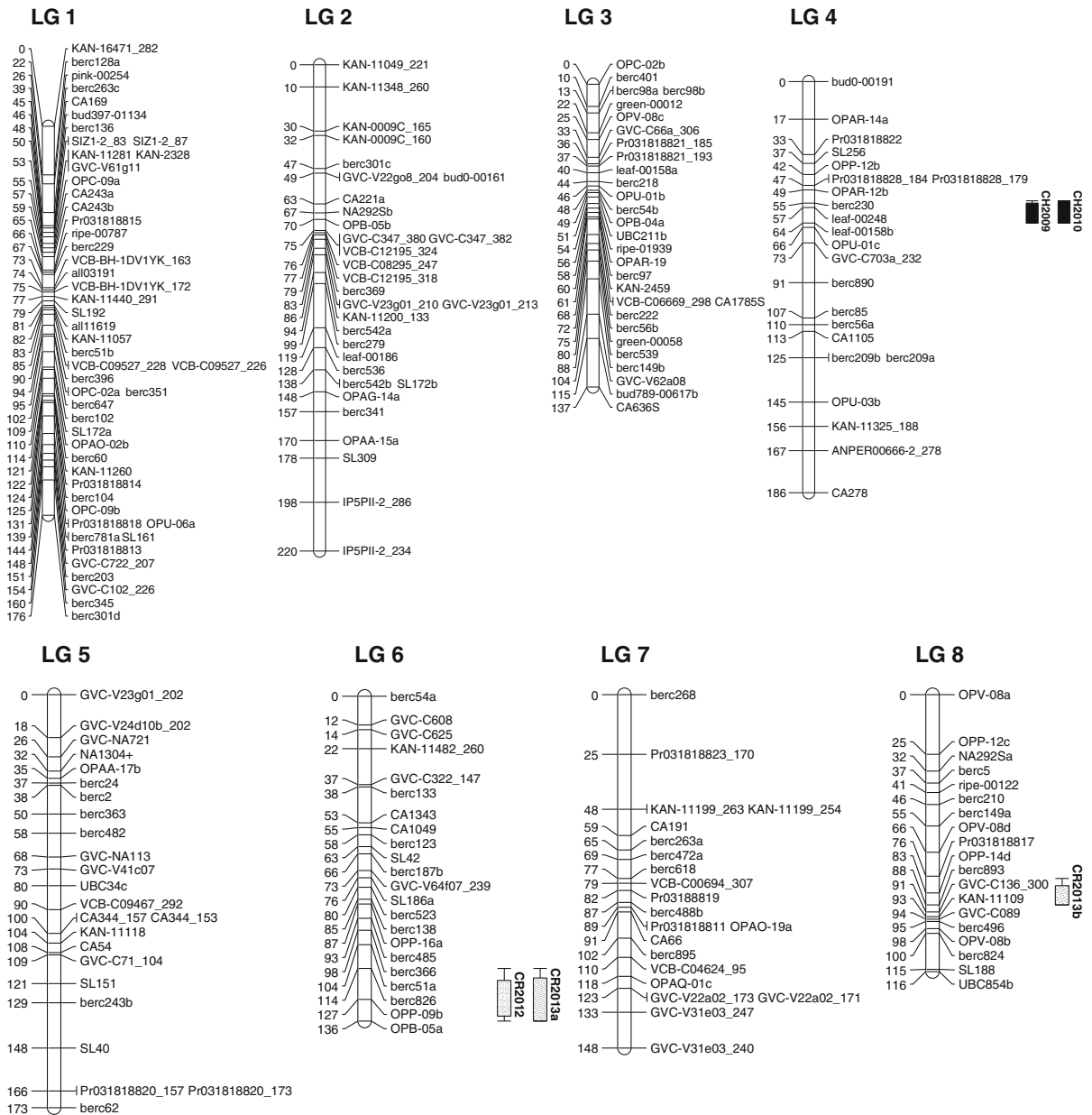


Fig. 2 Genetic linkage map of diploid blueberry population [(Fla4B × W85-20) F₁#10 × W85-23]. Markers are described in detail in Online Resource 1. Mapped markers include EST-PCR, SSR, SNP, and RAPD markers (Table 2). Location of QTL for cold hardiness (CH) and chilling requirement (CR) is

indicated to the *right* of the linkage groups by *different patterned bars*. The *bars* represent 2-LOD (logarithm of the odds) support intervals based on the results from rMQM (restricted multiple QTL mapping)

GW95 % 3.1). Percentage of phenotypic variance explained by the cold hardiness QTL was 32.9 and 20.1, in 2009 and 2010, respectively.

Chilling requirement QTL were identified in 2 of the 3 years. IM followed by rMQM analysis identified a QTL on LG 6 that coincided with the position of the

RAPD marker OPP-09b in 2012 (LOD 3.31, GW95 % 3.3) and in 2013 (LOD 3.5, GW95 % 3.0). With OPP-09b used as a cofactor in rMQM, a second QTL was identified near RAPD marker OPP-14d on LG 8 only in 2013 (LOD 2.84, GW90 % 2.7). The LG 6 QTL explained 18.6 and 16.7 % of the phenotypic variance

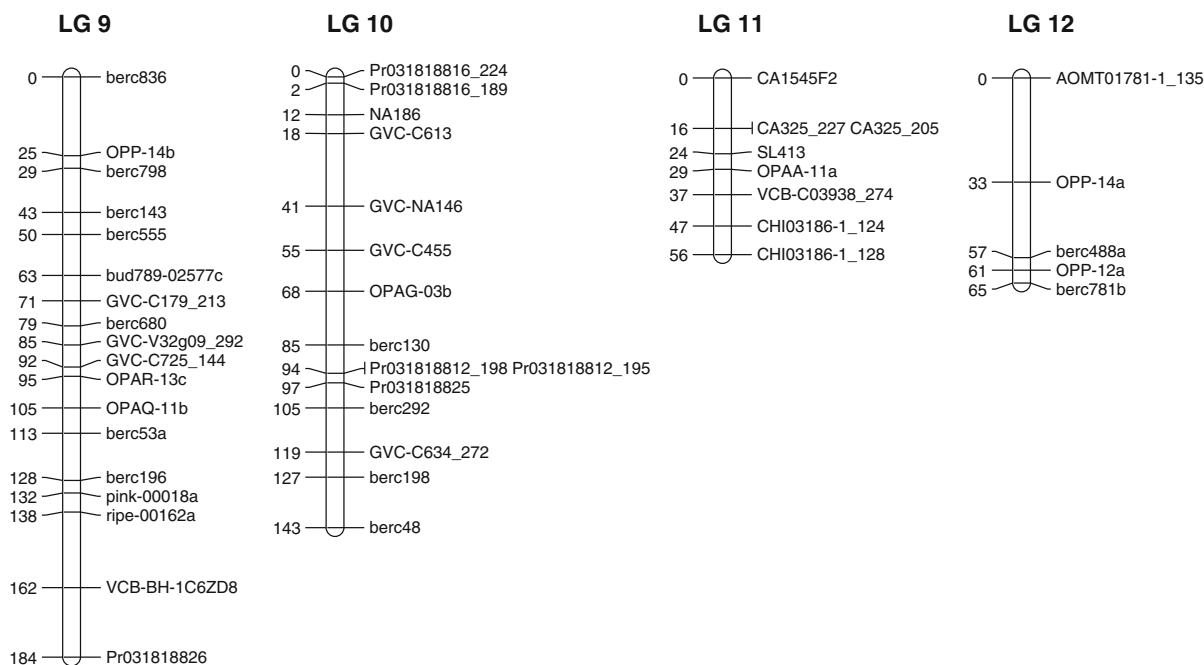


Fig. 2 continued

Table 2 Summary of mapped markers

Types of molecular markers scored	No. of markers successfully mapped out of total of each type scored	No. of primer pairs yielding mapped markers	No. of primer pairs yielding multiple mapped bands	No. of mapped multiples that were allelic	% of mapped markers with distorted segregation ratios
EST-PCR	128/169	113	15	2 pairs	42/128 = 33
SSR	90/114	70	18	15 pairs	23/90 = 26
SNP (based on HRM)	8/9	8	0	0	0/8 = 0
RAPD	39/48	28	7	0	9/39 = 23

in 2012 and 2013, respectively, while the LG 8 QTL from 2013 explained 14.6 %. In 2011, marker OPP-09b on LG 6 still contributed to the phenotypic variance (10.8 % explained) although the LOD score was below the threshold for 90 % GW significance.

Discussion

Here, we have described the first genetic linkage map of diploid blueberry based primarily on SSR and EST-PCR markers, and of sufficient density for QTL mapping. Relatively, low-density maps of diploid (Rowland and Levi 1994; Rowland et al. 1999, 2003a)

and tetraploid blueberry (Qu and Hancock 1997) have been published previously but were comprised mainly of RAPD markers. An earlier map of a related *V. darrowii* × *V. corymbosum* hybrid population (based on several subpopulations), composed of only about 100 RAPD markers (Rowland et al. 1999, 2003a), was used to identify one putative QTL for cold hardiness (Rowland et al. 2003a).

Here, blueberry progeny from a single population ($F_1\#10 \times W85-23$) was genotyped for over 300 total markers. Of these, 265 markers were mapped to 12 LG, equivalent to the haploid chromosome number for diploid blueberry. A couple of the LG were quite short, however, with only 5–8 markers. Perhaps coverage of

Table 3 Quantitative trait loci detected for cold hardiness (CH) and chilling requirement (CR)

Trait	Linkage group	LOD	% phenotypic variance explained	% genotypic variance explained	Associated markers ^a
CH2009	4	5.12 ^b	32.9	37.4	leaf00248—1.5 kb—from Fla4B-cold sensitivity
CH2010	4	3.14 ^b	20.1	22.9	leaf00248—1.5 kb
CR2012	6	3.31 ^b	18.6	21.6	OPP-09b—750 bp—from Fla4B-low CR
CR2013 a	6	3.5 ^b	16.7	19.4	OPP-09b—750 bp
CR2013 b	8	2.84 ^c	14.6	16.9	OPP-14d-550 bp—from W85-20—low CR

^a Nearest markers used as cofactor in rMQM

^b Significant at the 0.05 level based on 1,000 permutations

^c Significant at the 0.10 level based on 1,000 permutations

these LG was lacking because, so far, most of the mapped markers were derived from expressed genes (EST-PCRs and EST-SSRs). An effort to map more genomic SSRs may remedy this problem in the future. About 28 % of the mapped markers (74/265) had significantly distorted segregation ratios, even though 17 with the most highly distorted ratios (significant at $P < 0.0001$) had been removed before the analysis. Considering that F₁#10 is an interspecific hybrid, the percentage of markers with significantly distorted ratios was still not particularly high. Of the mapped markers with distorted ratios, 24 were clustered on LG 1 in a region between 67 and 144 cM, 13 clustered on LG 3 between 44 and 88 cM, and 5 clustered on LG 5 between 109 and 173 cM, suggesting these may be of biological significance.

A map of cranberry (*V. macrocarpon*), which is closely related to blueberry, consisting of 136 distinct loci was recently published (Georgi et al. 2013). The map is based on SSR markers, derived from blueberry ESTs and from cranberry genomic sequences, and cranberry-derived sequence-characterized amplified region (SCAR) markers. Some of the markers were designed from SSRs near sequences of interest based on the draft cranberry genome assembly, such as flavonoid biosynthetic pathway genes and defense-related genes. Markers in common between the blueberry and cranberry maps are shown in the Online Resource 2 table. Even though the cranberry map utilized 33 SSR primer pairs from blueberry, only four of these primer pairs gave markers that were also mapped in blueberry. In addition, markers generated from SSR primer sequences located near a chalcone isomerase gene were mapped in both blueberry and cranberry; the SSR primer sequences were different, however. With so few markers in common and the whole genome assemblies for

blueberry and cranberry still unpublished, little can be compared between the two maps at this point. None of the shared markers were linked on the blueberry map except for the pair of fragments, CA325_205 and CA325_227, and the pair, CHI03186-1_124 and CHI03186-1_128, which mapped to LG 11. In cranberry, the comparable markers, CA325 and Scf12916, mapped to LG 8, suggesting that the blueberry LG 11 corresponds to the cranberry LG 8. Another pair of shared markers, GVC-V24d10b_202 and 2ms4d10b, were located on LG 5 of the blueberry map and LG 2 of the cranberry map, respectively, suggesting that LG 5 of blueberry aligns with LG 2 of cranberry. Another marker ctg600 mapped to LG 2 of the cranberry map, while the comparable blueberry marker GVC-C600_132 grouped with LG 5 markers. This marker was not placed until the third round and therefore is not shown on the map. This also suggests, however, that LG 2 of the cranberry map is comparable to LG 5 of the blueberry map. Another pair of shared markers, GVC-V22a02_171 and GVC-V22a02_173, and 2ms2a02, was located on LG 7 of the blueberry map and LG 5 of the cranberry map, respectively, suggesting these LG correspond. Finally, the pair of shared markers, GVC-V32g09_292 and 3ms2g09, mapped to LG 9 of blueberry and LG 3 of cranberry, respectively, suggesting these also correspond with each other.

Our research to date has focused on identifying QTL for traits associated with climatic adaptation, cold hardiness, and chilling requirement, in the blueberry mapping population. In this study, we identified one QTL for cold hardiness on LG 4 that was significant at the 5 % error rate in 2009 and 2010. The closest markers to the QTL were EST-PCR marker leaf-00248, followed by berc230. The markers appeared to be located 3 and 5 cM from the QTL in 2009, and 0 and

2 cM from the QTL in 2010. This is the same region of the map that was identified previously as having a putative QTL for cold hardiness (LOD >2) using MAPMAKER/QTL (Rowland et al. 2003a). At that time, the RAPD marker OPAR12b was the closest marker to the QTL; EST-PCR markers leaf-00248 and berc230 had not yet been mapped. The identified QTL explained 17.9 % of the phenotypic variance of the population. In the present study, in 2009 and 2010, the QTL explained 32.9 and 20.1 % of the phenotypic variance, respectively. From our analyses, broad-sense heritability (H) of cold hardiness was high (0.88) in the blueberry mapping population. By dividing the percent of the phenotypic variance explained by H (De Vicente and Tanksley 1993; Lewers et al. 1999), it was determined that the cold hardiness QTL explained 37.4 and 22.9 % of the genotypic variance in 2009 and 2010, respectively (Table 3).

Contigs leaf-00248 and berc230 (from which the EST-PCR markers were derived) have been blasted against the all assembly of the blueberry 454 transcriptome database. Contig leaf-00248 had high similarity to contig all-12494 encoding a predicted peptidyl-prolyl *cis-trans* isomerase-like protein or cyclophilin with a CP12 (chloroplast protein 12) domain. Berc230 had high similarity to contig all-01955 encoding a hypothetical protein in the alpha/beta hydrolase superfamily. The leaf-00248 and berc230 markers were amplified in Fla4B and absent in W85-20, and presence of the markers was associated with cold sensitivity as would be expected considering the cold hardiness of the parent plants.

Mapping QTL for cold hardiness has been accomplished in several plant species including various cereals (Galiba et al. 1995; Francia et al. 2004; Fisk et al. 2013), legumes (Avia et al. 2013; Tayeh et al. 2013), forestry species (Howe et al. 2000, 2003; Jermstad et al. 2001, 2003; Tsarouhas et al. 2004; Wheeler et al. 2005), and citrus (Weber et al. 2003). Association mapping of cold hardiness has been carried out as well in several species including Douglas fir (Eckert et al. 2009a, b) and Sitka spruce (Holliday et al. 2010). In a few cases, the genes underlying cold hardiness QTL have been identified. For example, variation in the copy number of the *CBF* gene (Stockinger et al. 1997; Vogel et al. 2005) at *Frost Resistance-2* has been found to be associated with levels of freezing tolerance in temperate-climate cereals (Francia et al. 2007; Knox et al. 2010). C-repeat

binding factors or CBFs are transcription factors that regulate one, but not the only, cold acclimation pathway in plants (Stockinger et al. 1997; Fowler and Thomashow 2002; Vogel et al. 2005). The association mapping studies in Douglas fir and Sitka spruce found most notable associations of cold hardiness with genes involved in light signal transduction, in the case of autumn cold hardiness in Sitka spruce (Holliday et al. 2010), as well as genes involved in lignin biosynthesis, cell membrane and cell wall architecture, and chaperone function in Douglas fir (Eckert et al. 2009a, b).

To test whether the *CBF* gene previously isolated from blueberry flower buds during cold acclimation (Naik et al. 2007; Polashock et al. 2010) could be responsible for the cold hardiness QTL identified in our mapping population on LG 4, the gene sequence was blasted against the current blueberry whole genome assembly (Allan Brown, unpublished). The gene was located to one of the scaffolds, and SSR primers were designed to flank SSRs near the gene. The amplified SSR markers, KAN-0009C_160 and KAN-0009C_165, mapped to LG 2 (Fig. 2), however, suggesting that the *CBF* gene is not responsible for the identified QTL.

In our study, we also successfully identified QTL for chilling requirement. We found one QTL on LG 6 that coincided with the RAPD marker OPP-09b and was significant at the 5 % error rate in 2012 and 2013. A second QTL for chilling requirement was identified only in 2013 located 2 cM from RAPD marker OPP-14d on LG 8, which was significant at the 10 % error rate. Broad-sense heritability of chilling requirement was also high (0.86), like that of cold hardiness. In 2012 and 2013, the QTL at OPP-09b explained 18.6 and 16.7 % of the phenotypic variance, and 21.6 and 19.4 % of the genotypic variance, respectively. In 2013, the second QTL near OPP-14d explained 14.6 % of the phenotypic variance and 16.9 % of the genotypic variance.

Also, within the chilling requirement QTL, confidence region on LG 6 was the RAPD marker OPB-05a (9 cM from the QTL) and the EST-PCR marker berc826 (13 cM from the QTL). Contig berc826 was blasted against the all assembly of the blueberry 454 transcriptome database and found to have high similarity to contig all-02175, which encodes a putative proline-rich cell wall protein.

The marker OPP-09b, near the strongest QTL for chilling requirement on LG 6, was amplified in Fla4B,

absent in W85-20, and associated with lower chilling requirement. The markers further from the QTL, OPB-05a, and berc826, were amplified in W85-20 and associated with higher chilling requirement. The marker OPP-14d, closest to the weaker QTL on LG 8 (detected only in 2013), was amplified in W85-20, but surprisingly was associated with lower chilling requirement. This suggests that the parent W85-20 is heterozygous for at least one gene that contributes to chilling requirement.

Quantitative trait loci for chilling requirement have been identified in a few fruit crops, including peach (Fan et al. 2010), almond (Sanchez-Perez et al. 2012), and cherry (Quero-Garcia et al. 2010). Some of the identified chilling requirement QTL appear to be in common among these *Prunus* species, such as a significant QTL on LG 4. Probably the best studied, however, is a major QTL identified on LG 1 of peach (Fan et al. 2010), which coincides with the *EVG* (*evergrowing* mutation) locus (Wang et al. 2002; Bielenberg et al. 2004, 2008). The genes responsible for this QTL (and mutation) appear to be a group of *DAM* [*dormancy-associated Short Vegetative Phase* (*SVP*)-like *MADS*-box] genes (Li et al. 2009; Jimenez et al. 2010; Fan et al. 2010). In addition, the expression of *DAM4*, *DAM5*, and *DAM6* appears to be correlated with bud dormancy release in a group of peach cultivars with varying chilling requirements (Leida et al. 2012). On the other hand, in a recent association mapping study done on 104 peach landrace accessions from China, no association was found between chilling requirement and the region around the QTL identified in Fan et al. (2010) and the *EVG* locus (Cao et al. 2012).

To test whether *DAM*-like genes are responsible for the major chilling requirement QTL identified in our mapping population, *DAM4*, *DAM5*, and *DAM6* gene sequences from peach were blasted against the current blueberry whole genome assembly (Allan Brown, unpublished), and SSR primers were designed to flank SSRs near the genes. Of the SSR markers that amplified, some were not polymorphic and therefore could not be mapped, while others did not map near the chilling requirement QTL (data not shown). Although this work is ongoing, it so far suggests that these *DAM* genes are not responsible for the identified QTL.

Efforts are currently underway to anchor the genetic linkage map of our population to the assembly of the whole genome sequence of *V. corymbosum* W85-20 to allow us to directly identify genes in the

vicinity of QTL for cold hardiness and chilling requirement. In addition, we are continuing to map other candidate genes for these traits, like the E3 ubiquitin-protein ligase gene family found near a QTL for chilling requirement in apple (Celton et al. 2011). Efforts to map fruit quality traits that are segregating in the blueberry population are also in progress.

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Conflict of interest The authors declare that they have no conflict of interest.

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