Theor Appl Genet (1994) 87:863–868

THEORETICAL AND APPLIED GENETICS

RAPD-based genetic linkage map of blueberry derived from a cross between diploid species (*Vaccinium darrowi* and *V. elliottii*)

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Received: 10 May 1993 / Accepted: 23 July 1993

Abstract. An initial genetic linkage map for blueberry has been constructed from over 70 random amplified polymorphic DNA (RAPD) markers that segregated 1:1 in a testcross population of 38 plants. The mapping population was derived from a cross between two diploid blueberry plants: the F_1 interspecific hybrid (*Vaccinium darrowi* Camp × *V. elliottii* Chapm.) and another *V. darrowi* plant. The map currently comprises 12 linkage groups (in agreement with the basic blueberry chromosome number) and covers a total genetic distance of over 950 cM, with a range of 3–30 cM between adjacent markers. The use of such a map for identifying molecular markers linked to genes controlling chilling requirement and cold hardiness is discussed.

Key words: Molecular markers – Random amplified polymorphic DNA – Polymerase chain reaction

Introduction

Molecular markers, primarily restriction fragment length polymorphisms (RFLPs) and isozymes, have been used to create extensive linkage maps for many annual plants including maize (Helentjaris 1987; Burr et al. 1988), tomato (Bernatzky and Tanksley 1986), and rice (McCouch et al. 1988), among others. Genetic analysis and breeding of woody perennial fruit species, on the other hand, can be complicated by many factors including long periods of juvenility, high ploidy levels, lack of described Mendelian markers, self- and cross-incompatibility, and inbreeding depression (Janick and Moore 1975; Moore and Janick 1983). Genetic linkage maps recently reported for *Citrus* (Durham et al. 1992; Jarrell et al. 1992) based on RFLPs and isozymes were the first to be described for any tree fruit crop. The availability of more molecular marker-based maps of tree and shrub fruit species would facilitate the localization of genes controlling traits such as fruit quality, fruit size, plant vigor, disease resistance, and various environmental tolerances.

The polymerase chain reaction (PCR) method of DNA amplification (Saiki et al. 1988) has provided geneticists with new ways of generating molecular markers for mapping. One of these is the use of short 10-base primers to randomly amplify polymorphic DNA sequences (RAPDs) (Welsh and McClelland 1990; Williams et al. 1990). Molecular mapping based on RAPD markers is quickly becoming one of the most popular methods because it is technically simpler than using RFLPs. It does not require preparation of genomic or cDNA clones, restriction enzyme digestions of DNA, Southern blotting, or hybridization with radioactively-labeled probes. The technical simplicity of RAPD marker analyses as compared to RFLPs is an advantage not only to geneticists attempting to construct genetic maps but also to breeders attempting to use such markers to tag traits of horticultural value. Because RAPD analyses require much smaller amounts of DNA and less pure DNA than RFLP analyses, the RAPD method is particularly attractive to geneticists working with species recalcitrant to nucleic acid extraction, such as woody plant species.

Communicated by P. L. Pfahler
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A goal of our research is to identify molecular markers linked to genes which control winter dormancy and cold-hardiness in a woody perennial, blueberry (*Vaccinium*, section *Cyanococcus*). Blueberry plants, like other woody perennial plants of the temperate zone, are exposed to freezing temperatures each winter. Their ability to survive is dependent on the development of dormancy and cold-hardiness. Once plants are dormant, a period of exposure to low temperatures (approximately 0–7 °C), known as the chilling requirement, is necessary for vegetative and floral budbreak in the spring (Scalabrelli and Couvillon 1986).

There has been an emphasis in several blueberry breeding programs in the past decade to develop highbush blueberry varieties (*V. corymbosum* L., 4x) with low chilling requirements, suitable for growing in the southern United States (Austin and Draper 1987; Lyrene and Sherman 1988; Hancock and Draper 1989) because they are early ripening. Plants of the diploid species *V. darrowi* which occasionally produce unreduced gametes have been used extensively in breeding programs to introduce low-chilling requirement into the typically high-chilling highbush background (Austin and Draper 1987; Hancock and Draper 1989). Because genetic analyses of tetraploids require formidable population sizes, we have developed two populations of blueberry plants generated from interspecific hybrids of wild diploid species: (1) *V. darrowi* clone Fla4B (evergreen, low-chilling lowbush) × *V. elliottii* clone Knight (decidous, moderate-chilling highbush) and (2) *V. darrowi* clone Fla4B × *V. caesariense* Mackenz clone W85-20 (deciduous, high-chilling highbush) both of which are to be used initially for developing genetic linkage maps and subsequently for identifying molecular markers linked to genes which control chilling requirement and cold-hardiness in blueberry. The nomenclature of Camp (1945) and Galletta (1975), both of whom recognized nine diploid blueberry species, is used here. Diploid blueberry species are essentially self-sterile (Meader and Darrow 1944; Ballington and Galletta 1978). The probable cause, based on studies with the cultivated tetraploid species (*V. corymbosum*), is inbreeding depression (Krebs and Hancock 1988). True F_2 or backcross diploid populations cannot be easily generated for mapping. Very few seeds from F_2 or backcrosses will germinate, and of those that do the resulting plants are often severely stunted and deformed. Thus, we have crossed the F_1 interspecific hybrids to other plants of the same species as the original parents to generate testcross populations for mapping purposes. Here, we report an initial genetic linkage map for blueberry utilizing a small population of about 40 plants resulting from a testcross between the *V. darrowi* × *V. elliottii* hybrid and another *V. darrowi* plant.

Materials and methods

Plant material

A testcross population was evaluated for the segregation of 89 RAPD markers. The diploid population resulted from crosses between one interspecific hybrid US388 [*V. darrowi* clone Fla4B (evergreen, low-chilling lowbush) × *V. elliottii* clone Knight (deciduous, moderately high-chilling highbush)] and another *V. darrowi* clone, US799. Fla4B, Knight, US388, and US799 were kindly provided by A. Draper. Fla4B and US799 are both wild selections collected in Florida. Knight is a wild selection collected in North Carolina (A. Draper, personal communication). Crosses between US388 and US799 were made by ourselves during the winter of 1990 using US388 as the female parent.

DNA extractions

During the spring of 1992, 1–4 g of leaf tissue was collected from each of Fla4B, Knight, US388, US799, and 38 plants of the testcross population. These were the only seedlings that were sufficiently large for collecting leaf tissue. Leaf tissue was immediately frozen in liquid N_2 and stored at −70 °C until DNA was extracted. DNA was isolated using a CTAB (hexadecyltrimethylammonium bromide) procedure (Doyle and Doyle 1987) as modified by Rowland and Nguyen (1993) but with the polyethylene glycol precipitation omitted.

DNA amplification conditions and gel electrophoresis

Amplification reactions were performed in 25 µl volumes using a PCR buffer previously described by Barry et al. (1991) for amplification of the 16*s*/23*s* rDNA intergenic spacer region from eubacteria. Reaction conditions were as follows: 1x Barry buffer (20 mM NaCl, 50 mM Tris-Cl pH 9.0, 1% Triton-X-100, 0.1% gelatin), 1.5 mM $MgCl_2$, 200 µM each dATP, dCTP, dGTP, and dTTP (Sigma), 0.2 µM primer (Biotechnology Laboratory, University of British Columbia), 1 ng/µl genomic DNA, and 0.03 units/µl *Taq* DNA polymerase (Promega, supplied in storage buffer A). DNA was amplified using either the Hybaid Thermal Reactor HB-TR1 or the original Perkin Elmer thermal cycler and 45 cycles of the following program: 30 s at 94 °C, 60 s at 48 °C, and 120 s at 72 °C. The fastest attainable transitions between each temperature were employed. We have previously shown that the combined use of this buffer and an annealing temperature of 48 °C results in consistent phenotypes and much higher amplification of blueberry DNA than other standard PCR buffers and annealing temperatures often used for RAPD analysis (Levi et al. 1993). Amplification products were separated by electrophoresis through 1.4% agarose gels in 0.5 × Tris-borate electrophoresis buffer (Sambrook et al. 1989). DNA fragments were visualized by UV irradiation after staining with 0.5 µg/ml ethidium bromide.

Linkage analysis

The linkage map of the testcross population was generated using LINKAGE-1 computer program (Suiter et al. 1983). The program tests single-locus Mendelian segregation using chi-square goodness-of-fit values and performs contingency chi-square analyses to test for linkage between segregating locus pairs. The recombination fractions and their standard errors are calculated using Kosambi's (1944) formula. The linkage map was deduced from these data using the principle of the three-point-cross method (Suzuki et al. 1981). A probability of obtaining the calculated chi-square of < 0.05 for the single locus goodness-

of-fit data and < 0.01 for the linkage analysis was considered significant.

Results and discussion

RAPD marker selection

Initally 176 random 10-base primers with GC contents ranging from 50–90% were screened in amplification reactions using DNA isolated from Fla4B and Knight, their interspecific hybrid US388, and the testcross parent US799. Of the 176 primers screened, 118 resulted in amplification of at least one DNA fragment. These included 22, 75, 87, 97, and 100% of the primers screened with GC contents of 50, 60, 70, 80, and 90%, respectively. Generally, the higher the GC content of the primer, the higher was the likelihood of it resulting in amplification and the higher the number of amplified fragments. The GC content of a primer has been reported to be the best indicator for whether or not it will result in amplification (Fritsch et al. 1993). Forty-five of the 118 primers (see Table 1 for sequences) resulted in amplification of a total of 89 polymorphic DNA fragments which were suitable for mapping, i.e., fragments which were unique to one of the original parents, present in the F_1, and absent from the testcross parent. Because RAPDs are dominant markers, it was possible that the original parent which carried a particular marker was heterozygous for the marker. Thus, it was essential that we screen the F_1 for the inheritance of each marker. In fact, of a total of 583 fragments which were polymorphic between the original parents, 264 were not inherited by the F_1. A high level of heterozygosity is expected from typically outcrossing species like those used for this study.

Markers ranged in size from approximately 0.1 to 3 kb. Each marker was given a 4-character name as required by the LINKAGE-1 program. The marker name was based on the 3-digit name of the primer (assigned by the Biotechnology Laboratory, University of British Columbia) followed by a letter. Letters were assigned in alphabetical order beginning with the highest molecular weight marker generated from a particular primer and ending with the lowest molecular weight marker. The number of "mappable" markers generated from a single primer ranged from 0 to 5.

Table 1. Primers and primer sequences resulting in amplification of "mappable" markers

Primer	Primer sequence	Primer	Primer sequence
101	GCG GCT GGA G	296	CCG CTG GGA G
102	GGT GGG GAC T	299	TGT CAG CGG T
105	CTC GGG TGG G	300	GGC TAG GGC G
125	GCG GTT GAG G	504	ACC GTG CGT C
127	ATC TGG CAG C	508	CGG GGC GGA A
149	AGC AGC GTG G	515	GGG GGC CTC A
169	ACG ACG TAG G	516	AGC GCC GAC G
181	ATG ACG ACG G	521	CCG CCC CAC T
184	CAA ACG GCA C	523	ACA GGC AGA C
186	GTG CGT CGC T	534	CAC CCC CTG C
188	GCT GGA CAT C	536	GCC CCT CGT C
189	TGC TAG CCT C	540	CGG ACC GCG T
199	GCT CCC CCA C	541	GCC CCT TTA C
203	CAC GGC GAG T	542	CCC ATG GCC C
222	AAG CCT CCC C	546	CCC GCA GAG T
239	CTG AAG CGG A	559	GAG AAC TGG C
244	CAG CCA ACC G	580	GCG ATA GTC C
268	AGG CCG CTT A	584	GCG GGC AGG A
280	CTG GGA GTG G	586	CCG GTT CCA G
287	CGA ACG GCG G	592	GGG CGA GTG C
292	AAA CAG CCC G	595	GTC ACC GCG C
293	TCG TGT TGC T	598	ACG GGC GCT C
295	CGC GTT CCT G		

Segregation of RAPD markers

Selected primers were used to screen the segregating population. Typical gels are shown in Figs. 1 and 2. The most easily-scorable marker from each set of reactions is indicated. Only 1 of the 89 segregating markers (239b) deviated significantly ($P < 0.05$) from the expected 1:1 ratio (data not shown). This was surprising because deviations from expected ratios have been reported for some molecular markers, especially those analyzed in interspecific or intergeneric crosses (Zamir and Tadmore 1986; Bonierbale et al. 1988; Weeden 1989). For example, 12 of 36 RFLP markers exhibited distorted segregation ratios in an interspecific backcross population of *Citrus* (Durham et al. 1992). The lack of markers exhibiting skewed segregation ratios in our interspecific blueberry population may be due to a relatively high amount of homology between the *V. darrowi* and *V. elliottii* genomes. Hall and Galletta (1971) showed that the chromosome morphology of extant diploid *Vaccinium* species was very similar and concluded that the rich variation in the genus was most likely genic in origin and not due to chromosomal rearrangements. van Heemstra et al. (1991) found aberrant segregations of isozyme markers in different diploid blueberry populations to constitute only 6% of 65 segregation ratios observed. In addition, two crosses between closely-related parent plants accounted for most of the observed deviations. Thus, it was suggested that crosses between closely-related parent plants may have resulted in the unmasking of certain deleterious recessive genes.

Linkage analysis

Hanson (1959) estimated 38 as the minimum backcross (or testcross) population size required to detect linkage, so that the probability of concluding independence is 0.05 if the recombination value is 0.25. Furthermore,

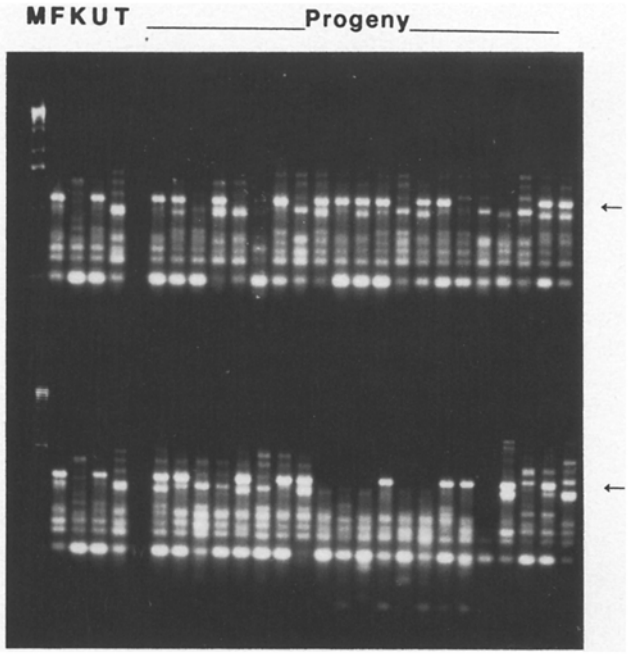

Fig. 1. Amplification reactions using 10-base primer #516 (from the University of British Columbia) and blueberry DNA as template. From left to right, on upper and lower halves of the gel, are shown: first, molecular weight markers (*M*) (1-kb ladder from BRL), followed by PCR using original parents Fla4B (*F*) and Knight (*K*), Fla4B × Knight F_1 US388 (*U*), testcross parent US799 (*T*), an empty lane, and then PCR using a total of 38 individuals of the mapping population. The *last four lanes* on the bottom half of the gel also contain DNA from PCR reactions using Fla4B, Knight, US388 and US799 DNA as template. However, these reactions were run using a different thermal cycler from the previous reactions. The *arrows* to the right of the gel point to the most easily scorable segregating RAPD marker present in reactions using Fla4B and US388 as template but not in reactions using Knight or US799 DNA

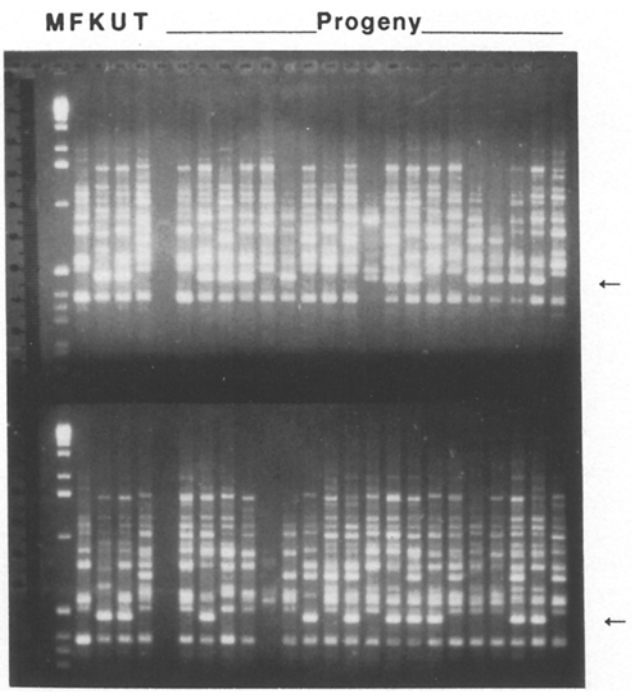

Fig. 2. Amplification reactions using 10-base primer #542 (from the University of British Columbia) and blueberry DNA as template. From left to right, on upper and lower halves of the gel, are shown: first, molecular weight markers (*M*) (1-kb ladder from BRL), followed by PCR using Fla4B (*F*), Knight (*K*), US388 (*U*), US799 (*T*), an empty lane, and then PCR using a total of 38 individuals of the mapping population. The *arrows* to the right of the gel point to a segregating RAPD marker present in reactions using Knight and US388 as template but not in reactions using Fla4B or US799 DNA

according to Hanson, the minimum population size required to detect linkage is 25 or 35 if the recombination value is 0.2 and the probability of concluding independence is 0.05 or 0.025, respectively. Since our population size was 38, we considered pairs of segregating loci to be linked only if the contingency chi-square analysis showed significant deviation from independent assortment at $P < 0.01$ and the maximum likelihood estimate of the recombination fraction was less than 0.25. There were six exceptions; six pairs of loci showed significant deviation from independent assortment at $P < 0.01$ (in fact, $0.003 < P < 0.006$) but the recombination fractions were 0.25–0.27. These were included on the linkage map.

Based on these criteria, the linkage map shown in Fig. 3 was constructed. Of 89 segregating RAPD markers analyzed, 72 mapped to 12 linkage groups. Ten of the remaining seventeen were difficult to score with confidence and, thus, were not included on the map. Of the remaining seven markers, some (102b, 105c, 584a, and 592c) did not appear to be linked to any of the other markers while others (299a, 546a, and 592a) appeared linked to several markers of a linkage group (linkage groups 4, 4, and 9, respectively) but their relative positions could not be determined from the available data. Linkage groups ranged in length from 19 to 165 cM with a total length for the map of 954 cM. The distance between adjacent markers ranged from 3 to 30 cM with an average distance of 16 cM. All pairs of markers placed at adjacent positions on the map, excluding the six pairs described in the previous paragraph, showed sigificant deviation from independent assortment at $0.000 \leqq P \leqq 0.003$. From the number of individuals and the number of markers analyzed thus far, the order of three pairs of markers (indicated by brackets in Fig. 3) could not be deduced with good certainty. Generally, however, adding map distances between adjacent markers agreed well with those determined for nonadjacent markers when these were within about 30 cM of each other. Two markers from the same primer, 559a and 559b, segregated together in all individuals of our population. Until more individuals are analyzed, these two markers cannot be distin-

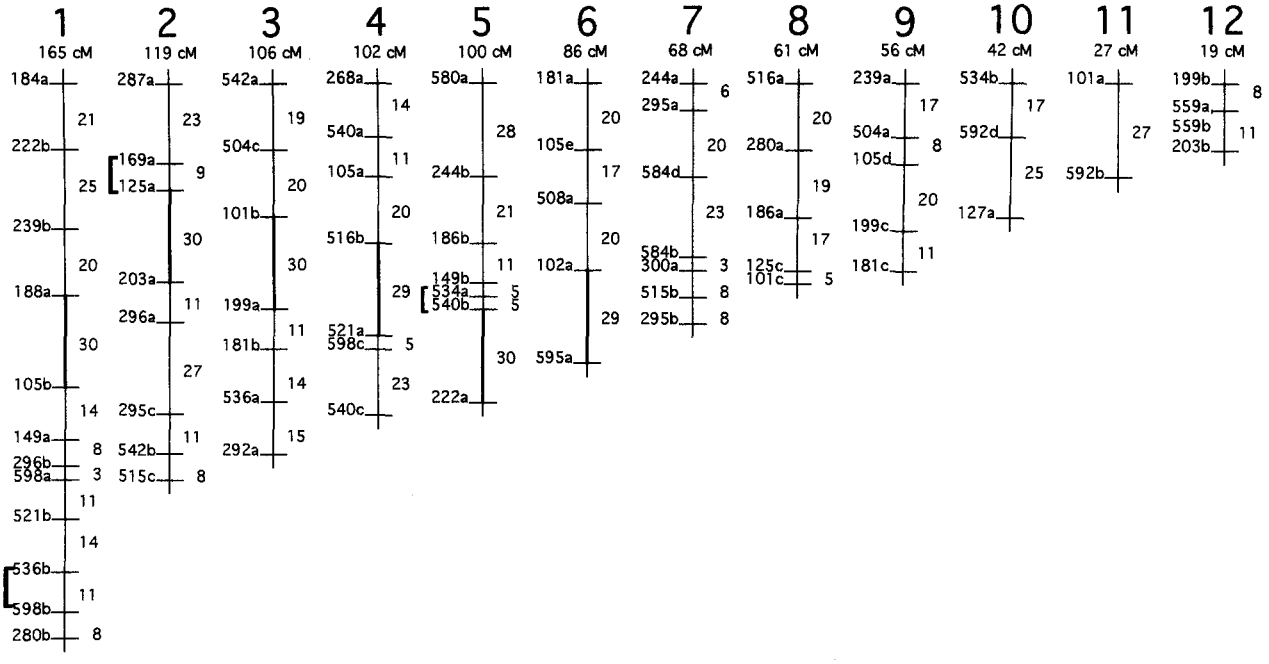

Fig. 3. RAPD-based genetic linkage map of blueberry derived from a cross between diploid *V. darrowi* × *V. elliottii* and *V. darrowi*. Linkage groups are numbered from longest to shortest. Marker names are shown to the left of each linkage group and distances between adjacent markers (in cM) are shown to the right. *Brackets* indicate markers whose order could not be deduced with good certainty. Loose linkages ($0.25 < r < 0.27$) are indicated with *bold lines*

guished and so were placed at the same location on the map.

Considering the basic chromosome number for blueberry ($n = 12$; Hall and Galletta 1971) and the number and length of the linkage groups identified thus far, it is conceivable that we have not identified linkage groups corresponding to all chromosomes. With the additon of more markers, the smaller linkage groups may converge or join with other linkage groups. Thus, we are continuing to identify and analyze more markers to add to the map.

Interestingly, a few pairs of marker loci segregated differently from that expected of both linked and unlinked loci. The contingency chi-square analyses of these marker pairs showed significant deviation from independent assortment at $P < 0.01$. However, an examination of the raw data revealed the deviation was not due to a significantly higher number of parental types than recombinant types, as expected of linked loci, but rather to a significantly higher number of recombinant types than parental types. The marker pairs, the linkage groups to which they were assigned, and the chi-square and probability values are presented in Table 2. Three pairs involved markers located on linkage groups 5 and 8. The three markers involved on linkage group 8 were within 10 cM of each other. Another three pairs consisted of markers located on linkage groups 3 and 4, while the final two pairs included markers on linkage groups 4 and 9. In these cases, markers on the same linkage groups were within 11 cM of each other. Segregation of these markers individually did not deviate significantly from the expected 1:1 ratio (data not shown). A possible biological interpretation of these findings is that certain genes closely linked to these markers are exposed to some form of selection pressure in pairs, but not individually, at either pre or post-zygotic stages of development and the recombinant types have a selective advantage over the parental types. Alternatively, given the large number of pairwise comparisons performed in the linkage study (3916) and even using $P < 0.001$ as the significance level, approximately four deviations from independent assortment

Table 2. Marker pairs present in the mapping population at a higher frequency in the recombinant configuration than the parental configuration

Marker pairs	Linkage groups	χ^2	P
181b/521a	3 and 4	7.797	0.005
199a/521a	3 and 4	7.836	0.005
199a/598c	3 and 4	7.836	0.005
105a/239a	4 and 9	8.395	0.004
239a/540a	9 and 4	7.446	0.006
149b/516a	5 and 8	11.906	0.001
516a/534a	8 and 5	11.906	0.001
516a/540b	8 and 5	7.797	0.005

are expected due to chance alone. Therefore, a definitive conclusion concerning these marker pairs will have to await the analysis of more individuals of this population and of our second (*V. darrrowi* × *V. caesariense* derived) mapping population.

Acknowledgements. We thank Dr. A. Draper for kindly providing the parent plants, Fla4B, Knight, US388, and US799, for this mapping project. We also thank Dr. J. Hartung for help in developing a protocol for generating reliable RAPD markers, E. Ogden for help in making crosses and maintaining plants, and Dr. G. Galletta for spending much time with us discussing blueberry genetics.

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