Utility of Blueberry-derived EST-PCR Primers in Related Ericaceae Species

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Abstract. Expressed sequence tag-polymerase chain reaction (EST-PCR) markers for DNA fingerprinting and mapping in blueberry (*Vaccinium* sp.) had previously been developed from expressed sequence tags (ESTs) produced from a cDNA library, derived from RNA from floral buds of cold acclimated plants. Because EST-PCR markers are derived from gene coding regions, they are more likely to be conserved across populations and species than markers derived from random regions of DNA, such as randomly amplified polymorphic DNA (RAPD) or amplified fragment length polymorphism (AFLP) markers. In this study, we tested whether many of the EST-PCR primer pairs developed for blueberry are capable of amplifying DNA fragments in other members of the family Ericaceae. In addition, we cloned and sequenced a selection of 13 EST-PCR fragments to determine if they showed homology to the original blueberry cDNA clones from which the EST-PCR primer pairs were derived. Closely related cranberry genotypes (two wild selections of *V. oxycoccus* L. and two cultivars of *V. macrocarpon* Aiton, 'Early Black' and 'Stevens') and more distantly related rhododendron genotypes (one wild selection each of *Rhododendron arboreum* Marsh, *R. maximum* L., and *R. ponticum* L. and three complex species hybrids, 'Sonata', 'Grumpy Yellow', and 'Roseum elegans') were used. Of 26 primer pairs tested in cranberry, 23 (89%) resulted in successful amplification and eight of those (35%) amplified polymorphic fragments among the cranberry genotypes. Of 39 primer pairs tested in rhododendron, 29 (74%) resulted in successful amplification and 21 of those (72%) amplified polymorphic fragments among the rhododendron genotypes. Approximately 50% of the 13 sequenced EST-PCR fragments were found to be homologous to the original blueberry cDNA clones. These markers should be useful for DNA fingerprinting, mapping, and assessing genetic diversity within cranberry and rhododendron species. The markers which are shown to be homologous to the blueberry cDNA clones by DNA sequencing should also be useful for comparative mapping and genetic diversity studies between some genera of the family Ericaceae.

Polymerase chain reaction (PCR)-based molecular markers, such as randomly amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), simple sequence repeat (SSR) markers, and sequence-tagged-site (STS) markers, have been widely used in recent years for DNA fingerprinting, genome mapping, and population genetic studies. Their popularity is due, in part, to the technical simplicity of setting up PCRs and the small amount of genomic DNA that is required in the reactions. The many expressed sequence tag (EST) databases available today are providing sequences for developing a subcategory

of STS markers, termed expressed sequence tag-polymerase chain reaction (EST-PCR) markers. There are a number of advantages to using EST-PCR markers or other cDNA-based markers over random-primed markers for genetic studies. First, they target expressed genes; thus, they should be particularly useful for quantitative trait loci (QTL) mapping. If an EST-PCR marker is found linked to a QTL, it is possible that the gene itself, from which the EST-PCR marker was derived, controls the trait in question. Second, because they are derived from gene coding regions, which are more likely to be conserved across populations and species than noncoding regions, EST-PCR markers should be useful for comparative mapping studies. Third, EST-PCR markers have the potential for being codominantly inherited, which allows the identification of two different alleles at heterozygous loci in diploid organ-

isms. Schubert et al. (2001) reported that six out of seven polymorphic EST-PCR markers developed for Norway spruce [*Picea abies* (L.) Karst.] were inherited in a codominant fashion.

In an effort to obtain markers with these advantages for use in blueberry (*Vaccinium* L., section *Cyanococcus* A. Gray, family Ericaceae), we have been producing ESTs using a cDNA library derived from floral buds of cold acclimated blueberry plants (Rowland et al., 2003a), and have developed a set of EST-based primer pairs with general utility for DNA fingerprinting and mapping (Rowland et al., 2003b). Recently, EST-PCR markers have been developed for many woody plant species, including loblolly pine (*Pinus taeda* L.) (Temesgen et al., 2001), black spruce [*Picea mariana* (Mill.) B.S.P.] (Perry and Bousquet, 1998), Norway spruce (Schubert et al., 2001), and sugi (*Cryptomeria japonica* D. Don) (Tsumura et al., 1997). Generally, amplification using EST-PCR primers must be followed by digestion with restriction enzymes to generate cleaved amplified polymorphic sequences (CAPS) markers, heteroduplex analysis, or single-stranded conformational polymorphism (SSCP) analysis to detect polymorphisms (Cato et al., 2001). In the case of blueberry, half (15 out of 30) of the tested EST-PCR primer pairs resulted in amplification of polymorphic fragments that were detectable directly after ethidium bromide staining of agarose gels (Rowland et al., 2003b). The fact that blueberry species are primarily outcrossing and exhibit low to moderate levels of self-fertility (Galletta and Ballington, 1996) may explain the high percentage of polymorphic EST-PCR markers observed without any need for additional manipulation or analysis.

Although there are many advantages to using EST-PCR markers, considerable cost is involved in terms of time, money, and other resources in developing this type of marker. RNA must be extracted from sometimes small amounts of the appropriate tissue, a cDNA library must be constructed, hundreds of clones must be picked and plasmid DNA isolated from them, the clones must be sequenced, and primers must be designed, synthesized, and tested. If the EST-PCR primers designed for one species can be used in related species, then the cost involved in developing markers for DNA fingerprinting, genetic relationship studies, mapping, etc. for those other species is significantly reduced. In addition, if the markers are shown to be homologous, they can be used for comparative mapping studies between the species. The ability to transfer mapped STS markers within the Poaceae family has been demonstrated, both between cereal species (Erpelding et al., 1996) and between cereal and forage grass species (Taylor et al., 2001). STS primers developed from black spruce cDNA sequences have been shown to direct amplification in several different conifer species. Nearly all (95% to 97%) of the black spruce STS primers worked in congeneric trials, while some (21% to 33%) worked in other Pinaceae genera as well (Perry and Bousquet, 1998). Here, we have investigated whether

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the EST-PCR primers originally developed for blueberry are useful in other Ericaceae, namely the closely related cranberry species [subfamily Vaccinioideae; tribe Vaccinieae; genus *Vaccinium*; section *Oxycoccus* (Hill) W.D.J. Koch] and the more distantly related rhododendron species (subfamily Ericoideae; tribe Rhodoreae; genus *Rhododendron* L.) (Anderberg, 1993). Many of the 30 original blueberry EST-PCR primer pairs and several new ones were tested in PCRs using four cranberry genotypes (two wild selections of *V. oxycoccus* and two cultivars of *V. macrocarpon*, 'Early Black' and 'Stevens') and six rhododendron genotypes (one wild selection each of *R. arboreum*, *R. maximum*, and *R. ponticum* and three complex species hybrids, 'Sonata', 'Grumpy Yellow', and 'Roseum elegans'). The percentage of primer pairs that resulted in successful amplification and in amplification of polymorphic fragments was determined. In addition, several of the EST-PCR products from cranberry and rhododendron were cloned and sequenced and their homology to the original blueberry cDNA clones, from which the primer sequences were derived, was assessed.

Materials and Methods

Plant material and genomic DNA extraction. Four cranberry genotypes [two wild selections of *V. oxycoccus* (NJ96-37 and NJ96-125) and two cultivars of *V. macrocarpon* ('Early Black' and 'Stevens')] and six rhododendron genotypes {three wild selections, one each of *R. arboreum* (accession no. 70/046), *R. maximum* (accession no. 77/564), and *R. ponticum* (accession no. 76/411) and three complex species hybrids, 'Sonata' [*Purple Splendour* (*R. ponticum* x unknown) x *R. dichroanthum* Diels], 'Grumpy Yellow' (*R. yakusimanum* Nakai x unknown), and 'Roseum Elegans' (*R. catawbiense* Michx. x unknown)} (Salley and Greer, 1992) were evaluated in this study. Several different blueberry genotypes were used in different experiments as positive controls. Wild rhododendron selections and the 'Sonata' hybrid were originally obtained as rooted cuttings from the Rhododendron Species Botanical Garden (Federal Way, Washington D.C., courtesy Dr. S.L. Krebs of the Holden Arboretum, Kirtland, Ohio), whereas the two cultivars, 'Grumpy Yellow' and 'Roseum elegans', were obtained as potted liners from Van Veen Nursery (Portland, Ore.) and Appalachian Nurseries (Waynesboro, Pa.). The rhododendron genotypes were maintained at the Dept. of Horticulture Research Farm, Iowa State Univ., Ames. The cranberry genotypes were maintained at the Blueberry and Cranberry Research Center, Rutgers Univ. (Chatsworth, N.J.). The blueberry genotypes were maintained at the Fruit Laboratory, USDA/ARS (Beltsville, Md.).

Blueberry and rhododendron DNA was extracted from frozen young leaves (≈5 g), collected from greenhouse-grown plants, using the CTAB procedure of Doyle and Doyle (1990). Cranberry DNA was isolated from 4–5 fresh, fully expanded leaves (≈30 mg) using the CTAB method of Stewart and

Via (1993) as modified by Novy and Vorsa (1995). DNA concentrations were estimated by agarose gel electrophoresis (1% w/v gel) using known concentrations of uncut lambda DNA (Invitrogen Life Technologies, Carlsbad, Calif.) as standards.

cDNA clones, sequence analysis, and primer design. Construction of the cDNA library for EST analysis, prepared from RNA expressed in floral buds of cold acclimated plants of the highbush blueberry cultivar Bluecrop, has been previously described (Levi et al., 1999). Clones were picked at random from the unamplified library. Plasmid DNA was isolated using the QIAprep® Spin Miniprep Plasmid Kit (Qiagen, Valencia, Calif.), quantified spectrophotometrically, digested with *EcoRI* and *XhoI* restriction enzymes (New England BioLabs, Beverly, Mass.) to release the inserts, and electrophoresed through 1% agarose gels to verify the presence of inserts and the quantity of DNA.

Single-pass nucleotide sequencing of recombinant plasmid DNAs was performed from both ends of the cDNA inserts by the

Univ. of Maryland, Center for Agricultural Biotechnology-DNA Sequencing Facility (College Park). After trimming vector sequences, each cDNA sequence was compared against known gene sequences contained in GenBank (<http://www.ncbi.nlm.nih.gov/BLAST>) (using the BLASTX algorithm (Altschul et al., 1990). ESTs with BLASTX scores >50 and/or E-values ≤10⁻³ ($P \leq 0.001$) were considered to have significant similarity to known sequences. Primer pairs were designed from sequence data using the P3 website (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Forward and reverse primers were designed from sequences near the end of each cDNA insert, for those inserts from which sequence was obtained from both 5' and 3' ends of the cDNA. If good sequence data (>300 bases) were obtained from only one end of the cDNA, then both forward and reverse primers were designed as far apart as possible from only that one end. Primers were synthesized by Invitrogen Life Technologies.

Generation of EST-PCR markers. DNA amplification reactions were performed as

Table 1. Summary of blueberry cDNA clones, including their names, origin of nucleotide sequences (5' or 3' end), putative identification from BLASTX searches, BLASTX E-values, and percent identities.

Clone name	Origin of nucleotide sequences	Putative identification	E-value ^z	% Identity ^y
4*	5' and 3'	Non-identified	NS ^w	
10*	3'	Phospholipid-hydroperoxide glutathione peroxidase	3 × 10 ⁻⁵⁹	68
14*	3'	Non-identified	NS	
15*	5' and 3'	Pyruvate decarboxylase isozyme 1	1 × 10 ⁻¹⁰²	83
20*	5' and 3'	Non-identified	NS	
21*	5' and 3'	Non-identified	NS	
22*	5'	Dessication-responsive protein 29B	2 × 10 ⁻⁴	36
23*	5' and 3'	Putative protein (<i>Arabidopsis thaliana</i>)	2 × 10 ⁻¹⁷	70
25*	5'	U6 snRNA-associated Sm-like protein	2 × 10 ⁻⁴⁵	90
28*	5'	Auxin-responsive protein IAA8	6 × 10 ⁻¹⁷	67
30*	5'	Putative protein (<i>Arabidopsis thaliana</i>)	1 × 10 ⁻²⁸	76
31*	5' and 3'	Aldehyde dehydrogenase	2 × 10 ⁻⁵³	51
32*	5' and 3'	Putative protein (<i>Arabidopsis thaliana</i>)	6 × 10 ⁻³⁰	56
33*	5' and 3'	MAP kinase kinase	6 × 10 ⁻⁴⁷	81
34*	5'	Low temperature-induced 65 kDa protein	2 × 10 ⁻¹³	29
38	5' and 3'	Non-identified	NS	
39*	5' and 3'	Putative protein (<i>Arabidopsis thaliana</i>)	9 × 10 ⁻²⁷	65
42*	5' and 3'	Putative pollen allergen, beta expansin	1 × 10 ⁻⁸⁷	65
43*	5' and 3'	Calmodulin-binding protein	6 × 10 ⁻⁵⁸	65
44*	5' and 3'	ATP synthase alpha chain	2 × 10 ⁻⁶⁷	95
45*	5' and 3'	Putative RNA helicase	7 × 10 ⁻⁵¹	50
47*	5' and 3'	Ubiquitin activating enzyme E1	1 × 10 ⁻⁹³	83
49*	5' and 3'	Non-identified	NS	
51*	5' and 3'	Dicyanin, blue copper protein	8 × 10 ⁻²⁷	46
52*	5' and 3'	ABI3-interacting protein	6 × 10 ⁻⁶⁶	38
53*	5' and 3'	Lysophosphatidic acid acyltransferase	1 × 10 ⁻⁷⁵	56
133	5' and 3'	Putative protein (<i>Arabidopsis thaliana</i>)	2 × 10 ⁻⁴⁵	56
136	5' and 3'	Putative protein (<i>Arabidopsis thaliana</i>)	4 × 10 ⁻⁶⁹	68
147	5' and 3'	RNA binding protein homolog	9 × 10 ⁻³⁶	75
148*	5' and 3'	Phosphatidylinositol transfer-like protein	2 × 10 ⁻⁹⁹	83
150	5' and 3'	DNA-directed polymerase 1 190K chain	2 × 10 ⁻⁴⁹	69
169	5' and 3'	Non-identified	NS	
172	5' and 3'	Putative protein (<i>Arabidopsis thaliana</i>)	1 × 10 ⁻³⁷	55
175	5' and 3'	Non-identified	NS	
191	5' and 3'	Sucrose synthase	7 × 10 ⁻⁷⁹	77
193	5' and 3'	Vacuolar H ⁺ -pyrophosphatase	1 × 10 ⁻¹⁰⁶	92
1043	5' and 3'	Adenine phosphoribosyltransferase	3 × 10 ⁻⁶²	84

^zProbability of a random association with a sequence in GenBank.

^yPercent identity at the amino acid level for the available sequence.

^xThe asterisks mark the clones that were used to develop the original set of EST-PCR markers for blueberry. A discussion of the development of these markers and a more detailed description of these clones, including size of cDNA inserts and length of nucleotide sequences, is available in Rowland et al. (2003b).

^wNon-significant ($P \geq 10^{-3}$) association with a sequence in GenBank.

described previously (Stommel et al., 1997). Briefly, amplification reactions were carried out at least twice in 25- μ L volumes containing reaction buffer (20 mM NaCl, 50 mM Tris-HCl pH 9, 1% Triton-X-100, 0.1% bovine serum albumin); 1.6 mM MgCl₂; 200 μ M each of dATP, dCTP, dGTP, and dTTP; 0.1 μ M each of the forward and reverse EST primers; 0.7 units Taq DNA polymerase (Promega, Madison, Wis.); and 25 ng template DNA. DNA was amplified by programming an MJ Research (Watertown, Mass.) PTC-100 thermal cycler for 40 cycles of a 40-s denaturation step at 92 °C, 70-s annealing step at a temperature of 10 °C below the T_m (1.0 M Na⁺) of the primer (forward or reverse) with the lowest T_m , and 120-s extension step at 72 °C. Amplification products and molecular weight standards (1-kb DNA ladder; Invitrogen Life Technologies) were separated by electrophoresis through 1.4% agarose gels.

Cloning and sequence analysis of EST-PCR products. A selection of EST-PCR amplification products, including both monomorphic and polymorphic fragments from cranberry and rhododendron, were excised from 1.4% TBE agarose gels and purified using the Ultraclean Gelspin Kit (MO BIO Laboratories, Solana Beach, Calif.) according to manufacturer's instructions. Purified fragments were cloned using the pGEM[®]-T Vector or the pGEM[®]-T Easy Vector System (Promega) according to the directions provided. Recombinant plasmids were isolated using the Ultraclean Miniprep Kit (MO BIO Laboratories). Cloned fragments were cycle-sequenced using Cy5 labeled M13 forward and reverse primers and the SequiTherm EXCEL II Long-Read[™] Kit (Epicentre Technologies, Madison, Wis.). Labeled primers were purchased from Integrated DNA Technologies (Coralville, Iowa). Sequencing reactions were separated and detected on an ALF Express Sequencer (Amersham Pharmacia Biotech, Piscataway, N.J.). Sequences were compiled using the Lasergene software package (DNASTAR, Madison, Wis.). To determine if the sequences of the PCR products were homologous to the sequences of the blueberry cDNA clones from which the EST-PCR primers were derived, sequences were compared to each other manually and by use of BLAST algorithms, Pairwise BLAST and BLASTX or BLASTN to search GenBank.

Results and Discussion

cDNA sequencing and gene identification.

The cDNA library used for generating ESTs represents genes expressed in blueberry floral buds during the winter (December), when plants have reached their maximum level of cold hardiness (Muthalif and Rowland, 1994). ESTs were produced previously from clones, numbered 4, 10, 14, 15, 20, 21, 22, 23, 25, 28, 30, 31, 32, 33, 34, 39, 42, 43, 44, 45, 47, 49, 51, 52, 53, and 148, and used in the development of the first set of EST-PCR markers for blueberry [described in Rowland et al. (2003b)]. For this study, additional ESTs were produced from single-pass sequencing of cDNAs, numbered 38, 133, 136, 147, 150, 169, 172, 175, 191,

Table 2. Summary of blueberry-derived EST-PCR primer pairs tested in cranberry and rhododendron. Each primer pair was scored for success (+) or failure (–) at amplifying fragments and for ability to amplify polymorphic-sized fragments (p) among the cranberry or rhododendron genotypes.

Clone name	Forward and reverse primer sequences (5'–3')	Annealing temp	Cranberry ^z	Rhododendron ^z
4	GCC GCT CTT CTC TTC CTA GC CCA TCA AAC ACC ACC TAT GC	58 °C	–	+
10	CTT TGC CAA GTT CTG GTT GA GCT AAA TGA AGT GCG GGA GA	56 °C	+	+
14	CTG GCC CGC AAT ATC ATA G GTA GAT TGC CTC CCA CGA GA	58 °C	+	+p
15	CTA GAG GCT GCA GTG GAA GC TTG CTC GTG TCG TCC TTA TG	58 °C	+p	–
20	AAA TTG CTC GCA AAC CAA TC TTT TCC AAG CAA GGG ATG G	54 °C	+	+
21	TCC GAT AAC CGT TAC CAA GC TAT ACA GCG ACA CGC CAA AA	56 °C	+p	–
22	CTG GTC ATA CCG GGC AAC GAT CAA CGG GGT TCA TGG T	58 °C	+	+p
23	GTT AGA GAG GGT TTC GAG GA AGC AAA AAC TTC ACG CCA AT	54 °C	+	–
	CGT CTC ACA GTC CCG TTT CT CAA AAA CTT CAC GCC AAT CA	54 °C	ND	+
25	TCT CTC GGA GTT GAT CCA AAA CAA CCT TTC CTC ACA CAC ACA	57 °C	+p	–
28	AAT GGA AAG AAG CTC TGA CA ATC AGC CTC AGA TCC ACC AC	54 °C	+p	+
	TGG GGT TTC TCT TTT CTT GG CCA ATT GTG AAG CAG CTG AA	56 °C	ND	+p
30	AAT GCT GAC CCA ATC AAA GC GGG TCA CGC TTG AGC TAG TG	56 °C	+	–
31	AGC ATT TGA CAC CAG TCA CG TTA CAG GAG GGG GAT TTT	52 °C	+p	+p
32	CGT GGC GAA TGT ATC ATC AC TGC CGC AGA TCA TGA ATA GA	56 °C	+	+
33	CTA ATG CAC CTG CTG ACC AA ACT AAT CTC CAC GCT TCC GA	58 °C	+	–
34	CTA AAG ACG GGC CTG AAG TG TCT GGT GAG AAC TGG TCG TG	60 °C	+p	+p
38	GAC CGC TAT ATA CAT GTG TGA AAA AAG GCA TCG CTT TTG AA	52 °C	ND	+p
39	TAA TGA GTC TGT GGC GAA CG AAC AAG ACC AAA CCC CAC AT	56 °C	+	+p
42	ATC TGG GGC TTG TGG ATA TG CAA CCT TCT TGT GCA ATG TCA	57 °C	+p	+
43	GGC ACG AGG TCA CAG AAT GT ACA ATC CCA CCC AAA AAC AA	54 °C	+	ND
44	AGC AGC GGT ATT CTC CTC AA CAC AGA ATC CAT TGA CAG CG	58 °C	+	+
45	CAA TGT TGG GGA GAA TGC TT AAG AGC AAA ATA CAC GCA CG	56 °C	+	+p
	AGA GAC TGC TGC TGG TGA CA CGC ACG TAC TTG GCT ATC AG	60 °C	ND	+p
47	CTG CTG ATC CTA GGC ACC TC AAA GGT TGC CCA AAA GTT CC	56 °C	+	–
49	GGG CAA AAC ATT TGA TTG GT CTT TCC GGC TTC TCA CAA AA	54 °C	–	+p
51	GCT GCT CTT GTA CAG GGC TC TTG CGC ACA CAT AAA CCT AAA	55 °C	+p	–
52	GGC AAG TGG GCA ATG ATA GT TCA CAA ACT GAC CCC GTA CA	58 °C	+	+p
53	TCC AGC GCT TCT TAT CCA CT GGC AGG CAT GAT TTT ACC AG	58 °C	+	+p
133	AAA GTT AAA CGG CAG CGT GT AGG CCC TGT TAA GTT TCA TCC	56 °C	ND	+p
136	CAC AAC TGA TGC TTG GGA AG ACC ATG CCC TCA CAG AAG AA	58 °C	ND	–
147	CTA AAT TGG GCG ACC TTC AG TGG TTG ACC CTC TAC TCC AAA	58 °C	ND	+p
148	CAA GGG TGC ACG TGA ACT TA AAA AGC ATT GCA GTC ACA CG	56 °C	–	+
150	ATT CAG CGA ATT GCA AAG GA AAA CCA GAG GGG GCT TAC AT	54 °C	ND	+p
169	TAG TGG AGG GTT TTG CTT GG TCT AAA TAA AGG GGC CAA AGG	57 °C	ND	+p

Table 2 continued on next page

193, and 1043; and good quality 5' and 3' sequences were obtained for all of them. The DNA sequences were 629 bases long on average, after removing vector sequences. Putative identities of the 11 cDNAs were assigned by searching Genbank for similar sequences. Their identities, together with the identities of the first 26 cDNAs, are summarized in Table 1. For eight of the cDNAs, no significant sequence similarity was found, while for seven more of the clones significant sequence similarity to putative proteins from *Arabidopsis thaliana* was found. The remaining 22 cDNAs appeared to encode a wide range of proteins including low temperature stress-related proteins, proteins possibly involved in signal transduction, hormone-responsive proteins, and basic structural and metabolic proteins.

Development and testing of EST-PCR markers in cranberry and rhododendron. The EST-PCR primer pairs, designed from the ends of the available nucleotide sequences, were tested in amplification reactions with DNA from four cranberry genotypes and six rhododendron genotypes (Table 2). For some of the sequences (cDNAs 23, 28, 45), primer pairs were redesigned, in an effort to obtain better amplification or more polymorphic fragments. Annealing temperatures ranged from 52 to 60 °C.

A total of 26 primer pairs were tested in cranberry and 39 pairs were tested in rhododendron. Fewer total primer pairs were tested in cranberry than rhododendron because a very high percentage of the primer pairs that were tested worked extremely well in cranberry; thus there did not appear to be a need for further testing. Of the 26 primer pairs tested, 23 (89%) resulted in successful amplification and eight of those (35%) amplified polymorphic fragments among the cranberry genotypes. Most of the polymorphisms occurred between the genotypes of the two species, *V. oxycoccus* and *V. macrocarpon*, and not within genotypes of the same species. Only primer pair 31 distinguished the *V. macrocarpon* cultivars while two primer pairs, 25 and 42, distinguished the *V. oxycoccus* selections. Amplification profiles, from PCRs using EST primer pair 42, are shown in Fig. 1A. Eighteen of the 23 primer pairs amplified similar-sized fragments to those amplified in blueberry, suggesting that most of the primer pairs result in amplification of homologous fragments.

Of the 39 primer pairs tested in rhododendron, 29 (74%) resulted in successful amplification and 21 of those (72%) amplified polymorphic fragments among the rhododendron genotypes (Fig. 1B). Twenty-one of the 29 primer pairs amplified at least some fragments that were of similar sizes to those amplified in blueberry. The higher percentage of primer pairs that were useful in cranberry (89%), as compared to rhododendron (74%), reflects the closer relationship between blueberry and cranberry than between blueberry and rhododendron. The higher percentage of polymorphic fragments observed among the rhododendron genotypes (72%) than among the cranberry genotypes (35%) may be due, at least

Table 2. Continued from previous page.

Clone name	Forward and reverse primer sequences (5'-3')	Annealing temp	Cranberry ^z	Rhododendron ^z
172	CAC AAC TCC ATA ACC AGA CCA A GCA GGG AAA ATT TGA CAT TCA C	58 °C	ND	—
175	GAC AGA TTG CGT AAC CCG TAA CCA ATC CGC TTT GTC TGT TT	56 °C	ND	+p
191	GCC TGG TGT TTG GGA ATA TG TGT ACA GTT CGC TCG GTG AG	58 °C	ND	+p
193	GAG GGA TTC AGC ACG AAG AG CAA CAT CAT CAA CCC CAA CA	56 °C	ND	+p
1043	TAC ATC CAT CCG CAC ATA CG ACC TGC ACG GAT TTA ACC AG	58 °C	ND	+p

^zND = not done.

Fig. 1. Testing blueberry-derived EST-PCR primer pairs in cranberry and rhododendron. (A) Amplification products resulting from PCRs of cranberry and blueberry DNA using EST-PCR primer pair 42. From left to right, lanes were loaded as follows: blueberry selections Fla4B (1) and W85-20 (2), cranberry genotypes 'Early Black' (3), 'Stevens' (4), NJ96-37 (5), and NJ96-125 (6), and 1-kb molecular weight ladder (7). (B) Amplification products resulting from PCRs of rhododendron and blueberry DNA using EST-PCR primer pair 147. From left to right, lanes were loaded as follows: 1-kb molecular weight ladder (1), rhododendron genotypes *R. arboreum* (2), 'Grumpy Yellow' (3), 'Sonata' (4), *R. maximum* (5), *R. ponticum* (6), and 'Roseum elegans' (7), and blueberry cultivars 'Bluecrop' (8) and 'Berkeley' (9).

Table 3. Summary of EST-PCR products that were cloned and sequenced from cranberry and rhododendron, including the primers and genotypes that were used, ~sizes of fragments in bp, genetic variation observed among cranberry or rhododendron genotypes, and whether the PCR products were homologous to the original blueberry cDNA clones from which the primer sequences were derived.

EST-PCR primer pair	Genotype	Approx. size of fragment ^a	Monomorphic (M) or polymorphic (P)	Homologous to cDNA
<i>Cranberry PCR products</i>				
28	Stevens	650	P	No
		300	M	Yes
31	Stevens	375	P	No
34	NJ96-125	600	P	Yes
42	Early Black	1900	P	Yes
52	Early Black	700	M	Yes
<i>Rhododendron PCR products</i>				
20	<i>R. arboreum</i>	520	M	Yes
28	Grumpy Yellow	300	M	Yes
34	Roseum Elegans	950	P	No
38	Grumpy Yellow	900	M	No
42	Sonata	600	M	Yes
45	<i>R. ponticum</i>	320	P	No
53	<i>R. ponticum</i>	700	P	No

^aSizes in bp.

in part, to the larger number of rhododendron genotypes examined and to the fact that each of the rhododendron genotypes represents a different species or complex interspecific hybrid.

Cloning and sequence analysis of EST-PCR products. Thirteen EST-PCR fragments, resulting from nine different EST primer pairs, were cloned and sequenced from the cranberry and rhododendron genotypes, a sufficient number to give a good indication of whether the fragments were generally homologous to the blueberry cDNAs from which the primer sequences were derived (Table 3). In making our selection of EST-PCR fragments to sequence, we chose approximately equal numbers of fragments from the cranberry and rhododendron genotypes and approximately equal numbers of monomorphic and polymorphic fragments. Specifically, six fragments (two monomorphic and four polymorphic) were cloned and sequenced from the cranberry genotypes, and seven fragments (four monomorphic and three polymorphic) were cloned and sequenced from the rhododendron genotypes. Overall, seven out of 13 or 54% of the sequenced fragments showed homology to the original blueberry clones. Four of the six (two monomorphic and two polymorphic fragments) or 67% of the fragments from the cranberry genotypes showed homology to the original cDNA clones, whereas three of the seven (all monomorphic) or 43% of the fragments from the rhododendron genotypes showed homology to the blueberry clones. Of 20 EST-PCR products cloned and sequenced from blueberry in the original study to develop the EST-PCR markers (Rowland et al., 2003b), all but three (or 85%) were homologous to the cDNAs from which the primer sequences were designed. Thus, although these EST-PCR primers are capable of amplifying fragments from a genus as distantly related as *Rhododendron*, their ability to amplify ho-

mologous fragments has dropped significantly from 85% in blueberry to 67% in cranberry to 43% in the rhododendron genotypes. These results highlight the importance of cloning and sequencing EST-PCR products that are to be used for comparative mapping and genetic relationship studies between species and genera and not assuming that they are homologous to the original clones from which the PCR primer sequences were derived. However, even EST-PCR markers that lack homology to the original cDNA clones may still be used for some DNA fingerprinting and genetic studies if they are reproducible, as are all the markers described in this study.

In conclusion, a high percentage of the blueberry-derived EST-PCR primer pairs resulted in successful amplification of DNA from cranberry and rhododendron, approximately 89% and 74%, respectively. Approximately 50% of 13 EST-PCR fragments that were cloned and sequenced from cranberry and rhododendron were actually homologous to the original cDNA clones from blueberry. Thus, these markers should be useful for DNA fingerprinting, developing genetic linkage maps, and assessing genetic diversity within cranberry and rhododendron species. The markers which are shown to be homologous to the blueberry cDNA clones by DNA sequencing should also be useful for comparative mapping and possibly for examination of genetic relationships between species of the genus *Vaccinium* and between some genera of the family Ericaceae.

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