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Short communication

# EST-PCR markers developed for highbush blueberry are also useful for genetic fingerprinting and relationship studies in rabbiteye blueberry

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ABSTRACT

The pedigrees of most rabbiteye blueberry (*Vaccinium virgatum*) cultivars can be traced back to four wild selections, 'Ethel', 'Clara', 'Myers', and 'Black Giant'; thus, they result from a very narrow germplasm base and are highly related. Until now randomly amplified polymorphic DNA (RAPD) has been the only type of molecular marker used in rabbiteye blueberry. Here we have tested whether a type of sequence-tagged site (STS) marker which utilizes specific ~20-mer primers from expressed sequence tags (ESTs) of highbush blueberry (*V. corymbosum*), called EST-PCR markers, are useful for genetic fingerprinting and relationship studies in rabbiteye blueberry. Of 44 EST-PCR primer pairs, from an assortment of genes expressed in flower buds of cold acclimated and non-acclimated plants, and shown to amplify polymorphic fragments among a collection of highbush genotypes, 40 (91%) resulted in successful amplification, and 33 of those (83%) amplified polymorphic fragments among the rabbiteye genotypes. The average number of scorable bands per primer pair was two. A dendrogram constructed from genetic similarity values, based on the EST-PCR marker data, tended to group siblings and parent/progeny together, generally agreeing with pedigree information. A group of 20 markers from five EST-PCR primer pairs distinguished all the genotypes in this study. These markers are as easy to generate and as affordable as RAPDs, but are based on actual gene sequences, and should have general utility for DNA fingerprinting, genetic diversity, and mapping studies.

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## 1. Introduction

Commercial production of blueberry utilizes multiple species in the section *Cyanococcus* of the genus *Vaccinium*. About two-thirds of blueberry production is from improved cultivars mainly of *V. corymbosum* L. (tetraploid highbush blueberry) and its hybrids and, to a lesser extent, *V. virgatum* Ait. (hexaploid rabbiteye blueberry). The other one-third of blueberry production is from wild, managed stands of *V. angustifolium* Ait. (tetraploid lowbush blueberry) (USDA Statistics, http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1113).

Not as widely grown as highbush and lowbush blueberry, the rabbiteye blueberry's natural range is the southeastern U.S., encompassing northern Florida, southern Georgia, and southern Alabama (Brightwell et al., 1955). Efforts to domesticate rabbiteye blueberry began around 1893 with the transplantation of native seedlings by M.A. Sapp to his farm in northwestern Florida (Hancock and Draper, 1989; Ballington, 2001). Rabbiteye blueberry breeding began in 1939 in Tifton, Georgia, at the Coastal Plain Experiment Station. Since then, breeding programs in Georgia, Florida, and North Carolina, in collaboration with the USDA/ARS, have all worked to develop new, improved cultivars (Austin, 1979). Rabbiteye blueberry is traditionally grown commercially in the southern regions of the U.S. However, because it is vigorous, high-yielding, and adaptable to upland soils, breeding efforts to develop northern-adapted rabbiteye cultivars are currently underway (Ehlenfeldt et al., 2007).

Although wild populations of rabbiteye blueberry contain much genetic diversity (Ballington et al., 1984), most current cultivars result from a very narrow germplasm base, and thus, are highly related. The pedigrees of most, but not all, rabbiteye cultivars can be traced back to four wild selections, 'Ethel' (thought to be identical to 'Satilla') from southeastern Georgia, 'Clara' and 'Myers' from north-central Florida, and 'Black Giant' from western Florida (Lyrene, 1981; Aruna et al., 1993). As in highbush and lowbush blueberry, inbreeding depression is a problem in progeny from self crosses and crosses of closely related genotypes of rabbiteye blueberry, resulting in reduced fruit set, smaller berries, later-

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maturing berries (Meader and Darrow, 1944; Hellman and Moore, 1983), and reduced seedling survival and vigor (Lyrene, 1983). The use of molecular markers to measure genetic relatedness among cultivars and selections, and identify more diverse germplasm to use in breeding is a recognized approach for widening the germplasm base of cultivated genotypes (Aruna et al., 1993).

Until now, the only type of molecular marker used extensively in rabbiteye blueberry has been randomly amplified polymorphic DNA (RAPD) markers. Aruna et al. (1993) used RAPD markers first to investigate the extent of genetic relatedness among 19 cultivars of rabbiteye blueberry, 15 improved cultivars, and the four original selections from the wild mentioned previously. As expected, results showed that the improved cultivars are progressing towards increased genetic similarity when compared with the four wild selections. Later, Aruna et al. (1995) developed a cultivar key for distinguishing the 19 rabbiteye cultivars based on 11 RAPD markers amplified from four RAPD primers.

RAPD markers have since been criticized for being difficult to reproduce between laboratories because of the need to duplicate the exact conditions for reproducible amplification from the 10-mer random-sequence primers (Jones et al., 1997). This has led many researchers to look for more robust marker systems to use, such as sequence-tagged site (STS) markers that utilize specific ~20-mer primers from sequenced DNA. We have developed an expressed sequence tag (EST) database comprised of about 5000 ESTs from flower bud cDNA libraries from the highbush blueberry cultivar Bluecrop (Dhanaraj et al., 2004, 2007; GenBank link: http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucest&cmd=search&term=vaccinium). In our initial efforts to develop markers from these sequences, we designed 30 PCR primer pairs from these ESTs and tested them in amplification reactions with genomic DNA from a collection of 15 highbush or highbush hybrid cultivars (Rowland et al., 2003b). Primers were designed near the ends of the ESTs to amplify as much of each gene as possible, to increase chances of detecting polymorphisms. Fifteen of the 30 primer pairs resulted in amplification of polymorphic fragments that were detectable directly after ethidium bromide staining of agarose gels. We are using these markers to further saturate a genetic linkage map of a diploid blueberry population (Rowland et al., 2003a). We have also shown that these highbush-derived EST-PCR markers are suitable for genetic relationship studies on wild lowbush blueberry (Bell et al., 2008). EST-based PCR markers have been developed for other plants as well, including Norway spruce (Schubert et al., 2001), sugi (Tsumura et al., 1997), and rhododendron (Wei et al., 2005).

Here we have tested the highbush-derived EST-PCR markers for their efficacy at distinguishing a collection of 28 rabbiteye cultivars and selections (many of which are closely related) and one highbush cultivar (included as a positive control and expected outlier). A dendrogram was constructed based on genetic similarity values calculated from number of shared bands for each pair of genotypes. In addition, the correlation between similarity coefficients, calculated from molecular marker data, and coefficients of coancestry, calculated from pedigree information, was evaluated.

## 2. Materials and methods

### 2.1. Plant material

Twenty-nine blueberry genotypes, including 28 rabbiteye or rabbiteye/highbush hybrid cultivars and selections and one highbush cultivar, were evaluated in this study. The highbush cultivar used was 'Bluecrop' because it is the one from which all the currently available blueberry ESTs were derived. Thus, 'Bluecrop' could serve as a positive control in the PCRs and as an outlier in the genetic relationship studies. Genotypes were maintained by the USDA/ARS (Blueberry and Cranberry Research Center, Chatsworth, NJ). The cultivars used are listed below [along with their parents]: Bluecrop [GM-37 (Jersey × Pioneer) × CU-5 (Stanley × June)], Aliceblue [Beckyblue O.P.], Austin [T-110 (Woodard × Garden Blue) × Brightwell], Baldwin [Tifblue × GA 6-40 (Myers × Black Giant)], Beckyblue [Fla 6-138 (*V. virgatum*, 6×) × E 96 (*V. corymbosum*, 4×)], Black Giant [native selection], Bluegem [Tifton 31 (Ethel × Callaway) O.P.], Bonita [Beckyblue O.P.], Brightwell [Tifblue × Menditoo], Callaway [Myers × Black Giant], Centurion [W-4 (native selection) × Callaway], Chaucer [Beckyblue O.P.], Choice [Tifton 31 (Ethel × Callaway) O.P.], Clara [native selection], Climax [Callaway × Ethel], Coastal [Myers × Black Giant], Delite [Bluebelle × T-15 [GA 10-144 (Myers × Black Giant) × W-8 (native selection)]], Ethel [native selection], Ira [Centurion × NC 911 (Tifblue × Menditoo)], Montgomery [NC 763 [GA 11-180 (Myers × Black Giant) × W-4 (native selection)] × Premier], Myers [native selection], Powderblue [Tifblue × Menditoo], Premier [Tifblue × Homebell], Satilla (=Ethel) [native selection], Snowflake [Fla K (Beckyblue O.P.) × NC 1830 (NC 7-63-3a *V. constablaei* × Premier)], Tifblue [Ethel × Clara], Windy [Fla 79-17 [Bluebelle × Fla M (Beckyblue O.P.)] × Fla 79-27 (pedigree lost)], Woodard [Ethel × Callaway], and Yadkin [Premier × Centurion].

### 2.2. Genomic DNA extraction

Young leaves were collected from field-grown plants of all the genotypes used in this study, ground with dry ice in a coffee grinder, and stored at −80 °C. DNA was extracted from leaf tissue (~5 g) using the CTAB procedure of Doyle and Doyle (1990) and quantified.

### 2.3. Generation of EST-PCR markers

Expressed sequence tag-polymerase chain reaction (EST-PCR) markers were initially developed for use in commercial highbush blueberry (Rowland et al., 2003b). EST/cDNA libraries were derived from cold acclimated and non-acclimated floral buds of the highbush cultivar Bluecrop, and a contig analysis was performed to identify unique genes (Dhanaraj et al., 2004). In unrelated projects, our laboratory is attempting to identify genes that are important for cold acclimation in blueberry, and are using these markers to map genes associated with cold hardiness in a diploid mapping population; therefore, many of the genes are related to cold stress. To generate the ESTs from these libraries, in some cases, single-pass nucleotide sequencing was performed from both ends, 5′ and 3′, of the cDNA inserts. However, in most cases, sequencing was performed from only the 5′ end of the cDNA inserts. Primer pairs were designed from sequence data from contigs using the P3 website (http://frodo.wi.mit.edu/primer3/input.htm), to allow for amplification of as much of each gene as possible from the available sequences. For instance, forward and reverse primers were designed from sequences near the 5′ and 3′ ends, respectively, for those cDNA inserts for which sequences were obtained from both 5′ and 3′ ends. If sequence data 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. Location near the ends of the ESTs was the only criterion used.

DNA amplification reactions were performed as described previously (Levi et al., 1993) with minor modifications as described by Stommel et al. (1997). Briefly, amplification reactions were carried out at least twice in 25 μL volumes containing reaction buffer (20 mM NaCl, 50 mM Tris–HCl pH 9, 1% Triton-X-100, and 0.1% bovine serum albumin), 1.6 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP, and dTTP, 0.1 μM each of the forward and reverse EST primers, 0.7 units *Taq* DNA polymerase (Promega, Madison, WI), and 25 ng template DNA. DNA was amplified in an MJ Research (Watertown, MA) PTC-100 thermal cycler, programmed for an ini-