High Resolution Melting Detects Sequence Polymorphism in *Rubus occidentalis* Monomorphic Microsatellite Markers

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**Keywords:** SSR, single nucleotide polymorphism, HRM, black raspberry

**Abstract**

Microsatellite, or simple sequence repeat (SSR), markers are valuable as codominant genetic markers with a variety of applications such as DNA fingerprinting, linkage mapping, and population structure analysis. However, primer pairs designed from the regions that flank SSRs often generate fragments with no size polymorphism. High resolution melting (HRM) technology provides a new method of identifying sequence variations in these amplified fragments. In this study, we used HRM to identify polymorphism in SSR PCR products that were monomorphic for size in a black raspberry (*Rubus occidentalis* subgenus *Idaeobatus*) population. When HRM revealed polymorphism between the parents of a mapping population, the seedlings were genotyped and scored. The HRM markers segregated in a Mendelian fashion. Comparison of DNA sequences of the parents revealed the presence of single nucleotide polymorphisms (SNP) in the amplicons. HRM can be a valuable tool for detecting polymorphism in PCR amplicons using existing primer pairs that would otherwise be of little use.

**INTRODUCTION**

Black raspberry (*Rubus occidentalis* L. subgenus *Idaeobatus*, 2n=2x=14) production in the United States is limited by a lack of suitable cultivars. Little progress in breeding new cultivars has been made in the last 40 years because of a lack of phenotypic variation in available germplasm. This problem has received relatively little attention until recent years, with renewed interest in understanding and finding new sources of black raspberry diversity for breeding improved cultivars. Microsatellite, or simple sequence repeat (SSR), markers are robust, highly polymorphic, codominant markers with applications in population genetics, genetic diversity studies and DNA finger-
printing. SSRs have been the focus of recent efforts to characterize genetic diversity in black raspberry, as well as develop linkage maps and other resources to aid in marker assisted selection and breeding (Dossett et al., 2010). In black raspberry and many other taxa, the development of SSR markers has relied heavily on transferability of markers from closely related taxa (Stafne et al., 2005; Dossett et al., 2010). This approach, in addition to the traditional methodology of developing SSR markers by identifying repeated sequences and developing primers from the flanking regions, often leads to a high proportion of markers that are monomorphic, or uninformative, based on fragment size alone.

High-resolution melting (HRM) is a relatively new technique that uses high resolution optics and fluorescent double-stranded DNA dyes such as LCGreen to precisely measure the melting temperature of, and detect the presence of heteroduplex DNA in, fragments generated by PCR (Gundry et al., 2003; Wittwer et al., 2003). The technique can be an effective and relatively inexpensive method for detecting mutations that change the melting temperature of a PCR amplicon (Muleo et al., 2009; Taylor, 2009). The objective of this study was to explore the use of HRM in black raspberry as a technique for the identification of melting point differences in SSR amplicons with no size polymorphism.

MATERIALS AND METHODS

DNA was extracted from freshly growing tissue of two black raspberry selections (ORUS 3021-2 and ORUS 4153-1) and 24 full-sib progeny from a cross of these two selections, using a modified PureGene kit (Gentra). This population is being studied for segregation of aphid resistance and a variety of adaptability and fruit quality traits (Dossett and Finn, 2010). Dossett et al. (2010) examined transferability of red raspberry and blackberry SSR primer pairs for use in black raspberry. Twenty primer pairs that generated PCR products monomorphic for size were selected and used in this study. The amplification and optimum annealing temperatures for 20 Rubus primer pairs was determined by gradient polymerase chain reaction (PCR) from 50 to 65°C in ORUS 3021-2. DNA was amplified for 40 cycles in a PTC-225 gradient thermal cycler programmed for a 30 s denaturation step at 94°C, a 30 s annealing step at the optimum annealing temperature of the primer pair and a 30 s extension step at 72°C. Following a final melting step at 95°C for 30 s, the PCR products were cooled to room temperature. The optimal annealing temperature for HRM analysis was determined by examination of HRM melting curves from the gradient PCR using the LightScanner (Idaho Technology Inc.) as illustrated by Vossen et al. (2009). To identify primers for which the parents showed scorable melting curve differences, PCR amplifications at the optimal annealing temperature for each primer pair were performed for the two parents in duplicate. PCR and HRM were performed on 24 seedlings after identification of melting curve differences between the parent clones, to examine segregation and suitability for linkage mapping. In addition, PCR amplicons from the two parents were cloned (TOPO TA, Invitrogen), and sequenced to confirm the number and types of SNPs present.

RESULTS AND DISCUSSION

Of the 20 Rubus primer pairs examined by HRM, five were tentatively selected as polymorphic because of consistent melting curve differences detected between the parents in duplicate samples (Fig. 1). Of these five primer pairs, three showed a 1:1 segregation ($P \geq 0.05$) in the 24 seedlings, with the seedling melting curves closely matching one or the other parent (Fig. 2). These three primers can be used for “blind mapping” of the segregating haplotypes with no further information needed as described by Studer et al. (2009) for SNP mapping. Sequencing of PCR fragments from the two parents identified up to four SNPs in the SSR fragments studied, and confirmed parental alleles that would lead to a 1:1 segregation for the three primers.

The remaining two primer pairs amplified products which showed the presence of more than two alleles and consequently resulted in segregation other than 1:1 in all or part
of the melting curve. This may be the result of the nature and number of the mutations present, or a need for further PCR optimization. Sequencing of PCR fragments in one of these cases confirmed the observed segregation based on the number and phase of SNPs present in the parents, simplifying deduction of the progeny genotypes by melting curve analysis for linkage mapping of one of the SSR primer pairs. In the other primer pair, sequencing of the parents indicated two areas to target for primer redesign. Redesigning the primers to generate a smaller PCR amplicon that more closely targets these SNPs and/or further optimization of PCR conditions is expected to lead to clear and reproducible results.

While the use of HRM has been shown to be effective in discerning differences in repeat number of SSR motifs (Mackay et al., 2008; Mader et al., 2008), there may be some challenges for the use of HRM for SNP detection in SSR fragments. For example, SNP detection may be more successful in smaller PCR amplicons where polymorphism is likely to have a larger effect on the melting temperature. Additionally, SSR stutter may introduce noise, as stutter fragments will have a different melting temperature and profile from each other and could lead to difficulties in determining segregation. This study illustrates the potential for HRM as a relatively inexpensive tool for detection of sequence differences in SSR amplicons with no size polymorphism, adding utility to existing primers that are otherwise uninformative.

ACKNOWLEDGEMENTS

We would like to thank the USDA-ARS Northwest Center for Small Fruits Research and the Oregon Raspberry and Blackberry Commission for funding as well as April Nyberg (USDA-ARS NCGR) and Jason McKinney (Idaho Technology Inc.) for their technical assistance with this project.

Literature Cited

Figures

Fig. 1. Normalized and temperature-shifted melting curves for six *Rubus* SSRs with no size polymorphism in duplicate samples of ORUS 3021-2 and ORUS 4153-1 black raspberry genotypes. Panels 1-5, SNPs detected from melting curve differences based on genotype. Panel 6, no melting curve differences present.
Fig. 2. Melting profiles for the *Rubus* SSR Gen003 for 24 progeny in the black raspberry population ORUS 3021-2 x ORUS 4153-1. Progeny with the haplotype matching ORUS 3021-2 are in black while those with the haplotype matching ORUS 4153-1 are in gray. Normalized and temperature-shifted melting curves (above) and normalized and temperature-shifted difference curves (below) illustrate clear segregation in the progeny.