Study of Transferability of *Rubus* Microsatellite Markers to Hybrid Boysenberry

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ABSTRACT Boysenberry, a *Rubus* hybrid between loganberry and a trailing blackberry, possesses distinctive polyphenol compounds, which have demonstrated positive biological effects on human health. Several new boysenberry genotypes have recently been developed from mutation breeding technology. In this study, a total of 103 SSR markers developed from expressed sequence tag (EST) and genomic libraries in blackberry and red raspberry were tested for cross-amplifications in 10 boysenberry genotypes. All primer pairs successfully produced amplification products, ranging from 1 to 4 loci per primer. Eleven polymorphic SSR markers (RH_MEa0007aB01, RH_MEa12cE03, RH_MEa14bF07, RH_MEa15aD04, RH_MEa13cF08, ERubLR_SQ01_N03, ERubLR_SQ053_H01, ERubLR_SQ191_A05, RubfruitG7, Rubusr43a, and RiM019) were detected among boysenberry genotypes, while polymorphic loci were not detected in 92 markers. Polymorphism information content (PIC) and genetic diversity (GD) values ranged from 0.160 to 0.580 and from 180 to 0.640, with average values of 0.359 and 0.407, respectively, in the 11 polymorphic markers. According to a cluster analysis, all the mutant boysenberry genotypes can be classified into one category. Although the level of genetic diversity revealed by SSR markers in 10 boysenberry genotypes was low, these SSR markers will be useful for future genetic diversity, cultivar identification, QTL mapping, and gene cloning studies in boysenberry.

Keywords Boysenberry, SSR marker, Genetic diversity, Mutation, Gamma-ray

INTRODUCTION

Fruits of *Rubus* species are a rich source of anthocyanins and phenolics that provide a broad spectrum of biomedical functions in human health (Zafra-Stone *et al.* 2007). Boysenberry (*Rubus ursinus* Chamisso & Schlenhtendal) is a *Rubus* hybrid between loganberry (*Rubus loganobaccus* Bailey) and a trailing blackberry (*Rubus baileyanus* Britt.) (Wood *et al.* 1999). Boysenberry fruits, which are usually consumed in fresh and processed products in human diet, contain a unique mixture of polyphenols including ellagitannins, short oligomeric proanthocyani-

dins, anthocyanins, and ellagic acid (Furuuchi *et al.* 2011). Recent studies have indicated that the intake of polyphenols in boysenberry might offer positive effects such as reduction of oxidative stress (Barnett *et al.* 2007), prevention of liver injury (Igarashi *et al.* 2004), anti-hypertension (Furuuchi *et al.* 2012), and anti-obesity effects (Mineo *et al.* 2015). In addition, boysenberry had the highest level of free ellagic acid content among *Rubus* species (Wada and Ou 2002). The ellagic acid is well known as a potential anticarcinogenic compound against colon cancer, breast cancer, prostate cancer, and skin cancer (Zhang *et al.* 2014).

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Generally, breeding progress in *Rubus* species has been limited by a lack of genetic variation for important agronomic traits in germplasms. Therefore, interspecific hybridization and mutation breeding constitute important breeding techniques in *Rubus* species. The inter-specific hybridization between red raspberry and blackberry have generated several new cultivars such as Loganberry, Nessberry, Phenomenal, and Brazos (Ipek *et al.* 2009). In addition, boysenberry is a “hybridberry.” Mutation breeding has been used to improve specific agronomic traits such as bigger fruit sizes, early maturation, higher disease resistance, and higher anthocyanin content in fruits (Ryu *et al.* 2012; Ryu *et al.* 2016). Recently, a new blackberry cultivar, Maple, with high sugar content in fruit was developed via gamma-irradiation (Kim *et al.* 2013).

The narrow range of variation of morphological traits in *Rubus* species has thus far limited the accurate characterization of *Rubus* germplasm and cultivars. DNA markers could be a useful tool for cultivar identification and for the assessment of genetic diversity in breeding programs. In red raspberry, highly polymorphic and co-dominant simple sequence repeat (SSR) markers have been developed from genomic and cDNA libraries (Graham *et al.* 2002; Graham *et al.* 2004; Woodhead *et al.* 2008). Castillo *et al.* (2010) also developed SSR markers from genomic libraries of red raspberry and blackberry. These SSR markers could be transferable across *Rubus* species and have been used to differentiate between wild and cultivated Andean blackberry (*Rubus glaucus*) (Marulanda *et al.* 2012), to study genetic diversity in wild and cultivated black raspberry accessions (Dossett *et al.* 2012), and to construct a genetic

map of tetraploid blackberry (Castro *et al.* 2013). However, there are no studies on the development or application of SSR markers in boysenberry. Only dominant AFLP markers have been applied to assess the genetic relationship between some blackberry cultivars and boysenberry (Ipek *et al.* 2009).

Recently, several boysenberry mutant lines showing high anthocyanin and ellagic acid contents have been developed via gamma-irradiation (Ryu *et al.* 2017). In this study, 103 SSR markers previously developed for other *Rubus* species were tested to determine their transferability in boysenberry and were used to assess the genetic diversity in boysenberry mutant genotypes.

MATERIALS AND METHODS

Plant materials and DNA extraction

Ten boysenberry genotypes were used in this study (Table 1). These genotypes were used to analyze fruit quality and antioxidant components in previous study (Ryu *et al.* 2017). The BS_PI genotype, which is spiny, was introduced from Japan, and others included stabilized lines from advanced generations, all of which are thornless. The BS_Hybrid was developed from the cross between the thornless blackberry (*R. fruticosus* L. ‘V3’) and the BS_PI which was introduced from Japan (Shin *et al.* 2008). Six mutant genotypes (BSA-036 to BSA-144) were developed from 20 Gy gamma-ray treatments on explants of the hybrid boysenberry. Two mutant genotypes (BSB-032 and BSB-127) were developed from 40 Gy gamma-ray treat-

Table 1. List of 10 boysenberry genotypes evaluated with 103 SSR markers (Ryu *et al.* 2017).

No.	Line	Origin	Treatment	Stem Spiny
1	BS_PI	Boysenberry from Japan		Spiny
2	BS_Hybrid	Cross breeding	Blackberry(V3) × BS_PI	Thornless
3	BSA-036	Hybrid Boysenberry	Gamma-ray 20 Gy	Thornless
4	BSA-065	Hybrid Boysenberry	Gamma-ray 20 Gy	Thornless
5	BSA-078	Hybrid Boysenberry	Gamma-ray 20 Gy	Thornless
6	BSA-101	Hybrid Boysenberry	Gamma-ray 20 Gy	Thornless
7	BSA-119	Hybrid Boysenberry	Gamma-ray 20 Gy	Thornless
8	BSA-144	Hybrid Boysenberry	Gamma-ray 20 Gy	Thornless
9	BSB-032	Hybrid Boysenberry	Gamma-ray 40 Gy	Thornless
10	BSB-127	Hybrid Boysenberry	Gamma-ray 40 Gy	Thornless

ments on explants of the hybrid boysenberry. Freshly growing young leaves from each genotype were harvested for DNA samples. DNA was extracted using a DNeasy plant mini kit (Qiagen, Hilden, Germany) and quantified using a UV-Vis spectrophotometer (NanoDrop 2000, Thermo Scientific, DE, USA).

SSR analysis

A total of 103 SSR markers previously developed from expressed sequence tag (EST) and genomic libraries in blackberry (*Rubus fruticosus* L.) and red raspberry (*Rubus idaeus* L.) were used in this study. These primers are summarized in Table 2 and Supplementary Table S1. PCR amplification used a 10 µL reaction mix containing 2 µL of 40 ng template DNA, 1.0 × PCR buffer, 3.0 mM MgCl₂, 100 µM of each dNTP, 0.2 µM each of forward and reverse primers, and 0.5 units of GoTaq DNA polymerase (Promega, Madison, USA). PCR cycling conditions were 30 second DNA denaturation step at 94°C, a 30 second annealing step at 50°C, and a 1 minute extension step at 72°C for 35 cycles on a TProfessional Basic thermocycler (Biometra, Göttingen, Germany). PCR amplifications were separated by capillary electrophoresis using a Fragment Analyzer system (Advanced Analytical Technologies, IA, USA) with dsDNA 900 Reagent Kit. After capillary electrophoresis separation, the SSR alleles were scored using PROSize 2.0 software (Advanced analytical Technologies, IA, USA).

Data analysis

Polymorphic validation and genetic diversity assessments, including polymorphism, gene diversity (GD), and

polymorphism information content (PIC) of the alleles revealed by each primer pair were calculated from the genotype data from 10 boysenberry individuals using PowerMarker v3.25 software (Liu and Muse 2005). A phylogenetic dendrogram was constructed using the unweighted pair-group method with arithmetical algorithms averages (UPGMA) method by NTSYS PC 2.1 software (Exeter Software, NY, USA). Genetic distance matrix was calculated using Nei (1978)'s unbiased genetic distance measure.

RESULTS

SSR marker selection

A total of 103 primer pairs were tested against 10 boysenberry genotypes (Table 2). These SSR markers were selected from previous works. Among those, 53 SSR markers were developed from a blackberry and red raspberry genomic library, and 50 SSR markers were derived from a blackberry and red raspberry EST library (Graham *et al.* 2004; Lewers *et al.* 2008; Castillo *et al.* 2010; Castro *et al.* 2013; Woodhead *et al.* 2008). All primer pairs amplified a product in each of the 10 boysenberry genotypes that showed 100% transferability in boysenberry. Of these, only eleven SSR markers (10.7%) revealed genetic differentiation among boysenberry genotypes, while polymorphic loci were not detected in 92 SSR primers (Table 2). Two out of 53 SSR markers (3.8%) derived from the genomic library were found to be polymorphic for the 10 genotypes. However, 9 out of 50 SSR markers (18%)

Table 2. Amplification profiles for 103 SSR markers tested in 10 boysenberry genotypes.

Type of SSR markers	Primer pairs tested	Amplification success (%)	No. polymorphic markers	Ratio of polymorphic markers (%)	References
Genomic SSR of blackberry	4	100	0	0	Castro <i>et al.</i> (2013)
Genomic SSR of blackberry and red raspberry	10	100	1	10	Castillo <i>et al.</i> (2010)
Genomic SSR of red raspberry	39	100	1	2.6	Graham <i>et al.</i> (2004)
EST SSR of blackberry	19	100	5	26.3	Lewers <i>et al.</i> (2008)
EST SSR of red raspberry	23	100	3	13.0	Woodhead <i>et al.</i> (2008)
EST SSR of red raspberry	8	100	1	12.5	Graham <i>et al.</i> (2004)
Total (Mean)	103	(100)	11	(10.7)	

derived from the EST library were polymorphic. Therefore, the polymorphic rate of EST-SSR markers was approximately 4.7 times higher than that of genomic-SSR markers in this study. In addition, the blackberry-derived EST-SSR markers showed the highest polymorphic rates (26.3%) among boysenberry genotypes.

The number of loci, number of polymorphic loci, and polymorphic rate of boysenberry genotypes are presented in Supplementary Table S1. Polymorphism analysis detected 123 loci generated from all 103 SSR primer set, of which 27 loci were polymorphic, representing 21.9% of the

total generated loci. The total number of loci per primer ranged from 1 to 4. Eight SSR primers (ERubLR_SQ01_N03, ERubLR_SQ191_A05, RH_MEa0007aB01, RiM019, Rubusr43a, RH_MEa12cE03, RH_MEa15aD04, and RH_MEa13cF08) gave the highest percentage of polymorphic loci (100%).

The observed number of alleles (N_a), effective number of alleles (N_e), gene diversity (GD) and PIC of boysenberry genotypes were analyzed with 11 polymorphic SSR markers (Table 3). The mean N_a value was 1.909 and ranged from 1.5 to 2.0. The mean N_e value was 1.421 and

Table 3. Characteristics of 11 SSR markers showing polymorphisms in 10 boysenberry genotypes.

No.	Marker name	Type of SSR	$N_a^{2)}$	$N_e^{3)}$	GD ^{x)}	PIC ^{w)}
1	RH_MEa0007aB01	EST SSR of blackberry	2.000	1.458	0.640	0.580
2	RH_MEa12cE03	EST SSR of blackberry	2.000	1.220	0.340	0.310
3	RH_MEa14bF07	EST SSR of blackberry	2.000	1.584	0.640	0.580
4	RH_MEa15aD04	EST SSR of blackberry	2.000	1.395	0.580	0.540
5	RH_MEa13cF08	EST SSR of blackberry	2.000	1.490	0.180	0.160
6	ERubLR_SQ01_N03	EST SSR of red raspberry	2.000	1.220	0.180	0.164
7	ERubLR_SQ053_H01	EST SSR of red raspberry	1.500	1.500	0.420	0.330
8	ERubLR_SQ191_A05	EST SSR of red raspberry	2.000	1.345	0.620	0.540
9	RubfruitG7	EST SSR of red raspberry	2.000	1.490	0.180	0.164
10	Rubusr43a	Genomic SSR of red raspberry	2.000	1.571	0.380	0.310
11	RiM019	Genomic SSR of blackberry and red raspberry	1.500	1.362	0.320	0.270
Mean			1.909	1.421	0.407	0.359

²⁾ N_a : Observed number of alleles, ³⁾ N_e : Effective number of alleles, ^{x)}GD: Gene diversity, ^{w)}PIC: Polymorphism information content.

Table 4. Putative function of EST-SSR markers showing polymorphisms in 10 boysenberry genotypes.

No.	Marker name	GenBank accession no.	Homology	E-value
1	RH_MEa0007aB01	FF683655	<i>Fragaria vesca</i> subsp. <i>vesca</i> E3 ubiquitin-protein ligase SIS3	1e-155
2	RH_MEa12cE03	FF684289	<i>Fragaria vesca</i> subsp. <i>vesca</i> uncharacterized LOC101302468	1e-119
3	RH_MEa14bF07	FF684762	<i>Fragaria vesca</i> subsp. <i>vesca</i> chlorophyll a-b binding protein of LHCII type 1-like	0.0
4	RH_MEa15aD04	FF684949	<i>Fragaria x ananassa</i> zeta-carotene desaturase protein (zds)	0.0
5	RH_MEa13cF08	FF684532	<i>Fragaria vesca</i> subsp. <i>vesca</i> ranBP2-type zinc finger protein At1g67325	2e-31
6	ERubLR_SQ01_N03	EX567290	<i>Fragaria vesca</i> subsp. <i>vesca</i> major allergen Pru av 1-like (LOC101298594)	3e-121
7	ERubLR_SQ053_H01	EX567274	<i>Rosa x bourboniana</i> xyloglucan endotransglucosylase/hydrolase 8 (XTH8)	1e-78
8	ERubLR_SQ191_A05	EX567282	<i>Prunus mume</i> RING-H2 finger protein ATL20-like	6e-40

ranged from 1.220 (ERubLR_SQ01_N03) to 1.584 (RH_MEa14bF07). The GD for 11 polymorphic SSR markers ranged from 0.180 to 0.640, with a mean of 0.407. The PIC of the 11 polymorphic SSR markers ranged from 0.160 to 0.580, with an average of 0.359. The maximum values of GD (0.640) and PIC (0.580) occurred with the RH_MEa0007aB01 and RH_MEa14bF07 primers that were derived from the EST library for blackberry.

EST-SSR markers associated with expressed genes are useful to study functional diversity in populations. The putative function of 8 EST-SSR markers showing high PIC values were determined by using BLAST against the non-redundant GenBank database at the National Center for Biotechnology Information (NCBI) (Table 4). The 8 EST sequences showed significant similarities to known genes including E3 ubiquitin-protein ligase, chlorophyll a-b binding protein, zeta-carotene desaturase protein, allergen Pru av1-like, xyloglucan endotransglucosylase/hydrolase 8, and RING-H2 finger protein. The most homologies were from *Fragaria vesca*.

Genetic distance and cluster analysis

Genetic distance matrix (GDM) obtained from 27 polymorphic loci were shown in Table 5. The scale used for the genetic distance runs from 0 (meaning no genetic difference) to 0.898 with an average of 0.268. BSA-119 (No. 7), BSA-114 (No. 8), and BSB-127 (No. 10) exhibited no genetic difference among genotypes. Original cultivar (Hybrid boysenberry) and BSA-036 were the most distantly related.

Based on the simple-matching coefficient between boysenberry genotype, a cluster analysis was carried out and a dendrogram was generated with 27 polymorphic loci via the average linkage method for genetic relationships (Fig. 1). According to cluster analysis of the SSR markers, boysenberry genotypes could be divided into a gamma-ray-treated group (BSA-036, BSA-065, BSA-078, BSB-032, BSA-101, BSB-127, BSA-119 and BSA-144) and a non-treatment group (boysenberry and hybrid boysenberry), divided at the genetic distance of 0.485. However, a gamma-ray-treated group was not classified according to the dosage of gamma rays in the dendrogram.

Fig. 1. Phylogenetic tree based on boysenberry genotypes of 11 polymorphic SSR markers.

Table 5. Matrix of Nei (1978)'s genetic distances between boysenberry genotype.

Line No. ²⁾	1	2	3	4	5	6	7	8	9	10
1	0.000									
2	0.160	0.000								
3	0.898	0.898	0.000							
4	0.463	0.463	0.588	0.000						
5	0.406	0.406	0.523	0.205	0.000					
6	0.588	0.588	0.588	0.251	0.205	0.000				
7	0.588	0.588	0.463	0.351	0.118	0.077	0.000			
8	0.588	0.588	0.463	0.351	0.118	0.077	0.000	0.000		
9	0.731	0.731	0.588	0.463	0.205	0.160	0.077	0.077	0.000	
10	0.588	0.588	0.463	0.351	0.118	0.077	0.000	0.000	0.077	0.000

²⁾Line No.: Line number is listed in Table 1.

DISCUSSION

In berry crops, mutation breeding has already been used to introduce many useful traits affecting fruit quality, phytochemicals, disease resistance, resistance to low temperature, and the presence or absence of thorns (Basaran and Kepenek 2011; Ryu *et al.* 2016). In this study, the 'BS_Hybrid' genotype, obtained from crosses between the thornless blackberry (*R. fruticosus* L. 'V3') and boysenberry, has unfavorable fruit characteristics such as malformed fruit, small size, and lower sugar content. However, the mutant genotypes, obtained from gamma-ray mutations with 'BS_Hybrid', showed improved agronomic characteristics such as higher fruit yields, sugar contents, and antioxidant components when compared with the original parent (Ryu *et al.* 2017). These mutant genotypes can be directly released as new cultivars.

Molecular markers were useful tools for the evaluation of genetic diversity, marker-assisted breeding, DNA fingerprinting, and cultivar identification in crops. However, there are many challenges in developing molecular markers in *Rubus* species for which genomic information is not available. Therefore, previous studies have analyzed the genetic diversity of boysenberry and blackberry using AFLP markers (Ipek *et al.* 2009; Ryu *et al.* 2014). While no sequence information is needed, the AFLP marker system is not as easy to perform as SSR markers, which show highly reproducible, co-dominant, multi-allelic, PCR-based, and medium throughput to assay (Dossett *et al.* 2012; Stafne *et al.* 2005). In particular, SSR markers developed for one plant species can be transferred between related species (Varshney *et al.* 2005). The high level of transferability of SSR markers could reduce the costs of marker development in boysenberry. In this study, a total of 103 SSR markers previously developed from EST and genomic libraries in blackberry and red raspberry showed 100% transferability to 10 boysenberry genotypes. The high level of transferability of SSR markers among three *Rubus* species means that they have a close genetic relationship with each other (Ipek *et al.* 2009; Lee *et al.* 2015). Similarly, high rates of transferability of SSR markers were reported in pine (94.2% between *Pinus taeda* and *Pinus radiata*), buckwheat (97.1% between *Fagopyrum esculen-*

tum and *Fagopyrum homotropicum*), and onion (93.5% between *Allium fistulosum* and *Allium altaicum*) (Araki *et al.* 2010; Chagné *et al.* 2004; Ma *et al.* 2009).

Screening for polymorphism of SSR markers rendered the 11 polymorphic markers (10.7%) in boysenberry genotypes. This rate is low compared to the 18% to 30% polymorphisms between the two blackberry genotypes using SSR markers derived from *Rubus* and *Fragaria* L. (Castro *et al.* 2013; Stafne *et al.* 2005). The lower rate of polymorphism may be the result of the eight boysenberry genotypes developed from one BS_Hybrid through gamma-ray mutation techniques, as well as BS_Hybrid derived from the cross with the BS_PI. However, the blackberry-derived EST-SSR markers showed the 26.3% polymorphisms that had similar rates to the previous two studies of Castro *et al.* (2013) and Stafne *et al.* (2005).

The EST-SSR markers representing transcribed genes can be used for assaying functional diversity in accessions as well as yielding a higher rate of polymorphism (Varshney *et al.* 2005). Nine polymorphic SSR markers were developed from a blackberry leaf and raspberry root and fruit EST library (Graham *et al.* 2004; Lewers *et al.* 2008; Woodhead *et al.* 2008). The putative functions of two EST-SSR markers (RH_MEa0007aB01 and RH_MEa14bF07) showing the highest PIC value (0.580) were similar with *Fragaria vesca* subsp. *vesca* E3 ubiquitin-protein ligase and chlorophyll a-b binding protein, respectively (Table 4). Interestingly, the putative function of RH_MEa15aD04 marker was similar to zeta-carotene desaturase protein essential for carotenoid biosynthesis (Nisar *et al.* 2015). Avendaño-Vázquez *et al.* (2014) reported that the mutation of zeta-carotene desaturase caused severe alterations in leaf morphology. However, any abnormal leaf morphology was not detected within our boysenberry genotypes. In addition, the ERubLR_SQ01_N03 marker showing the putative function of major allergen Pru av1 (*Prunus avium*) can be used as a potential diagnostic marker for severe allergic reactions to boysenberry genotypes (Scheurer *et al.* 1997).

In the clustering analysis, the eight gamma-ray mutagen-treated genotypes and the original genotypes (BS_PI and BS_Hybrid) were divided into independent groups. Ryu *et al.* (2014) reported that blackberry mutants could be

divided into gamma-ray and MNU-treated groups and crossbred (*R. fruticosus* × *R. parvifolius*) lines by AFLP markers. Also, individuals of *Cymbidium* spp. were divided into control and electron beam treatment groups using both ISSR and RAPD markers (Ryu *et al.* 2013). These results suggest that radiation could induce significant genetic variability in boysenberry genotypes. The GDM among boysenberry genotypes ranged from 0 to 0.898 with an average 0.268, which was similar to 11 cultivars of boysenberry and blackberry spanning from 0 to 0.85 by AFLP analysis (Ipek *et al.* 2009).

Analysis of genetic diversity and relationships may be helpful for QTL mapping and for gene cloning studies. This study also provides 11 polymorphic SSR markers among the boysenberry genotypes that would be useful for identification of cultivars. Therefore, these results may contribute to the selection of parental lines for both traditional and mutational breeding of boysenberry.

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