Microsatellite-Based Fingerprinting of Western Blackberries from Plants, IQF Berries and Puree

N.V. Bassil[1, a], M. Muminova[2] and W. Njuguna[3]
[1]USDA-ARS, National Clonal Germplasm Repository (NCGR), 33447 Peoria Road, Corvallis, Oregon 97333, USA
[2]Tashkent Chemical-Technology Institute, 23 Navoi Street, 100011, Tashkent, Uzbekistan
[3]Dept. of Horticulture, Oregon State University, ALS 4017, Corvallis, OR 97331, USA

Keywords: individually quick-frozen (IQF), simple sequence repeat (SSR) markers, *Rubus* species

Abstract

The blackberry industry needs a reliable method to ensure trueness-to-type of blackberry products. Microsatellite markers or simple sequence repeats (SSRs) are ideal for cultivar fingerprinting, paternity testing and identity certification. Fingerprinting is valuable for variety identification, quality control and as a legal method to protect against infringement by competitors. The objectives of this study were to develop a DNA extraction protocol and SSR-based identification for individually quick-frozen (IQF) 'Marion' and 'Kotata' whole berries and concentrate and to generate genetic fingerprints for 16 important western blackberry cultivars. IQF berries and frozen concentrate of 'Marion' and 'Kotata' were generously provided by reliable commercial sources. The FAST ID Kit worked better than two other DNA extraction methods for isolating DNA from IQF berries and from frozen and thawed concentrate. Out of twenty-nine SSRs tested, ten polymorphic SSRs differentiated between 'Marion' and 'Kotata' leaves and were chosen for subsequent analyses. SSR-based fingerprinting of individual IQF berries (using the receptacle for DNA extraction) revealed a mixture of 'Kotata' and 'Marion' berries in the commercial 'Marion' bag while fingerprinting of frozen 'Marion' concentrate identified 'Kotata' in the small frozen puree sample evaluated and possible contamination from seed DNA. The ten SSRs differentiated between each of the 16 western cultivars included in this study. In fact, one SSR marker, Rubus 275a was sufficient to distinguish these 16 cultivars. In summary, blackberry can be reliably identified with SSR markers, using leaves and frozen berries as sources of DNA. Fingerprinting from concentrate does not appear reliable for identity certification due to possible contamination from seed DNA.

INTRODUCTION

Blackberry, an aggregate fruit of *Rubus* species subgenus *Rubus* in the Rosaceae family, has long been a favorite wild fruit. *Rubus* species are native to several countries where they are picked for personal or commercial use. The US is the leading producer of cultivated blackberries in the world. Oregon dominates the US production and the Corvallis-based breeding program is the oldest continuously active blackberry breeding program in the world (Clark et al., 2007). Most of the cultivars released from that program and grown for the processing industry are of the trailing type. They are largely derived from the western dewberry (*Rubus ursinus* Cham. & Schlt.), but have several other species in their background. Over 95% of the blackberries in Oregon are harvested for processing, most commonly as individually quick-frozen (IQF) or pureed product. In 2008, Oregon produced 39.2 million pounds of blackberries valued at 3.8 million dollars as fresh fruit and 19 million dollars in processed products (USDA-NASS, 2009). Over 4000 acres of this production is in the cultivar Marion, believed to

[a] Nahla.bassil@ars.usda.gov

have ideal processing characteristics such as: high soluble solids, high titratable acidity, excellent flavor, low perception of seediness, and good color and size (Yorgey and Finn, 2005).

The blackberry industry is in critical need of a reliable method for ensuring trueness-to-type of their fruit products. Molecular markers and, in particular, microsatellite or simple sequence repeat (SSR) markers are ideal for cultivar fingerprinting, paternity testing and identity certification. When this study was initiated, the number of SSR markers in blackberry was limited. Eight SSRs from 'Marion' blackberry and four markers from 'Meeker' raspberry were developed in our lab and used to develop genetic fingerprints for 48 blackberry cultivars (Castillo et al., 2009). Additional microsatellite markers were developed mostly from *R. idaeus* (Graham et al., 2002, 2004, 2006). A small number of SSRs were isolated from other *Rubus* species: eight from *R. alceifolius* Poir. (Amsellem et al., 2001) and 15 from *R. hochstetterorum* Seub. (Lopes et al., 2006). A minimum of 14 *R. idaeus* and one *R. alceifolius* SSRs were polymorphic in parents of a mapping blackberry population (Stafne et al., 2005) and 13 of 15 microsatellite markers cross-amplified in *R. fruticosus* auct. aggr. (Lopes et al., 2006). The objectives of this study were to evaluate existing SSRs for cultivar identification in blackberry. Our goals were to develop a microsatellite-based identification protocol for IQF 'Marion' and 'Kotata' berries and puree using polymorphic SSR markers; and to fingerprint 16 of the most important western cultivars.

MATERIALS AND METHODS

Fresh leaves and berries of 'Marion' and 'Kotata' were collected from the USDA-ARS blackberry breeding field in Corvallis, Oregon. Actively growing leaves of the remaining fourteen cultivars were obtained in the spring from the NCGR screenhouse (Table 1). IQF 'Marion' and 'Kotata' berries and frozen concentrate were obtained from reputable commercial sources. Tissue samples were homogenized with an MM 301 Mixer Mill (Retsch International, Haan, Germany) and DNA was extracted using three different methods including: a modified Puregene (Gentra Systems Inc., Minneapolis, MN) protocol used routinely in the NCGR lab (Proteinase K and RNase A digestion steps were included in the extraction and protein precipitation was repeated twice); a Qiagen DNeasy mini kit (Qiagen Inc., Valencia, CA); and a Fast ID kit (Genetic ID NA, Inc., Fairfield, IA). The samples used to identify 'Marion' and 'Kotata' included leaves, receptacles from fresh berries, drupelets from fresh berries, receptacles from IQF berries, drupelets from IQF berries and frozen concentrates. For fingerprinting the 16 western cultivars, DNA was extracted from actively-growing leaves.

Thirty-two primer pairs were tested for polymorphism between 'Marion' and 'Kotata' by visual inspection of ethidium bromide-stained agarose (3%) gel electrophoresis of the PCR products. The primers included: six SSRs from 'Marion' (RhM001, RhM011, RhM021, RhM023, RhM031, and RhM043) (Castillo et al., 2009); three primers pairs from the red raspberry 'Meeker' (RiM017, RiM019, and RiM036) (Castillo et al., 2009); thirteen *R. idaeus* SSR markers (Graham et al., 2004) that cross-amplified in the parents of a blackberry mapping population (Rubus 105b, Rubus 107a, Rubus 117b, Rubus 119a, Rubus 145a, Rubus 194h, Rubus 270a, Rubus 275a, Rubus 262b, RubfruitC1 and RubfruitE4) (Stafne et al., 2005); and ten microsatellite primer pairs developed in *Rubus hochstetterorum* that generated two or more alleles in *Rubus fruticosus* (ssrRhCBA5, ssrRhCBA6, ssrRhCBA12, ssrRhCBA15, ssrRhCBA16, ssrRhCBA18, ssrRhCBA21, ssrRhCBA23, ssrRhCBA27, and ssrRhCBA28) (Lopes et al., 2006). Ten SSRs clearly differentiated between 'Marion' and 'Kotata' and were subsequently used (Table 2).

PCRs were performed in 10 µl volume containing 1× reaction buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM of each primer, 0.25 U of Biolase *Taq* DNA polymerase (Bioline), and 2.5 ng genomic DNA. The PCR protocol consisted of initial

denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 93°C for 40 s, annealing at optimum T_a for 40 s, and extension at 72°C for 40 s, and a final extension cycle at 72°C for 30 min. DNA was amplified in an Eppendorf Gradient thermocycler (Eppendorf, Westbury, NY) or an MJ Research Tetrad thermocycler (BioRad, Hercules, CA). PCR products were separated in 3% agarose gels and scored for amplification and polymorphism after visualization by ethidium bromide staining. PCR products were pooled for fragment analysis prior to separation on a Beckman CEQ 8000 genetic analyzer (Beckman Coulter Inc., Fullerton, CA). Allele sizing and visualization were performed using the fragment analysis module of the CEQ 8000 software. Due to the multiple ploidy levels of the blackberry cultivars, individuals were scored for the presence or absence of each allele and each allele was treated as a separate locus. PowerMarker (Liu and Muse, 2004) was used for Neighbor Joining clustering and bootstrap analysis.

RESULTS AND DISCUSSION

Three DNA extraction methods were used to isolate DNA from leaf, frozen and fresh receptacle, frozen and fresh berry homogenate, and frozen puree. The DNA was tested for its ability to amplify with the PCR conditions described above. Leaf tissue generated amplifiable DNA with each of the three methods. DNA extracted from berry tissue failed to amplify when isolated with the modified Puregene protocol but generated products with the other two methods. The FAST ID kit was the only DNA isolation technique that generated easy to amplify DNA irrespective of tissue source. Therefore, we recommend the FAST ID kit for extracting DNA from blackberry berry tissue, leaves or concentrates.

In ‘Kotata’, the genetic fingerprint of berry receptacle was identical to that obtained from leaves irrespective of fresh or frozen status. However, additional alleles, not amplified from leaves, were generated when the DNA was extracted from berries (data not shown), indicating possible contamination from seed DNA. The genetic profile of frozen ‘Marion’ berry receptacle was different from that of leaves and fresh berries of ‘Marion’. DNA was then separately extracted from receptacles of three individual berries from the IQF ‘Marion’ berries bag. The genetic profile of two out of the three berries was identical to ‘Kotata’ while the remaining berry generated a ‘Marion’ fingerprint, thus indicating that the ‘Marion’ berries bag contained a mix of ‘Kotata’ and ‘Marion’ berries.

At least two independent DNA extractions were performed from each of the ‘Marion’ and ‘Kotata’ puree bags (Table 3). When compared to the genetic profiles obtained from the corresponding leaves of ‘Kotata’ and ‘Marion’, fingerprints of the resulting puree contained: additional alleles not present in leaves of the characteristic cultivar (e.g. at Rubus 145a: 134 bp in Marion Puree-1; 125, 132 and 139 bp in Kotata Puree-2); or missing alleles (e.g. at ssrRhCBA18: 171 and 209 in Kotata Puree-1; and 118 in Marion Puree-2) (Table 3). Additional alleles were either characteristic of ‘Kotata’ or ‘Marion’, indicating possible contamination of puree from these two common cultivars; or were not present in either cultivar indicating possible contamination from seed or from other cultivars that are grown in the Northwest. Each of the puree samples taken from the same bag had a different fingerprint, leading us to conclude that sampling strategies using varying concentrations of pure puree from each cultivar must be optimized for accurate detection of its genotypic composition. Alternatively cultivar certification from concentrate might not be possible due to contamination from seed.

The ten SSRs used in this study were highly polymorphic, an expected outcome given that they were chosen based on their ability to differentiate between ‘Marion’ and ‘Kotata’. The number of alleles ranged from nine (Rubus 259f) to 21 (Rubus 275a and ssrRhCBA18) in the 16 Western blackberry cultivars evaluated (Table 2). They differentiated among each of the 16 Western cultivars as illustrated in the UPGMA dendrogram (Fig. 1). In fact, one SSR marker, Rubus 275a was sufficient to distinguish

these 16 cultivars (data not shown). These 16 cultivars were grouped based on breeding programs into an Oregon Group of high ploidy (> 6x-9x) released from the USDA-ARS Corvallis breeding program, and a Non-Oregon Group that contained the Brazilian cultivar Tupy that was sister to the tetraploid, semi-erect thornless cultivars Hull Thornless (Beltsville, MD), Cacanska Bestrna (Serbia), Loch Ness (Scotland) or erect Navaho (Arkansas, US). In the consensus tree generated based on the proportion of shared allele distance, considerable bootstrap support (>75) was obtained for two groups: parent 'Kotata' and its offspring 'Black Diamond'; and the Non-Oregon Group (Fig. 1).

CONCLUSIONS

In summary, blackberry can be identified with SSR markers, using leaves, receptacles from frozen berries, and concentrate as sources of DNA. Further studies are needed to optimize DNA sampling from puree in order to reliably detect contamination in concentrate. Alternatively cultivar certification from concentrate might not be possible due to contamination from seed. The ten SSRs presented in this study can easily differentiate between blackberry cultivars.

ACKNOWLEDGEMENTS

We would like to thank the Oregon Raspberry and Blackberry Commission and the USDA-ARS CRIS Project 5358-21000-038-00D for funding this project. We are grateful to industry members whose names shall remain anonymous for promptly providing us with IQF berries and puree. We would also like to acknowledge laboratory technical assistance provided by April Nyberg.

Literature Cited

Amsellem, L., Dutech, C. and Billotte, N. 2001. Isolation and characterization of polymorphic microsatellite loci in *Rubus alceifolius* Poir. (Rosaceae), an invasive weed in La Réunion island. Mol. Ecol. Notes 1:33-35.

Castillo, N.R.F., Reed, B., Graham, J., Fernández-Fernández, F. and Bassil, N.V. 2009. Microsatellite markers for raspberries and blackberries. Mol. Breed. (submitted)

Clark, J.R., Stafne, E.T., Hall, H.K. and Finn, C.E. 2007. Blackberry breeding and genetics. Plant Breed. Rev. 29:19-144.

Graham, J., Smith, K., MacKenzie, K., Jorgenson, L., Hackett, C. and Powell, W. 2004. The construction of a genetic linkage map of red raspberry (*Rubus idaeus* subsp *idaeus*) based on AFLPs, genomic-SSR and EST-SSR markers. Theor. Appl. Genet. 109:740-749.

Graham, J., Smith, K., Tierney, I., MacKenzie, K. and Hacket, C.A. 2006. Mapping gene H controlling cane pubescence in raspberry and its association with resistance to cane botrytis and spur blight, rust and cane spot. Theor. Appl. Genet. 112:818-831.

Graham, J., Smith, K., Woodhead, M. and Russell, J. 2002. Development and use of simple sequence repeat SSR markers in *Rubus* species. Mol. Ecol. Notes 2:250-252.

Lopes, M.S., Maciel, G.B., Mendonca, D., Gil, F.S. and Da Camara Machado, A. 2006. Isolation and characterization of simple sequence repeat loci in *Rubus hochstetterorum* and their use in other species from the Rosaceae family. Mol. Ecol. Notes 6:750-752.

Stafne, E.T., Graham, J., Lewers, K.S., Clark, J.R. and Weber, C.A. 2005. Simple sequence repeat (SSR) markers for genetic mapping of raspberry and blackberry. J. Amer. Soc. Hort. Sci. 130:722-728.

USDA-NASS. 2009. Washington and Oregon berry crops: acres, yield, production, price, and value, 2006-08. http://www.nass.usda.gov/Statistics_by_State/Washington/Publications/Berries/berry09.pdf

Yorgey, B.M. and Finn, C.E. 2005. Comparison of 'Marion' to thornless blackberry genotypes as individually quick-frozen and puree products. HortScience 40:513-515.

Tables

Table 1. Sixteen important western cultivars fingerprinted in this study. Plant Introduction Number, pedigree, origin, growth habit, thorniness and ploidy where available in the literature are listed. Habit is trailing (L), semi-erect (S) or erect (E). Plants are thorny (Y) or thornless (T).

Cultivar	PI no.	Pedigree	Origin	Habit	Thorniness	Ploidy
Black Diamond	PI 638257	Kotata x NZ 8610L-163	Oregon, US	L	T	
Black Pearl	PI 638260	ORUS 1117-11 × ORUS 1122-1	Oregon, US	L	T	9x
Boysen	PI 553336	Presumably from a hybridization of *R. ursinus* and *R. idaeus*	California, US	L	Y	7x
Cacanska Bestrna	PI 643970	Dirksen Thornless × Black Satin	Serbia	S	T	4x
Hull Thornless	PI 553299	SIUS 47 × Thornfree	Maryland, US	S	T	4x
Kotata	PI 553293	OSC 743 × OSC 877	Oregon, US	L	Y	7x
Loch Ness	PI 638182	SCRI 74126RA8 × SCRI 75131D2	Scotland, UK	S	T	4x
Marion	PI 553254	Chehalem × Olallie	Oregon, US	L	Y	6x
Navaho	PI 553343	Ark.583 × Ark.631	Arkansas, US	E	T	4x
Nightfall	PI 638263	Marion × Waldo	Oregon, US	L	T	6x
Obsidian	PI 638259	ORUS 828-43 × ORUS 1122-1	Oregon, US	L	Y	6x
Silvan	PI 553308	OCS 742 × Marion	Victoria, Australia	L	Y	6x
Tupy	PI 638226	Uruguay blackberry × Comanche	Rio Grande do Sul, Brazil	L	Y	
Wild Treasure	PI 638265	GP 9-24 × Waldo	Oregon, US	L	T	8x
Young Thornless	PI 618391	Thornless mutant of Youngberry	California, US	L	T	7x
ORUS 1324-1		ORUS 834-5 × ORUS 1045-14	Oregon, US	L	Y	

Table 2. List of 10 microsatellite primer pairs used in this study, SSR motif, optimum annealing temperature (T_a) used in PCR and alleles in bp observed in the 16 western cultivars evaluated.

SSR name	SSR motif	T_a (°C)	Alleles
RhM021	$(TC)_6$	50	254, 271, 274, 278, 280, 282, 284, 286, 288, 290, 292
Rubus 107a	$(AG)_8$	54	151, 153, 159, 171, 173, 177, 179, 181, 187, 191, 234
Rubus 117b	$(CATA)_6$-$(GA)_8$	58	111, 113, 117, 121, 127, 129, 131, 135, 137, 144, 150, 152
Rubus 145a	$(GT)_7$	58	124, 126, 128, 130, 132, 134, 137, 139, 143, 145
Rubus 259f	$(CT)_4$-$(AG)_8$	56	241, 243, 245, 247, 249, 255, 257, 259, 267
Rubus 270a	$(GA)_{10}$	52	135, 137, 139, 141, 151, 159, 161, 163, 165, 167, 171, 177, 179, 184, 186, 190, 196
Rubus 275a	$(AG)_{27}$	56	107, 114, 116, 118, 120, 126, 128, 132, 134, 136, 140, 142, 144, 146, 149, 155, 163, 165, 173, 190, 203
ssrRhCBA18	$(CT)_{26}$	60	108, 110, 112, 114, 116, 118, 120, 151, 161, 163, 165, 171, 209, 211, 224, 226, 232, 234, 252, 332, 338
ssrRhCBA23	$(GA)_{10}G(GA)_5$	60	86, 90, 92, 96, 98, 100, 102, 104, 108, 115, 120, 122, 124, 132, 143, 145
ssrRhCBA27	$(AC)_2(GA)_{24}$	52	180, 205, 215, 217, 219, 228, 230, 252, 263, 273, 275, 281, 296, 298, 300, 312, 332

Table 3. Comparison of allelic composition of ‘Kotata’ and ‘Marion’ from leaves to that obtained from two independent DNA samples of puree generated from four microsatellite primer pairs: Rubus 145a, ssrRhCBA18, ssrRhCBA27, and ssrRhCBA23.

Name	Rubus 145a						ssrRhCBA18					ssrRhCBA27		ssrRhCBA23				
Kotata	128	130	134	141			116	118	161	171	209	312		98	104	108	120	
Kotata Puree-1	128	130	134	141			116	118	161			312		98	104	108	120	
Kotata Puree-2	**125**	128	130	**132**	**139**	141	118	161	171			**224**	**228**	86	**98**	104	108	120
Marion	125	130	132	139			118	161	171			228	300	86	100	104	124	132
Marion Puree-1	125	130	**134**	139			161	171				**312**		98	104	**108**	**120**	
Marion Puree-2	125	130	132	139			118	161	171			228	**250**	86	104	**111**	**117**	**120**

Figures

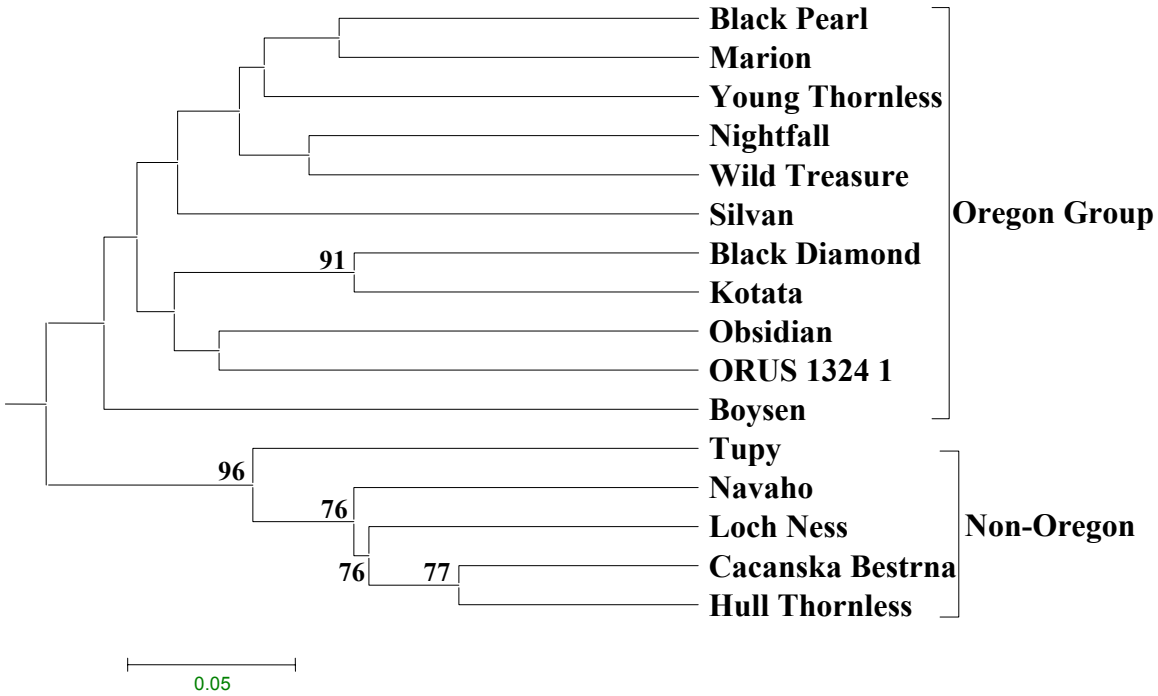

Fig. 1. UPGMA cluster analysis of 16 western blackberry cultivars based on microsatellite analysis using 10 SSR primer pairs. Bootstrap support at >75 is illustrated where found.