A Universal Fingerprinting Set for Red Raspberry

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Abstract
        Red raspberry, *Rubus idaeus* L., is the most economically important fruit crop in the highly diverse *Rubus* subgenus *Idaeobatus*. This subgenus also includes black raspberry *R. occidentalis* L. The USDA-ARS National Clonal Germplasm Repository (Corvallis, Ore.), is responsible for preserving a *Rubus* collection of 1940 accessions that includes 370 red raspberry genotypes originating from 26 countries. These red raspberry clones are maintained as potted plants in screenhouses. Microsatellite or simple sequence repeat (SSR) markers can be used for rapid identity verification. The objective of this study was to develop a universal SSR fingerprinting set for establishing genetic profiles for red raspberry accessions and enabling comparison of genotypes between collections. We tested 24 SSRs for ease of scoring and polymorphism in 35 red raspberry accessions common to both the NCGR and the James Hutton Institute. Ten additional species genotypes with edible berries, including *Rubus* subsp. *Rubus* and *R. trivialis* Mich., the American black raspberry (*R. occidentalis*), purple raspberry (*R. ×neglectus* Peck) and two Asian black raspberry species (*R. biflorus* Buch.-Ham. ex Sm. and *R. niveus* Thunb.) were also examined. Six SSRs were easy to score, polymorphic, and mapped to five of the seven red raspberry linkage groups. They were amplified in two multiplexes and were successful in comparing fingerprints from eight red raspberry accessions at both genebanks. The fingerprinting set differentiated between the unique accessions. This protocol is recommended for scientists and industry for raspberry identity verification.

INTRODUCTION
        Red raspberry, *Rubus idaeus*, is the most economically important fruit crop in the highly diverse *Rubus* subgenus *Idaeobatus*. This subgenus also includes black raspberry *R. occidentalis*. Major world raspberry production regions include Europe, South and North America including central highlands of Mexico, California (U.S.), the Pacific Northwestern U.S., and British Columbia (Canada). The USDA-ARS National Clonal Germplasm Repository (Corvallis, Ore.), is responsible for maintaining the *Rubus* genebank for the United States. This collection includes 1940 total accessions, about a third of which are 370 red raspberry genotypes, originating from 26 countries. GENBERRY is a project funded by the European Commission (DG-AGRI) that promotes conservation and characterization of genetic diversity of small berries, particularly strawberry and raspberry (Denoyes-Rothan et al., 2008). The goals of Work Package 3 (WP3) are to characterize genetic diversity of a representative part of the

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83

European collection using microsatellite markers.

Red raspberries as well as other cultivated species in this genus are propagated vegetatively. They are maintained at the Corvallis genebank as potted plants in screenhouses. Inflorescences are removed to prevent seedling contamination to maintain genotypic integrity. Genotype or cultivar identification in these crops can be difficult solely using elastic morphological vegetative traits. Molecular markers and, in particular, microsatellite or simple sequence repeat (SSR) markers have been successful in other fruit crops for cultivar fingerprinting, paternity testing and identity confirmation. Many microsatellite markers were developed from *R. idaeus* (Graham et al., 2002, 2004, 2006; Castillo et al., 2010). The objectives of this study were to evaluate 24 existing red raspberry SSRs for characteristics that are crucial for reliable fingerprinting such as polymorphism, ease of fluorescence-based automated scoring and reproducibility across labs.

MATERIALS AND METHODS

Fresh leaves from 35 *R. idaeus* genotypes and 10 representatives of other *Rubus* species with edible berries (Fig. 1) were collected from the USDA-ARS NCGR screenhouses in Corvallis, Oregon. DNA was extracted using a modified Puregene (Gentra Systems Inc., Minneapolis, MN) protocol used routinely in the NCGR lab. DNA from the eight reference accessions selected was obtained from the James Hutton Institute.

Twenty-four primer pairs were initially tested. The primer pairs included: RhM003, RhM011, RiM019 (Castillo et al., 2010), Rubus1b, Rubus26a, Rubus102c, Rubus116a, Rubus118b, Rubus126b, Rubus153a, Rubus223a, Rubus228a, Rubus242, Rubus262b, Rubus264b, Rubus270a, Rubus275a, RubfruitE4, RubfruitE8 and RubLeaf97 (Graham et al., 2004), Rub5a, Rub222e, Rub244a and Rub284a (Graham et al., 2006). An M13 tail was added to the 5' end of most of the forward primers and fluorescently labeled universal complementary M13 primers were included in the PCR reaction to allow inexpensive labeling of PCR products as described by Schuelke (2000) and the PCR reaction and protocol was modified accordingly (Schuelke, 2000). After ensuring PCR success by 2% agarose gel electrophoresis, PCR products were pooled for fragment analysis prior to separation on a Beckman CEQ 8000 genetic analyzer (Beckman Coulter Inc., Brea, CA).

After identifying the 6 SSRs to include in the fingerprinting set (Table 1), multiplex PCR was performed in 15 µl volume with the Type-it Microsatellite PCR Kit$^{®}$ (Qiagen Inc., Valencia, CA) as recommended by the manufacturer. Allele sizing and visualization were performed using the fragment analysis module of the CEQ 8000 software. For unweighted pair group method with arithmetic mean (UPGMA) cluster analysis, individuals were scored for the presence or absence of each allele and PowerMarker (Liu and Muse, 2004) was used for cluster analysis.

RESULTS AND DISCUSSION

The 24 SSR primer pairs generated products in each of the *Rubus* species included in this study. For use in a fingerprinting panel, each SSR should be highly polymorphic, distributed throughout the genome and easy to score for automated allele calling. The first phase of this study rated each of the 24 SSRs for absence of PCR artifacts such as stutter, amplification of up to two products in diploid red raspberry as well as high polymorphism as indicated by the number of alleles. Four of the SSRs were easy to score and highly polymorphic in the 34 unique red raspberry accessions: Rubus275a, Rubus126b, RiM019 and RhM011 with 12, 13, 14 and 20 alleles, respectively. They also mapped to linkage groups (LG) 5, 4, 5 and 7, respectively, in the 'Glen Moy' x 'Latham' genetic map (Graham et al., 2004). They were also polymorphic and easy to score in the 10 accessions that represented additional *Rubus* species with edible berries (Fig. 1). Since the PCR products produced by these primer pairs overlapped in size and required amplification in two different multiplexes, we added two SSRs (RhM003 and RubLeaf97) that were easy

to score, polymorphic (each with 6 alleles), mapped to other linkage groups (LG 2 and 6, respectively) and produced PCR products outside the range of these four SSRs.

UPGMA cluster analysis based on these 6 SSRs identified two pairs of cultivars with identical fingerprints: 'Chilliwack' and 'Comox' (results not shown); and 'Glen Clova' and 'Malling Orion' (Fig. 1). Leaf samples of 'Chilliwack' and 'Comox' were obtained from red raspberry breeder Dr. Patrick Moore (WSU, WA) and these were also fingerprinted. 'Comox' and 'Chiliwack' plants that grew in neighboring pots on the NCGR screenhouse bench had identical fingerprints and matched that of 'Chilliwack'. The previous 'Comox' was therefore discarded and a new source was requested. Tissue samples of true-to-name 'Glen Clova' and 'Malling Orion' will be obtained and their fingerprints compared to those of the NCGR accessions to clear up this unexpected result. A single allele difference at RiM019 was found between 'Willamette' (186) and its clonal variant 'Spinefree Willamette' (184), allowing us to distinguish them.

Eight reference cultivars in common between the James Hutton Institute and the NCGR genebanks were chosen to allow allele standardization across different collections. SSR-based fingerprints for six of the eight cultivars were identical (Table 1). However, a 4 bp-difference between the two sources of 'Meeker' was observed in one of the Rubus275 alleles (Table 1). Such small variations have been reported to be due to the high rate of mutation of microsatellite loci. It would be interesting to determine if 'Meeker' is prone to somaclonal variation during micropropagation. However, 'Cuthbert' was different at each of the 6 loci (Table 1), indicating that one of these cultivars is not true-to-name.

CONCLUSIONS

In summary, we identified a 6-SSR fingerprinting panel that has proved useful for identifying misidentified red raspberry cultivars and that is easy and economical to use for fingerprinting. Further studies are needed to optimize multiplex PCR amplification and to replace 'Meeker' and 'Cuthbert' as reference accessions. This panel also cross-amplified in other *Rubus* species with edible berries including cultivated blackberry and black raspberry where it differentiated between the small number of tested cultivars. We recommend these markers for raspberry identification verification for research and industry needs.

ACKNOWLEDGEMENTS

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Tables

Table 1. Comparison of SSR-based fingerprints of 8 reference red raspberry cultivars from the Hutton Institute and from the NCGR using the proposed 6-SSR fingerprinting set. Genetic profiles of 'Cuthbert' and 'Meeker' from these two genebanks were not identical and are provided.

| Reference cultivars	Multiplex 1			Multiplex 2		
SSR	Rubus275a	Rubus126b	Rubleaf97	RiM019	RhM011	RhM003
Meeker_NCGR	119/191	169/189	210/210	173/185	293/293	208/216
Meeker_Hutton	119/**187**	169/189	210/210	173/185	293/293	208/216
Carnival	129/181	143/145	210/210	187/187	289/295	208/222
Cuthbert_NCGR	119/**147**	**153**/169	210/**216**	**185/201**	293/**299**	**212/216**
Cuthbert_Hutton	119/**129**	169/**189**	**195**/210	**173/189**	293/**297**	**208/222**
Malling Jewel	145/181	143/169	195/210	185/189	293/295	204/222
Latham	133/135	143/169	210/222	187/197	293/295	204/222
Heritage	119/149	153/189	210/228	187/189	291/299	206/212
Tulameen	153/187	169/189	210/210	173/189	289/297	204/222
Glen Ample	135/187	143/169	210/210	185/185	293/297	204/208
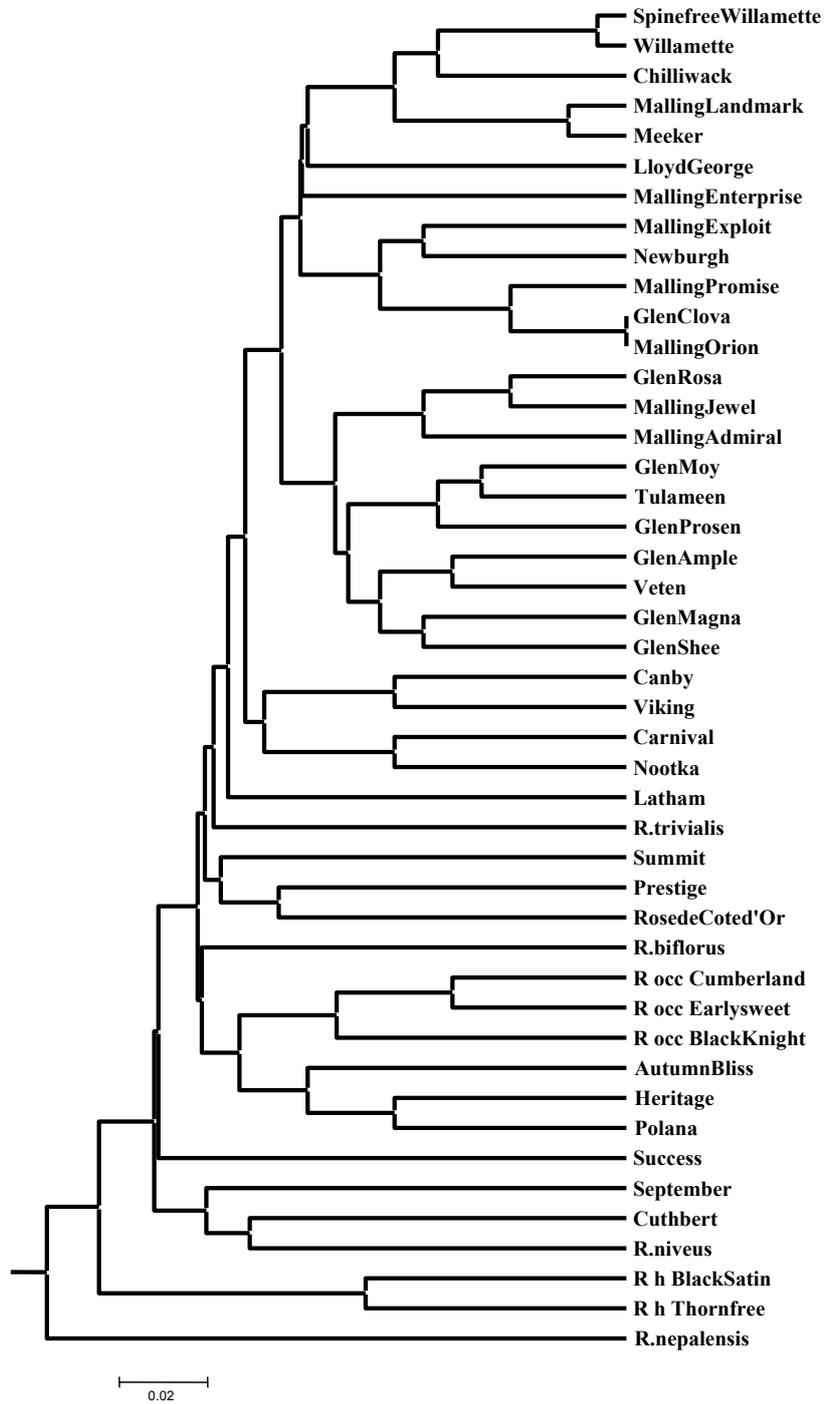
Fig. 1. UPGMA cluster analysis of 35 red raspberry cultivars and 10 representatives of other *Rubus* species with edible berries based on microsatellite analysis using the recommended 6-SSR fingerprinting panel.