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RESEARCH ARTICLE

Genetic diversity in wild and cultivated black raspberry (*Rubus occidentalis* L.) evaluated by simple sequence repeat markers

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Abstract Breeding progress in black raspberry (*Rubus occidentalis* L.) has been limited by a lack of genetic diversity in elite germplasm. Black raspberry cultivars have been noted for showing very few phenotypic differences and seedlings from crosses between cultivars for a lack of segregation for important traits. Despite these challenges, little molecular work has been done to explore genetic diversity and relationships in wild and cultivated black raspberry germplasm. Microsatellite, or simple sequence repeat (SSR), markers are highly polymorphic codominant markers useful for studying genetic diversity, population genetics, genetic fingerprinting and other applications. We examined genetic diversity in 148 wild and cultivated black raspberry accessions using 21 polymorphic SSR markers. Black raspberry cultivars clustered tightly and showed higher than expected heterozygosity while that of wild accessions was low. Relationships between wild black raspberry accessions were poorly resolved and regional clusters were mostly absent from our analysis. Our results indicated that wild black raspberry germplasm is a relatively untapped resource available for future breeding.

Keywords Blackcap · Microsatellite · Molecular markers · *Rubus occidentalis* · SSR

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Introduction

The black raspberry, commonly called "blackcap", was first domesticated in the 1830s (Hedrick 1925). A member of the Rosaceae, it is diploid ($2n = 2x = 14$) and is native to eastern North America from New Brunswick to the Carolinas and west into Kansas and Nebraska. West of the Rockies, it is supplanted by *R. leucodermis* Dougl. ex Torr. et Gray (Hitchcock and Cronquist 1973), which is similar in appearance but with more coarsely toothed leaves, spinier canes, and softer, purplish fruit. Both species are somewhat unusual among diploid members of the subgenus

Idaeobatus for their self-compatible flowers (Jennings 1988).

Black raspberry production in North America has undergone a slow but steady reduction since the 1920s due in large part to disease and a lack of adapted, disease-resistant cultivars. Today, growers in Oregon, the leading production region, typically see a decline in production after the second harvest and remove fields after only three or four seasons because of decreased profitability (Halgren et al. 2007). At the same time, demand for black raspberry fruit has increased in recent years in large part because of studies outlining the potential health benefits of black raspberry consumption (Kresty et al. 2001; Seeram et al. 2006; Seeram 2008; Stoner et al. 2005, 2008). These factors have combined to create a renewed interest in breeding better cultivars that meet the demands of growers and consumers.

Historically, progress in breeding black raspberry has been limited by a lack of variation and segregation for important traits in elite germplasm. Attempts to broaden the genetic base of black raspberry breeding populations by using other *Rubus* species date back to the 1950s (Drain 1956; Slate and Klein 1952; Williams 1950). The lack of genetic diversity is so acute that Ourecky (1975) felt that no future progress would be made in breeding black raspberry without the use of other species. However, in contrast to red raspberry, in which interspecific hybridization has played a major role in the introgression of new traits of interest, this approach has been of limited success in black raspberry. 'Earlysweet', released in 1996, is the first, and only, black raspberry cultivar reported to have another species, *R. leucodermis*, in its background (Galletta et al. 1998).

Few recent studies have attempted to quantify the genetic variation present in black raspberry germplasm. Weber (2003) examined genetic diversity in 14 cultivars and two wild selections from New York using random amplified polymorphic DNA (RAPD) markers. Genetic diversity was quite low; on average, there was 81% similarity among polymorphic markers, however, more than half of this variability was accounted for by 'Black Hawk', 'Cumberland', 'John Robertson', and the two wild selections. The remaining 11 genotypes had a collective marker similarity of 92%. Weber (2003) asserted that many cultivars that originated as chance seedlings were probably from open pollination of other cultivars. While this work yielded valuable information about the apparent lack of variability and relationships between black raspberry cultivars, RAPD markers lack the reproducibility desired for genetic fingerprinting and large scale population studies. Nybom and Schaal (1990) used restriction fragment length polymorphism (RFLP) markers to document genetic diversity in a wild black raspberry population in Missouri. They found 15 unique genotypes among 20 plants sampled along a 600 m stretch of roadside, and suggested that the main mode of plant recruitment in this population was through sexually produced seed leading to intrapopulation diversity.

Simple sequence repeat (SSR) or microsatellite markers are robust, highly polymorphic, codominant markers giving them an advantage over RAPD and RFLP markers for applications in population genetics, genetic diversity studies, and DNA fingerprinting. Microsatellite markers have been developed from expressed sequence tag (EST) and genomic libraries in red raspberry (*Rubus idaeus* L.; Castillo et al. 2010; Graham et al. 2004) and blackberry (*Rubus* L. subgenus *Rubus*) (Amsellem et al. 2001; Castillo et al. 2010; Lewers et al. 2008; Lopes et al. 2006). More recently, work to develop SSRs from black raspberry ESTs is underway (unpublished data). Using SSR markers, Dossett et al. (2010) found 12 black raspberry cultivars to be more closely related to each other than to any of the four wild accessions examined. This result, along with those of Weber (2003) and Nybom and Schaal (1990), suggests that wild populations have more genetic diversity than do current cultivars.

Surprisingly, beyond a few selections made in the late 19th and early 20th centuries, there is little record of the use of wild *R. occidentalis* as a source of genetic diversity for breeding improved black raspberry cultivars, and no record of attempts to systematically collect and evaluate germplasm from across the species' range. Dossett et al. (2008) found increased vigor and adaptability in progeny of a wild black raspberry selection from North Carolina. Dossett and Finn (2010) found aphid resistance in wild black raspberry germplasm, a trait that will be of great benefit in developing new virus resistant cultivars. It appears that wild black raspberry germplasm could be beneficial in developing better adapted and more disease resistant cultivars. The objective of this study was to investigate the level of genetic variation present in wild and cultivated black raspberry.

Materials and methods

Plant materials

During the summer of 2006, friends and colleagues in eastern North America, within the native distribution of *R. occidentalis*, were solicited to send seed or fruit from wild plants in their area. Additional seed was obtained in 2007 through a similar request and from collecting trips across the southern and western edges of the native range (Hall et al. 2009; Hummer et al. 2008a, b). Through these efforts, seeds were obtained from more than 150 locations across the range, including 27 states and two Canadian provinces. Upon arrival in the lab, seeds were extracted from the fruit, dried, and stored in a cool dry place until scarification. Additional seed was obtained from *R. occidentalis* seed lots held at the National Clonal Germplasm Repository (NCGR) in Corvallis, OR. Seeds were treated to promote germination using the methods of Dossett and Finn (2010), and a single seedling from each population from which seed was successfully germinated was randomly selected for inclusion in this study. In addition, each of the black raspberry cultivars and wild accessions currently available as clones at the NCGR were included in this study, for a total of 21 cultivars and 137 wild accessions (Table 1, Fig. 1). Naturally occurring hybrids with wild red raspberry (as recognized by their densely spined canes, and differences in leaf shape and leaflet number) were noted in seedlings of a few populations and were deliberately avoided when sampling seedlings for this study. A few plants showing morphology consistent with polyploidy (primarily leaf shape and appearance of leaf venation, see Hull and Britton 1956) were identified in two of the populations and these seedlings were also excluded from sampling for this study. Two wild seedlings of *R. leucodermis*, one from Washington, the other from Oregon, were included for comparison and dendrogram construction, but were not included in measurements of allelic diversity.

DNA extraction and amplification

DNA was extracted from freshly growing young leaf tissue with the Gentra Puregene kit (Qiagen, Valencia, CA) using the optional RNAse A treatment.

Rubus SSR primer sequences were selected from published reports in red raspberry (Graham et al. 2004) and blackberry (Castillo et al. 2010; Lopes et al. 2006; Lewers et al. 2008). Dossett et al. (2010) described the transferability of many of these *Rubus* SSR primers to black raspberry. These primers, and two previously unreported black raspberry EST SSR primer pairs, are summarized in Table 2. Optimum annealing temperatures was determined by gradient polymerase chain reaction (PCR) from 50 to 65 °C in 'Munger' using non-fluorescent primers. After an initial denaturation at 94 °C for 3 min, DNA was amplified for 35 cycles in a PTC-225 gradient thermal cycler (Bio-Rad, Hercules, CA) programmed for a 40 s denaturation step at 94 °C, a 40 s annealing step at the optimum annealing temperature of the primer pair and a 40 s extension step at 72 °C. A final extension step at 72 °C for 30 min was included. Non-fluorescent PCR reactions were performed in a volume of 10 µl and bands visualized by ethidium bromide staining after separation by 2% agarose gel electrophoresis. PCR was then performed on all samples with fluorescently labeled (WellRed D2, D3, or D4) forward primers at the appropriate annealing temperature in a volume of 15 µl. For some SSRs, instead of fluorescently labeling all forward primers, the M13 sequence TGTAAAACGACGGCCAGT was added to the 5′ end of the forward primer (Table 2) and a fluorescently labeled (WellRed D2, D3, or D4; Integrated DNA Technologies, Inc. Coralville, IA) M13 primer was used in the PCR, following the procedure outlined by Schuelke (2000). Fluorescently labeled PCR products were separated by capillary electrophoresis using a Beckman CEQ 8000 genetic analyzer (Beckman Coulter, Fullerton, California) for all samples. The reverse primer for Rub1C6 was pigtailed (Brownstein et al. 1996) to minimize the occurrence of split peaks and difficulties encountered in fragment analysis following capillary electrophoresis.

Data analysis

The data were compiled and analyzed with PowerMarker (Liu and Muse 2005). Expected and observed heterozygosity (H_e, H_o, Nei 1987) and polymorphism information content (PIC, Botstein et al. 1980; Liu 1998) were estimated for all black raspberry genotypes together, as well as separately for cultivated and wild genotypes. A neighbor-joining (NJ) dendrogram (Fig. 2) was constructed based on the proportion of shared alleles distance measure (Bowcock et al. 1994).

Table 1 US Department of Agriculture- Agricultural Research Service plant introduction (PI) number, accession name, origin, and type, for 137 wild and 21 cultivated *Rubus occidentalis* and two *R. leucodermis* accessions studied

PI no.	Name	Origin
Wild accessions		
653296	ORUS 4123	Mentone, AL
653327	ORUS 3779	Litchfield County, CT
652978	HDF-039	Appalachian Trail, GA
652975	ORUS 4117	Clayton, GA
652976	ORUS 4119	Clayton, GA
653294	ORUS 4120	Clayton, GA
653298	ORUS 4122	Dahlonega, GA
652977	ORUS 4121	Union County, GA
653328	ORUS 3780	Story County, IA
NA[a]	ORUS 3789	Arenzeville, IL
653329	ORUS 3781	Iroquois County, IL
553949	ORUS 3946	Waukegan County, IL
553949	CRUB 641.002	Waukegan County, IL
553950	CRUB 642.001	Waukegan County, IL
653331	ORUS 3796	Greene County, IN
653335	ORUS 3800	Greene County, IN
653332	ORUS 3797	Hendricks County, IN
653330	ORUS 3794	Putnam County, IN
653333	ORUS 3798	Sullivan County, IN
NA	ORUS 3795	Vigo County, IN
653334	ORUS 3799	Vigo County, IN
653336	ORUS 3801	Southern IN
652984	ORUS 4126	Alma, KS
653299	ORUS 4124	Bonner Springs, KS
653303	ORUS 4129	Fort Riley, KS
653301	ORUS 4127	Manhattan, KS
651846	ORUS 4130	Minneapolis, KS
653302	ORUS 4128	Ogden, KS
653300	ORUS 4125	Perry Lake, KS
651848	ORUS 3802	Fayette County, KY
653337	ORUS 3803	Berkshire County, MA
653338	ORUS 3804	Berkshire County, MA
653343	ORUS 3811	Allegany County, MD
653344	ORUS 3812	Anne Arundel County, MD
NA	ORUS 3809	Dorchester County, MD
653341	ORUS 3808	Harford County, MD
653342	ORUS 3810	Howard County, MD
NA	ORUS 3806	Howard County, MD
653339	ORUS 3805	Washington County, MD
653340	ORUS 3807	Washington County, MD
653350	ORUS 3821	Camden, ME
653349	ORUS 3820	East Vassalboro, ME
653347	ORUS 3817	Gardiner, ME
653348	ORUS 3819	Hallowell, ME

Table 1 continued

PI no.	Name	Origin
651849	ORUS 3815	Monmouth, ME
653345	ORUS 3814	Orono, ME
653346	ORUS 3816	West Kennebunk, ME
NA	ORUS 4109	Bath, MI
NA	ORUS 4110	Benton Harbor, MI
553765	ORUS 3948	Fred Russ State Forest, MI
553766	ORUS 3949	Fred Russ State Forest, MI
NA	ORUS 4111	Grand Ledge, MI
553764	ORUS 3947	Oak Grove, MI
NA	ORUS 4112	Okemos, MI
653323	ORUS 4149	Belgrade, MN
653321	ORUS 4148	Big Stone Lake National Wildlife Refuge, MN
651847	ORUS 4147	Big Stone Lake State Park, MN
653351	ORUS 3823	Cass County, MN
651851	ORUS 3827	Dakota County, MN
653354	ORUS 3828	Dakota County, MN
653324	ORUS 4150	Hasty, MN
651850	ORUS 3824	Ramsey County, MN
653353	ORUS 3826	Ramsey County, MN
NA	ORUS 3833	Cassville, MO
651852	ORUS 3830	Fordland, MO
653356	ORUS 3832	Fordland, MO
653357	ORUS 3835	Madison County, NC
653358	ORUS 3837	Rutherford County, NC
653359	ORUS 3838	Rutherford County, NC
553755	NC 84-10-3	Zebulon, NC
653311	ORUS 4139	Chadron, NE
653310	ORUS 4138	Chadron State Park, NE
653308	ORUS 4136	Halsey, NE
618482	CRUB 1732.001	Nebraska City, NE
653305	ORUS 4133	North Loup State Recreation Area, NE
653306	ORUS 4134	Pibel Lake State Recreation Area, NE
653309	ORUS 4137	Valentine, NE
653307	ORUS 4135	Victoria Springs State Recreation Area, NE
638243	ORUS 3955	Manasquan Reservoir, NJ
638244	ORUS 3956	Tom's River, NJ
653363	ORUS 3843	Columbia County, NY
653362	ORUS 3842	Dutchess County, NY
653360	ORUS 3839	Ontario County, NY
653361	ORUS 3840	Ontario County, NY
NA	ORUS 3841	Ontario County, NY
618560	ORUS 3951	Poughkeepsie, NY
653364	ORUS 3844	Yates County, NY
653368	ORUS 3849	Clermont County, OH
NA	ORUS 4107	Hilliard, OH

Table 1 continued

PI no.	Name	Origin
NA	ORUS 4108	Newton Falls, OH
653372	ORUS 3854	Centre County, PA
653373	ORUS 3856	Centre County, PA
653369	ORUS 3851	Chester County, PA
653370	ORUS 3852	Greene County, PA
653371	ORUS 3853	Somerset County, PA
NA	ORUS 4185	Charlestown, RI
652971	ORUS 4113	Glassy Mountain, SC
652973	ORUS 4114	Glassy Mountain, SC
652974	ORUS 4115	Rich Mountain, SC
653315	ORUS 4142	Clay County State Park, SD
653318	ORUS 4145	East Sioux Falls, SD
653317	ORUS 4144	Newton Hills State Park, SD
653319	ORUS 4146	Oakwood Lakes State Park, SD
652988	ORUS 4140	Pease Creek State Recreation Area, SD
653316	ORUS 4143	Union Grove State Park, SD
653314	ORUS 4141	Yankton, SD
653389	ORUS 3904	Cannon County, TN
653395	ORUS 3915	Cheatham County, TN
653374	ORUS 3857	Davidson County, TN
653375	ORUS 3863	Davidson County, TN
653376	ORUS 3864	Davidson County, TN
618286	NC 98-12-1	DeKalb County, TN
653377	ORUS 3867	DeKalb County, TN
653378	ORUS 3869	DeKalb County, TN
653379	ORUS 3871	DeKalb County, TN
653380	ORUS 3873	DeKalb County, TN
653381	ORUS 3878	DeKalb County, TN
653384	ORUS 3889	Grundy County, TN
653385	ORUS 3893	Grundy County, TN
653398	ORUS 3919	Henderson County, TN
618287	NC 98-7-1	Roane County, TN
653396	ORUS 3916	Unicoi County, TN
653397	ORUS 3918	Unicoi County, TN
653382	ORUS 3883	Van Buren County, TN
653390	ORUS 3906	Van Buren County, TN
653392	ORUS 3910	Van Buren County, TN
653393	ORUS 3911	Van Buren County, TN
653394	ORUS 3912	Van Buren County, TN
653383	ORUS 3884	Warren County, TN
653386	ORUS 3898	Warren County, TN
653387	ORUS 3902	Warren County, TN
653399	ORUS 3926	Columbia County, WI
653401	ORUS 3930	Inwood, WV
653402	ORUS 3931	Preston County, WV

Table 1 continued

PI no.	Name	Origin
653400	ORUS 3929	Shepherdstown, WV
653325	ORUS 3777	Mactaquac, NB, Canada
653326	ORUS 3778	Simcoe, ON, Canada
NA	ORUS 4150	*R. leucodermis*—Mt. Rainier National Park, WA
553680	CRUB 647.001	*R. leucodermis*—Curry County, OR
Cultivars		
553733	'Allen'	'Bristol' × 'Cumberland', 1957
553734	'Black Hawk'	'Quillan' × 'Black Pearl', 1955
553754	'Black Knight'	'Johnson Everbearing selfed', 1973
553735	'Bristol'	'Watson Prolific' × 'Honeysweet', 1934
553739	'Cumberland'	Wild selection from Pennsylvania, 1890s
553770	'Dundee'	'Smith1' × 'Palmer', 1927
657877	'Earlysweet'	('Haut' × *R. leucodermis*) × open-pollinated, 1996
553773	'Ebonee'	'Cumberland' × open-pollinated, 1961
658341	'Explorer'	Wild parents from New York and Arkansas, 2004
553768	'Hanover'	Unknown, perhaps from Indiana
553769	'Haut'	'Manteo' selfed × 'Bristol' selfed, 1987
553742	'Jewel'	('Bristol' × 'Dundee') × 'Dundee', 1973
553736	'Huron'	'Rachel' × 'Dundee', 1965
553772	'John Robertson'	Wild selection from near Hot Springs, SD, 1934
618387	'Mac Black'	Unknown
553740	'Munger'	Reputed to be 'Schaefer' open-pollinated
553741	'New Logan'	Unknown wild parentage
553737	'Plum Farmer'	Chance seedling from Ohio, 1892
553738	'Shuttleworth'	Developed in New York, 1933
618505	'Somo'	Unknown, from wild parents, 1956
618458	'White Chimera'	Sport of a 'Munger' seedling, 1993

[a] Accessions not yet available through the USDA, ARS, National Genetic Resources Program, *Germplasm Resources Information Network* (GRIN)

A separate NJ dendrogram was constructed from a cluster within the UPGMA tree comprised of most black raspberry cultivars and a few wild black raspberry accessions (Fig. 3). The bootstrap option of PowerMarker was used to create 1,000 dendrograms and MEGA version 4 software (Tamura et al. 2007) was used to generate and edit a consensus dendrogram. Principal component analysis (PCA) was performed using a similarity matrix based on Euclidean distances with NTSYS-pc (version 2.1; Exeter Software, Setauket, NY).

Results and discussion

Twenty-one SSR primer pairs amplified one or two alleles in each of the 21 cultivated and 125 wild *R. occidentalis* accessions. In 12 additional wild accessions, more than two alleles were amplified by one or more of the primer pairs studied. This may be the result of introgression of alleles from red raspberry or other *Rubus* species, duplication of some genome regions, or polyploidy. Individuals amplifying more than two alleles for any primer pair (ORUS 3779, ORUS 3789, ORUS 3795, ORUS 3803, ORUS 3823, ORUS 3827, ORUS 3910, ORUS 4111, ORUS 4122, ORUS 4141, ORUS 4142, and ORUS 4147) were excluded from the analysis and the remaining data were treated as though each SSR primer pair amplified a single locus.

Allelic diversity among the 21 black raspberry cultivars was very low, with three or fewer alleles present at 15 of 21 loci, and only a single locus having more than four alleles present (Table 3). The 21 SSR

Fig. 1 Geographical distribution of 137 wild *Rubus occidentalis* populations surveyed

loci were unable to distinguish between six of the cultivars: Bristol, Cumberland, Munger, New Logan, Plum Farmer, and Shuttleworth (Fig. 3). This is in contrast to previous work (Dossett et al. 2010) that found differences between some of these cultivars using 19 of the same SSRs. During the course of this study, we found that differences in primer stocks led to some fragments having been incorrectly sized in a subset of the data from Dossett et al. (2010). Once this problem was discovered, PCRs for samples at the suspect loci were repeated and the correct alleles were verified. The most recently named cultivar of this group (Bristol) was released nearly 80 years ago, and it is possible that mislabeling of plants at some point in the past led to this result. While Hedrick (1925) considered several of these clones to be distinct, Ourecky (1975) noted difficulty in distinguishing between black raspberry cultivars as well as a lack of segregation for important traits in black raspberry seedlings. This may have been due in part to identical clones being evaluated under different names. Alternatively, it is possible that these genotypes may be distinct but cannot be distinguished with existing SSR markers. Using RAPD markers, Weber (2003) was able to distinguish between each of the 14 black raspberry cultivars examined. In that study, 'Bristol', 'Munger', 'New Logan', and 'Plum Farmer' had very high marker similarity (average = 97%); 'Cumberland' was somewhat less similar (average 86% similarity); and 'Shuttleworth' was not included. It is unlikely that the differences observed by Weber (2003) can be attributed solely to the lack of reproducibility of RAPD markers that has been previously noted (Büscher et al. 1993; Jones et al. 1997; MacPherson et al. 1993). In either case, our data highlights the need for better genomic resources and markers to reliably distinguish between closely related black raspberry genotypes, as well as a need for greater genetic diversity in material used in breeding. Further study will be needed to determine whether there are real performance differences between these six clones in the field. Clones from alternate sources should also be fingerprinted. Unfortunately, 'New Logan', 'Plum Farmer', and 'Shuttleworth' are no longer widely available and may be among the many black raspberry cultivars that have been lost over the last 100 years.

Based on the similarity of their alleles, the majority of black raspberry cultivars clustered tightly in one relatively well-defined group in the NJ dendrogram

Table 2 Summary information for 21 SSR primer pairs used for studying genetic diversity in wild and cultivated black raspberry genotypes

Primer name	Primer sequence	Motif	Allele size range in *R. occidentalis* (bp)	Allele size range in *R. leucodermis* (bp)	Source
ssrRhCBA23[ab]	F: ATCGGGGATTTGGTGTGGGTTTAGG	$(GA)_{10}$-G$(GA)_5$	98–154	110–114	Lopes et al. (2006)
	R: ATTGTGTGCATCACTCTGAGAACCG				
Rubus 110a[bc]	F: AAACAAAGGATAAAGTGGGAAGG	$(TC)_8$	163–215	161	Graham et al. (2004)
	R: TGTCAGTTGGAGGGAGAACA				
RhM003[ab]	F: CCATCTCCAATTCAGTTCTTCC	$(TG)_{10}$	211–229	194–198	Castillo et al. (2010)
	R: AGCAGAATCGGTTCTTACAAGC				
Rub1C6[ab]	F: TCTGCCTCTGCATTTTACACAG	$(CT)_{15}$	237–268	235	Dossett et al. (2010)
	R: GTTTAGGTAAGCAATGGGAAAGCTC				
Rh_ME0013bG01[ad]	F: CCCTCCATCTCCACCATAAA	$(GA)_{38}$	243–251	255–259	Lewers et al. (2008)
	R: GTAAGGCCACCCCATTGAG				
RubFruitC1[bd]	F: CACGAGCTTCATCCTCTTCC	CTT-$(CCT)_7$	161–164	158	Graham et al. (2004)
	R: ATCCAAAGCTTTTGCGATTG				
Rubus 275a[bc]	F: CACAACCAGTCCCGAGAAAT	$(AG)_{27}$	112–162	156–170	Graham et al. (2004)
	R: CATTTCATCCAAATGCAACC				
Rubus 270a[bc]	F: GCATCAGCCATTGAATTTCC	$(GA)_{10}$	153–171	159–161	Graham et al. (2004)
	R: CCCACCTCCATTACCAACTC				
RO_CBEa010N20[d]	F: GGGGGCTTTACATCATCATT	$(GA)_9$	114–118	114–118	Unpublished
	R: TTCGTAGTCTTGCCCTTGCT				
Rh_ME0013cE02[ad]	F: AGGGTGGGTCTGAGATTGTG	$(TA)_8$	318–326	325	Lewers et al. (2008)
	R: AACAGTGCACAGGGGCTAAT				
Rubus 262b[bc]	F: TGCATGAAGGCGATATAAAGG	$(AG)_{15}$	203–209	203	Graham et al. (2004)
	R: TCCGCAAGGGTTGTATCCTA				
Rubus 123a[bc]	F: CAGCAGCTAGCATTTTACTGGA	$(AG)_8$	158–188	162	Graham et al. (2004)
	R: GCACTCTCCACCCATTTCAT				
Rh_ME0015cH02[ad]	F: TGGATTTCCACACGCACATA	$(TC)_9$	212–216	214–216	Lewers et al. (2008)
	R: TGTTGGATTTGCCTCCTTTC				
Rh_ME0013cF08[ad]	F: TTTGTCTCCGTCTTTTTGCC	$(TC)_{15}$	248–278	252–256	Lewers et al. (2008)
	R: CCTCCGAAGAAAAACAGCAG				
RO_CBEa011M1[d]	F: TCGAACCTGTTGCCTTCTCT	$(AG)_{14}$	233–247	231	Unpublished
	R: TCCATTTCCAAAACACATTGA				

Table 2 continued

Primer name	Primer sequence	Motif	Allele size range in *R. occidentalis* (bp)	Allele size range in *R. leucodermis* (bp)	Source
Rh_ME0007aB01[ad]	F: TGGTGGTTCACCGTTCACTA	$(CT)_{15}$	145–155	147–149	Lewers et al. (2008)
	R: GAAATGCTTGAAGCCGAGAG				
Rubus 223a[bc]	F: TCTCTTGCATGTTGAGATTCTATT	$(AT)_4$-$(TA)_8$-$(AT)_{10}$	156–166	160–162	Graham et al. (2004)
	R: TTAAGGCGTCGTGGATAAGG				
Rubus 26a[bc]	F: AACACCGGCTTCTAAGGTCT	$(CT)_{11}$-$(CA)_{29}$	123–143	129–133	Graham et al. (2004)
	R: GATCCTGGAAAGCGATGAAA				
Rubus 126b[bc]	F: CCTGCATTTTTCTGTATTTTGG	$(CT)_{31}$-$(CA)_{22}$	152–174	180–182	Graham et al. (2004)
	R: TCAGTTTTCTTCCCACGGTTA				
Rubus 107a[bc]	F: GCCAGCACCAAAAACCTACA	$(AG)_8$	160–164	160	Graham et al. (2004)
	R: TTTCACCGTCAAGAAGAAAGC				
Rubus 194h[bc]	F: TGTGTTGTTCTCTGCAACCA	$(GA)_{12}$	127–133	131–133	Graham et al. (2004)
	R: AGCCCTTACTTTTCCTGCAA				

Names of primers evaluated with the M13 sequence at the start of the forward primer are italicized

[a] SSR marker developed in blackberry

[b] SSR marker developed from a genomic library

[c] SSR marker developed in red raspberry

[d] SSR marker developed from an expressed sequence tag (EST) library

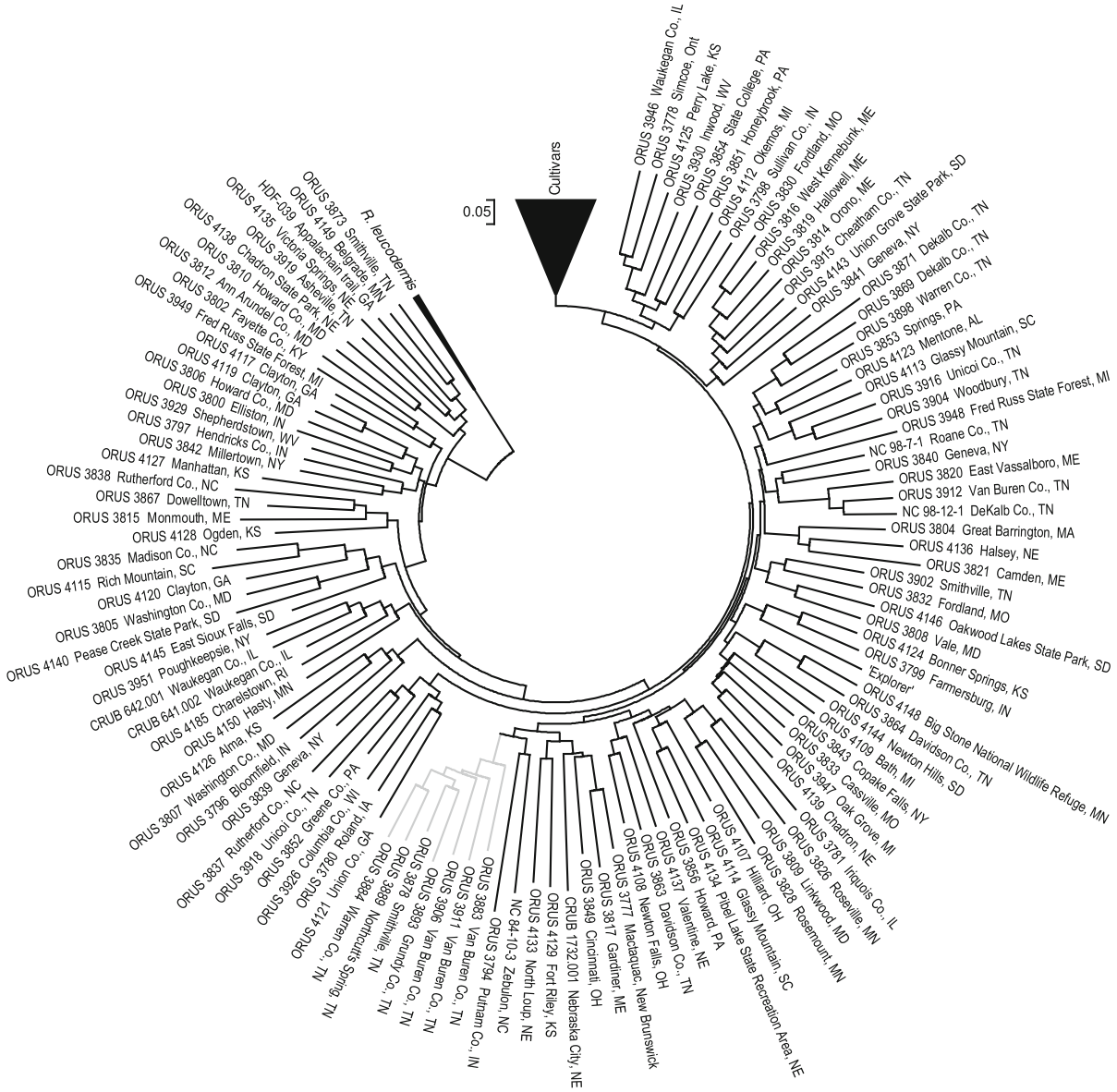

Fig. 2 Neighbor-joining (NJ) dendrogram depicting all black raspberry genotypes studied. A cluster containing most black raspberry cultivars and a few wild accessions has been collapsed and is depicted in Fig. 3. Branches for a cluster of seven accessions from Tennessee, discussed in the text, are depicted in a lighter shade of gray

(Fig. 2). The average branch length (distance of shared alleles) separating all of the black raspberry cultivars was 0.26. A NJ dendrogram depicting the genotypes within this group also shows good bootstrap support for several of the pairings (Fig. 3). 'Explorer', the one cultivar falling outside of this group, was selected from crosses of wild plants from New York and Arkansas for its unusual fall-fruiting habit (Tallman 2007) and was therefore not expected to show a close relationship to the other cultivars. Within the cluster of 20 cultivars (Fig. 3), there were also 10 wild black raspberry accessions (ORUS 3801, ORUS 3811, ORUS 3824, ORUS 3844, ORUS 3857, ORUS 3931, ORUS 3955, ORUS 3956, ORUS 4110, and ORUS 4130), some of which consistently paired with cultivars (e.g. ORUS 3956 with 'Jewel'). With the exception of ORUS 3811, and ORUS 3931, which was noted in the field for its distinct morphology, each

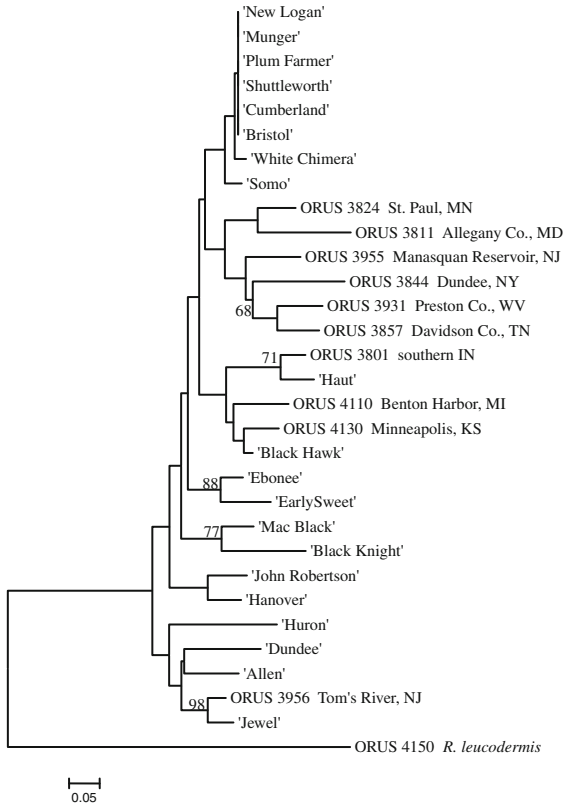

Fig. 3 Neighbor-joining dendrogram of black raspberry cultivars and closely paired wild accessions from a condensed cluster in Fig. 2. Numbers near nodes show bootstrap support for pairings (percent of 1,000 trees). One accession of *R. leucodermis* was used to root the dendrogram

of these had larger than average fruit and/or came from seed lots that segregated for plants lacking the normal waxy, glaucous bloom on their canes (data not shown). Dossett (2007) noted segregation for non-glaucous canes in progeny of some black raspberry cultivars, and the presence of one or both of these traits in these populations suggests that they may be derived from escaped cultivated germplasm.

Despite the low allelic diversity found among black raspberry cultivars, novel alleles not found in the wild genotypes were present at three loci (Table 3). Further examination revealed discrepancies between SSR fingerprint and the stated pedigrees of some cultivars. The published pedigree of 'Jewel' is ('Bristol' × 'Dundee') × 'Dundee'. However, in our study, 'Jewel' had alleles at multiple SSR loci that are not carried by either 'Bristol' or 'Dundee' (as illustrated by 112 at ssrRhcBA23 and 169 at Rubus110a, Table 4). This indicates that either the published pedigree is incorrect, or the identity of the 'Jewel', 'Dundee', or 'Bristol' used in this study is incorrect (Table 4). 'Jewel' and 'Huron' ('Rachel' × 'Dundee') were the only two individuals sharing a 112 bp allele at ssrRhCBA23, the most polymorphic locus in this study (Table 4), suggesting that either 'Huron' or 'Rachel' may be an ancestor of 'Jewel'. The identity of 'Huron' in this study also does not match its reported pedigree as it does not share an allele with 'Dundee' at Rubus 275a (Table 4). 'Allen' ('Bristol' × 'Cumberland') and 'Haut' [('Cumberland' selfed × selfed) × 'Bristol' selfed] also have alleles that cannot be traced to either of their reported parents (as shown by 158 at Rubus126b for 'Allen' and 128 at Rubus275a and 187 at Rubus110a for 'Haut', Table 4). 'Haut' and 'Huron' were the only two cultivars sharing a 128 bp allele for Rubus 275a (Table 4).

Similarly, 'Earlysweet' is reported to have *R. leucodermis* as one of its grandparents (Galletta et al. 1998). Alleles observed in the two *R. leucodermis* accessions fell outside the size range of *R. occidentalis* at seven loci (Table 2) and were unique to *R. leucodermis* at six other loci where there was size overlap (data not shown). While only two *R. leucodermis* genotypes were available in this study for comparison, SSR alleles found in 'Earlysweet' were characteristic of *R. occidentalis* cultivars at every locus, and alleles in the size range of *R. leucodermis* were not observed. This, combined with its close clustering within the group of other cultivars, suggests that 'Earlysweet' may not be one quarter *R. leucodermis* as reported. 'Earlysweet' [('Haut' × *R. leucodermis*) × open-pollinated] may have instead originated from contamination of the pollen used in the cross, or from contamination of the open-pollinated seed lot. In this study, 'Earlysweet' grouped closely with 'Ebonee' ('Cumberland' open-pollinated), possibly due to shared alleles from 'Cumberland', a common ancestor. 'Earlysweet' and 'Dundee' were the only two individuals in the study with a 188 bp allele at Rubus 123a, indicating that 'Dundee' may be a parent of 'Earlysweet' (Table 4). This close relationship is also supported by RAPD markers (Weber 2003). Similarly, 'Mac Black' and 'Black Knight' were the only two individuals that shared a 209 bp allele at Rubus 262b (Table 4). While the pedigree of 'Mac Black' is unknown, 'Black Knight' ('Johnson Everbearing' selfed) predates 'Mac Black' by about 20 years and may be in its lineage. Because of its

Table 3 Allelic diversity, expected heterozygosity (H_e), observed heterozygosity (H_o) and polymorphism information content (PIC) for 21 *Rubus* SSR primer pairs in 21 cultivars and 125 wild *R. occidentalis* accessions

Primer name	Cultivars (n = 21)				Wild accessions (n = 125)				All genotypes (n = 146)			
	Allele #	H_e	H_o	PIC	Allele #	H_e	H_o	PIC	Allele #	He	Ho	PIC
ssrRhCBA23	3	0.54	0.67	0.44	23	0.91	0.35	0.90	24	0.90	0.40	0.89
Rubus 110a	4	0.68	0.76	0.63	22	0.88	0.32	0.87	22	0.88	0.38	0.87
RhM003	3	0.56	0.81	0.49	6	0.47	0.22	0.42	6	0.52	0.31	0.45
Rub1C6	4	0.57	0.71	0.50	18	0.90	0.41	0.90	18	0.89	0.45	0.88
Rh_ME0013bG01	2	0.13	0.14	0.12	3	0.23	0.08	0.21	3	0.22	0.09	0.20
RubFruitC1	2	0.24	0.29	0.21	2	0.23	0.10	0.20	2	0.23	0.13	0.20
Rubus 275a	5	0.66	0.76	0.60	20	0.91	0.35	0.90	20	0.90	0.41	0.89
Rubus 270a	2	0.44	0.57	0.35	9	0.78	0.23	0.74	9	0.77	0.28	0.73
RO_CBEa010N20	2	0.17	0.19	0.16	2	0.39	0.16	0.31	2	0.36	0.16	0.30
Rh_ME0013cE02	3	0.50	0.67	0.40	5	0.56	0.17	0.46	5	0.55	0.24	0.45
Rubus 262b	2	0.09	0.10	0.09	1	0.00	0.00	0.00	2	0.01	0.01	0.01
Rubus 123a	2	0.09	0.10	0.09	4	0.51	0.21	0.41	5	0.49	0.19	0.39
Rh_ME0015cH02	1	0.00	0.00	0.00	3	0.10	0.07	0.09	3	0.09	0.06	0.08
Rh_ME0013cF08	4	0.48	0.52	0.43	16	0.81	0.32	0.79	16	0.78	0.35	0.76
RO_CBEa011M11	3	0.48	0.57	0.38	5	0.61	0.19	0.54	5	0.61	0.25	0.55
Rh_ME0007aB01	4	0.54	0.62	0.44	6	0.57	0.27	0.52	6	0.57	0.32	0.52
Rubus 223a	1	0.00	0.00	0.00	6	0.54	0.25	0.51	6	0.48	0.21	0.46
Rubus 26a	4	0.64	0.86	0.57	9	0.71	0.22	0.66	9	0.70	0.32	0.65
Rubus 126b	3	0.56	0.67	0.49	10	0.66	0.30	0.62	10	0.66	0.36	0.61
Rubus 107a	1	0.00	0.00	0.00	3	0.10	0.04	0.10	3	0.09	0.03	0.08
Rubus 194h	2	0.17	0.19	0.16	5	0.46	0.14	0.38	5	0.43	0.15	0.36
Mean	2.7	0.36	0.44***	0.31	8.5	0.54	0.21***	0.5	8.6	0.53	0.24***	0.49

*** Significant at $P \leq 0.0001$

erect growth habit and very late fruit maturity, as compared to other black raspberry cultivars, there has been speculation that 'Mac Black' may have *R. idaeus* in its ancestry (Makielski, personal communication). However, SSR alleles in 'Mac Black' were characteristic of *R. occidentalis*, matching those found in other cultivars at every locus. This, along with its clustering with the other black raspberry cultivars, casts some doubt on this hypothesis.

Simple sequence repeat analysis also revealed a surprising level of heterozygosity in black raspberry cultivars. At every polymorphic SSR locus examined, observed heterozygosity in the cultivars was higher than expected. This shouldn't be a big surprise since the process of selection and breeding, particularly in a clonally propagated crop such as black raspberry, can lead to highly heterozygous breeding populations. What is slightly surprising, however, is that this heterozygosity has been maintained despite some level of inbreeding in a few cultivars that should lead to a loss of heterozygosity. While pedigree information is missing or sparse for many cultivars, several are known to be parents and/or grandparents of others and to have related clones in both their maternal and paternal pedigrees. This is suggestive of inadvertent selection for heterozygosity in the process of selecting for the best performers, and leads one to suspect that homozygosity may lead to inbreeding depression in black raspberry despite "conventional wisdom" that black raspberries do not suffer from inbreeding depression (Haskell 1960; Ourecky 1975). Dossett (2007) and Dossett et al. (2008) noted that progeny of a wild black raspberry selection from North Carolina, NC 84-10-3, when crossed to cultivars, outperformed and had higher vigor than progeny of crosses among cultivars despite observations that NC 84-10-3 had very low vigor and never grew to be large in the field (Dossett 2007).

Table 4 Microsatellite alleles (fragment size in bp) at six loci in 'Jewel', 'Haut', 'Allen', 'Earlysweet', 'Black Knight', 'Mac Black' and related black raspberry cultivars, illustrating shared rare alleles and discrepancies in reported pedigrees

Cultivar	ssrRhcBA23	Rubus 275a	Rubus 262b	Rubus 123a	Rubus 126b	Rubus 110a
Bristol	124, 126	116, 144	203	158	154, 168	183, 185
Dundee	124	**116**, **132**	203	158, 188	158, 168	183
Huron	**112**, 124	**128**, **144**	203	158	168	169, 183
Jewel	**112**, 124	144	203	158	158, 168	**169**, 183
Bristol	124, 126	116, 144	203	158	154, 168	183, 185
Cumberland	124, 126	116, 144	203	158	154, 168	183, 185
Huron	112, 124	**128**, 144	203	158	168	169, 183
Haut	124	**128**, 132	203	158	168	**187**
Allen	124	144	203	158	**158**	183, 185
Ebonee	124, 126	116	203	158	154, 168	187
Haut	124	128, 132	203	158	168	187
Dundee	124	116, 132	203	158, **188**	158, 168	183
Earlysweet	124, 126	116, 144	203	158, **188**	154, 168	183, 187
Black Knight	126	134, 144	203, **209**	158	154, 168	169, 183
Mac Black	124, 126	144	203, **209**	158	168	169, 183

Names of cultivars with pedigree discrepancies are in bold and are presented in a block with their reported parents and other clones sharing unique alleles. Alleles specifically mentioned in the text are in bold and underlined. Fingerprints for some cultivars (i.e. Dundee, Huron and Haut) are repeated in different blocks for ease of comparison

In this study, NC 84-10-3 was heterozygous at only one of the 21 loci examined (data not shown), suggesting a degree of inbreeding. In fact, the wild genotypes had lower than expected heterozygosity at every polymorphic SSR locus (Table 3). This is not entirely unexpected; subdivision of wild black raspberry populations may lead to an apparent deficiency of heterozygotes and the sampling method violates Hardy–Weinberg expectations. Despite this, the rate of observed heterozygosity (0.21) is less than half that observed in the cultivars (Table 3). The reasons for this are unclear, but may be due to bottlenecking and/or isolation of wild populations from one another. Further sampling from within these populations is needed to better understand the reasons for the observed homozygosity before firm conclusions can be made about the causes.

With the high level of homozygosity in mind, inbreeding depression may be a limiting factor in the field performance of some of these seedlings and their value in breeding may only become evident by evaluating the performance of their progeny from crosses with unrelated germplasm. Further study should be undertaken to examine the impact of inbreeding on black raspberry performance.

In contrast to black raspberry cultivars, wild black raspberry germplasm is diverse. Branch lengths separating the wild genotypes are longer than those separating the cultivars and bootstrap support for groups of wild accessions was poor, indicating that these accessions are more distantly related to each other and that their relationships were not well resolved. The average branch length (distance of shared alleles) separating wild black raspberry accessions in this study was 0.53, more than twice that of the cultivars. NJ clustering (Fig. 2) illustrates a lack of grouping based on geographical location. For example, wild plants from Nebraska (ORUS 4134) and South Carolina (4114) grouped together as did plants from Maryland (ORUS 3808) and South Dakota (ORUS 4146). A few groups of accessions from neighboring locations were scattered throughout the dendrogram. For example, ORUS 4117 and ORUS 4119 from Clayton, Georgia grouped with each other. However, ORUS 4120 which was collected in the same area, fell in a different cluster. A group of seven accessions from Tennessee cluster together, although other wild accessions from the same areas of Tennessee are scattered throughout the dendrogram (Fig. 2).

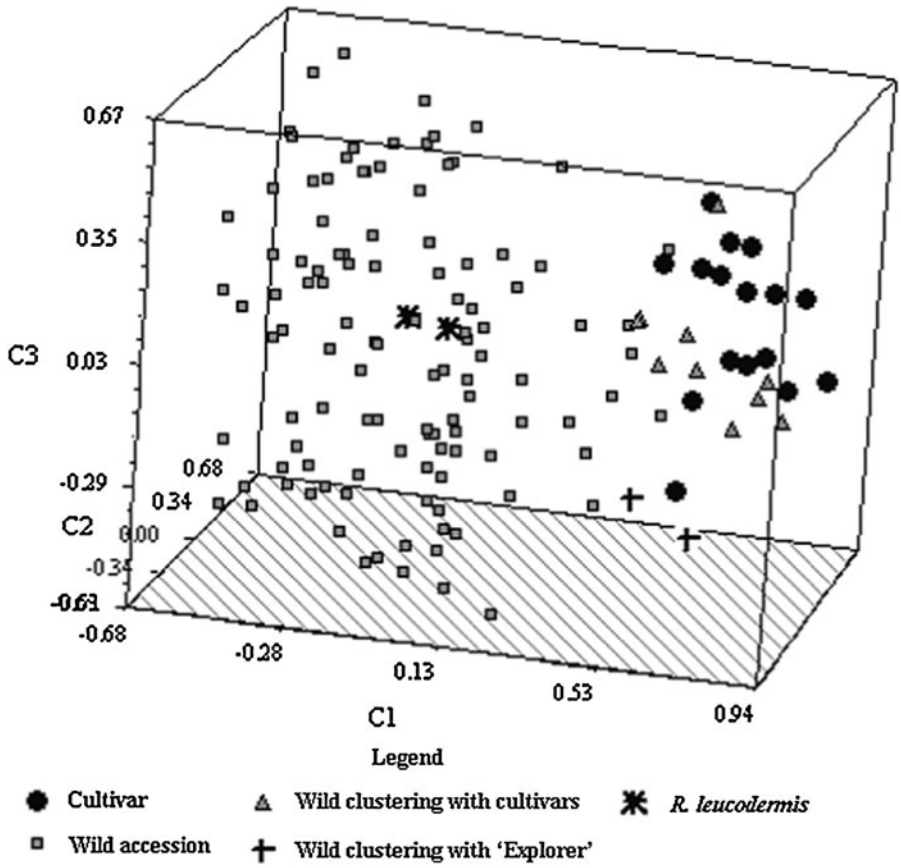

Fig. 4 Principal component analysis (PCA) plot of wild and cultivated black raspberry based on Euclidean distance measured from 21 polymorphic SSR loci and illustrating PCA clustering of wild accessions, black raspberry cultivars, wild accessions clustered with cultivars in Fig. 3, and wild accessions clustering with 'Explorer' in Fig. 2

The deep branching and lack of resolution in relationships among wild black raspberry populations, combined with the general lack of strong regional clusters, may be an indication that black raspberry diversity has not been exhaustively sampled. This also suggests that black raspberry populations may be well-differentiated from each other but not in a strongly geographical manner. Future work investigating the extent of diversity and relationships within and between many of these wild populations should provide insight into the degree of differentiation between wild populations and answer questions regarding whether certain areas of the range contain more allelic diversity than others. This information would be useful for future efforts to collect and preserve genetic diversity in wild black raspberry germplasm.

Principal component analysis did not provide better resolution of the data but did support some of the clusters already observed in the dendrogram. The first three eigenvalues collectively explain 9.6% of the variance. The first, however, separated black raspberry cultivars from the majority of the wild germplasm (Fig. 4). The wild accessions that clustered with the cultivars (Fig. 2) also grouped with the cultivars on the positive side of this axis. 'Explorer' and the wild accessions that clustered with it in the NJ dendrogram (ORUS 3799 and ORUS 4124) also fell near these on the positive end of the first axis. ORUS 4124 has been noted in field evaluations for larger than average fruit weight, and seed lots of ORUS 3799 and ORUS 4124 segregate for non-glaucous canes (data not shown) that may be an indication of cultivated ancestry. A few additional wild accessions fell in this area, including ORUS 3819, ORUS 3851, and ORUS 3947. The second PCA axis provides some separation between the rest of the black raspberry cultivars and the NJ cluster that includes 'Explorer' and two wild accessions (ORUS 3799, and ORUS 4134). These were located towards the negative end of axis 2 with the rest of the cultivars spread out along this axis. Otherwise, separation of groups along the second and third axes was relatively poor and groups of wild accessions

were not well resolved. Eigenvalues four and five (data not shown) each explain only about 2% of the variance, and plotting these does not help further resolve relationships in black raspberry germplasm.

Conclusions

The vast majority of genetic diversity available in *R. occidentalis* remains untapped in the development of new cultivars. While several cultivars that have not been lost over the last 100 years are reputed to have originated as wild seedlings that were discovered and brought into cultivation because of their superior horticultural traits (Hedrick 1925), it is now clear that the black raspberry cultivars remaining today are very closely related to each other and many of the "wild" selections named as cultivars were probably seedlings of cultivated plants. The few apparently wild accessions that clustered with cultivars have traits such as larger than average fruit, suggesting that they may be the offspring of cultivated plants. Conversely, this also shows that characterization of wild-collected black raspberry germplasm with SSR markers could be a useful tool in the future for identifying whether wild plants with good horticultural attributes are truly wild or are closely related to cultivated material.

Even the most recently developed black raspberry cultivars are not more than a few generations removed from truly wild ancestors. This knowledge, combined with the apparent diversity among wild plants available today, suggests that significant progress in breeding improved cultivars may be possible from a few generations of crossing and selection from between these wild populations without requiring further use of cultivated black raspberry germplasm. The use of this wild germplasm combined with existing cultivars should lead to faster gains for commercially important traits, such as large fruit.

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