A genetic linkage map of black raspberry (*Rubus occidentalis*) and the mapping of *Ag*₄ conferring resistance to the aphid *Amphorophora agathonica*

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Abstract

**Key message** We have constructed a densely populated, saturated genetic linkage map of black raspberry and successfully placed a locus for aphid resistance.

**Abstract** Black raspberry (*Rubus occidentalis* L.) is a high-value crop in the Pacific Northwest of North America with an international marketplace. Few genetic resources are readily available and little improvement has been achieved through breeding efforts to address production challenges involved in growing this crop. Contributing to its lack of improvement is low genetic diversity in elite cultivars and an untapped reservoir of genetic diversity from wild germplasm. In the Pacific Northwest, where most production is centered, the current standard commercial cultivar is highly susceptible to the aphid *Amphorophora agathonica* Hottes, which is a vector for the *Raspberry mosaic virus* complex. Infection with the virus complex leads to a rapid decline in plant health resulting in field replacement after only 3–4 growing seasons. Sources of aphid resistance have been identified in wild germplasm and are used to develop mapping populations to study the inheritance of these valuable traits. We have constructed a genetic linkage map using single-nucleotide polymorphism and transferable (primarily simple sequence repeat) markers for F₁ population ORUS 4305 consisting of 115 progeny that segregate for aphid resistance. This is the first linkage map of black raspberry and will aid in developing markers for marker-assisted breeding, comparative mapping with other *Rubus* species, and enhancing the black raspberry genome assembly.

Introduction

Genetic linkage map construction of rosaceous crops has been used to understand genetics and as a precursor to enabling molecular breeding for about 20 years. The earliest maps made during the 1990s were constructed mainly by using isozymes, random amplification of polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), and morphological markers (Chaparro et al. 1994;
Foolad et al. 1995; Hemmat et al. 1994; Rajapakse et al. 1995; Stockinger et al. 1996; Viruel et al. 1995). Advancements in DNA technology in the 2000s led to the rapid development of simple sequence repeat (SSR) markers for de novo map construction (Castro et al. 2013; Celton et al. 2009; Dirlewanger et al. 2004; Fernández-Fernández et al. 2008; Gisbert et al. 2009; Graham et al. 2004; Hibrand-Saint Oyant et al. 2008; Olmstead et al. 2008) as well as their incorporation into existing maps (Aranzana et al. 2003; Dirlewanger et al. 2006; Etienne et al. 2002; Liebhard et al. 2003; Paterson et al. 2013; Pierantoni et al. 2004; Silfverberg-Dilworth et al. 2006; Stafne et al. 2005; Vilanova et al. 2008; Woodhead et al. 2008, 2010). Additional technological advances in high-throughput detection of single-nucleotide polymorphic (SNP) loci using arrays, or genotyping by sequencing (GBS), and the associated improvement of data analysis have made SNP markers increasingly useful for genetic map construction. Recently, linkage maps for several members of the Rosaceae have been constructed using SNP array technology (Anta-Campo et al. 2012; Clark et al. 2014; Frett et al. 2014; Klagges et al. 2013; Montanari et al. 2013; Pirona et al. 2013; Yang et al. 2013).

The genus Rubus L. (Rosaceae, Rosoideae) has an estimated 750 species distributed world-wide (Alice and Campbell 1999; Thompson 1995). Of these, three are of particular commercial importance, red raspberry (R. idaeus L., subgenus Idaeobatus Focke), blackberry (Rubus sp., subgenus Rubus L.), and black raspberry (subgenus Idaeobatus). Genetic linkage maps have been constructed for tetraploid blackberry (Castro et al. 2013), diploid red raspberry (Sargent et al. 2007; Ward et al. 2013; Woodhead et al. 2010), and an interspecific cross between diploid red raspberry and diploid black raspberry (Bushakra et al. 2012). While blackberry and red raspberry are highly heterozygous, black raspberry, particularly R. occidentalis, is highly homozygous (Dossett et al. 2012b). Genetic improvement of blackberry and red raspberry through breeding has been a continual process for decades. For example, from 1994 to 2014, the American Pomological Society’s Fruit and Nut Variety Registers List 38–47 (Clark and Finn 1999, 2002, 2006; Clark et al. 2008, 2012; Daubeney 1997a, b, 1999, 2000, 2002, 2004, 2006, 2008; Finn and Clark 2000, 2004, 2014; Finn et al. 2010; Moore and Kempler 2010, 2012, 2014) records the release of 75 blackberry and hybrid berry and 189 red raspberry cultivars and only three black raspberry cultivars (‘Pequot’, ‘Niwort’, and ‘Explorer’). In addition, ‘Earlysweet’, a selection derived from a purported cross between R. occidentalis and the western black raspberry, R. leucodermis Dougl. ex Torr. & Gray, was released in 1998 (Galletta et al. 1998). Black raspberry figures prominently in the pedigrees of many of the red raspberry cultivars released between 1994 and 2014. Difficulties in improving black raspberry through breeding were first reported by Slate (1933) while attempting to improve purple raspberries. Crossing with other species was proposed as a way to increase genetic diversity in cultivated black raspberry (Drain 1956; Hellman et al. 1982; Slate and Klein 1952), but has met with limited success. Low genetic diversity was proposed by Orecky (1975) as the main reason for lack of development of improved cultivars.

More recent interest in improving black raspberry has been driven by research and commercial interest into its bioactive compounds and their influence on human health, specifically modulation of cancer cell proliferation, inflammation, cellular death, oxidation, etc. (Stoner et al. 2007). Since the 1940s, Oregon has been the primary commercial production region of black raspberry in North America. In 2014, 1650 acres were harvested that earned growers a utilized production value of over US$16.8 million (Anonymous 2015). One hindrance to expanding production is susceptibility of the predominant cultivar Munger to the Raspberry mosaic virus complex vectored by the North American large raspberry aphid, Amorphophora agathonica Hottes (Dossett and Finn 2010). Infection causes a rapid decline of plantings, often with field replacement necessary after only three or four growing seasons (Halgren et al. 2007). In contrast, under perennial production in open fields for processed fruit, plantings of current cultivars of red raspberry are typically kept in the field for 7–8 growing seasons, and plantings of blackberry cultivars can last many decades (C.E. Finn, personal communication). Selection for cultivars with resistance to A. agathonica could significantly increase the longevity of the plants, reduce insecticide use, and therefore improve profitability for the grower and quality of the environment.

A low level of genetic diversity in cultivated black raspberry has been found using molecular tools. Weber (2003), using RAPD markers in 16 black raspberry cultivars, determined a level of similarity of 81%. Two wild accessions and five elite genotypes accounted for more than 50% of the similarity, while the remaining 11 cultivars shared 92% similarity compared to 70% similarity among red raspberry cultivars found by Graham et al. (1994). In 2005, Lewers and Weber used SSR markers from red raspberry and strawberry to evaluate an F₂ population of a red raspberry × black raspberry cross and found that the homozygosity of the black raspberry clone used was 80% and only 40% in the red raspberry clone used. However, wild populations of black raspberry show greater genetic diversity. For example, Nybom and Schaal (1990) sampled black raspberry plants along a roadside in Missouri that were then analyzed by RFLP. They found 17 informative fragments that identified 15 genotypes in the 22 samples collected. Dossett et al. (2012b) used SSR markers to examine...
the genetic diversity among cultivars and wild germplasm. They found that the diversity at 21 loci was much higher among wild germplasm than in the elite cultivars, and that six elite cultivars were identical at these 21 loci.

Genetic diversity in wild black raspberry germplasm as detected by molecular tools (Dossett et al. 2012b; Nybom and Schaal 1990) and through breeding experiments (Dossett et al. 2008) is currently untapped. To address this, Dossett and Finn (2010) canvassed the native range of *R. occidentalis* collecting seed, which was subsequently germinated and evaluated for multiple traits including aphid resistance. From this study, three of 132 wild populations were determined to segregate for resistance to *A. agathonica*. Two populations, ORUS 3817 collected from Maine, and ORUS 3778 collected from Ontario, Canada, were subsequently used to develop populations for genetic mapping and phenotypic analysis. F1 progeny of susceptible cultivars Munger and Jewel crossed with individuals from ORUS 3778 and ORUS 3817 were all resistant to aphids under greenhouse conditions suggesting that the alleles for resistance are dominant and that ORUS 3778 (*Ag4*) and ORUS 3817 (*Ag3*) are homozygous for their respective alleles. Dossett and Finn (2010) originally identified one of the susceptible cultivars used in the crosses as ‘Black Hawk’, however, subsequent fingerprinting work found it to be ‘Jewel’ (Dossett et al. 2012a).

In this paper, we report the analysis of population ORUS 4305, an F1 black raspberry population, raised as one of several populations to investigate genetic sources of resistance to the aphid *A. agathonica* with the intent of mapping the aphid resistance allele *Ag4*. To quickly and efficiently generate markers for mapping we have employed GBS following the protocol established by Elshire et al. (2011) with modifications for *Rubus* (Ward et al. 2013), and anchored the map with SSR markers from a variety of sources. We have placed the phenotypic character of aphid resistance on this linkage map which covers the seven *Rubus* linkage groups (RLG) as defined by Bushakra et al. (2012).

**Methods**

**Plant material**

A full-sib (F1) family of 115 individuals was developed from the controlled cross of ORUS 3021-2 (female, susceptible to aphids, postulated genotype *ag4ag4*) × ORUS 4153-1 (male, resistant to aphids, postulated genotype *Ag4Ag4*, Fig. 1). The source of this resistance is from ORUS 3778-1, an accession from wild seed collected in Ontario, Canada (Dossett and Finn 2010). Progeny from this cross were screened for aphid resistance as small seedlings in the greenhouse as described by Dossett and Finn (2010) and followed the expected 1:1 segregation ratio (56 resistant, 59 susceptible, \( \chi^2 = 0.08, P = 0.78 \)).

**DNA extraction**

Leaf samples were collected, bagged, kept cool, and transported to the laboratory. Leaf tissue aliquots of 30–50 mg were placed in a cluster tube (Corning Life Sciences, Tewksbury, MA, USA) containing a 4-mm stainless steel bead (McGuire Bearing Company, Salem, OR, USA). The samples were frozen in liquid nitrogen and stored at −80 °C until extraction. Frozen tissue was homogenized using the Retsch® MM301 Mixer Mill, (Retsch Inc., Hann, Germany) at a frequency of 30 cycles/s using three 30 s bursts. The E-Z 96® Plant DNA extraction kit (Omega Bio-Tek, Norcross, GA, USA) was used as previously described (Gilmore et al. 2011).

**DNA quantification and quality**

Genomic DNA was quantified using Quant-iT™ Pico-green® dsDNA Assay kit (Invitrogen, Eugene, OR, USA) following manufacturer’s instructions modified to 100 μl and compared against a λ standard DNA dilution series with a *Victor*V 1420 Multilabel Counter (PerkinElmer, Downers Grove, IL, USA), followed by visualization on 1% agarose gel in 1× TBE (Tris/Borate/EDTA) stained
with ethidium bromide. Samples were stored at −20 °C prior to use.

**Marker sources**

SSR primer pairs were selected from multiple sources (Table 1). Markers derived from GBS were coded as S with a number indicating the scaffold followed by an underscore and a number indicating the physical SNP position on the scaffold (i.e., S75_381030) (Bryant et al. 2014). Markers developed from the sequencing of paired-end short reads were coded with Ro (R. occidentalis) or Ri (R. idaeus) followed immediately by a number (i.e., Ro11481, Ri13528) (Dossett et al. in press). All other markers are from published sources as indicated in Table 1. Ag4_AphidR is a phenotypic marker for aphid resistance.

An additional 26 SSR and two high-resolution melting (HRM) markers that mapped in multiple populations were identified from the literature (Bushakra et al. 2012; Castillo et al. 2010; Castro et al. 2013; Fernández-Fernández et al. 2013; Graham et al. 2004; Sargent et al. 2007) with the intention of anchoring and orienting the linkage groups to published maps (Table 2).

**DNA amplification of SSR markers**

DNA amplification was performed on a PTC-225 gradient thermalycler (Bio-Rad, Hercules, CA, USA), a Dyad Peltier thermalycler (Bio-Rad, Hercules, CA, USA), an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany), or a Nexus (Eppendorf, Hamburg, Germany). A fluorescent labeling polymerase chain reaction (PCR) protocol (Schuelke 2000) was used for most SSR primer pairs. The forward (F) primer of each pair was extended on the 5′-end with an M13-TGTAAAACGACGGCCAGTAGC sequence tag to which a universal M13-tagged fluorescent dye label (WellRed D2, D3, D4; Integrated DNA Technologies, Inc., Coralville, IA, USA) was annealed. The touch-down PCR protocol began with an initial denaturation for 3 min at 94 °C followed by 10 cycles of 94 °C for 40 s, 65 °C (decreasing 1 °C every cycle) for 45 s, 72 °C for 45 s; 20 cycles of 94 °C for 40 s, 52 °C for 45 s, 72 °C for 45 s; 10 cycles of 94 °C for 40 s, 53 °C for 45 s, 72 °C for 45 s; followed by a final extension of 72 °C for 30 min. Reactions were performed in a final volume of 10 μl consisting of 6 ng DNA, 1× LightScanner Master Mix, 1 μM each forward and reverse primer. Each well was topped with one drop of mineral oil. The PCR amplification protocol was 94 °C for 30 s, followed by 30 s at the appropriate annealing temperature (57 or 58 °C) and extension at 72 °C for 30 s for 40 cycles. Following a final melting step at 95 °C for 30 s, the samples were cooled to 4 °C until HRM analysis. Amplicon melting occurred on the LightScanner where samples were heated to 98 °C over a period of 8 min with default settings. Analysis was performed using the LightScanner® Instrument & Analysis Software small amplicon genotyping module.

**GBS library construction and sequencing**

GBS libraries were constructed following Ward et al. (2013) and Elshire et al. (2011). Briefly, 100 ng of genomic DNA per sample were digested with 4 U of ApeKI (New England Biolabs, Ipswich, MA, USA) and then ligated with T4 ligase to 1.8 ng of combined common and unique barcode adapters (Elshire et al. 2011). Annealed and quantitated unique barcode and common adapters were provided by the Buckler Lab for Maize Genetics and Diversity, Cornell University (Ithaca, NY, USA) and Clemson University (Clemson, SC, USA) (Supplementary Table 1).

The GBS libraries were submitted to the Oregon State University Center for Genome Research and Biocomputing...
Table 1 Transferable locus primer sequences used to construct the genetic linkage maps for black raspberry F1 population ORUS 4305

| Locus | RLG | 3021-2 allele sizes | 4153-1 allele sizes | Repeat motif | Forward primer sequence | Reverse primer sequence | Source |
|-------|-----|---------------------|---------------------|-------------|------------------------|------------------------|--------|
| ERubLR_SQ07-3_C07_HRM_RLG7 | 7 | NA | NA | NA | GGATCAAGGAGTGAGATGG | CCGTGTTGGTTATGTTG | Bushakra et al. (2012) |
| Ri_1B16_HRM_RLG7 | 7 | NA | NA | NA | CTTGGGACAGCTTATGCTTTT | AAGAAGAGGGTGGGTTTCA |
| Ru_EE284382_HRM_RLG5 | 5 | NA | NA | NA | AGGGAGTGAGGAGAAC | AGGTGAGGAGGAGATGAT |
| Ro_CBEa0001L24_SSR | 2 | 275, 277 | 277, 279 | (CCA)₆-(TA)₃ | M13-TAAAGAGAAGGAGTTTGGG | GACGTCCTACATTGGGAAGAA |
| Ro_CBEa0002P01b_SSR | 1 | 226, 228 | 228 | (TC)₆-(CT)₅ | M13-CCTCTCTCTCTCTGGTTTGGG | GCGCTGACACCTAAATGTA |
| Ro_CBEa0003K17_SSR | 1 | 307 | 307, 309 | (TC)₆ | M13-CCAGGAAAGAAACCTAAGGCG | CTTACCGCTCTTGCTTCAC |
| Ro_CBEa0004G23_SSR | 4 | 509 | 507, 509 | (AG)₈ | M13-GGCGGCTGAGATTTGTTG | GTCCTCCTGCTTCTGAGC |
| Ro_CBEa0009J505_SSR | 4 | 292 | 292, 294 | (CA)₆-(GA)₃ | M13-CCACGTCACACCTACAC | TTGCGTCCTGCTACTACAC |
| RhM003_SSR_RLG3 | 3 | 227, 236 | 236, 238 | (TG)₁₀ | M13-CCATCTCAATTCAGTTTCA | AGCGAATCGGTTCTACAGC |
| RiM017_SSR | 4 | 212, 214 | 212 (TG)₉ | M13-GAAACAGGTGGAAAGAACTG | CATGTTGACCTAATGTTTGG |
| Rh_MEa0006bG05_SSR | 6 | 294, 303 | 294 | (AAG)₈ | M13-GAACGACACAGCAAGACTT | GTTCAGGCAAGTCAATGCA |
| Rub1C6_SSR | 6 | 244, 260 | 262, 268 | (CT)₁₅ | D4-TCTGCTCCTGCATTCTAAGAG | GTTTCAGTAAGCAATGGGAAAGG |
| Ro_CBEa0010N20_SSR | 4 | 114, 118 | 118 | (GA)₁₂ | M13-GGGGGCTTACATCATCATT | TTCGAGTAAGCTTACCTG |
| Ro_CBEa0011M11_SSR | 5 | 243, 245 | 241, 243 | (AG)₁₄ | M13-GGCGATACGACACATAAAGG | TCCATTTCCAAAACACATG |
| Ri10139_SSR | 7 | 295 | 295, 314 | (TC)₈ | M13-GTCTCGGCAAAATATCAAACAA | CAGCAGAAACAGAGAAGAAG |
| Ri11086_SSR | 2 | 268, 300 | 268, 302 | (TA)₉ | M13-3AATTCTGATGTTGCGGAC | ACAACAGGAAACAGAGAAG |
| Ri11795_SSR | 3 | 299, 313 | 299, 313 | (GA)₉ | M13-ATCAGACCCATTCTGCTTGGT | GAGGACAGGAGGAAATGAT |
| Ri12319_SSR | 3 | 304 | 292, 304 | (TCA)₉ | M13-GAGTCTGATACAGACTGAGCCTT | AAGGTGAAAAAGGGAGAAC |
| Ri13528_SSR | 7 | 460, 463 | 463 | (TC)₉ | M13-CTCTGCTCAACCCATACAG | GGCATTTGGAGATTCTTG |
| Ri14075_SSR | 2 | 351, 355 | 357, 359 | (TC)₈ | M13-ACAATTTGACCTAGTCATG | CAGACCCATATAATCGT |
| Ri16959_SSR | 7 | 283, 286 | 286 | (TC)₆ | M13-AAAATGTTGAATGACGGCAG | GGGAAACTGAGAACAGGAAG |
| Ri18886_SSR | 1 | 308, 317 | 314, 317 | (ATG)₁₀ | M13-CCCAAAAGGAGAAGATTGAC | CGGCTTCTAACAGCGAGTA |
| Ri20047_SSR | 5 | 376, 393 | 376, 405 | (CT)₉ | M13-CCTGTTTCATATATTTCACCTC | GAGGACAGGCTTGGAGAT |
| Ri20466_SSR | 2 | 369 | 369, 374 | (GA)₁₀ | M13-GTGGTTTCTGGGGAAAACAGAAG | CGGCTTTCAGTCTCAAG |
| Ri3758_SSR | 2 | 392, 406 | 400, 406 | (AT)₁₀ | M13-GTGGTTTCTGGGGAAAACAGAAG | CGGCTTTCAGTCTCAAG |
| Ri5037_SSR | 2 | 336, 339 | 359, 359 | (GA)₁₀ | M13-CAGACGAAACTCCCAACTATA | TCTTGGAAATTTGGGATTTGG |
| Ro10488_SSR | 2 | 119 | 121, 131 | (TC)₆-(AC)₄ | M13-AGGTGTCATGATGACGAAT | GCTGTAGATTTGGGACTGAA |
| Ro1079_SSR | 4 | 223, 225 | 223 | (TA)₉ | M13-AAAATGAGACTAGCATCGAC | GGCGAGAATTTGAGGGTTT |
| Ro11481_SSR | 6 | 156 | 156, 158 | (AG)₁₀-(TA)₆-(AT)₅ | M13-AAGATAAAGGGAAGATGTTGG | CTGTTTCCAGCAAACTACCT |
| Ro12112_SSR | 6 | 157, 167 | 140 | (TC)₆ | M13-TACTCCAAAACCTCAGATTTG | GTCCGAGACAAATAGGAAAT |
| Ro14925_SSR | 7 | 117 | 114, 117 | (TG)₁₀ | M13-AGCGGATGGAAGAGTTTTAGT | AACCTTCCTCCGCTTCCAG |
| Ro15590_SSR | 6 | 179, 211 | 201, 205 | (GA)₁₀ | M13-GAGACGAAAGCCTGTTAGAATA | GTTGCCTGCTGATGTTTAT |
| Ro16697_SSR | 4 | 152, 160 | 160 | (AT)₁₀-(TA)₆ | M13-CCAGGTCAGTGGACTCGAGATA | ATTTGGAAGAATACGGGAA |
| Ro1682_SSR | 3 | 119, 125 | 121 | (AT)₉ | M13-AGGAGCGATGTTAGTACGAT | TAGAGGAGAAACGAGGAGTA |

Dossett and Finn (2010)
Table 1 continued

| Locus          | RLG 3021-2 allele sizes | 4153-1 allele sizes | Repeat motif | Forward primer sequence | Reverse primer sequence | Source       |
|----------------|-------------------------|---------------------|--------------|-------------------------|-------------------------|--------------|
| Ro17045_SSR    | 3 164                   | 164, 167            | (TGA)$_3$    | M13-TCCAACATGGTGACAGTTTC | ACTTTTGCACTGCTTCACTTG  |
| Ro17803_SSR    | 2 139, 147              | 139, 141            | (TA)$_{10}$  | M13-GGCGCATGATTTAAAAGGAA | GTTCAAGATGGCAGTTGAAACCA |
| Ro18036_SSR    | 1 104, 119              | 119                 | (CCT)$_3$    | M13-CCTTTGAGGACGAAACATTAC  | CTGTTGGATTCAGACGAAGATGA |
| Ro19042_SSR    | 6 201                   | 196, 201            | (GA)$_3$     | M13-GGTTATTTCCAAGCCCATAT  | TGCTTTTCAAGAGTTCACTCT  |
| Ro20267_SSR    | 4 159                   | 153, 159            | (TGA)$_3$    | M13-GAAACAGCTTGTGATGTTTC  | GTTTGATTTCTAGGAAGTTGC  |
| Ro2173_SSR     | 4 199, 240              | 203, 221            | (TTA)$_3$    | M13-TATTGGGGATTTAGAGAGCTA  | GGGTTGATTTCAGTTGACACCA |
| Ro2432_SSR     | 5 114, 116              | 114                 | (TC)$_3$     | M13-GCTTTAGTAATTTAGGCTG   | CTTCTCAAGACAGCGGAT  |
| Ro2579_SSR     | 4 181                   | 179, 181            | (CA)$_{10}$  | M13-TTTTATATGCTTGTCACCCAC  | ATTTAGAATAGGGGCACTC  |
| Ro2827_SSR     | 5 133, 141              | 137, 141            | (CT)$_3$     | M13-GGCCTTGTTTTCTCTAGCT  | AGAGGGCAAGAGAGCTTAC   |
| Ro3003_SSR     | 5 145, 199              | 145, 152            | (GA)$_3$     | M13-AGGTGTGATCATGCTTCAAT  | CTTCCATAGCAACTCTATCCC  |
| Ro3017_SSR     | 3 159, 173              | 161                 | (GA)$_3$     | M13-CAACCGCTTTATGAAAGTGTG  | GCACAGATGACCAACTCAACA  |
| Ro3237_SSR     | 1 131, 135              | 133, 135            | (TA)$_3$     | M13-AACCCAAAGCCTTGTCTTTGT  | ATTTGGGCTGTTTCTATGAGAT |
| Ro3981_SSR     | 6 115, 117              | 115                 | (TG)$_3$     | M13-GATCTCTGATCTCCGCTATAT  | AAATGTCTTTCTAGTATGAGAT |
| Ro4104_SSR     | 7 181, 185              | 181, 183            | (TA)$_3$     | M13-AAAAGCTTCTCCTAATTTTGAGC  | ATGATTAGACGCAGTGACATCA  |
| Ro4261_SSR     | 4 204, 219              | 204, 219            | (TTC)$_3$    | M13-ATATAGCAGGAGACACTCACC  | TCTCATTGACAGGTGGATTAC  |
| Ro4345_SSR     | 5 108, 114              | 114                 | (TC)$_3$     | M13-TTACAGCAATTTAGAGGAGC  | AAGAAATAGGGGAAGGGGAGG  |
| Ro4532_SSR     | 6 210, 213              | 210, 213            | (TGT)$_3$    | M13-AGTGTACATTTAGGAGATG  | TCTGATGATCATCATTACACC  |
| Ro5263_SSR     | 6 201, 203              | 201                 | (GA)$_3$     | M13-AACCTTTGCTGTTTGAATCT  | TTTTGTTGGAACCTTTCCTCC  |
| Ro5378_SSR     | 4 207                   | 191, 207            | (TA)$_3$     | M13-TCTTCCACATGTCCACTGGT  | TCAGTGATTTTGCCAGAGAT  |
| Ro6594_SSR     | 1 171, 177              | 171                 | (TTC)$_3$    | M13-TGTGAGAAGAGAGATGTGTA   | GTTGAATGACTGCTCCAGCAG  |
| Ro7020_SSR     | 3 168, 177              | 177                 | (GA)$_3$     | M13-CTGCAAGAACCCTCTAATCC  | TGGTTCTTCACAAATCTTTAG  |
| Ro8167_SSR     | 6 94, 96                | 96                  | (TC)$_3$     | M13-CAATTGCACATACCCCTACT  | GAGGAGATGCAAAACCGAAGAG  |
| Ro8486_SSR     | 2 172, 178              | 172                 | (CT)$_3$     | M13-TGGCGTCTGATGTCTTACAT  | AAGGTAAGAATAGGGGAGTGT  |
| Ro9206_SSR     | 5 135, 139              | 126, 137            | (AT)$_3$     | M13-ACAGTTCTCACAAGGATCGGA  | CGAGATGTTACAGTACTCGGGA  |
| Ro9324_SSR     | 1 156, 164              | 164, 201            | (AG)$_3$     | M13-CCTACCTTCAAGGCCATTTTG  | GCAATCAGACATAAAAAGGTC  |
| Ro942_SSR      | 1 155, 161              | 161, 181            | (GA)$_3$     | M13-AATCGTGTGTGCTGCAATTTC  | CAAATGCACACACCTATCAGG  |
| Rubus110a_SSR_RLG4 | 4 187, 207              | 203, 205            | (TC)$_3$     | M13-AAAAAACAGTAAATGGGAGAAGG  | GTGTCAGTTTGGAGGAGAACA Graham et al. (2004) |
| Rubus116a_SSR_RLG4 | 4 222, 224              | 218, 224            | (CT)$_3$     | M13-CCAACCCAAAACCTCTCAAC  | GTTGTTGACATGCCTTCTTTAT  |
| Rubus126b_SSR_RLG2 | 2 171                   | 157, 171            | (CT)$_3$     | M13-CCTGCAATTTCTGATTTCTG  | TCAGTTTTTCTTCCACCGTGA  |
| Rubus156a_SSR_RLG6 | 6 170, 172              | 164, 172            | (AT)$_3$(GT)$_3$ | M13-TGTTGATCCTGGTGCTTTC  | GGGTTGTTGCGACTTCTAGT  |
| Rubus223a_SSR  | 6 158, 162              | 158                 | (AT)$_3$(T)$_3$(AT)$_3$ | M13-TCTTTGCGTGTTAGATTTCTAT  | TTAAGGCGTCTGAGTATAAGG |
| Rubus26a_SSR   | 4 139, 141              | 137, 143            | (CT)$_3$     | M13-ACACCGCTCTAAGGTTATC  | GATTTCTGGAAGAGTGAAGAA  |
| Rubus270a_SSR_RLG3 | 3 182, 184              | 184, 186            | (GA)$_3$     | D3-GCATCAGCATGTTACCTC   | CCAACCTCCATGACACATCAC  |
| Rubus275a_SSR_RLG5 | 5 139, 165              | 165                 | (AG)$_3$     | M13-CACAACAGTCCGAGAATGA   | ATTTCTCACAAATGCAACC  |
core facilities (Corvallis, OR, USA) for quantitation using a Qubit® fluorometer (Invitrogen, Carlsbad, CA, USA). The size distribution of the library was confirmed by checking 1000 pg of DNA with the Bioanalyzer 2100 HS-DNA chip (Agilent Technologies, Santa Clara, CA, USA). Libraries were diluted to 10 nM based on Qubit® readings and quantitative PCR (qPCR) was used to quantify the diluted libraries. For each pooled library, 15.5 pM were loaded for single-end Illumina® sequencing of 101 cycles with the HiSeq™ 2000 (Illumina, Inc.) and analyzed using the Version 3 cluster generation and sequencing kits (Illumina, Inc.).

The libraries were sequenced in three lanes at three different times. The first sequencing lane included 95 samples (91 progeny, and two replicated samples per parent). The second sequencing run included 88 samples (26 black raspberry including parents, grandparents, standards and progeny, and 62 unrelated strawberry samples). The third sequencing run included 64 samples (ORUS 3021-2 repeated 4 times, ORUS 4153-1 repeated 5 times and 55 progeny). Over all three runs, the parents ORUS 3021-2 and ORUS 4153-1 were sequenced at least twice in each lane. Forty-four progeny were sequenced more than once due to low initial quality and numbers of reads per individual.

### GBS SNP calling

Version 3.0 of the TASSEL GBS discovery software pipeline (Li et al. 2009) was used to call SNP loci using a repeat-masked version of the genome sequence. Three GBS runs representing 112 individuals as described above were analyzed simultaneously. Data were initially subjected to sequence and nucleotide read quality control using Trimmomatic (Bolger et al. 2014) (http://www.usadellab.org/cms/?page=trimmomatic) and were then analyzed with TASSEL.

### Genetic linkage map construction

All loci were converted into segregation codes for JoinMap® v. 4.1 (Van Ooijen 2006). Loci were then organized into parental sets and subjected to the maximum likelihood (ML) mapping algorithm. Independence Likelihood of Odds (LOD) threshold of 5 was used for establishing the linkage groups (LG). All other settings were default. Five progeny (ORUS 4305-38, 39, 41, 59, and 65) were excluded based on incongruous SNP data occurring from 30 (ORUS 4305-39) to 90 (ORUS 4305-65) times. GBS data were not available for ORUS 4305-7, 19, 45, 54, 58, 75, 95, 97, 103, and 110 due to poor DNA quality. The consensus map of seven linkage groups was generated by combining the parental linkage maps of ORUS 3021-2 and
ORUS 4153-1 using the regression algorithm of the mapping software JoinMap v. 4.1. Linkage map visualization was accomplished with MapChart 2.2 (Voorrips 2002).

The quality of genotype calls and of each map were evaluated with a graphical genotyping approach in Microsoft Excel (Redmond, WA, USA) as previously described (Bassil et al. 2015; Young and Tanksley 1989).

Results

Transferable markers

In total, 552 SSR markers from new and published sources were evaluated for the amplification of polymorphic PCR products in the parents and one progeny. Of these, 118 failed to amplify, 235 were homozygous in both parents or gave ambiguous results, 138 were heterozygous in both parents, 29 were heterozygous in ORUS 3021-2, and 32 were heterozygous in ORUS 4153-1 (Table 2).

A total of 30 primer pairs (SSR and HRM) for 28 anchor loci were assessed for the production of a polymorphic PCR product in the parents and six progeny of population ORUS 4305. Twelve of these loci were successfully mapped (Table 2).

Eighty HRM primer pairs (Bushakra et al. 2012) were evaluated for the amplification of polymorphic PCR products on the parents and 14 progeny. Of these 80 HRM primer pairs, 57 were monomorphic, 12 were unclear or had poor amplification, and 11 were evaluated in the full population. Three of these HRM markers were mapped successfully, two in ORUS 3021-2 and one in ORUS 4153-1 (Table 2). Out of 660 transferable markers evaluated, a total of 72 (11 %) were successfully mapped. BLAST analysis (Altschul et al. 1990) of the forward and reverse primer and nucleotide sequences (when available), allowed scaffold assignment of most mapped transferable markers (Supplementary Table 2).

GBS SNP markers

The first sequencing run of 95 samples generated 596 K sequence clusters/mm² (optimal density is 750–850 K clusters/mm²; MyIllumina Support Bulletin); the second and third sequencing runs were within the optimum range at 825 and 752 K clusters/mm², respectively. These cluster densities provided raw reads ranging from approximately 165 million to 310 million. Over the three sequencing runs, 112 progeny and the two parents were sequenced to generate an average number of reads per individual of 3,105,333, with 20,317,182 (5.8 %) of reads unaligned. Default TASSEL filtering parameters using the parent information identified 57,238 SNP positions. Further filtering of the SNP data to remove those loci with more than 10 % missing data resulted in a data set of 7911 SNP loci, of which 3472 were monomorphic or ambiguous, 921 were heterozygous in both parents, 318 were heterozygous in ORUS 3021-2, and 326 were heterozygous in ORUS 4153-1 (Table 2).

A total of 100 progeny were used to construct the seven linkage groups for the parental linkage maps, the

| Table 2 Summary of loci mapped in F1 population ORUS 4305 |
|-----------------------------------------------------------|
| **Genotyping by sequencing (GBS)**                       |
| Total number of GBS SNP identified over three sequencing  |
| runs                                                      |
| Number of monomorphic or ambiguous loci                  |
| Number of loci heterozygous in both parents              |
| Number of loci heterozygous in ORUS 3021-2               |
| Number of loci heterozygous in ORUS 4153-1               |
| Total scaffolds represented                             |
| Scaffolds mapping to multiple RLG                        |
| Total number of GBS SNP mapped                           |
| **Simple sequence repeat (SSR)**                         |
| Total number of loci screened                            |
| Number of monomorphic or ambiguous loci                  |
| Number of loci that failed                               |
| Number of loci that are heterozygous in both parents      |
| Number of loci heterozygous in ORUS 3021-2               |
| Number of loci heterozygous in ORUS 4153-1               |
| Number of loci mapped                                    |
| **High-resolution melting (HRM)**                        |
| Total number of loci screened                            |
| Number of monomorphic or ambiguous loci                  |
| Number of loci that failed                               |
| Number of loci heterozygous in ORUS 3021-2               |
| Number of loci heterozygous in ORUS 4153-1               |
| Number of loci mapped                                    |
| Anchor loci (26 SSR + 2 HRM)                             |
| Not mapped                                               |
| Mapped                                                   |

Genotyping by sequencing (GBS) single nucleotide polymorphic (SNP) loci were generated by DNA digestion and subsequent high-throughput sequencing. Data were analyzed for mapping using the TASSEL computer software provided through the Buckler Lab for Maize Genetics and Diversity. Simple sequence repeat (SSR) and high-resolution melting (HRM) loci were derived from a number of sources.

ORUS 4153-1 using the regression algorithm of the mapping software JoinMap v. 4.1. Linkage map visualization was accomplished with MapChart 2.2 (Voorrips 2002).

The quality of genotype calls and of each map were evaluated with a graphical genotyping approach in Microsoft Excel (Redmond, WA, USA) as previously described (Bassil et al. 2015; Young and Tanksley 1989).
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characteristics of which are summarized in Table 3. To construct the linkage map for ORUS 3021-2, five GBS-generated SNP markers were removed for skewed segregation ratios, four were removed for creating double recombination events within a distance of 10 cM or less, and one was removed due to unsuccessful linkage phase determination. For ORUS 3021-2 (Supplementary Fig. 1) the resulting 274 markers comprising the seven LGs spanned 779.4 centi-Morgans (cM) with an average distance of 2.9 cM between markers. RLG7 had the greatest number of markers (56), and was also the longest (134.5 cM) with an average distance of 2.4 cM between markers. RLG2 was the shortest at 84.1 cM, with an average distance of 2.8 cM between the 30 markers, and two gaps of 11.4 and 11.9 cM. The largest gap for the map of ORUS 3021-2 was 22.2 cM on RLG6. Of the 222 GBS SNP markers used for map construction, 200 (90 %) segregated as expected, either 1:1 or 1:2:1; two loci (1 %) varied from expected at a significance level of 0.01, 11 loci (5 %) varied from expected at a significance level of 0.05, and nine loci (4 %) varied from expected at a significance level of 0.1. To construct the linkage map for ORUS 4153-1, 18 GBS-generated SNP markers were removed for skewed segregation ratios, 14 were removed for creating double recombination events within a distance of 10 cM or less, and one SSR marker was removed due to unsuccessful linkage phase determination. For ORUS 4153-1 (Supplementary Fig. 2) the resulting 292 markers comprising the seven LGs spanned 892.1 cM with an average distance of 3.2 cM between markers. RLG7 had the greatest number of markers (64) and was also the longest (151.4 cM) with an average distance of 2.4 cM between markers, and three gaps greater than 10 cM, the largest of which was 12.2 cM; RLG2 was the shortest at 101.7 cM with 23 markers, an average distance of 4.4 cM between markers, and three gaps greater than 10 cM, the largest of which was 14.8 cM. The largest gap for the map of ORUS 4153-1 was 14.8 cM at the end of RLG1. Of the 249 GBS SNP markers used for map construction, 230 (92 %) segregated as expected, either 1:1 or 1:2:1; a single locus (0.4 %) varied from expected at a significance level of 0.01, nine loci (4 %) varied from expected at a significance level of 0.05, and nine loci (4 %) varied from expected at a significance level of 0.1.

Transferable markers for the parental maps ranged from a low of three markers on ORUS 3021-2 RLG7 and 4153-1 RLG6 to a high of 12 on ORUS 4153-1 RLG4. A total of 72 transferable markers were mapped in this population. BLAST analysis of the transferable markers against the draft genome assembly allowed scaffold assignment for 65 of 72 markers (90 %) so that 356 scaffolds were represented. The phenotypic marker for aphid resistance, Ag4_AphidR, was located on RLG6 of the aphid-resistant parent ORUS 4153-1 and maps to the same location as S99_32802 (Fig. 2).

The seven consensus RLGs (Table 3; Fig. 3) assembled from merging the parental maps consisted of 438 markers spanning 546.4 cM with an average distance between markers of 1.3 cM. Consensus RLG6 was the longest (90.2 cM) with an average distance between the 69 markers of 1.3 cM, and one gap of 10.4 cM. Consensus RLG7 had the most markers (77) that spanned 81.0 cM with an average distance of 1.1 cM between markers. RLG2 was the shortest at 70.8 cM with an average distance between the 59 markers of 1.2 cM. The 12 anchor markers identified from the literature (Table 1; Supplementary Figs. 1, 2, markers in italics) allowed the positive identification of consensus RLG 2-7, with the last, RLG1, identified by default.

Thirteen of the 356 represented scaffolds (3.6 %) map to more than one linkage group (Table 4); 33 of the loci are SNPs and five are SSRs. Four scaffolds (S10, S26, S134, and S142) are represented by SNP loci on more than two linkage groups. Four scaffolds (S14, S71, S78, and S279) are represented by at least one SNP and a single SSR locus on more than one linkage group.

Discussion

We present the first linkage map constructed from a pure black raspberry cross. The first attempt at genetic linkage mapping using SSR markers on an F2 generation of a black raspberry × red raspberry cross identified high homozygosity as well as severe segregation distortion and did not result in a linkage map (Lewers and Weber 2005). The linkage map constructed using non-anonymous DNA sequences for black raspberry selection 96395S1 comprises 29 markers spaced on average at intervals of 10 cM over six LG spanning 306 cM (Bushakra et al. 2012). The first published red raspberry map of ‘Glen Moy’ × ‘Latham’ consisted of 273 markers derived from amplified fragment length polymorphic and genomic-SSR markers and spanned 789 cM over nine LG (Graham et al. 2004). Over the next 6 years as more markers were developed and added, the improved ‘Glen Moy’ × ‘Latham’ map reported by Woodhead et al. (2010) consisted of 228 markers over seven LG spanning 840.3 cM with transferable markers present on each LG. Paterson et al. (2013) subsequently added gene-based markers to the linkage map constructed by Woodhead et al. (2010) by mining Rubus transcriptome and EST databases for candidate genes in the fruit volatiles pathway. The efficiency of marker generation used here is a vast improvement over previous marker development techniques in Rubus. The saturated consensus linkage map presented here spans
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546.2 cM and is composed of 374 GBS-generated SNP markers and 68 transferable markers with an average of 1.3 cM between markers. The transferable markers are distributed among the LG and can be used for alignment to other Rubus maps. The scaffold assignment allows for

| Table 3 | Summary of genetic linkage maps for female parent ORUS 3021-2, male parent ORUS 4153-1, and the consensus map for F1 population ORUS 4305 |
|---------|----------------------------------------------------------------------------------------------------------------------------------|
| ORUS 3021-2 (female parent) | ORUS 4153-1 (male parent) | Consensus Map |
| Locus number | Number of transferable loci | Average distance between loci in cM | Locus number | Number of transferable loci | Average distance between loci in cM | Locus number | Number of transferable loci | Average distance between loci in cM |
| RLG1 | 29 | 8 | 99.5 | 3.4 | 2 | 23 | 6 | 101.7 | 4.4 | 3 |
| RLG2 | 30 | 8 | 84.1 | 2.8 | 2 | 40 | 7 | 115.9 | 2.9 | 1 |
| RLG3 | 44 | 5 | 113.6 | 2.6 | 1 | 33 | 4 | 102.9 | 3.1 | 1 |
| RLG4 | 38 | 8 | 115.3 | 3.0 | 1 | 43 | 12 | 143.0 | 3.3 | 1 |
| RLG5 | 45 | 10 | 134.0 | 3.0 | 1 | 43 | 4 | 127.8 | 3.0 | 2 |
| RLG6 | 32 | 10 | 98.4 | 3.1 | 1 | 46 | 3 | 149.4 | 3.2 | 3 |
| RLG7 | 56 | 3 | 134.5 | 2.4 | 0 | 64 | 6 | 151.4 | 2.4 | 3 |
| **Total** | **274** | **52** | **779.4** | **2.9** | | **292** | **42** | **892.1** | **3.2** | | **438** | **70** | **546.4** | **1.3** |

Each Rubus linkage group (RLG) details the number of loci mapped, the number of loci that are transferable (either simple sequence repeat or high-resolution melting), the length of the linkage group in centiMorgans (cM), the average distance between each locus in cM, and the number of gaps larger than 10 cM

Fig. 2. Rubus linkage group (RLG) 6 for black raspberry mapping population parent ORUS 4153-1. The morphological locus for Ag4 aphid resistance against the North American large raspberry aphid is shown in blue bold font. The linkage map is constructed of single-nucleotide polymorphic (SNP) loci generated by sequencing (GBS) (prefaced with S) and simple sequence repeat (SSR) loci from various Rubus sources (prefaced with Ro, Ri, Rh, Ru, Rub, and SQ). Transferable loci are indicated in bold font; anchor loci for comparisons with other Rubus linkage maps are indicated in bold italic font (color figure online)
Consensus_RLG1

Consensus_RLG2

Consensus_RLG3

Consensus_RLG4

Fig. 3 Consensus linkage map for population ORUS 4305. Each of the linkage groups consists of single-nucleotide polymorphic (SNP) loci generated by genotyping by sequencing (GBS) (prefaced with S) and simple sequence repeat (SSR) loci from various Rubus sources (prefaced with Ro, Ri, Rh, Ru, Rub, and SQ). Transferable loci are indicated in bold font; anchor loci for comparisons with other Rubus linkage maps are indicated in bold italic font. The morphological locus for $A_g$ aphid resistance against the North American large raspberry aphid is shown in blue bold font (color figure online).

Lu et al. (2013) performed GBS in tetraploid switchgrass (Panicum virgatum) and were able to map 88,217 SNP loci; Truong et al. (2012) used GBS to generate SNP in Arabidopsis thaliana and lettuce (Lactuca sativa) and were able to map 1200 and 1113 SNP loci, respectively; Russell et al. (2014) mapped 790 SNP loci in blackcurrant (Ribes nigrum). This is the first use of GBS on black raspberry, a future fine mapping, QTL analysis, and improved black raspberry genome assembly.

The reduced-representation sequencing accomplished with GBS has generally been used in crop plants with high levels of heterozygosity. For example, Poland et al. (2012) were able to map 20,000 and 34,000 GBS-generated SNP loci in wheat and barley reference linkage maps, respectively;
Fig. 3 continued
trait loci (QTL) having to do with resistance to aphids and fungal-like (Graham et al. 2011) pathogens in red raspberry and we hope to use our linkage map to better understand the underlying reasons for these associations.

**Conclusions**

We present here the first genetic linkage map of black raspberry comprised of GBS-generated SNP and transferable markers. The presence of SSR and HRM markers selected from the literature, along with the other transferable markers allowed us to positively identify all RLG as well-populated map, it, along with the low mapping success rate of transferable markers, illustrates the low level of heterozygosity found in black raspberry. In contrast, the linkage maps constructed of GBS-derived SNP and SSR markers for red raspberry parents ‘Heritage’ and ‘Tulameen’ comprise 4521 markers spaced on average at intervals of 0.1 cM over seven LG spanning 462.7 cM and 2391 markers spaced on average at intervals of 0.1 cM spanning 376.6 cM, respectively (Ward et al. 2013). While digestion by a more frequent restriction enzyme cutter for GBS may be a way to increase the number of SNP loci identified, this does not guarantee mapping success as segregation within the population is essential for linkage mapping.

Up to 97% of the mapped scaffolds were placed on a single linkage group indicating high quality assembly of the draft genome. The 13 scaffolds that map to multiple LGs will need to be investigated further to assess whether these inconsistencies represent errors in the genome assembly; however, initial observations could indicate regions of high chromosome homology or possible regions of genome duplication especially between RLG3 and RLG7.

**Table 4** Summary of the genomic scaffolds with loci on more than one *Rubus* linkage group (RLG)

| Scaffold | RLG | Parent | SNP, SSR |
|---------|-----|--------|----------|
| S10     | 1, 2, 4 | 4153-1 | 3, 0     |
| S14     | 1, 2  | 3021-2 | 2, 0     |
| S14     | 2, 6  | 4153-1 | 1, 1     |
| S21     | 3, 7  | 3021-2 | 2, 0     |
| S26     | 3, 5, 7 | 3021-2 | 3, 0     |
| S71     | 1, 2  | 3021-2 | 1, 1     |
| S78     | 5, 6  | 3021-2 | 1, 1     |
| S115    | 3, 7  | 3021-2 | 2, 0     |
| S134    | 2, 5  | 3021-2 | 2, 0     |
| S134    | 1, 2, 5 | 4153-1 | 3, 0     |
| S142    | 1, 3  | 3021-2 | 2, 0     |
| S142    | 3, 7  | 4153-1 | 2, 0     |
| S161    | 7     | 3021-2 | 1, 0     |
| S161    | 4     | 4153-1 | 1, 0     |
| S279    | 3, 7  | 4153-1 | 1, 1     |
| S380    | 3     | 3021-2 | 1, 0     |
| S380    | 6     | 4153-1 | 1, 0     |
| S472    | 1, 7  | 3021-2 | 2, 0     |

Each entry details the linkage group and the parental map on which the loci are found and the type of locus, either single-nucleotide polymorphic (SNP) or simple sequence repeat (SSR).
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