Microsatellite Markers for *Aronia melanocarpa* (Black Chokeberry) and Their Transferability to Other *Aronia* Species

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Abstract. This study reports the development, characterization, and cross-species transferability of 20 genomic microsatellite markers for *Aronia melanocarpa*, an important nutraceutical fruit crop. The markers were developed with Illumina paired-end genomic sequencing technology using DNA from Professor Ed cultivar that was originally collected from the wild in New Hampshire. The markers were highly polymorphic and transferable to *Aronia arbutifolia* and *Aronia prunifolia* genomes. The average number of alleles per locus was 9.1, 4.5, and 5.6 for *A. melanocarpa*, *A. arbutifolia*, and *A. prunifolia*, respectively. The polymorphism information content (PIC) of loci ranged from 0.38 to 0.95 for all taxa, with an average of 0.80, 0.68, and 0.87 for *A. melanocarpa*, *A. arbutifolia*, and *A. prunifolia*, respectively. This is the first study to develop microsatellite markers in the *Aronia* genus. These markers will be very useful in studying the genetic diversity and population structure of wild *Aronia* and expediting the breeding efforts of this emerging fruit crop through marker-assisted selection.

The genus *Aronia* (Rosaceae) is composed of multistemmed deciduous woody shrubs native to eastern North America (Brand, 2009; Hardin, 1973). Generally, the genus is considered to contain three species, *A. melanocarpa* (L.) Elliot (black

chokeberry), *A. arbutifolia* (Michx.) Elliot (red chokeberry), and *A. prunifolia* (purple chokeberry) (Hardin, 1973). There is a continuum of morphological and phenological characteristics among the three species and evidence suggests that *A. prunifolia* may be an interspecific hybrid between *A. arbutifolia* and *A. melanocarpa* (Brand, 2009; Hardin, 1973). The large-fruited forms of *Aronia* grown for fruit production represent a fourth species called *Aronia mitschurinii* (Jeppsson, 2000; Skvortsov and Maitulina, 1982; Skvortsov et al., 1983). *Aronia mitschurinii* has been shown to be an intergeneric hybrid between *Aronia* and *Sorbus* (Leonard et al., 2013).

For many years, dark-fruited forms of *Aronia* have been targeted for breeding as a fruit crop because of their valuable nutraceutical properties. Phytochemical analyses have shown that *Aronia* berries have the highest content of phenolic compounds of any temperate fruit (Zheng and Wang, 2003) with *A. melanocarpa* having the

highest content of anthocyanins, exceeding levels found in *A. arbutifolia* or *A. prunifolia* fruits (Taheri et al., 2013). The nutraceutical potential of *Aronia* berries (see reviews by Kokotkiewicz et al., 2010; Kulling and Rawel, 2008) has sparked great interest in the development of *Aronia* as a fruit crop in the United States. To support *Aronia* agriculture and ensure its viability, there is a need to quickly develop new high-yielding cultivars using molecular breeding techniques and genetically diverse germplasm from *Aronia*'s native range (Persson Hovmalm et al., 2004). Some cultivars of *A. mitschurinii* have been selected, but these cultivars are phenotypically indistinguishable from other plants of *A. mitschurinii* and have limited molecular variation due to apomictic seed production (Brand, 2009; Persson Hovmalm et al., 2004).

The success of molecular plant breeding hinges on availability of genome-specific molecular tools such as microsatellites or simple sequence repeat (SSR) markers. SSR markers are hypervariable, codominantly inherited, and widely distributed in the genome making them very informative for plant studies (Kalia et al., 2011). Furthermore, SSR markers are sometimes transferable to closely related taxa making it possible to study other species whose genome sequence data are not available. The objective of this study was to develop microsatellite markers for *A. melanocarpa* and assess their transferability to *A. arbutifolia* and *A. prunifolia* genomes.

Materials and Methods

Plant materials and DNA extraction. A total of 22 *Aronia* germplasm accessions (13 *A. melanocarpa*, 4 *A. arbutifolia*, and 5 *A. prunifolia*) were used in this study (Table 1). All accessions are maintained as live plants at the University of Connecticut, Plant Science Research Farm, Storrs, CT. Genomic DNA was extracted from fresh young leaves following the protocol outlined in Lubell et al. (2008), with the exception that the initial homogenate was not filtered through miracloth. The quality and concentration of extracted DNA were determined using a NanoDrop-1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

Isolation of microsatellite loci and primer design. DNA from 'Professor Ed' (accession number UC023) was used to isolate microsatellite loci. An Illumina paired-end shotgun library was prepared by shearing 1 µg of DNA using a Covaris S220 ultrasonicator (Covaris, Inc., Woburn, MA) and following the standard protocol of the Illumina MiSeq Reagents kit v2 (Illumina, San Diego, CA). Illumina sequencing was conducted on the MiSeq system (Illumina) with 150 bp paired-end reads. The resulting reads were analyzed with the program PAL_FINDER_v0.02.03 (Castoe et al., 2012) to identify reads that contained di-, tri-, tetra-, penta-, and hexanucleotide microsatellites. Once reads were identified, they were batched to the program

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Table 1. Species, accession number, cultivar name, source, and place of collection of *Aronia* genotypes used in this study.

Species	Accession no.	Cultivar name	Source of accession	Place of collection
<i>Aronia melanocarpa</i>	UC004	'Morton' Iroquois Beauty	RareFind Nursery	New Jersey
	UC020		Wild	Maine
	UC023	'Professor Ed'	Wild	New Hampshire
	UC024	var. <i>elata</i>	Forest Farm	Oregon
	UC031		Wild	Connecticut
	UC049		Wild	Connecticut
	UC054		Wild	New Jersey
	UC064		Wild	New York
	UC095		Wild	Pennsylvania
	UC110A		Wild	Tennessee
	UC160		Wild	Vermont
	PI545686		USDA collection, Ames, IA	Ontario, Canada
	PI603106		USDA collection, Ames, IA	Tennessee
<i>Aronia arbutifolia</i>	UC001	'Brilliantissima'	Spring Meadow Nursery	Michigan
	UC021	'Erecta'	Forest Farm	Oregon
	UC080		Wild	New York
	UC088		Wild	Florida
<i>Aronia prunifolia</i>	UC019		Wild	Maine
	UC045		Wild	Rhode Island
	UC090		Wild	Massachusetts
	UC092		Wild	Massachusetts
	PI603107		USDA collection, Ames, IA	Virginia

Primer3 v2.0.0 for primer design. Only sequences that occurred one to two times were selected to avoid issues with the copy number of primer sequences in the genome. Forty-eight of the 6219 loci that met this criterion were selected for primer development. One primer in each pair was modified at the 5'-end with an engineered CAG tag sequence (5'-CAGTCGGGCGTCATCA-3'), and the other primer was pigtailed with GTTT sequence at the 5'-end. The CAG tag enabled use of a third fluorescent dye-labeled primer in the polymerase chain reaction (PCR) to allow visualization of fragments following capillary electrophoresis (Schuelke, 2000).

PCR and marker analysis. The forty-eight primer pairs selected were tested for PCR amplification and polymorphism using DNA from eight additional samples of *A. melanocarpa*. PCR amplifications were performed in 12.5 µL volume containing 10 mM Tris (pH 8.4), 50 mM KCl, 25.0 µg/mL BSA, 3.0 mM MgCl₂, 0.4 µM unlabeled primer, 0.04 µM tag-labeled primer, 0.36 µM fluorescent dye-labeled primer, 0.8 mM dNTPs, 0.5U AmpliTaq Gold® Polymerase (Applied Biosystems, Foster City, CA), and 20 ng DNA template using GeneAmp 9700 thermal cycler (Applied Biosystems) (Allen et al., 2012). A touchdown thermal cycling program (Don et al., 1991) was used for all PCR amplifications. The program included an initial denaturation step at 95 °C for 5 min followed by 20 cycles of 95 °C for 30 s, 65–55 °C annealing temperatures (decreasing 0.5 °C per cycle) for 30 s, and an extension step at 72 °C for 30 s; followed by 20 cycles of 95 °C for 30 s, 55 °C for 30 s,

and 72 °C for 30 s, and a final extension step at 72 °C for 5 min.

Amplified fragments (amplicons) were separated by capillary electrophoresis using ABI-3130xl sequencer (Applied Biosystems) and were sized using Naurox internal size standard prepared as described in DeWoody et al. (2004) with the exception that nonfluorescent primers had GTTT pigtailed on their 5'-ends. Twenty primer pairs that successfully amplified and were polymorphic in the eight samples were further tested on five more *A. melanocarpa* samples and evaluated for their cross-amplification in *A. arbutifolia* and *A. prunifolia* accessions. Amplicons were analyzed using GeneMapper version 3.7 software (Applied Biosystems). Given the polyploid nature of the *Aronia* genus, genetic parameters such as observed and expected heterozygosity could not be reliably determined. Instead, PIC values were calculated to evaluate the genetic informativeness of each locus. Alleles at each locus were treated as dominant markers and were scored as present (1) or absent (0) across all accessions. The frequency of alleles at each locus was determined based on binary scores, and PIC values were calculated using the formula $PIC = 1 - \sum (P_i)^2$; where P_i is the frequency of the i th allele at each locus (Anderson et al., 1993).

Results and Discussion

Twenty of the 48 primer pairs tested amplified high-quality PCR products and exhibited polymorphism in *A. melanocarpa*

accessions tested. The characteristics of the 20 microsatellite loci are shown in Table 2, and the BioSample metadata are available in the NCBI BioSample database (<http://www.ncbi.nlm.nih.gov/biosample/>) under accession number SAMN05325189. Together, the 20 polymorphic SSR loci yielded 182 alleles in *A. melanocarpa*. The number of alleles per locus ranged from three to twenty, with an average of 9.1. The allele sizes ranged from 147 to 451 bp, and PIC values ranged from 0.62 to 0.93, with an average of 0.80 (Table 2). All 20 SSRs were transferable to *A. arbutifolia* and *A. prunifolia* genomes (Table 2). In *A. arbutifolia*, the number of alleles per locus ranged from 1 to 9, with an average of 4.5. The allele sizes ranged from 155 to 423 bp, and PIC values ranged from 0.38 to 0.88, with an average of 0.68. In *A. prunifolia*, the number of alleles per locus ranged from 2 to 10, with an average of 5.6. The allele sizes ranged from 159 to 443 bp, and PIC values ranged from 0.59 to 0.95, with an average of 0.87 (Table 2).

Several studies have demonstrated cross transferability of SSR markers in plants; however, the ratio of marker transferability between taxa is influenced by the closeness of their relationship. In a study by Vanwynsberghe et al. (2009), transferability of apple SSR markers to other taxa within Maloideae subfamily ranged from 58% to 94%. Higher transferability ratios were observed between species within genera (94%) compared with between genera (58% to 81%). Similarly, Fan et al. (2013) reported that 58.2% of the 67 pear SSR markers tested were transferable to apple compared with only 1.5% in strawberry. Apple and pear belong to Maloideae subfamily and strawberry belongs to Rosoideae subfamily, both in the Rosaceae family. On the basis of these transferability percentages, it is apparent that the pear genome is more closely similar to that of apple than strawberry. In our study, the high percentage of transferability (100%) of *A. melanocarpa* SSR markers to *A. arbutifolia* and *A. prunifolia* obviously reflects the close similarities of their genomes. It will be interesting, however, to see if these newly developed *Aronia* SSR markers are transferable to other taxa in the Rosaceae family.

This is the first study to develop and characterize microsatellite markers in *A. melanocarpa*, and to test for their transferability to other *Aronia* species. Given the growing interest to develop *Aronia* as a nutraceutical fruit crop, these SSR markers will be a great resource for the scientific community interested in studying *Aronia* and other members of Rosaceae. In particular, these markers could be used in assessing the genetic diversity and structure of *Aronia* populations in the wild, evaluating the diversity of *Aronia* accessions currently held in different germplasm collections to better understand and manage existing genotypes, identifying important horticultural

Table 2. Characteristics of the 20 polymorphic microsatellite loci markers developed in *Aronia melanocarpa* and their cross-amplification in *Aronia arbutifolia* and *Aronia prunifolia*.

Locus ^z	Primer sequence (5'–3')	Repeat motif	<i>A. melanocarpa</i> (n = 13)			<i>A. arbutifolia</i> (n = 4)			<i>A. prunifolia</i> (n = 5)		
			Size range (bp)	No. of alleles	PIC	Size range (bp)	No. of alleles	PIC	Size range (bp)	No. of alleles	PIC
Arme02	F: *GCAGGTTGGATAATGCTAAGGG R: GTTATCTTGGCTTATGTCTCCTTCG	(ATAC) ₄₀	287–321	6	0.76	305–313	3	0.64	309–321	4	0.85
Arme03	F: *CATCTACTTTGGCCTCATTTGG R: GTTTGATTGATGACGAATTTCAAACC	(ATAC) ₄₄	292–348	10	0.84	296–320	7	0.83	292–320	6	0.91
Arme04	F: *ACGACCGTCTCGACAATTCC R: GTTTCATTTGGTCTCTGCAATCG	(AAAG) ₃₂	264–321	13	0.89	279–321	9	0.87	275–305	8	0.93
Arme05	F: *TTAATAAACATAGAACTCGAAAGTGGG R: GTTCTTCGAATCCGAGAGCC	(TTGG) ₂₈	147–183	7	0.71	155–163	3	0.59	159–163	2	0.59
Arme09	F: *CAAACTTCCATGGTGATTGTGG R: GTTCCGAGACTGAATCAAACGC	(ATAC) ₃₂	176–214	9	0.77	176–190	3	0.57	182–210	4	0.84
Arme10	F: *CTGTTCCAGAACCCTCTCCC R: GTTGGCATGACGCAAGAAAGGG	(AGTG) ₃₆	280–352	7	0.82	282–292	2	0.38	280–300	6	0.84
Arme14	F: *ATTTGGGATCCCTCAACG R: GTTTCATTTGCTCCCACTCCG	(ATAC) ₂₈	309–345	12	0.89	305–323	6	0.81	309–345	8	0.94
Arme15	F: *ATTGGACCGTGATTAGGGC R: GTTGAAGGTGGTTGTAGTGCCG	(AGGG) ₂₈	187–203	4	0.66	187–195	2	0.50	187–199	4	0.84
Arme16	F: *GAGAGGCGTCTTCTACCG R: GTTAAAGCAAATCGCTTGACAATCC	(ATAC) ₂₈	323–359	8	0.81	337–353	4	0.72	323–371	4	0.81
Arme23	F: *TTCTACTTTGGATGATGGATCG R: GTTGTCTGTGTTCATAGGAGGAGG	(ATGG) ₂₈	263–278	5	0.67	262–278	4	0.72	266–274	3	0.81
Arme25	F: *AGCCTTCAATTATCGACCC R: GTTAAACCAGAAAGGTGGTGCC	(ATAC) ₃₆	253–273	7	0.75	261–269	3	0.61	261–283	4	0.88
Arme26	F: *GCTTGATTAAACTGTGTGATTGTGC R: GTTTGAGGCGTGAGGTGAAACC	(ATAC) ₄₀	194–224	8	0.84	198–248	5	0.77	190–248	7	0.93
Arme29	F: *GGAGATTGTAGAGAGAATGATTGC R: GTTGTAGCTATTGTCCACCACACG	(AACT) ₃₂	229–273	8	0.78	225–233	3	0.59	229–241	3	0.87
Arme30	F: *AATTCGCACACTATAAATTCATGC R: GTTAAAGCTCGGCTTAGTACAGTTTCC	(ATAC) ₄₀	395–451	13	0.90	395–423	2	0.49	395–443	8	0.90
Arme32	F: *TCCTTCAGGGTCAAATTCAGG R: GTTGTCTCTGCAACAAAGGGAAGG	(ATAC) ₃₂	237–266	11	0.88	241–257	6	0.78	245–257	7	0.92
Arme36	F: *GCTTAATCTTCCTTAACATGTTGTCC R: GTTAAAGTCTTTGCATGACGGCG	(AAAT) ₂₈	180–192	3	0.62	180–188	2	0.49	188–192	2	0.80
Arme39	F: *TAAACCACGCACTCAATCG R: GTTCTGGTAGAGTTTGCTAGAAAGTGCC	(AAAG) ₃₂	208–268	9	0.84	212–264	9	0.87	212–268	9	0.93
Arme41	F: *CAGGGTCTGGATGGACAAGG R: GTTCCCACATTGAATAATATCCATCCC	(AAAG) ₃₂	194–246	7	0.74	194	1	—	194–238	3	0.88
Arme45	F: *GAGAAATCATATGGTTAGCAGACCG R: GTTTCATCATGACCAAACCTGTGC	(ATAC) ₂₈	219–303	15	0.88	239–307	7	0.84	238–293	10	0.95
Arme46	F: *TGCACGATGCACCTACATACC R: GTTTATTCTCCACATGCCTTGGG	(ATAC) ₃₂	188–274	20	0.93	204–276	9	0.88	192–260	9	0.93
Average				9.1	0.80		4.5	0.68		5.6	0.87

PIC = polymorphism information content; F = forward primer; R = reverse primer, all reverse primers had a GTTT pigtail added at the 5'-end.

^zA touchdown protocol spanning annealing temperatures 65–55 °C was used for all loci.

^yCAG tag label used: 5'-CAGTCGGGCGTCATCA-3'.

^xCAG tag label used: 5'-CAGTCGGGCGTCATC-3'.

^wCAG tag label used: 5'-CAGTCGGGCGTCAT-3'.

characteristics in different *Aronia* species, and expediting *Aronia* breeding efforts through marker-assisted selection.

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