Sexual and Apomictic Seed Reproduction in *Aronia* Species with Different Ploidy Levels

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Additional index words. Rosaceae, Pyrinae, apomixis, meiosis, polyploid, AFLP

Abstract. The genus *Aronia* Medik., also known as chokeberry, is a group of deciduous shrubs in the Rosaceae family, subtribe Pyrinae. The four commonly accepted species include *A. arbutifolia* (L.) Pers., red chokeberry; *A. melanocarpa* (Michx.) Elliott, black chokeberry; *A. prunifolia* (Marshall) Reheder, purple chokeberry; and *A. mitschurinii* (A.K. Skvortsov & Maitul). Wild and domesticated *Aronia* species are found as diploids, triploids, and tetraploids. Genetic improvement of polyploid *Aronia* genotypes has been limited by suspected apomixis, which may be widespread or distinct to tetraploids. The objectives of this study were to elucidate the reproductive mechanisms of *Aronia* species and reveal the occurrence of apomixis within the genus and along ploidy lines. Twenty-nine *Aronia* accessions (five *A. melanocarpa* (2x), five *A. melanocarpa* (4x), eight *A. prunifolia* (3x), four *A. prunifolia* (4x), six *A. arbutifolia* (4x), and one *A. mitschurinii* (4x)) were used in this study. Intra-accession variability was evaluated by growing out progeny from each open-pollinated maternal accession and comparing plant phenotypes, ploidy levels, and amplified fragment length polymorphism (AFLP) marker profiles between the progeny and maternal accession. Progeny of diploid and tetraploid maternal plants had ploidy levels identical to maternal plants, except for UC009 (*A. melanocarpa*, 2x) which produced a mix of diploids and tetraploids. UC143 and UC149 (*A. prunifolia*, 3x) produced all triploid offspring, whereas all other triploid accessions produced offspring with variable ploidy levels including 2x, 3x, 4x, and 5x. Pentaploid *Aronia* has not been previously reported. Diploid accessions produced significant AFLP genetic variation (0.68–0.78 Jaccard’s similarity coefficient) in progeny, which is indicative of sexual reproduction. Seedlings from tetraploid accessions had very little AFLP genetic variation (0.93–0.98 Jaccard’s similarity coefficient) in comparison with their maternal accession. The very limited genetic variation suggests the occurrence of limited diplosporous apomixis with one round of meiotic division in tetraploid progeny. Triploid accessions appear to reproduce sexually or apomictically, or both, depending on the individual. These results support our understanding of *Aronia* reproductive mechanisms and will help guide future breeding efforts of polyploid *Aronia* species.

Native to eastern regions in North America, the genus *Aronia* is a group of deciduous shrubs in the Rosaceae family, subtribe Pyrinae. The Pyrinae subtribe has a base chromosome count of n = 17 (Postman, 2011), and *Aronia* species are commonly found as diploids (2n = 2x = 34) and tetraploids (2n = 4x = 68) with some occurrence of triploids.

(Received for publication 26 Nov. 2018. Accepted for publication 24 Jan. 2019. This research was partially supported by the U.S. Department of Agriculture Multistate Hatch NC007 Plant Germplasm and Information Management and Utilization.


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nearly all *A. mitschurinii* cultivars, an intergeneric hybrid involving *A. melanocarpa* × *Sorbus aucuparia* L. (Leonard et al., 2013; Skvortsov and Mautulina, 1982), are genetically identical and likely renares of a single genotype (J.D. Mahoney and M.H. Brand, unpublished data). Interest in *Aronia* is high because their fruits contain high levels of antioxidants and polyphenols (Brand et al., 2017; Wu et al., 2004; Zheng and Wang, 2003), they are valuable as replacements for invasive exotic ornamental plants (Brand, 2010), and they are widely adapted to various geographic regions (Dirr, 2009; McKay, 2001).

*Aronia* flowers are thought to be protogynous and self-compatible (Connolly, 2014). Polyploid *Aronia* species have been reported to reproduce apomictically, via gametophytic apomixis, resulting in embryos that are identical or nearly identical to maternal plants (Brand, 2010). Hovmalm et al. (2004) reported that diploid *A. melanocarpa* produced highly heterogeneous offspring and tetraploid plants produced homogeneous offspring, suggesting that polyploid *Aronia* reproduce apomictically. Gametophytic apomixis occurs when a progenitor cell in the megasporangium forms a megagametophyte (Grossniklaus et al., 2001; Richards, 2003). Gametophytic apomixis is further classified into two categories: diplospory and apospory. In diplosporous apomictic plants, the megagametophyte forms from an unreduced or partially reduced megaspore. When a partially reduced megaspore is involved, meiosis is initiated but fails before completion and cell division continues mitotically (Bicknell and Koltunow, 2004). The result is an unreduced megagametophyte derived from a megaspore in which homologous recombination and one round of segregation may have occurred. Apospory refers to an unreduced megagametophyte arising from nucellar or integument tissue (Koltunow and Grossniklaus, 2003). Talent (2009) mentions that pseudogametic gametophytic apospory is common in the Maloide Rosaceae (Pyrinae), where seed development requires pollination, but the embryo has no paternal inheritance and only the endosperm is fertilized. Both diplospory and apospory have been reported to occur in the same species, including the Pyrinae genera *Crateagus* (Muniyamma and Phipps, 1979, 1984a, 1984b) and *Sorbus* (Jankun and Kovanda, 1988).

In normal sexual reproduction, genetic uniformity and hybrid vigor are lost after the F1 generation, but with apomixis these traits can be maintained through many generations due to a fixed heterozygosity (Koltunow et al., 1995; Ortiz et al., 2013; Richards 2003). For this reason, seed propagation of apomictic selections is possible and allows growers to achieve high yields while avoiding more expensive vegetative propagation methods (Barcaccia and Albertini 2013). In apomictic temperate fruit crops, it is advantageous to use vegetative propagation from mature phase plants rather than regenerate from juvenile seed. *Aronia*
and sand and placed under cool white medium with a ratio of 5:3:1 screened clear plastic salad trays containing potting mix and eventually transferred to 1-gallon pots. Four clonal softwood stem cuttings from *A. mitschurinii* (4x) were rooted in mid-June and served as a control.

**Phenotypic observations.** Within accessions, maternal plants and 2-year-old progeny were compared for overall plant form or habit, branching structure, leaf shape and size, phyllotaxy, degree of pubescence, and leaf and stem color. Floral and fruit traits were not used due to the juvenile and non-reproductive nature of the plants. Progeny were scored as either homogeneous or heterogeneous in comparison with each other and their maternal parent based on the composite characteristics of each plant. Homogeneity/heterogeneity scores were the combined opinions of five researchers knowledgeable about *Aronia*.

**Flow cytometry.** A modified version of the protocol in Arumuganathan and Earle (1991), summarized in Lehrer et al. (2008), was followed. Two to three newly emerged leaves were macerated using a fresh razor blade in nuclei suspending solution in a 55-mm petri dish on a freeze pack. The suspending solution was filtered, centrifuged to form a nuclei pellet, and resuspended in nuclei staining solution containing propidium iodide. Relative fluorescence of total DNA was measured using a Becton-Dickson FACs Calibur Dual Laser Flow Cytometer (Becton, Dickson and Co., Franklin Lakes, NJ) at the Flow Cytometry and Confocal Imaging Facility at the University of Connecticut in Storrs, CT. The cytometer was equipped with an argon ion laser emitting radiation at 488 nm. For each sample, 10,000 to 20,000 particles were measured. Data were logged and displayed in histograms by BD Cellquest TM software (Becton, Dickson and Co.). Histogram peaks from known ploidy levels of *Aronia* were compared with samples of unknown ploidy.

**DNA extraction and AFLP procedure.** A subset of 20 *Aronia* accessions (five *A. melanocarpa* (2x), five *A. melanocarpa* (4x), eight *A. prunifolia* (3x), six *A. prunifolia* (4x), four *A. arbutifolia* (4x), and one *A. mitschurinii* (4x)) were used for AFLP analysis. Genomic DNA was extracted following the protocol outlined in Leonard et al. (2013). The quality and concentration of extracted DNA were determined using a NanoDrop-1000 spectrophotometer (Thermo Scientific, Willington, DE). The AFLP steps including restriction digestion, adaptor ligation, and preselective and selective amplification reactions were carried out as outlined in the AFLP plant mapping protocol (Anonymous, 2007). Preselective primers had one selective nucleotide (EcoRI-A + MseI-C). Seven primer combinations were used for selective amplification (EcoRI-AGG + Msel-CAC, EcoRI-ACT + Msel-CAT, EcoRI-AGG + Msel-CAT, EcoRI-ACT + Msel-CTC, EcoRI-ACT + Msel-CTA, and EcoRI-ACT + Msel-CTG) and were fluorescently labeled EcoRI and unlabeled MseI probes. The DNA fragments from selective polymerase chain reaction were visualized by capillary electrophoresis on an ABI3730xl DNA analyzer (Applied Biosystems, Foster City, CA) using the 500 LIZ® Size Standard (Thermo Fisher Scientific, Waltham, MA).

**AFLP data analysis.** AFLP fragment files were processed into binary matrices using

### Materials and Methods

**Plant material.** Twenty-nine *Aronia* accessions (five *A. melanocarpa* (2x), five *A. melanocarpa* (4x), eight *A. prunifolia* (3x), six *A. prunifolia* (4x), four *A. arbutifolia* (4x), and one *A. mitschurinii* (4x)) were used in this study as maternal genotypes (Table 1). Plants were maintained at the University of Connecticut Research Farm in Storrs, CT. The maternal accessions were grown in a randomized field planting consisting of 120 *Aronia* accessions (with three replicates) and representing all four species and various ploidy levels. Intra-accession progeny variability was evaluated by growing out seedlings from their open-pollinated maternal accessions. Each maternal plant had the opportunity to be pollinated by any other accession in the field collection. Seeds were collected from a single maternal accession plant of each by cleaning them from the fruits and air drying them before placement in cool, dark storage (13 ± 2 °C, relative humidity 55 ± 5%) until further use. Seeds were cold stratified in moist sand for 90 d in 50-mL conical centrifuge tubes at 5 °C. Stratified seeds were sown in 32-oz. clear plastic salad trays containing potting medium with a ratio of 5:3:1 screened composted pine bark, sphagnum peatmoss, and sand and placed under cool white fluorescent light (80 μmol·m⁻²·s⁻¹) at 24 °C. Seedlings were transferred to standard 50-cell plug trays with the same 5:3:1

| Accession | Species | Maternal ploidy | Germplasm origin | No. of progeny |
|-----------|---------|-----------------|------------------|---------------|
| UC007     | A. melanocarpa | 2x              | Connecticut     | 10            |
| UC009     | A. melanocarpa | 2x              | Maine           | 10            |
| UC012     | A. melanocarpa | 2x              | Maine           | 10            |
| UC020     | A. melanocarpa | 2x              | Maine           | 10            |
| UC023     | A. melanocarpa | 2x              | New Hampshire   | 10            |
| UC011     | A. prunifolia | 3x              | Maine           | 10            |
| UC141     | A. prunifolia | 3x              | Cultivated origin | 12        |
| UC143     | A. prunifolia | 3x              | Cultivated origin | 17        |
| UC145     | A. prunifolia | 3x              | Cultivated origin | 10        |
| UC146     | A. prunifolia | 3x              | Cultivated origin | 10        |
| UC149     | A. prunifolia | 3x              | Cultivated origin | 10        |
| UC150     | A. prunifolia | 3x              | Cultivated origin | 4          |
| UC156     | A. prunifolia | 3x              | Cultivated origin | 8          |
| UC001     | A. arbutifolia | 4x              | Cultivated origin | 7          |
| UC021     | A. arbutifolia | 4x              | Cultivated origin | 10         |
| UC052     | A. arbutifolia | 4x              | New Jersey      | 10          |
| UC057     | A. arbutifolia | 4x              | New Jersey      | 6           |
| Ames27010 | A. melanocarpa | 4x              | Michigan        | 10          |
| UC005     | A. melanocarpa | 4x              | Cultivated origin | 10          |
| UC006     | A. melanocarpa | 4x              | Cultivated origin | 10          |
| P1603106  | A. melanocarpa | 4x              | Tennessee       | 11          |
| P1545060  | A. melanocarpa | 4x              | Ontario, Canada | 10          |
| UC003     | A. mitschurinii | 4x              | Cultivated origin | 9          |
| UC003 clonal | A. mitschurinii | 4x              | Cultivated origin | 4          |
| UC013     | A. prunifolia | 4x              | Cultivated origin | 10          |
| UC019     | A. prunifolia | 4x              | Maine           | 10          |
| UC062     | A. prunifolia | 4x              | Massachusetts   | 10          |
| UC048     | A. prunifolia | 4x              | Connecticut     | 7           |
| P1603107  | A. prunifolia | 4x              | Virginia        | 10          |
| UC070     | A. prunifolia | 4x              | New York        | 9           |

*aVegetative cutting propagated plants.*
The majority of triploid A. prunifolia accessions produced progeny that exhibited heterogeneous phenotypes (Table 2). UC143 and UC149 were exceptions and produced progeny with homogeneous phenotypes. In addition, eight of the 10 progeny from UC011 were phenotypically indistinguishable, but two seedlings exhibiting distinct phenotypes were shown by flow cytometry to be pentaploids (Table 2).

Ploidy level. Diploid accessions of A. melanocarpa produced diploid progeny with the single exception of accession UC009 (Table 2). Accession UC009 produced mostly diploid seedlings but also produced two tetraploid seedlings.

Triploid A. prunifolia accessions UC143 and UC149 produced only triploid progeny and UC011 produced mostly triploid progeny (Table 2). UC011 also produced two pentaploid plants sharing a distinct phenotype. Triploid A. prunifolia accessions UC141, UC145, and UC146 produced a mix of ploidy levels within their progeny, including 2x, 3x, 4x, and 5x plants. The triploids UC150 and UC156 produced seedlings at the 2x, 4x, and 5x ploidy levels but did not produce 3x seedlings. The relatively small progeny sample size may be responsible for the absence of 3x plants, or these accessions may not produce 3x progeny.

Tetraploid accessions, whether they were A. melanocarpa, A. prunifolia, A. arbutifolia, or A. mitschurinii, only produced tetraploid progeny (Table 2). The single exception was one pentaploid seedling that was produced by the tetraploid A. melanocarpa accession PI545682.

AFLP. Seedlings from diploid accessions produced a significant amount of intra-accession genetic variation in progeny (Jaccard’s similarity coefficient of 0.64 to 0.82), which was indicative of sexual reproduction (Fig. 2). These sets of progeny from diploid maternal plants also exhibited variable phenotypes that corroborate sexual seed production within this taxonomic group.

Maternal triploid plants that produced heterozygous phenotypes in their progeny, such as UC141 and UC150, also showed significant intra-accession genetic variation (Jaccard’s similarity coefficient of 0.65 to 0.80), providing support for sexual seed development in these accessions (Fig. 2). In contrast, maternal triploid plants that produced phenotypically homogeneous progeny, such as UC143, showed very little intra-accession genetic variation (Jaccard’s similarity coefficient of 0.90 to 0.99).

Seedlings from tetraploid accessions of A. melanocarpa, A. prunifolia, A. arbutifolia, and A. mitschurinii all exhibited small amounts of intra-accession genetic variation (Jaccard’s similarity coefficient of 0.89 to 0.98) (Fig. 2). These tetraploid accessions also displayed homogeneous phenotypes. In contrast, clonally propagated plants of UC003 (A. mitschurinii, 4x) propagated by cuttings produced AFLP profiles that were identical to the stock plant (Jaccard’s similarity coefficient of 1.00).

Discussion

In this study, we found very limited genetic and phenotypic variation in most polyploid offspring, providing strong evidence of diplosporous apomixis. Diploid and several triploid accessions produced significant variation in offspring, demonstrating that seed reproduction occurred sexually. In diplosporous apomicts, the small amount of genetic recombination that occurs during seed formation may produce phenotypic variability in progeny only after many generations (Campbell and Dickinson, 1990). For example, morphological variation has been detected in offspring of heavily cultivated apomictic lineages of Taraxacum (Sorensen and Gudjonsson, 1946 cited by Baarlen et al., 2000). However, it would require a significant amount of time to observe this variation in temperate shrubs because of their generation times.

There are several explanations for the variation in ploidy levels we observed in diploid and triploid Aronia progeny. As mentioned previously, the Aronia maternal accessions were planted in a randomized field design, which allowed open-pollination to occur and potential outcrossing between different species and ploidy levels. UC009 (A. melanocarpa, 2x) produced eight diploid offspring but also produced two tetraploid offspring that could have been either self-fertilized with an unreduced 2n gamete or out-crossed with a reduced 2n gamete from a

Table 2. Ploidy level and phenotypic observations of open-pollinated progeny, from maternal accessions of Aronia arbutifolia, A. melanocarpa, A. prunifolia, and A. mitschurinii with varying ploidy levels.

| Accession | Maternal ploidy | No. 2x | No. 3x | No. 4x | No. 5x | Progeny ploidy level | Progeny appearance |
|-----------|-----------------|--------|--------|--------|--------|----------------------|-------------------|
| UC007     | 2x              | 10     | 0      | 0      | 0      | Heterogeneous        |                   |
| UC009     | 2x              | 8      | 0      | 2      | 0      | Heterogeneous        |                   |
| UC012     | 2x              | 10     | 0      | 0      | 0      | Heterogeneous        |                   |
| UC020     | 2x              | 10     | 0      | 0      | 0      | Heterogeneous        |                   |
| UC023     | 2x              | 10     | 0      | 0      | 0      | Heterogeneous        |                   |
| UC011     | 3x              | 0      | 8      | 0      | 2      | Homogeneous<sup>a</sup> |                   |
| UC141     | 3x              | 1      | 3      | 5      | 3      | Homogeneous<sup>b</sup> |                   |
| UC143     | 3x              | 0      | 17     | 0      | 0      | Homogeneous<sup>c</sup> |                   |
| UC145     | 3x              | 1      | 3      | 1      | 5      | Homogeneous<sup>d</sup> |                   |
| UC146     | 3x              | 1      | 2      | 3      | 4      | Homogeneous<sup>e</sup> |                   |
| UC149     | 3x              | 10     | 0      | 0      | 0      | Homogeneous<sup>f</sup> |                   |
| UC150     | 3x              | 2      | 0      | 1      | 1      | Heterogeneous        |                   |
| UC156     | 3x              | 6      | 0      | 1      | 1      | Heterogeneous        |                   |
| UC001     | 4x              | 0      | 0      | 7      | 0      | Homogeneous<sup>g</sup> |                   |
| UC021     | 4x              | 0      | 0      | 10     | 0      | Homogeneous<sup>h</sup> |                   |
| UC052     | 4x              | 0      | 0      | 10     | 0      | Homogeneous<sup>i</sup> |                   |
| UC057     | 4x              | 0      | 0      | 6      | 0      | Homogeneous<sup>j</sup> |                   |
| Ames27010 | 4x              | 0      | 0      | 10     | 0      | Homogeneous<sup>k</sup> |                   |
| UC005     | 4x              | 0      | 0      | 10     | 0      | Homogeneous<sup;l</sup> |                   |
| UC006     | 4x              | 0      | 0      | 10     | 0      | Homogeneous<sup>m</sup> |                   |
| PI603106  | 4x              | 0      | 0      | 11     | 0      | Homogeneous<sup>n</sup> |                   |
| PI545682  | 4x              | 0      | 0      | 9      | 1      | Homogeneous<sup:o</sup> |                   |
| UC003     | 4x              | 0      | 0      | 9      | 0      | Homogeneous<sup>p</sup> |                   |
| UC003 clonal<sup>q</sup> | 4x | 0 | 0 | 4 | 0 | Homogeneous<sup>r</sup> |                   |
| UC013     | 4x              | 0      | 0      | 10     | 0      | Homogeneous<sup>s</sup> |                   |
| UC19      | 4x              | 0      | 0      | 10     | 0      | Homogeneous<sup>t</sup> |                   |
| UC062     | 4x              | 0      | 0      | 10     | 0      | Homogeneous<sup>u</sup> |                   |
| UC084     | 4x              | 0      | 0      | 7      | 0      | Homogeneous<sup>v</sup> |                   |
| PH603107  | 4x              | 0      | 0      | 10     | 0      | Homogeneous<sup>w</sup> |                   |
| UC070     | 4x              | 0      | 0      | 10     | 0      | Homogeneous<sup>x</sup> |                   |

<sup>a</sup>With the exception of two 5x plants.
<sup>b</sup>With the exception of one 5x plant.
<sup>c</sup>Vegetative cutting propagated plants.

With the exception of two 5x plants.
tetraploid Aronia spp. Similarly, UC141, UC145, UC146, UC150, and UC156 (A. prunifolia, 3x) produced variable ploidy levels in their offspring (diploids, triploids, tetraploids, and pentaploids). This suggests that the megagametophyte was reduced, partially reduced, or unreduced and then self-fertilized or out-crossed with reduced or unreduced paternal gametes from diploids, triploids or tetraploids.

In this study, we focused our analysis on within-intra-accession variability, whereas other studies have looked at within- and among-population variability. Hovmalm et al. (2004) evaluated eight native populations of Aronia for genetic variation and ploidy. They found populations that had high within-population diversity and high among-population similarity were all diploid, whereas tetraploid populations tended to have lower within-population diversity and significantly greater among-population diversity. This suggests that tetraploid populations were restricted in outcrossing with other populations because of their apomictic reproductive mechanisms. We found tetraploid and several triploid Aronia accessions to have a high genetic similarity to their maternal accession, which provides additional evidence to support apomictic seed reproduction in tetraploid and at least some triploid Aronia. Since Aronia apomicts reproduce through pseudogamy, fusion of the polar nuclei with one of the sperm nuclei may produce endosperm that contains some paternal inheritance. The sexually reproducing accessions in this study produced a wide range of Jaccard’s similarity coefficients from 0.64 to 0.82, which suggests that progeny from these accessions were the outcome of out-crossing or selfing.

Sources of genetic variation during meiosis include homologous recombination of chromosomes (crossing over) and random separation of homologous chromosomes. Homologous recombination, which takes place during prophase I, involves the exchange of genes between nonsister chromatids. The process of diplospory involves the apomeiotic formation of an unreduced female gametophyte from an apomictic initial cell, which will either omit or abort meiosis (Schmidt et al., 2015). During meiotic restitution, homologous recombination still takes place, creating a limited amount of genetic variation, which may explain the AFLP variation we observed in the polyploid apomictic offspring. In this study, we found that tetraploid and several triploid Aronia produced progeny with high Jaccard’s similarity coefficients of 0.89 to 0.98, which was indicative of apomitic diplospory. These similarity coefficients are significantly greater than the 0.64 to 0.82 we observed for diploid, sexually reproducing Aronia. In a study similar to ours with apomictic Cotoneaster, Bartish et al. (2001) reported some differences between RAPD marker profiles within progeny populations grown out from three maternal accessions and found Jaccard’s similarity coefficient of 0.97, 0.98, and 1.00. When comparing RAPD similarity coefficients for plants from different accessions, but still belonging to the same taxon, they obtained values of 0.77, suggesting sexual reproduction. Bartish et al. (2001) describe the very small marker differences as “in most likelihood, artefactual…” or derived from somatic mutations. An alternative explanation is that the similarity values are the result of diplospory and apospory apomixis occurring in the same species. Both forms of apomixis have been reported to occur within the same species of a closely related genus, Crataegus (Muniyamma and Phipps, 1979, 1984a, 1984b). In our study, we included vegetatively propagated material as a control to check for error. We did not find any genetic differences between clonal vegetative cuttings, providing evidence that AFLP markers were reliable and that the genetic differences we detected were a product of diplosporous apomixis.

The combination of homogeneous phenotypes and small amounts of genetic variation suggests that tetraploid and some triploid Aronia species produced seed through gametophytic diplosporous apomixis with one round of meiosis occurring. In contrast, heterogenous phenotypes and large amounts of genetic variation suggest that diploid and some triploid accessions reproduce sexually. Aronia has the potential to be a valuable study system because of its relatively short juvenile phase, compared with other temperate fruit crops, along with the ability to develop autotetraploids via somatic doubling from diploid material. This would allow for further investigation into the genetic
mechanisms involved with sexual and apomorphic reproduction in Aronia using nearly isogenic diploid and tetraploid genotypes, respectively. Furthermore, our results will enhance and guide future breeding efforts for genetic improvement of polyploid Aronia.

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