Insights into the genetic diversity of cranberries (*Vaccinium macrocarpon*), its wild relatives, and their relationship with other *Vacciniums*

By

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“Caminante son las huellas el camino y nada mas

Caminante no hay camino se hace camino al andar”

“Wayfarer, the only way
Is your footprints and no other.
Wayfarer, there is no way.
Make your way by going farther.”

Antonio Machado

Abstract

Cranberry breeding is evolving as consumer demand is increasing for specialty products. Therefore, new varieties are needed not only with better adaptations to biotic (e.g., fungal fruit rot) and abiotic (e.g., cold tolerance) stressors, but also with increased fruit quality. Cranberry is one of the few species in the genus *Vaccinium* utilized as a crop. However, the genus is composed of non-cultivated species, such as wild blueberries and cranberries. Many of these species are cross-compatible and possess an array of traits of high agronomic value that may be commercially exploited through intersectional breeding. In order to increase genetic resources for *Vaccinium* breeders, we tested the cross-transferability of 507 *V. macrocarpon* simple sequence repeat (SSR) markers on 17 different *Vaccinium* species. We found 61 SSR markers that consistently amplified and produced scorable bands across all 17 species tested. These markers will help us fingerprint the potential hybrids we obtained from *V. macrocarpon* x blueberry and *V. oxycoccos* x blueberry crosses. Some of the offspring of these crosses exhibit an intermediate phenotype between *V. oxycoccos*, *V. elliottii*, and a *V. corymbosum* (4x) that has *V. elliottii* in its background. When studying the genetic diversity of wild populations in the US, we found high levels of heterozygosity in wild populations of cranberries in Wisconsin and Minnesota, as well as in locations throughout the US located in National Forests. We found that there is much more diversity in the wild that needs to be explored and incorporated into breeding programs. Our results could be useful for making *in-situ* conservation decisions to protect valuable CWR of one of the few crops native to North America and for advancing breeding.

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General Introduction

For thousands of years berries have been an important source of nutrients for humans. Birch-bark containers from the Bronze Age (1500-1300 BC) have been found containing the remains of cowberries and bog cranberries used as ingredients in fermented beverages (McGovern 2009). Native Americans believed blueberries were gifts from the "great spirits of the night of heaven" so they could feed their children (Rock 2011). Cranberries were used in *pemmican*, a concentrated mixture of fat and protein used as a nutritious food, probably as a preservation agent, due to the high concentration of benzoic acid in cranberries (Eck 1990). Moreover, all Native people around the world have used berries to add variety to their diets, especially in winter when diets would consist mainly of seal, fish, and other meat. It is believed that the tart flavor of the berries was a good addition to a diet mostly based in proteins and fats (Darrow and Camp 1944, Vander Kloet 1988).

Berries were adopted by the Pilgrims when they arrived in North America as an abundant source of sustenance. In the 1800s the first commercial plantings of American cranberry (*Vaccinium macrocarpon* Ait.) were established using wild selections such as Early Black (1835), Howes (1843), McFarlin (1874), and Searles (1893) (Eck 1990). These cultivars are still widely used today for commercial production and breeding. Shortly after the first cranberry cultivars were domesticated, the first blueberry cultivars, Brooks (1908; highbush; *V. corymbosum* L.) and Russell (1909; lowbush; *V. angustifolium* Ait.), were also selected from the wild and established commercially. Since then, cranberries and blueberries have become the sole two commercially-grown members of the *Vaccinium* genus, and both species have active research and breeding programs (Song and Hancock 2011).

The genus *Vaccinium* consists of more than 400 species, most of them still in their wild state. Besides cranberry and blueberry, only a few other species, such as lingonberries (*V. vitis-idea*), are cultivated by small scale farmers for personal consumption as fresh fruit, jams, or in baked goods. *Vaccinium* species have a rich geographical distribution in temperate and circumboreal areas, but some species can also be found in Central and South America, as well as in some tropical areas in Asia and Africa. With such a wide range of distribution, the members of this genus provide an extensive toolbox full of undiscovered traits for breeders to use. According to Vorsa et al. (2012), a large number of non-cultivated *Vaccinium* species could be extremely useful as new crops or as a source of useful traits into cultivated species. For example, wild berries, such as lingonberry, have been historically used to treat colds and stomach ailments, improve vascular health, and relieve urinary tract infections.

The American cranberry

The American cranberry (*V. macrocarpon*) is a diploid perennial species that can grow as a shrub or vine up to 2 meters long and 20 cm in height (Song and Hancock 2011). Its natural distribution ranges from Newfoundland down to the Appalachian Mountains, Tennessee, and North Carolina, and west to central Minnesota. This popular berry is widely cultivated the US, Canada and parts of Europe where it was commercially introduced (Vander Kloet 1988). The top cranberry-producing countries are the US (304,089 tonnes), Canada (76,071 tonnes) and Chile (33,424 tonnes) (FAOSTAT 2013). The cranberry industry is valued at around 3.5 billion US dollars and is expected to keep growing due to high demand from consumers (Alston, 2014). With the increased demand for cranberry products, breeding programs are actively searching for large collections of wild accessions and genetic pools to expand the genetic base and to find

new useful traits. Thus, the cranberry breeder ideotype will possess higher yield and increased fruit quality and nutrition, coupled with better adaptations to biotic (e.g., fungal fruit rot) and abiotic (e.g., cold tolerance) stressors.

Cranberries have been shown to possess high nutritional and antioxidant properties. Extracts of cranberry juice contain elevated levels of apolar phenolic compounds, flavonoids, and antioxidants that possess high free radical-scavenging activity, which is believed to reduce or prevent cancer (Caillet *et al.* 2011). Flavonoids such as the procyanidins found in cranberries have been shown to possess antimicrobial, antihypertension, and antioxidant activity. Cranberries also possess the highest level of antioxidant phenols among other fruits such as grapes, blueberries, and strawberries (McKay and Blumberg 2007). Polyphenols, produced in plants such as cranberries, are known to provide protection against pathogens and ultraviolet radiation (Pandey *el al.* 2009). Cranberries are low in calories, sodium, and fat and are a great source of soluble fiber (McKay and Blumberg 2007).

Currently, the most widely grown cranberry cultivars share a very similar genetic background that could make them susceptible to challenges such as pathogens, climate change, and volatile commercial and economic conditions. In fact, all hybrid cultivars released in the history of cranberry breeding are derived from Early Black, Howes, McFarlin, and Searles, the "Big Four," and a few other native selections, such as Potters Favorite, Ben Lear, and LeMunyon, collectively denominated as the "Big Seven". Additionally, the most widely grown cranberry cultivar, 'Stevens,' which accounts for over 40% of all acreage grown worldwide, was also developed based on this narrow genetic pool. Due to the narrow genetic diversity base in cranberry, the introduction of new genotypes from wild populations into breeding programs

could add much needed genetic diversity and new useful traits such as adaptability to different temperatures and environments, resistance to diseases and pests, and increased yield, quality, and nutrition. As a starting point to breeding with new wild cranberry materials, breeding programs must broaden their collection of germplasm, gather trait and adaptation data, and perform studies to understand the genetic diversity, population structure, and genetic pools of the plant materials to ensure the expansion of the genetic base.

The small cranberry

The small cranberry*, Vaccinium oxycoccos*, is a sister species to cultivated *Vaccinium macrocarpon*. The species is morphologically very similar to the commercial cranberry and grows as a small evergreen perennial vine up to 80cm long (Jacquemart *el al.*, 1997a). The species propagates asexually, but it also produces overwintering berries with seed numbers that range from 3 to 11 (Vander Kloet 1988). *V. oxycoccos* has a circumboreal distribution that can extend to temperate areas (Jacquemart *el al.*, 1997b). Diploid (also known as *V. microcarpum* (Smith et al. 2015, Diaz-Garcia et al. 2019)) populations have been documented from Alaska to Alberta and Hudson Bay (Mahy *el al.*, 2000). Tetraploids can be found extending from Southern Alaska to Labrador, and as far south as the northern parts of California, Idaho, Minnesota, Wisconsin, Michigan, West Virginia, Tennessee, North Carolina and Northern Europe (Mahy *el al.*, 2000). The species can be found in humid and acidic environments such as basin-filled raised bogs and peatlands, sharing the same ecological niches as several *Sphagnum* species. *V. oxycoccos* is not commercially grown, even though in Europe it is widely consumed and harvested from wild stands. Since the fruit is mainly collected from natural environments,

conservation is a concern (Cesoniene *el al.* 2013), as peatlands are very delicate ecosystems that can be destroyed by even slight disturbances (Dorrepaal *el al.* 2006). It is believed that a constant flow of humans to harvest wild *V. oxycoccos* stands can destroy this environment, putting at risk all the microbial, animal, and plant communities living there. In parts of England, several populations of *V. oxycoccos* were lost and peatlands were destroyed, in part due to excessive human activity and lack of conservation strategies (Jacquemart *el al.*, 1997a).

Plant breeding is crucial for food production, and together with the strategic protection and conservation of wild genetic resources, can lead to improved sustainability and food security. For example, a greater understanding of plant genetic diversity resources will allow gauging the risk of genetic erosion and inbreeding within wild populations that are important for food security. Some authors such as Allendorf (2009) state that there are three types of diversity: genes, species and ecosystems, and all three are vital to food security and conservation. One of the limiting factors conservation programs face is being able to locate wild populations to study them and develop policy to protect them (FAO 2015). With ongoing urbanization, valuable wild areas and their endemic species are continually lost to cultivated crops. Plants at these sites could possess important traits useful in crop improvement and bio-fortification programs. These plants could also be important for the conservation of genetic resources to maintain a healthy genetic pool and prevent genetic erosion, which could ultimately impact food production and the economy. There is much uncertainty as to whether the increase in world food production could be met without genetic diversity and wild genetic resources (FAO 2010).

In the US, little has been done to analyze and understand the wild genetic diversity of native crops such as cranberry species, i.e., *V. oxycoccos* and *V. macrocarpon*. Knowledge about the state and distribution of genetic diversity in the wild will allow the development of efficient conservation strategies to protect and utilize wild genetic resources that may harbor valuable pockets of diversity. For this reason, in collaboration with the USDA Agriculture Research Service and Forest Service, this project will provide the most comprehensive analysis of the state of the genetic diversity of wild cranberries ever conducted using modern codominant genetic markers. The goal is to genetically study several locations containing cranberries across the US and provide information to create *in situ* and *in vitro* conservation strategies to protect these resources. Also, this study will produce a self F1 populations of *V. microcarpum* and an interspecific crosses among *V. macrocarpon* x blueberry and *V. oxycoccos* X blueberry. Finally, we will have a greenhouse collection and the first *in-vitro* germplasm collection of wild cranberries from Wisconsin and Minnesota. This project will be part of the germplasm foundation for cranberry breeding in Wisconsin.

References

Allendorf FW, Luikart G (2009) Conservation and the Genetics of Populations. John Wiley & Sons

Alston JM, Medellín-Azuara J, Saitone TL (2014) Economic impact of the North American cranberry industry. University of California: Davis, CA, USA

Caillet S, Côté J, Doyon G, et al (2011) Antioxidant and antiradical properties of cranberry juice and extracts. Food Res Int 44:1408–1413. doi: 10.1016/j.foodres.2011.02.019

Česonienė L, Daubaras R, Paulauskas A, et al (2013) Morphological and genetic diversity of European cranberry (Vaccinium oxycoccos L., Ericaceae) clones in Lithuanian reserves. Acta Soc Bot Pol 82:211–217. doi: 10.5586/asbp.2013.026

Darrow GM, Camp WH (1944) Vaccinium Hybrids and the Development of New Horticultural Material. Bull Torrey Bot Club 72:1–21. doi: 10.2307/2481260

Diaz-Garcia L, Rodriguez-Bonilla L, Rohde J, et al (2019) Pacbio Sequencing Reveals Identical Organelle Genomes between American Cranberry (Vaccinium macrocarpon Ait.) and a Wild Relative. Genes 10.: doi: 10.3390/genes10040291

Dorrepaal E, Aerts R, Cornelissen JHC, et al (2006) Sphagnum modifies climate-change impacts on subarctic vascular bog plants. Funct Ecol 20:31–41

Eck P (1990) The American Cranberry. Rutgers University Press

Food And Agriculture Organization Of The United Nations. (2015). The state of food insecurity in the world 2014: strengthening the enabling environment for food... security and nutrition. Food & Agriculture Org.

Food and Agriculture Organization of the United Nations. (1998). FAOSTAT statistics database. [Rome] :FAO, Accessed 2019

Jacquemart AL (1997a) Vaccinium oxycoccos L. Oxycoccus palustris 381–396

Jacquemart A-L (1997b) Vaccinium Oxycoccos L. (Oxycoccus Palustris Pers.) and Vaccinium Microcarpum (Turcz. ex Rupr.) Schmalh. (Oxycoccus Microcarpus Turcz. ex Rupr.). J Ecol 85:381–396. doi: 10.2307/2960511

Mahy G, Bruederle LP, Connors B, et al (2000) Allozyme evidence for genetic autopolyploidy and high genetic diversity in tetraploid cranberry, Vaccinium oxycoccos (Ericaceae). Am J Bot 87:1882–1889

McGovern PE (2009) Uncorking the Past: The Quest for Wine, Beer, and Other Alcoholic Beverages. University of California Press

McKay DL, Blumberg JB (2007) Cranberries (Vaccinium macrocarpon) and cardiovascular disease risk factors. Nutr Rev 65:490–502

Pandey KB, Rizvi SI (2009) Plant polyphenols as dietary antioxidants in human health and disease. Oxid Med Cell Longev 2:270–278. doi: 10.4161/oxim.2.5.9498

Rock T (2011) Blueberry Fool: Memory, Moments, and Meaning. Wipf and Stock Publishers

Smith TW, Walinga C, Wang S, et al (2015) Evaluating the relationship between diploid and tetraploid Vaccinium oxycoccos (Ericaceae) in eastern Canada. Botany 93:623–636. doi: 10.1139/cjb-2014-0223

Song G-Q, Hancock JF (2011) Vaccinium. In: Kole C (ed) Wild Crop Relatives: Genomic and Breeding Resources: Temperate Fruits. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 197–221

Vander Kloet SP (1988) The genus Vaccinium in North America. Agriculture Canada

Vorsa N, Johnson-Cicalese J (2012) American Cranberry. In: Badenes ML, Byrne DH (eds) Fruit Breeding. Springer US, Boston, MA, pp 191–223

Thesis objectives

The general objectives of my PhD research were to understand the genetic diversity and population structure of wild populations of cranberries across their native range and to explore the relationship among other berries in the genus *Vaccinium* and cranberries.

(1) Few studies have focused on understanding and exploring the genetic diversity of wild populations of cranberries across their native range. In Wisconsin and Minnesota, there are multiple populations of cranberries that can be found in the wild. Therefore, we found ourselves in a great location to work and study with cranberries. Most cranberry cultivars share a very narrow genetic base, and there is a need to increase the germplasm available for breeding. For this, we traveled to multiple locations across Wisconsin and Minnesota to collect samples of *V. macrocarpon* and *V. oxycoccos* to understand their genetic diversity and incorporate them into our breeding program (Chapter II, Chapter IV, and Chapter V). Before being able to do this, we needed to identify a set of markers that would work on cranberries, its wild relatives, and other species of *Vaccinium*. For this, we used a set of markers developed in cranberry and tested their cross-transferability among other non-cultivated *Vaccinium* species (Chapter I).

(2) One of our most ambitious goals for this research was to understand the genetic diversity and population structure of wild populations of cranberries growing in National Forests. The main goal of this was to designate protected areas inside the forests that

would safeguard the genetic diversity of one of the few crops native to the US that has crop wild relatives (CWR) in nature. For this we joined forces with USDA ARS and the Forest Service and collected samples of over 30 location across the US in the east, central and west regions. The results obtained from this study will provide science-based information that will impact zoning in National Forests and be a model used for the conservation of other CWR (Chapter III).

Chapter I

Title: Cross-transferability analysis of SSR markers developed from the American Cranberry to other *Vaccinium* species of agricultural importance.

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Abstract

Cranberry breeding is evolving as consumer demand increases for specialty products such as sweetened and dried cranberries (SDC). New varieties are needed with not only higher yield and better adaptations to biotic (e.g., fungal fruit rot) and abiotic (e.g., cold tolerance) stressors, but also increased fruit quality for processing and for human nutrition. *Vaccinium macrocarpon* Ait. is one of the few species in the genus *Vaccinium* that is commercially produced. However, the genus is composed of many understudied and underused species, such as wild blueberries and cranberries, lingonberries, and deerberries. Many of these species are cross-compatible and possess an array of traits of high agronomical value that may be commercially exploited through intrasectional or intersectional interspecific breeding. In order to provide a toolset for *Vaccinium* breeders, we tested the cross-transferability of 507 *V. macrocarpon* simple sequence repeat (SSR) markers on 17 different *Vaccinium* species. We found 61 SSR markers that consistently amplified and produced scorable bands across all 17 species tested. We tested the ability of the markers to discriminate species based on their genetic relationships for future use in phylogenetics. We were able to discriminate the different species and sections of the genus, providing an insightful look into the genetic relationship of species in this genus. These markers represent a working set of SSRs to use for the development of *Vaccinium* interspecific hybrids and to allow the development of population genetic studies of poorly understood species.

Keywords: *Vaccinium*, Marker Cross-transferability, SSR Markers, *Vaccinium macrocarpon*

Introduction

The genus *Vaccinium* consists of approximately 450 species, of which 35% are native to North America (Luby et al. 1991). These species are widely distributed throughout the world, from tropical to circumboreal areas, and they have been taxonomically divided into sections (Vander Kloet 1988). Cranberries and blueberries are the most widely studied, produced, and consumed species in this genus, belonging to the sections *Oxycoccus* and *Cyanococcus* (FAOSTAT 1998). Although very few of the hundreds of species with berries in the genus *Vaccinium* are the focus of production, there is great diversity of useful traits and many of these species are cross-compatible (Song and Hancock 2011). Thus, it could be possible to exploit these traits in *Vaccinium* breeding through intrasectional or intersectional interspecific hybridization, including developing new American cranberry (*Vaccinium macrocarpon*) cultivars using traits from other less-cultivated and non-cultivated species, such as lingonberry, huckleberry, and deerberry (Vorsa 1996).

Cranberry (*Vaccinium macrocarpon)* is a diploid perennial plant that can grow as a shrub or vine (Song and Hancock 2011). The industry has a value of approximately 3.5 billion dollars and is expected to keep growing due to the high demand for products such as juices, mocktails, fresh fruit and recently-developed high-demand products such as sweetened and dried cranberries (SDC) (Alston et al. 2014). Currently, increases in SDC consumption, along with climate change concerns such as abrupt changes in temperature, have necessitated breeding programs to actively increase both germplasm and genetic resources to diversify current cultivars. The introduction of desirable traits from other *Vaccinium* species may help improve cranberry cultivars to meet market demands and environmental issues while providing a better

livelihood for growers and superior products for consumers. To date, some of the cranberry breeding goals include increasing production, achieving higher TACy (total anthocyanin content) and higher Brix (soluble sugar content), maintaining fruit quality, and increasing tolerance to cold (Song and Hancock 2011, Vorsa and Johnson-Cicalese 2012). Overall, the cranberry variety ideotype will possess higher yield with increased fruit quality and nutrition, coupled with better adaptations to biotic (e.g., fungal fruit rot) and abiotic (e.g., cold tolerance) stressors (Schlautman 2016).

One source of new traits for cranberry breeding are sister species, such as *V. oxycoccos* (2x, 4x, 6x) (Vander Kloet, 1983, Vander Kloet and Dickinson2009). In particular, diploid *V. oxycoccos*, also named *V. microcarpum*, is considered a different species based on genetic and morphological data (Suda and Lysak 2001; Smith et al. 2015), and could be useful in cultivated cranberry breeding programs (Vorsa and Johnson-Cicalese, 2012). Both *V. microcarpum* and *V. oxycoccos* are widely adapted to temperate climates with circumboreal distributions, including extremely cold environments. These sister species are morphologically similar to the cultivated cranberry, growing as small, evergreen, perennial vines (Jacquemart 1997), and are also clonally propagated, as cultivated cranberries are. Although they are smaller-fruited, they produce overwintering berries with superior antioxidant profiles (Vander Kloet 1988, Jacquemart 1997; Česonienė et al. 2011). Due to their superior cold-hardiness, close relationship to cranberries, and beneficial fruit chemical composition, both *V. oxycoccos* and *V. microcarpum* are ideal candidates to improve cultivated cranberries genetically (Song and Hancock 2011). Other candidates for cranberry breeding include the blueberries, huckleberries, deerberries, and lingonberries. These species exhibit useful traits, such as high levels of Vitamin C in *V. vitis-*

idaea, adaptation to mineral soils in *V. tenellum* and *V. ovatum*, winter-hardiness in *V. angustifolium* and *V. boreale*, and drought tolerance in *V. stamineum*, to mention a few (Song and Hancock 2011).

The genus *Vaccinium* is rich in traits and very diverse, but there are many constraints for the development of interspecific hybrids among species, including the wide variety of ploidy levels, ranging from diploids to hexaploids, as well as the lack of known, clear taxonomic relationships (Vorsa and Johnson-Cicalese 2012). However, despite differences in ploidy, as well as challenges due to pre- and post-fertilization barriers, interspecific hybridization between several *Vaccinium* species has been successful (Song and Hancock 2011). The presence of 2n gametes provides a possible way to overcome ploidy barriers, and these unreduced gametes have been found in the pollen of 2x, 4x, and 6x species (Ortiz et al. 1992). Hybrids such as Northern Highbush X Darrow's Evergreen are thought by Sharp and Darrow (1959) to be the result of unreduced gametes. Other examples of successful interspecific hybrids in blueberry include the cross of *V. constablei* (6x) and *V. ashei* (6x). From this cross, a hexaploid hybrid was produced, which was then crossed with *V. darrowii* (2x) to produce a tetraploid hybrid (Draper, 1977). Another example is the cross between *V. ashei* (6x) and *V. tenellum* (2x) that resulted in the hybrid known as Beltsville 17 (Darrow, Scott and Derman, 1954) and the very successful development of southern highbush varieties which have *V. darrowi, V. elliotti, V. corymbosum* and *V. virgatum* in their background as is the case of the variety "Snowchaser" (Ballington 2008). Wide crosses have also been performed between cranberries and blueberries, using cranberry sister species *V. oxycoccos* as a bridge, since direct crosses between *V. macrocarpon* and *V. corymbosum* have failed to produce viable offspring (Vorsa et al. 2008). The crosses

between *V. macrocarpon* and *V. oxycoccos* have been successful, and have yielded hybrids that were able to successfully cross with *V. darrowii*, producing an offspring displaying an intermediate phenotype (Vorsa and Polashock 2005, Vorsa et al 2009). Another constraint is the lack of molecular tools available for non-cultivated species. Currently, blueberry and cranberry breeding programs have generated multiple genetic resources such as molecular markers (Boches 2005, Boches et al. 2005, Bassil et al. 2008, Zalapa et al. 2012, Fajardo et al. 2013, Bian et al. 2014, Schlautman et al. 2015, Bidani et al. 2016, Schlautman et al. 2017b), linkage maps (Rowland et al. 2014, McCallum et al. 2016, Covarrubias-Pazaran et al. 2016, Schlautman et al. 2017a, Daverdin et al. 2017, Schlautman et al. 2018), and sequencing data sets (Polashock et al. 2014, Fajardo et al. 2014, Diaz-Garcia et al. 2019). Additionally, a recent study found 93% synteny agreement between blueberry and cranberry simple sequence repeat (SSR) maps, suggesting that species in this genus could be more closely related than previously believed (Schlautman et al. 2018). Thus, molecular resources in *Vaccinium* species could be applicable or transferrable to different species with desirable traits, making the development of interspecific breeding programs possible.

Despite the proliferation of new sequence-based molecular markers, SSR markers continue to be an inexpensive, simple, fast, and reliable method to assess diversity, test identity (fingerprinting), and confirm pedigrees in both intraspecific and interspecific breeding programs (Zalapa et al. 2015). Also, SSR markers are well known to be transferable between closely related species due to their highly-conserved flanking regions (Scott et al. 2003). In 2015, Schlautman et al. developed a total of 697 *V. macrocarpon* novel polymorphic genomic and Expressed Sequence Tags(EST) SSR markers, from which 541 SSRs were incorporated into a

cranberry SSR linkage map (Schlautman et al. 2017a), and 147 SSRs were found to be shared between blueberry and cranberry maps (Schlautman et al. 2018). Based on these and previously developed markers (Boches et al. 2005, Zhu et al. 2012, Georgi et al. 2013), this study seeks to test the transferability of the 507 moderate to highly polymorphic *V. macrocarpon* SSR markers mapped by Schlautman et al. (2017a) in 15 other *Vaccinium* species. Our goals are to increase the use and understanding of these other non-cultivated species and provide a set of working markers for other breeders to use in these valuable and underused species.

Materials and Methods

Plant Materials

This study used 46 individuals total (Table 1), representing sections *Oxycoccus* (12), *Pyxothamnus* (4), *Polycodium* (1), *Herpothamnus* (2), *Vitis-idaea* (4) and *Cyanococcus* (25) of the genus *Vaccinium*. All these individuals possess interesting breeding traits or share morphological characteristics with species in other sections, as is the case of *V. crassifolium*, which shows an intermediate phenotype among cranberries, lingonberries, and blueberries. The plant materials consisted of frozen leaf tissue obtained from the Phillip E. Marucci Center for Blueberry and Cranberry Research and Extension of Rutgers University, Chatsworth, NJ, the United States Department of Agriculture National Clonal Germplasm Repository Corvallis, OR, and the Cranberry Breeding Program at the University of Wisconsin-Madison, Madison, WI.

DNA extraction, PCR and Fragment Analysis

Genomic DNA extractions were performed using leaf tissue per a CTAB method (Doyle and Doyle 1987) modified in our laboratory to isolate high quality, clean DNA to ameliorate amplification problems due to phenolic compounds present in *Vaccinium* species. Briefly, 10-20mg of fresh tissue were ground by hand using liquid nitrogen and transferred to a 2.0mL tube, and 700µL of 2% CTAB extraction buffer [20 mM EDTA, 0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, 2%CTAB] was added and mixed by inversion. The solution was then incubated at 65 °C for 45 min. After incubation, 700 µL of a chloroform-isoamyl alcohol (24:1) solution was added to the tubes and gently mixed by inversion for 30s. Samples were then centrifuged for 5 min at 14,000 rpm, 500 µL of the supernatant was transferred to a fresh 2.0 mL tube. A volume of 700µL of the chloroform-isoamyl alcohol (24:1) and 50µL 10% CTAB buffer was added to the supernatant, and the solution was gently mixed by inversion. The mixture was then centrifuged for 5 min at 14,000 rpm, and the supernatant was then transferred to a fresh 1.5mL tube with 750µL of cold isopropanol (70%), and then incubated from 2 to 24 hours at a temperature of -20 °C. Samples were gently mixed by inversion and centrifuged at 14,000 rpm for 30 min. After centrifugation, the supernatant was discarded and the resulting pellet was air-dried for 5 minutes. The pellet was then washed with 700 µL of cold 70% ethanol (- 20 °C) to clean the DNA. The solution was then vortexed and centrifuged at 14,000 rpm for 4 min, the ethanol was discarded, and the pellet was air-dried for 5 minutes to 24 hours. The DNA was then re-suspended in 100 µL TE 10:1 buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) plus 5 µL of ribonuclease (RNAse 10 mg mL–1) in each tube, and was incubated at 37 °C for 2 hours prior to storage at -20 °C. DNA was then quantified using a Nanodrop ND-1000 spectrophotometer.

Samples were then diluted to 10ng/µL with deionized distilled water for simple sequence repeat (SSR) amplifications.

All SSR markers and sequences used in this study were previously designed and analyzed by Schlautman et al. (2015). Forward primers were appended with the M13 sequence (5'-CACGTTGTAAAACGAC-3') to allow for indirect fluorescent labeling of PCR products (Schuelke, 2002). The PIG sequence (5'-GTTTCTT-3') was appended to the reverse primers to promote uniform non-templated “A” addition and to facilitate downstream genotyping (Brownstein et al. 1996). PCR reactions were performed in 8 uL total volume using 3.5 µL 1× JumpStart REDTaq ReadyMix (Sigma, St. Louis, MO, USA), 1.0 µL of 15 ng/µL DNA, 2.0 µL of ddH2O, 0.5 µL of 5 µM forward primer, 0.5 µL of 50 µM reverse primer, and 0.5 µL of 0.5 µM M13-FAM, M13-HEX, or M13-NED primer. Thermocycling conditions included a 3 min melting step of 94 °C, followed by 33 cycles of 94 °C for 15 s, 55 °C for 90 s, and 72 °C for 2 min, and a final extension step of 72 °C for 30 min. One microliter each of FAM, HEX, and NED-labeled PCR product was mixed with 10 uL formamide and a carboxy-X-rhodamine (ROX) ladder, and the pool-plexed mix was sent to the University of Wisconsin Biotechnology Center DNA Sequencing Facility for fragment analysis using an ABI 3730 fluorescent sequencer (Pop-6 and a 50 cm capillary array; Applied Biosystems, Foster City, CA, USA). Allele genotyping was performed using the GeneMarker software v 1.91 (SoftGenetics LLC, State College, PA, USA).

Data Analysis

The data obtained from the allele genotyping was formatted as a GenAlEx (Peakall and Smouse 2012) input file and then converted to a geneclone object to run in the R statistical software (R Core Team 2018) package Population Genetics in R (poppr) (Kamvar et al. 2014). Using poppr, we estimated the observed number of private alleles (PA), number of alleles (N_A), mean number of alleles ($\overline{N_A}$,) and Bruvo's (Bruvo et al. 2004) genetic distance, as well as the ploidy information and tree visualizations. To help analyze and visualize our data, the packages adegenet (Jombart 2008), ade4 (Dray et al. 2007), magrittr (Bache and Wickham 2014), pegas (Paradis 2010) and ape (Paradis et al. 2004) were also used.

Results and Discussion

Cross transferability of markers across 46 species of the genus Vaccinium

A total of 507 SSR markers (Supplementary Materials File) developed from *V. macrocarpon* (Boches et al. 2005, Zhu et al. 2012, Georgi et al. 2013, and Schlautman et al. 2015; Schlautman et al. 2018) were selected for this study based on their high polymorphism information content value or their presence in a cranberry linkage map. This array of markers was used to test their transferability among 17 species from six different sections of the genus *Vaccinium* (Table 1). Liu et al. (2014) considered a cross-transfer successful if one or more different size PCR products were observed in at least one accession per species. Based on these criteria, our results show that out of 507 SSR markers 90% worked in *Cyanococcus*, 59% in *Herpothamnus*, 78% in *Pyxothamnus*, 48% in *Polycodium*, 64% in *Vitis-idaea*, 99% across all *V. oxycoccos*, and 98% in *V. microcarpum* (Table 2). These results are comparable to those found by Bassil et al.

(2008) in a reverse analysis, in which 84% of markers developed in blueberry transferred to cranberry and 84.6% to *V. oxycoccos*. Amplification success percentages and other statistics by loci and species are provided in Table 2 and a Supplementary Materials File, respectively. A total of 61 (12%) SSR markers amplified consistently in all 46 individuals tested (Table 3). A full list of marker transferability by species can be found in the Supplementary Materials File. These markers amplified in 75% or more of the samples within each species tested. The high proportion of transferred markers among the *Oxycoccus* section suggests a very close genetic relationship between these species, as has been demonstrated before by Zalapa et al. (2015), Smith et al. (2015), Schlautman et al. (2018), and Diaz-Garcia et al. (2019). The number of markers transferred for other *Vaccinium*, although relatively high, could have been affected by problems during PCR amplification caused by high levels of phenolic compounds present in *Vaccinium* species, which make it difficult to isolate clean and high-quality DNA (John 1992, Bujor et al. 2018, Hokkanen et al. 2009, Česonienė et al. 2006). Another problem that could affect the transferability of markers to other *Vaccinium* species in our study is the use of markers designed specifically for cranberry for the amplification of several *Vaccinium* species, across multiple levels of ploidy (2x to 6x), which may result in changes in the original amplification patterns and lower amplification rates due to deletions or point mutations (Barbara et al. 2007).

Table 1. List of plant materials used for the transferability of *Vaccinium macrocarpon* simple sequence repeat (SSR) markers in 46 individuals representing 17 *Vaccinium* species *Vaccinium*

Section/Species Id	PI[1]	Accession/Cultivar name	Location	Traits of interest^
Section *Cyanococcus*				
V. angustifolium CAN2	PI 554666	Augusta	NCGR[2]	Tolerance to blossom frost, Cold hardiness.
V. angustifolium CAN1	PI 554665	Brunswick	NCGR	
V. angustifolium 1	N/A	Blomidon	PEMCBCRE[3]	
V. ashei	N/A	Rabbiteye blueberry	PEMCBCRE	Tolerance to drought. Fruit firmness.
V. corymbosum US-E3	PI 554789	Ashworth	NCGR	Low chilling requirements, Resistance to stem canker.
V. corymbosum US-E2	PI 554804	Grover	NCGR	
V. corymbosum US-E1	PI 554831	Harding	NCGR	
V. corymbosum 2	PI 618033	Bladen	NCGR	
V. corymbosum 1	N/A	N/A	PEMCBCRE	
V. darrowii US-E2	PI 638325	Johnblue	NCGR	Tolerant to heat, exhibit late ripening.
V. darrowii US-E1	PI 638326	Everblue	NCGR	
V. darrowii US-E3	PI 554904	Florida 4B	NCGR	
V. darrowii 1	N/A	N/A	PEMCBCRE	
V. elliottii US-E2	PI 554912	Oleno Yellow (*V. elliottii*)	NCGR	Adapted to high pH, Tolerant to drought.
V. elliottii US-E3	PI 613650	*V. elliottii* NC 95-8-4	NCGR	
V. elliottii 1	N/A	NJ88-07-41	PEMCBCRE	
V. virgatum US-S1	PI 554695	Hagood	NCGR	Low chilling requirements, Tolerant to drought.
V. virgatum US-S4	PI 554710	Myers	NCGR	
V. virgatum US-S3	PI 554714	Suwannee	NCGR	
V. boreale	N/A	NJ89-3-19	PEMCBCRE	Cold hardiness, Tolerand to blossom

				frost.
V. hirsutum	N/A	NJ90-54-13	PEMCBCRE	Fruit color*
V. tenellum	N/A	NJ87-52-61	PEMCBCRE	Adaptability to mineral soils, Late ripening.
V. myrtilloides	N/A	N/A	PEMCBCRE	Cold hardiness, Low acidity.
Section *Herpothamnus*				
V. crassifolium 2	PI 554900	Well's Delight	NCGR	Intermediate morphology interesting for breeding*
V. crassifolium 1	N/A	NC83-9-8	PEMCBCRE	
Section *Oxycoccus*				
V. oxycoccos US-M	PI 555194	*V. oxycoccos* Minnesota	NCGR	Cold hardiness
V. oxycoccos RS	PI 641321	*V. oxycoccos* HVSC-050	NCGR	
V. oxycoccos Jp	PI 638486	*V. oxycoccos* J31	NCGR	
V. oxycoccos US-W1	PI 660961	*V. oxycoccos* Dinger Lake	NCGR	
V. oxycoccos 20	N/A	*V. oxycoccos* WI Wild population	UWMCB[4]	
V. oxycoccos 270	N/A	*V. oxycoccos* WI Wild population	UWMCB	
V. oxycoccos 137	N/A	*V. oxycoccos* WI Wild population	UWMCB	
V. oxycoccos 156	N/A	*V. oxycoccos* WI Wild population	UWMCB	
V. macrocarpon-Ben Lear	N/A	Ben Lear	UWMCB	Large leaf, fruit, and seed number. Exhibit high antioxidant content
V. macrocarpon-Crimson Queen	N/A	Crimson Queen	PEMCBCRE	
V. macrocarpon-Stevens	N/A	Stevens	UWMCB	
V. microcarpum	N/A	*V. oxycoccos* diploid	PEMCBCRE	Cold hardiness.
Section *Polycodium*				
V. stamineum	N/A	B-59	PEMCBCRE	Tolerant to drought, adaptability to mineral

				soils.
Section *Vitis-Idaea*				
V. vitis-idaea SWE1	PI 657175	Ida	NCGR	High benzoic acid, resistance to bacterial fruit rot.
V. vitis-idaea USAL2	PI 613680	*V. vitis-idaea* KHCW 96-04-02	NCGR	
V. vitis-idaea USAL1	PI 555374	*V. vitis-idaea* Finland	NCGR	
V. vitis-idaea 1	N/A	'Koralle'	PEMCBCRE	
Section *Pyxothamnus*				
V. ovatum US-W2	PI 618200	Morris-1	NCGR	Adaptability to mineral soils, late ripening.
V. ovatum US-W1	PI 618203	Morris-4	NCGR	
V. ovatum US-S	PI 660969	Trentberry	NCGR	
*V .ovatum*1	N/A	N/A	PEMCBCRE	

[1]Plant Introduction Number, [2]National Clonal Germplasm Repository, Corvallis OR, [3]Phillip E. Marucci Center for Blueberry and Cranberry Research and Extension, The State University of New Jersey, Chatsworth, NJ, [4]University of Wisconsin Madison, Cranberry Breeding Program, Madison, WI . ^*Source:* Ballington (1990), Luby et al. (1991), Galletta and Ballington (1996), Jacquemart (1997), Vander Kloet and Dickinson (1999), Suda (2003), Hancock et al. (2008), Song and Hancok (2011) * UWMCB particular interest.

The 507 SSR markers used in this study revealed a total of 11,735 amplification fragments (hereinafter referred to as alleles) ranging from 3 to 61 and with a total $\overline{N_A}$ of 23.14. When analyzing the data by sections, we found that *Cyanococcus,* which was represented by (n=23) species with different ploidy levels (2x-6x), had alleles ranging from 1 to 46 with a $\overline{N_A}$ of 13.56 and a total N_A of 6157. For section *Herpothamnus* (n=2), represented by *V. crassifolium*, we encountered alleles ranging from 1 to 7 with a $\overline{N_A}$ of 2.15 and a total N_A of 652 alleles. In the section *Polycodium* (n=1) for *V. stamineum*, the number of alleles ranged from 1 to 4 with a $\overline{N_A}$ of 1.65 and a total N_A of 405. Section *Pyxothamnus,* which was represented by *V. ovatum* (n=4)*,* exhibited alleles ranging from 1 to 11 with a $\overline{N_A}$ of 4.16 and a total N_A of 1667. In section *Vitis-idaea* (n=4) the N_A ranged from 1 to 11 with an $\overline{N_A}$ of 3.12 and a total N_A of 1021 (Supplementary Materials File). The high numbers of alleles observed per locus for the different

sections in our study are comparable to those found by Boches (2005), where a total of 627 alleles were detected after analyzing wild and domesticated blueberry germplasm. Other studies reporting high number of alleles in *Cyanococcus* (Liu et al. 2014) and other *Vaccinium* sections can also be explained by the polyploid and outcrossing nature of the species analyzed (Schlautman et al. 2017b).

Table 2. Number of *Vaccinium macrocarpon* simple sequence repeat (SSR) successfully transferred across seventeen *Vaccinium* species

	Number of Working SSR	**%**
Cyanococcus	445	90
Pyxothamnus	400	78
Vitis-idaea	327	64
Polycodium	245	48
Herpothamnus	303	60
Oxycoccos		
V. microcarpum	499	98
V. oxycoccos	504	99
V. macrocarpon	507	100
Overall across all species		
	61	**11**

Additionally, we are particularly interested in transferrable and consistent markers amplifying in section *Oxycoccus*, since it includes cultivated cranberry and its two wild relatives,

V. microcarpum (2x) (n=1) and *V. oxycoccos* (4x) (n=8). Therefore, we further analyzed the representatives of the section *Oxycoccos* by species. *Vaccinium oxycoccos* N_A ranged from 1 to 24 alleles per loci with a $\overline{N_A}$ of 10.74 and a total N_A of 5415. *V. microcarpum,* for which we only had one individual, alleles ranged from 1 to 3 with a $\overline{N_A}$ of 1.73 and a total N_A of 867. For *V. macrocarpon*, which the SSRs were originally designed on, based on three samples analyzed here, alleles ranged from 1-5 per loci with a $\overline{N_A}$ of 2.79 and a total N_A 1410.

Overall, our results are comparable to those obtained by small scale transferability studies conducted by Zalapa et al. (2015) and Bian et al. (2014), who found similar allele counts in both wild and cultivated cranberries, with species with larger sample sizes and polyploid species such as *V. oxycoccos* naturally possessing more alleles. An important impetus for testing the transferability of markers across several species was to provide a working set of useful SSR markers across *Vaccinium* sections for fingerprinting, breeding, and diversity studies. Table 3 provides the sequences of the 61 SSRs that consistently amplified and produced scorable bands across seventeen *Vaccinium* species.

Table 3 List of 61 *Vaccinium macrocarpon* simple sequence repeat (SSR) markers which amplified consistent and scorable DNA fragments across seventeen *Vaccinium* species tested

Primer ID	PIC	Forward Primer	Reverse Primer
1TRIMCONTIG326802	0.82	TTTTCAGAGCAAGAGGAAAG	CTGTCTGTATCATGGAACTCAT
1TRIMCONTIG443603	0.92	TGCACCTCCTCTCTCTCTAA	GGTTATGATGGTGGGAAAG
214102_K63	0.45	GGTAATAGCTTTGTGATCTTGC	GGGTAAAATGACTGCCAAC
313928_K70	0.89	CAATTATCAAGGAGGCAATC	TCACAAATGAGGATCTACACAC

ct130570	0.74	GTTCACAATCTGCATCTCCT	ACGTAATAGATCAAGAACAGGG
ct174735	0.9	CTTATTTGTATGGCCTTCCT	GCAGCATATATTGTCCAGTTC
SCF109660	0.87	CCCCAAACTGTCGTATAAAA	TAGAGTACAGGAAAAGCCCTAA
SCF112540	0.69	CAGTAGTGGTATTTCACAATCG	TTTAATGCTTTTGGAAGAGG
SCF132922	0.93	TTAGACGCTTTATGTCCATTC	GAGTGTCCTTGTCTTTGTTGTA
SCF138394	0.92	AAGCCCAGAAGAAATAACCTA	TGCAAATGTTAGGAACTGTGT
SCF138607	0.96	CATATAGAATACTGGACGGACA	TTCTGCCATCTCCTTTCTC
SCF144748	0.95	ATTTCCAATCCTTTCCTCTC	CTCTGACACCTTCTGACACATA
SCF192715	0.94	CTCTGCCTTGTTCGTCTCT	AACCAATCGAAGGTGACAA
SCF208883	0.89	GAGGAGTGAAGAGCCAGTAA	GACATTTCAAGTCCCACACT
SCF30816	0.71	GTCCAAAATAGCATCGAAAG	CGCATTACTTCTTCACTATACG
SCF32727	0.95	ATGTAACGGTCTCCACTTTCT	TAGTATCTTCGTGGTCAGAGGT
SCF34513	0.69	TACTAATCTTCTGGTTTGGGC	GTACACCACTCCTGATGGC
SCF43145	0.77	TGGTTTTGGATACACACTTG	AAGAACAAGATCACCACTCTG
SCF74458	0.96	GCAGGAAGCTATGATTAAGGTA	TTGAATAGTGTCAGTGGAGAAG
SCF804	0.94	CAGTCAACAGAGAATACACCAC	TTCCCTATGAAAATCCACAC
SCF92414	0.92	GTTATCCTCCCTTTGATATGTG	AAGAGCAACAAGATGGGTACT
SCF965	0.39	GTAAACTAACAAGCAACGATCC	GATTTAGCTGATGCAGAGTCAT
172672_K70	0.95	GATAGTTGTATGCGCTGTAAGA	GTTACCCGAATGAACAGGT
198358_K70	0.93	AATCGTCTGTTGCTCAATGT	AACCATACTTACCACAACCAGT
1TRIMCONTIG440230	0.94	ACACTTTGTAGGTGGTGGTTAT	ATTAGCAGTAGTCCAATCGGT
409500_K63	0.95	GATTCCTGGGTGTAGTTCTGT	CTTAGTCTTTAATGCTGGCTCA
416328_K63	0.72	GTATGCCCAGAATATCCATTAC	TAGTCACGAGGAAAGCTAAAGT
SCF111145	0.93	TTAGTCTGGCTGGTTTTAGTTT	TTGTACCTATTGTTGGATTGTG
SCF124927	0.9	CGAGTGTCATTAGCAACAGA	TATCACTTTAGATCGAGCAGAC

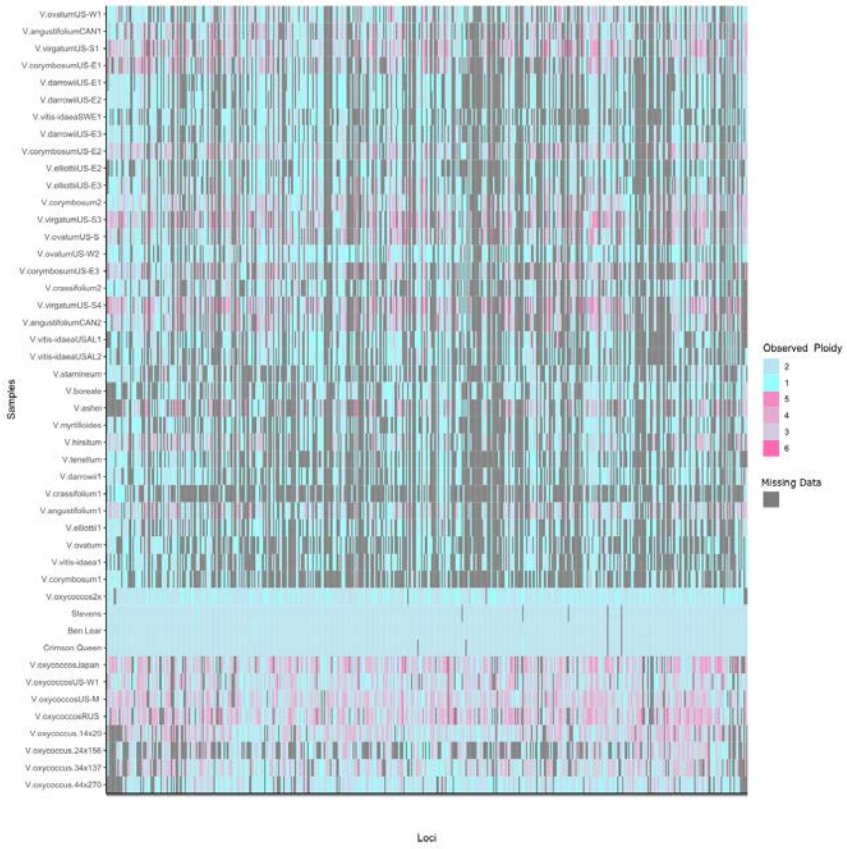
SCF145739	0.57	AAATCCTCCTGTTTTAGACTCC	CCTCAAGTCATCATTCCCT
SCF213102	0.42	GTGAAGATACAGTGGAGAGCA	ATGGTAGTTGTTGACCTGATG
scf21n	0.91	ACCAATTCCCTCCCAAGTTC	CCCTGGATATTTGCTTGCAT
scf275d	0.94	GCTTTTCTGAAGCGATTTGC	CCGCATACACGGCGTACTA
scf2882	0.93	CGCTACCATTGTCAGCTTCA	ACACTCAAAAGCAGGTGGCT
scf306f	0.94	GGGCAAGGATAAAGGGTTGT	TGCATGCAACTTCCTAGTCCT
SCF3427	0.96	GCAAGACATCATCACAAACA	CTTATCCCAGTCCTTCAACTTA
SCF37628	0.94	ACCAGCTCAGATAACAATGC	GAGTAGGATACCTCCACACCTA
SCF6926	0.91	ACATGCACTTCAAATAGTACCC	TTACAACTTACACAGGAAGCAG
SCF85946	0.94	TGTGAACAGAACCTACCACTAA	AAAGAGCCCCGTAGATAGAT
281884_K70	0.94	TCCACTATCTTTAGAATCCCAC	AGAGGATGGAGTTCCTTGATA
409618_K63	0.9	CTTCTCCTTCCCTTCACTTTA	TTAGTGTTAGTGTTGGTGTTGG
418596_K63	0.94	CGTGAGTTTGAGTGAGTAATTG	AGGACATGGTGAGTTGAGAAT
SCF10514	0.92	GTACTCTTTGTCGGATGTTTTC	GTTTCACTCCCACCTCTTAAT
SCF110757	0.94	TCATATCAACCTAACAATCGG	CACAAACAAGGAAATTAAGACC
SCF150919	0.93	TTGTTAGCACTTAGCATAACCC	GCTTCATCTCCACCAATACAT
SCF153094	0.94	TGTCATTAGGGTTCCTCAAA	CACCTAGACAACATCGAAACTA
SCF192219	0.9	GAATTTTGTCGTTCCAGAGA	AAAAGAAGAAGAGGAATGGC
SCF26697	0.84	TCGTAACTATTCAGTGGGTGT	GGAGCAGTAGAGATTAAACGAC
SCF27755	0.93	GAAGTGAGAGTAGGAATCGAAG	CCACAACACAAAACCCTAAT
SCF28509	0.89	GCAAACACCACACTATATGAGA	ATAGAGAACCACAGAACAGGAC
SCF30010	0.96	CTCAAATCAACGATCAAGAC	GAAAGAGACAACAAAACCCT
SCF48414	0.96	GTAGGGAAACAAGAATTGGAC	ACTGTGAGATTGGTGTGATATG
SCF53750	0.96	GTTTCATAGAGATGGGTTTCTG	CTTGGTTCCCTAAGCTACATT
SCF56816	0.61	CGGATTGACTAATTTCTGTCTC	CTCTTATTCCACCAAACGAA

SCF58861	0.88	GTTGACTAAAAGGCATTGGA	GACTACTATTTTCTGCACAGGG
SCF60761	0.91	ACTTAAACATCGGTCCATAGAG	AGAGTCGTGTCCTTTCTTTTC
SCF88396	0.94	ATAGAGGTTAATTGGTCCTCG	GACGAAGAACGACAGGTAGAT
SCF88902	0.94	GTGTTGTAGGATGAACCGAT	GATTTCCAGCATTTGATCTC
SCF89447	0.92	TAAATAAGACCTTCTGCTGACC	AATATGCTCACCACCAGTAAAG
SCF95754	0.97	CAGTGAGACTTCAGCTTGATAC	ATTGGTGACTTAGGAGTGAGAC
SCF9815	0.77	CATAGGAAGATTGCCTTGAG	GCCTGTTCACATAGATGGAG

One of the biggest interests of our breeding program is to better understand the genetic relationship between *V. macrocarpon* and its wild relatives *V. oxycoccos* and *V. microcarpum*. These species easily intercross and produce fruits and viable seeds, and they also share multiple morphological traits such as vegetative and reproductive growth, berry color, and tart taste (Rodriguez-Bonilla L, unpublished). Due to these similarities, we were interested in learning more about their genetic relationship in terms of private alleles (P_A) and other unique diversity in each species. We found that *V. oxycoccos* (4644 P_A) possessed the largest number of alleles, followed by *V. macrocarpon* (1181 P_A), and *V. microcarpum* (639P_A). When more closely looking at the private allele data, *V. macrocarpon* and *V. microcarpum* shared more alleles with each other than with *V. oxycoccos.* Although our sampling of alleles was limited by the samples size in each species, our data does not support the hypothesis that *V. oxycoccos* arose as a hybrid of the two diploid taxa, *V. macrocarpon* and *V. microcarpum* (Darrow and Camp, 1945) or that *V. oxycoccos* derived as an autopolyploid descendant of the diploid *V. microcarpum* species (Mahy et al., 2000). Moreover, a recent *V. macrocarpon* and *V. microcarpum* comparative analysis revealed that the two species are more similar than expected, with little to no variation in their organelle whole genome sequences, likely due to common ancestry (Diaz-Garcia et al. 2019).

Looking at the differences in ploidy level (Fig.1) present in our samples, we found that, based on SSR data, they ranged from diploid to hexaploid, as was expected. We also observed that some of the SSRs in species such as *V. ovatum, V. elliottii, V. darrowii*, *V. vitis-idaea*, and some instances in *V. corymbosum* demonstrated an abnormal amplification pattern, with more alleles per locus than expected for their ploidy. These could be due to issues during the amplification process in which other similar non-target sequences may have amplified, resulting in non-specific products, as in plants such as *Aracauria* (Scott et al. 2003; Hosseinzadeh-Colagar et al. 2016).

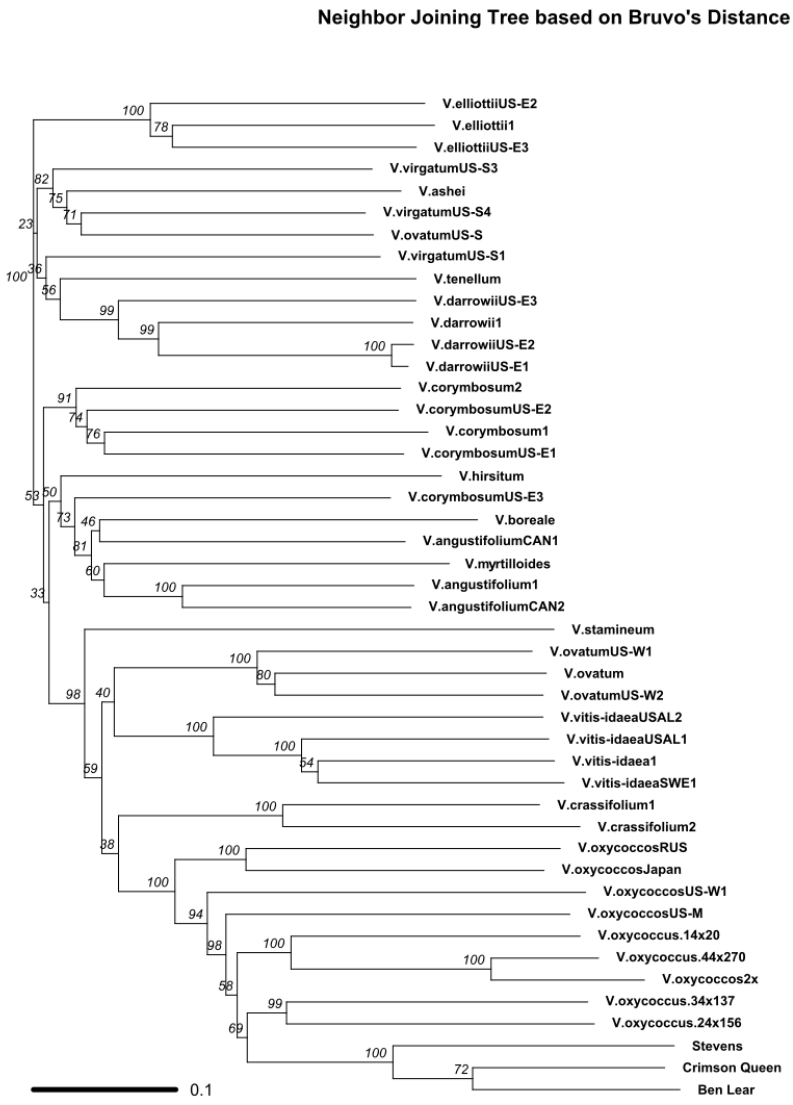
Fig. 1 Plot depicting the observed ploidy level of 46 *Vaccinium* species based on 507 *Vaccinium macrocarpon* simple sequence repeat (SSR) markers



Genetic distance

The markers used to analyze these accessions were found to be highly informative and transferrable (Schlautman et al. 2015); therefore, we were interested in testing their ability to also provide a clear picture of the genetic relationship among individuals of different sections in the *Vaccinium* genus. To do this, a Neighbor-Joining cluster analysis, based on Bruvo's genetic distance (Bruvo et al. 2004) was performed (Fig.2). This distance was chosen due to the model's capability to compare and estimate genetic distances of individuals from different ploidy. In the tree, we can observe two main groups, one containing the majority of the *Cyanococcus* (blueberry) species, and a second group subdivided into *Polycodium* (deerberry), *Pyxothamnus* (huckleberry), *Vitis-idaea* (lingonberry), and *Oxycoccus* (cranberry). These results show a similar grouping pattern as observed by Liu et al. (2014) and Schlautman et al. (2017b). The analysis placed the blueberries atop the tree, further away from members of the section *Oxycoccus*. Also, section *Vitis-idaea* clustered closer to *Oxycoccus* than to *Cyanococcus* as previously reported by Kron et al. (2002), results which could explain the morphological similarities between *Vitis-idaea*, *Oxycoccus*, and *Cyanococcus*. Interestingly, *V. crassifolium*, which is the only representative of the *Herpothamnus* section, and is described as a blueberry, also clustered close to *Vitis-idaea* and *Oxycoccus* species; this species exhibits intermediate morphological traits similar to cranberries and lingonberries.

Fig. 2 Neighbor Joining Tree based on Bruvo's Genetic Distance of 46 species of *Vaccinium* based on 507 *Vaccinium macrocarpon* simple sequence repeat (SSR) markers



Additionally, the SSR markers clearly separated cranberries from their wild relatives *V. oxycoccos* and *V. microcarpum,* while still clustering them close together, as has been described before (Zalapa et al. 2015; Schlautman et al. 2017b). The proximity of these species could

provide breeders a guide for which species could be useful and more successful for interspecific hybrid development and trait introgression into cranberry.

Conclusion

This analysis provides a set of transferrable markers between species from different sections of the genus *Vaccinium*. The 61 markers shared across species can facilitate more in-depth genetic diversity studies in multiple non-cultivated or understudied *Vaccinium* species that possess unique and interesting traits, but for which resources are still limited, as is the case of huckleberries, deerberries, and lingonberries. For species such as *V. oxycoccos* and *V. microcarpum*, the transferred markers will serve for future comparative mapping, trait introgression, and genetic fingerprinting, along with efforts to develop linkage mapping populations and experiments that aim to increase the genetic base for cranberry breeding programs.

Author contributions

LRB and JZ design the study; JZ provided plant material; LRB performed the experiment; LRB DM and JR analyzed the data; LRB and JZ wrote the manuscript.

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References

Alston JM, Medellín-Azuara J, Saitone TL (2014) Economic impact of the North American cranberry industry. University of California: Davis, CA, USA

Bache SM, Wickham H (2014) magrittr: a forward-pipe operator for R. R package version 1:1

Barbara T, Palma-Silva C, Paggi GM, Bered F, Fray MF, Lexer C (2007) Cross-species transfer of nuclear microsatellite markers: potential and limitations. Mol Ecol 16:3759–3767

Ballington JR (2008) The role of interspecific hybridization in blueberry improvement. XI International *Vaccinium* Symposium 810:49-60

Bassil N, Oda A, Hummer KE (2008) Blueberry microsatellite markers identify cranberry cultivars. Acta Hortic 810:181–187

Bian Y, Ballington J, Raja A, Brouver C, Reid R, Burke M, Wang X, Rowland LJ, Bassil N, Brown A (2014) Patterns of simple sequence repeats in cultivated blueberries (*Vaccinium* section Cyanococcus spp.) and their use in revealing genetic diversity and population structure. Mol Breed 34:675–689.

Bidani A, Hummer KE, Rowland LJ, Bassil NV (2016) Development of an efficient DNA test for genetic identity confirmation in blueberry. In: XI International *Vaccinium* Symposium 1180. actahort.org, pp 363–368

Boches P (2005) Microsatellite marker development and molecular characterization in highbush blueberry (*Vaccinium* corymbosum L.) and *Vaccinium* species. M.S. Dissertation, Oregon State University, Corvallis, USA.

Boches PS, Bassil NV, Rowland LJ (2005) Microsatellite markers for *Vaccinium* from EST and genomic libraries. Mol Ecol Notes 5:657–660.

Brownstein MJ, Carpten JD, Smith, JR (1996) Modulation of non-templated nucleotide addition by taq DNA polymerase: Primer modifications that facilitate genotyping. Biotechiques 20:6 1004-1010

Bruvo R, Michiels NK, D'Souza TG, Schulenburg H (2004) A simple method for the calculation of microsatellite genotype distances irrespective of ploidy level. Mol Ecol 13:2101–2106.

Bujor O-C, Ginies C, Popa VI, Dufour C (2018) Phenolic compounds and antioxidant activity of lingonberry (*Vaccinium* vitis-idaea L.) leaf, stem and fruit at different harvest periods. Food Chem 252:356–365.

Česonienė L, Daubaras R, Areškevičiūtė J, Viškelis P (2006) Evaluation of morphological peculiarities, amount of total phenolics and anthocyanins in berries of European Cranberry (Oxycoccus palustris). Baltic For 12:59–63

Česonienė L, Daubaras R, Jasutienė I, Venclovienė J, Miliauskienė I (2011) Evaluation of the Biochemical Components and Chromatic Properties of the Juice of *Vaccinium* macrocarpon Aiton and *Vaccinium* oxycoccos L. Plant Foods Hum Nutr 66:238–244.

Covarrubias-Pazaran G, Diaz-Garcia L, Schlautman B, Deutsch J, Salazar W, Hernandez-Ochoa M, Gryglesk, E, Steffan S, Iorizzo M, Polashock J, Zalapa JE, Vorsa, N (2016) Exploiting genotyping by sequencing to characterize the genomic structure of the American cranberry through high-density linkage mapping. BMC Genomics 17:451.

Darrow G M, Scott D H and Dermen H (1954) Tetraploid blueberries from hexaploid x diploid species crosses. Proceedings of the American Society for Horticultural Science 63:266-270

Draper A D (1977) Tetraploid hybrids from crossed of diploid, tetraploid and hexaploid *Vaccinium* species. Acta Horticulturae 61:33-37

Darrow GM, Camp WH (1945) Vaccinium Hybrids and the Development of New Horticultural Material. Bull Torrey Bot Club 72:1–21

Daverdin G, Johnson-Cicalese J, Zalapa J, Vorsa N, Polashock J (2017) Identification and mapping of fruit rot resistance QTL in American cranberry using GBS. Mol Breed 37:3 38.

Doyle JJ, Doyle JL (1987) CTAB DNA extraction in plants. Phytochemical Bulletin 19:11–15

Dray S, Dufour A-B, Others (2007) The ade4 package: implementing the duality diagram for ecologists. J Stat Softw 22:1–20

Fajardo D, Morales J, Zhu H, Steffan S, Harbut R, Bassil N, Hummer K, Polashock, Vorsa N, Zalapa JE (2013) Discrimination of American Cranberry Cultivars and Assessment of Clonal Heterogeneity Using Microsatellite Markers. Plant Mol Biol Rep 31:2 264–271.

Fajardo D, Schlautman B, Steffan S, Polashock J (2014) The American cranberry mitochondrial genome reveals the presence of selenocysteine (tRNA-Sec and SECIS) insertion machinery in land plants. Gene 536(2):336–343.

FAOSTAT (1998) Food and Agriculture Organization of the United Nations. FAOSTAT database

Georgi L, Herai RH, Vidal R, et al (2012) Cranberry microsatellite marker development from assembled next-generation genomic sequence. Mol Breed 30:227–237

Hokkanen J, Mattila S, Jaakola L, Pirttilä AM, Tolonen A (2009) Identification of phenolic compounds from lingonberry (*Vaccinium* vitis-idaea L.), bilberry (*Vaccinium* myrtillus L.) and hybrid bilberry (*Vaccinium* x intermedium Ruthe L.) leaves. J Agric Food Chem 57:20 9437–9447.

Hosseinzadeh-Colagar A, Haghighatnia MJ, Amiri Z, Mohadjerani M, Tafrihi M (2016) Microsatellite (SSR) amplification by PCR usually led to polymorphic bands: Evidence which shows replication slippage occurs in extend or nascent DNA strands. Mol Biol Res Commun 5:3 167–174

Jacquemart AL (1997) *Vaccinium* Oxycoccos L. (Oxycoccus Palustris Pers.) and *Vaccinium* Microcarpum (Turcz. ex Rupr.) Schmalh. (Oxycoccus Microcarpus Turcz. ex Rupr.). J Ecol 85:381–396.

John ME (1992) An efficient method for isolation of RNA and DNA from plants containing polyphenolics. Nucleic Acids Res 20:2381

Jombart T (2008) adegenet: a R package for the multivariate analysis of genetic markers. Bioinformatics 24:1403–1405.

Kamvar ZN, Tabima JF, Grünwald NJ (2014) Poppr: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. PeerJ 2:e281.

Kron KA, Judd WS, Stevens PF, Crayn AA, Anderberg PA, Gadek PA, Quinn CJ, Luteyn JL (2002) Phylogenetic Classification of Ericaceae: Molecular and Morphological Evidence. Bot Rev 68:3 335–423

Liu Y-C, Liu S, Liu DC, Wei YX, Liu C, Yang YM, Tao CG, Liu WS (2014) Exploiting EST databases for the development and characterization of EST-SSR markers in blueberry (*Vaccinium*) and their cross-species transferability in *Vaccinium* spp. Sci Hortic 176: 319–329.

Luby JJ, Ballington JR, Draper AD, Kazimierz P, Austin ME (1991) Blueberries and cranberries (*Vaccinium*). Genetic Resources of Temperate Fruit and Nut Crops 290 393–458

Mahy G, Bruederle LP, Connors B, et al (2000) Allozyme evidence for genetic autopolyploidy and high genetic diversity in tetraploid cranberry, Vaccinium oxycoccos (Ericaceae). Am J Bot 87:1882–1889

McCallum S, Graham J, Jorgensen L, Rowland LJ, Bassil NV, Hancock JF, Wheeler EJ, Vining K, Poland JA, Olmstead JW, Buck E. (2016) Construction of a SNP and SSR linkage map in autotetraploid blueberry using genotyping by sequencing. Mol Breed 36:4 41.

Meirmans PG, Van Tienderen PH (2004) GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. Mol Ecol Notes 4:792-794

Ortiz R, Vorsa N, Bruederle LP, Laverty T (1992) Occurrence of unreduced pollen in diploid blueberry species, *Vaccinium* sect. Cyanococcus. Theor Appl Genet 85:55–60.

Paradis E (2010) pegas: an R package for population genetics with an integrated–modular approach. Bioinformatics 26:419–420.

Paradis E, Claude J, Strimmer K (2004) APE: Analyses of Phylogenetics and Evolution in R language. Bioinformatics 20:289–290

Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research--an update. Bioinformatics 28:2537–2539.

Polashock J, Zelzion E, Fajardo D, Zalapa JE, Georgi L, Bhattacharya D, Vorsa N (2014) The American cranberry: first insights into the whole genome of a species adapted to bog habitat. BMC Plant Biol 14:1 165.

R Core Team (2018) R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing

Rowland LJ, Ogden EL, Bassil N, Buck EJ, McCallum S, Graham J, Brown A, Wiedow C, Campbell AM, Haynes KG, Vinyard BT (2014) Construction of a genetic linkage map of an interspecific diploid blueberry population and identification of QTL for chilling requirement and cold hardiness. Mol Breed 34:4 2033–2048.

Schlautman B, Covarrubias-Pazaran G, Diaz-Garcia L, Iorizzo M, Polashock J, Grygleski E, Vorsa N, Zalapa J (2017a) Construction of a high-density American cranberry (*Vaccinium* macrocarpon Ait.) composite map using genotyping-by-sequencing for multi-pedigree linkage mapping. G3: Genes, Genomes, Genetics:g3-116

Schlautman B, Covarrubias-Pazaran G Fajardo D, Steffan S, Zalapa J(2017b) Discriminating power of microsatellites in cranberry organelles for taxonomic studies in *Vaccinium* and Ericaceae. Genet Resour Crop Evol 64:3 451-466

Schlautman B, Diaz-Garcia L, Covarrubias-Pazaran G, Schlautman N, Vorsa N, Polashock J, Ogden EL, Brown A, Lin YC, Bassil N, Buck EJ (2018) Comparative genetic mapping reveals synteny and collinearity between the American cranberry and diploid blueberry genomes. Molec Breed 38:1 9

Schlautman B, Fajardo D, Bougie T, Wiesman E, Vorsa N, Steffan S, Zalapa J (2015) Development and validation of 697 novel polymorphic genomic and EST-SSR markers in the American cranberry (*Vaccinium* macrocarpon Ait.). Molecules 20:2 2001-2013

Schlautman B (2016) Accelerating Domestication and Genetic Gain in the American Cranberry (*Vaccinium* Macrocarpon Ait.): New Genetic and Genomic Resources for the Cranberry Breeders' Toolbox. Ph.D. thesis University of Wisconsin-Madison.

Schuelke, M (2002) An economic method for the fluorescent labeling of PCR fragments. Nature biotechnology 18:2 233.

Scott LJ, Shepherd M, Henry RJ (2003) Characterization of highly conserved microsatellite loci in Araucaria cunninghamii and related species. Plant Syst Evol 236:115–123

Sharp RH and Darrow GM (1960) Breeding blueberries for the Florida Climate. Proceedings of the Florida State Horticultural Society 72:308-11

Smith TW, Walinga C, Wang S, Kron P, Suda J, Zalapa J (2015) Evaluating the relationship between diploid and tetraploid *Vaccinium* oxycoccos (Ericaceae) in eastern Canada. Botany 93:10 623-636

Song G-Q, Hancock JF (2011) *Vaccinium*. In: Wild Crop Relatives: Genomic and Breeding Resources: Temperate Fruit

Vander Kloet SP (1983) The taxonomy of Vaccinium oxycoccus. Rhodora 85:1–43

Vander Kloet SP (1988) The genus *Vaccinium* in North America. Agriculture Canada.

Vander Kloet SP, Dickinson TA (2009) A subgeneric classification of the genus Vaccinium and the metamorphosis of V. section Bracteata Nakai: more terrestrial and less epiphytic in habit, more continental and less insular in distribution. J Plant Res 122:253–268s. Springer Berlin Heidelberg, Berlin, Heidelberg 197–221

Vorsa N (1996) On a wing: The genetics and taxonomy of *Vaccinium* species from a pollination perspective. In: VI International Symposium on *Vaccinium* Culture 446: 59–66

Vorsa N and Polashock J (2005) Alteration of anthocyanin glycosylation in cranberry through interspecific hybridization. *Journal of the American Society for Horticultural Science* 130 5: 711-715.

Vorsa N, Johnson-Cicalese J and Polashock J (2008) A blueberry cranberry hybrid derived from a V*accinium darrowii x* (*V.macrocarpon x V. oxycoccos*) intersectional cross. IX International *Vaccinium* Symposium 810:187-190

Vorsa N, Johnson-Cicalese J (2012) American Cranberry. In: Fruit Breeding. Springer US, Boston, MA, 191–223

Zalapa JE, Bougie TC, Bougie TA, Schlautman BJ, Wiesman E, Guzman A, Fajardo DA, Steffan S, Smith T (2015) Clonal diversity and genetic differentiation revealed by SSR markers in wild *Vaccinium* macrocarpon and *Vaccinium* oxycoccos. Annals of applied biology 166:2 196-207

Zalapa JE, Cuevas H, Zhu H, Steffan S (2012) Using next-generation sequencing approaches to isolate simple sequence repeat (SSR) loci in the plant sciences. American journal of botany 99:2 193-208

Zhu H, Senalik D, McCown BH, et al (2012) Mining and validation of pyrosequenced simple sequence repeats (SSRs) from American cranberry (Vaccinium macrocarpon Ait.). Theor Appl Genet 124:87–96

Chapter II

Unraveling the genetic diversity and breeding implications of wild cranberry populations in Wisconsin and Minnesota

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Abstract

Successful breeding programs utilize available germplasm to increase yield, biotic and abiotic stress tolerance, and other desirable traits. Knowledge of the range, genetic relationships, and diversity among crop wild relatives (CWR) is crucial for more efficient use. In cranberries, most cultivars or experimental populations share genetic background based on few wild selections. With increasing challenges due to climate change and pest pressures, there is a need to incorporate new and enhanced traits to the cranberry breeding pool. Therefore, we studied 36 wild populations of *Vaccinium macrocarpon* and *Vaccinium oxycoccos* across Wisconsin and Minnesota using 32 simple sequence repeat (SSR) markers. Although early molecular marker studies to understand the diversity of these species found low genetic variation, we found high levels of heterozygosity for both species. In *V. macrocarpon*, we found a total number of 294 alleles and moderate to high levels of heterozygosity (H_O=0.51, H_T=0.66). As expected for an outcrossed polyploid species, we found higher levels of heterozygosity (H_O=0.81, H_T=0.83) in *V. oxycoccos* (4x) than in *V. macrocarpon*. We compared a group of cultivated and experimental hybrids with wild *V. macrocarpon* and found that there is a clear demarcation between both groups suggesting there is much more diversity in the wild that needs to be explored and incorporated into breeding programs. Our results could be useful for making *in-situ* conservation decisions to protect valuable CWR of one of the few crops native to North America.

Keywords: cranberry breeding, crop wild relatives, SSR, genetic diversity

Introduction

Plant breeding is crucial for food production and food security; however, multiple cycles of selection can result in the loss of genetic diversity (Cheema 2018). This genetic diversity loss leads to crop homogeneity, which makes food systems vulnerable to biotic and abiotic stresses (National Research Council 1993; Dempewolf et al. 2017). An option to minimize this threat is to find, characterize, and use wild plant genetic resources as a source of crop traits (Brozynska et al. 2016). Crop wild relatives (CWR) have provided wild germplasm with many valuable traits for breeding cultivated crops such as resistance to pests (Anh et al. 2018), tolerance to biotic and abiotic stress (Zhang et al. 2017), and traits influencing yield and fruit quality (Brozynska et al. 2016).

In the US, very few species used for food production are native to North America and even fewer have CWR in nature (Khoury et al. 2013). The American cranberry (*Vaccinium macrocarpon* Ait.) is a diploid, woody perennial species native to North America with vast amounts of unexplored wild populations and wild relatives sharing overlapping ranges and environments (Darrow and Camp 1944; Vander Kloet 1988). *V. macrocarpon* can be found growing in swamps, upland meadows, and sphagnum bogs (Aaseng 2003), among other acid environments, and has a distribution extending from eastern Canada to parts of the Midwest and north-central US and as far south as North Carolina and Tennessee (Hummer et al. 2019).

Cranberry domestication and cultivation began in the 1800s with several documented wild selections (Chandler and Demoranville 1958; Dana 1983; Eck 1990), and with the first hybrids developed from wild selections in the 1950s (Eck 1990). Most cultivated cranberries share a similar genetic background; in fact, all hybrid cultivars released in the history of cranberry

breeding have been derived from ‘Early Black’, ‘Howes’, ‘McFarlin’, and ‘Searles’, the “Big Four,” and a few other native selections (Eck 1990). Recently, a survey published by Gallardo et al. (2018) identified the needs and goals for cranberry breeding. Breeding needs varied considerably by growing location, but some of the most salient needs across locations included disease resistance and fruit quality, including anthocyanin content, fruit size, and flavor due to acid and sugar content. Additionally, in contrast with other cranberry growing regions, Wisconsin, which is the most important cranberry producer in the world with 20,000 acres from a total of 50,000 acres worldwide, faces pests such as the cranberry fruit worm (*Acrobasis vaccinii*) and temperature challenges in terms of extreme summer heat and winter cold, and fluctuating winter temperatures leading to permanent frost risk (Dana 1983; Roper and Vorsa 1997).

Vaccinium oxycoccos is mostly a tetraploid species, which grows as a small evergreen perennial vine that produces small over-wintering berries with a similar flavor profile to cranberries and exhibit a superior antioxidant profile (Vander Kloet 1988; Jacquemart 1997; Česonienė et al. 2011; Brown et al. 2012). As one of the *V. macrocarpon* wild relatives in the continental US, *V. oxycoccos* provides great potential for developing interspecific hybrids to transfer desirable traits into cranberry, such as tolerance or resistance to abiotic stressors. Because *V. oxycoccos* has a circumboreal distribution, adaptability to higher latitudes and the ability to endure harsh winters (Areškevičiūtė et al. 2006), the species could be an excellent source of cold hardiness genes, as is the case of other wild relatives to crops such as rice (Shakiba et al. 2017) and potato (Hamernik et al. 2009).

Exploring, studying, and using wild cranberry diversity can impact cranberry breeding by identifying and transferring needed traits into cultivated cranberries. Identifying diversity in *V. oxycoccos* could lead to the development of cultivated hybrids with novel traits (Vorsa et al. 2008). In the US, little has been done to analyze and understand the wild genetic diversity of native crops such as *V. macrocarpon* and *V. oxycoccos*. This study is the most comprehensive analysis of wild populations of cranberries in Wisconsin and the first insight into the diversity present in Minnesota. Knowledge about the distribution of genetic diversity in the vastly unexplored wild populations in Wisconsin and Minnesota will allow us to identify valuable diversity pockets to collect, preserve, and use germplasm in breeding programs. The germplasm collected in this study will be kept *ex-situ* and housed in the USDA-ARS, Vegetable Crop Research Unit in Madison, WI and selected accessions will be added to the National Clonal Germplasm Repository (NCGR) collection.

Materials and Methods

1. **Plant Materials**

Plant material of 572 samples of wild cranberries (putatively collected as *V. macrocarpon* or *V. oxycoccos*) was obtained from 36 locations across Wisconsin (22) and Minnesota (14) (Table 1). The locations visited were public lands, Department of Natural Resources State Natural Areas, State Scientific Areas, or Forest Service lands. Permits were obtained to sample and collect in locations in both states. Using satellite imagery in Google Earth Pro and data provided by the DNR of both states, we located potential collection sites. In the sites, coordinates were logged per sample and cuttings of a single vine of about 20 cm long were obtained to ensure that plant tissue collected for each sample came from a single plant. Plant tissue was stored in a bag with

wet towels, labeled, and kept in a cold bag. A total of 20 plants were collected by location when possible based on the geography and environmental conditions. Leaf material was then stored at -4C for DNA extractions. Plant material from cultivated *V. macrocarpon* was obtained from the USDA-ARS, Vegetable Crop Research Unit, Cranberry Genetics and Genomics Laboratory at the University of Wisconsin-Madison.

Table 1. Cranberry (*Vaccinium macrocarpon* and *V. oxycoccos*) populations and individuals collected in Wisconsin and Minnesota

Pop. name	Pop. code	N	Species	Latitude	Longitude	State
Botany Bog	BB	20	*V. oxycoccos*	47.36802402	-93.60217603	MN
Big Lake Minnesota	BLM	13	*V. oxycoccos*	47.51002704	-94.59074297	MN
Beltrami Road	BRR	20	*V. oxycoccos*	48.26355502	-94.10271504	MN
Beltrami Road M	BRRM	20	*V. oxycoccos*	48.30934301	-94.24009804	MN
Chippewa National Forest MN	Chip	20	*V.macrocarpon*	47.65851497	-94.47189102	MN
Hole in the Bog	HB	19	*V. oxycoccos*	47.31079901	-94.21872402	MN
Iron Springs Bog	ISB	3	*V. oxycoccos*	47.25367801	-95.24143497	MN
Lova Lake	LL	15	*V.macrocarpon / V. oxycoccos*	46.57545104	-94.55575397	MN
Long Lake B	LOLB	20	*V. oxycoccos*	46.650917	-93.46198099	MN
Long Lake M	LOLM	20	*V.macrocarpon*	46.647796	-93.46040502	MN
Little Rice Lake	LRL	20	*V.macrocarpon / V. oxycoccos*	47.70827103	-92.439135	MN
Pennington Bog	PB	20	*V. oxycoccos*	47.49936501	-94.47868899	MN
PF-River Road	PRF	20	*V. oxycoccos*	48.42440198	-94.90923098	MN
TF-River Road	TR	21	*V. oxycoccos*	48.42439402	-94.89678302	MN

Bear Lake	BL	18	*V.macrocarpon / V. oxycoccos*	45.609398	91.81301	WI
Chippewa River	CHPR	1	*V. oxycoccos*	46.00559	-90.8275	WI
Flambeau Forest	FBF	7	*V. oxycoccos*	45.69066002	-90.77997297	WI
Frog Lake	FRLK	16	*V.macrocarpon / V. oxycoccos*	46.12128	-90.0073	WI
Goblers Lake	GL	18	*V. oxycoccos*	45.6328	-89.8744	WI
Gypsy Lake R	GYPL	33	*V.macrocarpon / V. oxycoccos*	45.80585	-89.49159499	WI
Hayward	HW	1	*V.macrocarpon*	46.05793	-91.7306	WI
Jaquet Lake	JL	12	*V. oxycoccos*	45.793367	-88.625817	WI
Kelley Lynn Bog	KLB	21	*V. oxycoccos*	45.45832	-89.2752	WI
Kemp Road	KMP	12	*V. oxycoccos*	45.845886	-89.662259	WI
Lincoln/Oneida	Locp	30	*V. oxycoccos*	45.46933	-89.3226	WI
Land O' Lakes	LOL	3	*V. oxycoccos*	46.1414	-89.4917	WI
Manitowish Waters	MWB	16	*V. oxycoccos*	46.13512	-89.7373	WI
N-Bradley	NB	1	*V. oxycoccos*	45.5397	-89.814333	WI
Powell Marsh SNA	PMS	20	*V. oxycoccos*	46.09658	-89.909	WI
Powell Marsh Wildlife Refuge	PMW	23	*V.macrocarpon / V. oxycoccos*	46.08051	-89.8815	WI
Spread Eagle Barrens	SEB	17	*V.macrocarpon / V. oxycoccos*	45.86595	-88.18705	WI
Shelp Lake	SHPL	11	*V.macrocarpon / V. oxycoccos*	45.776667	-89.020617	WI
Swamp Road	SR	17	*V. oxycoccos*	45.406667	-89.796233	WI
Wabaso Lake	WABL	39	*V.macrocarpon / V. oxycoccos*	45.9726	-90.0009	WI

Winchester	WIN	2	*V. oxycoccos*	46.19062	-89.9051	WI

2- DNA Extraction

Genomic DNA extractions were performed using leaf tissue per a CTAB method (Doyle and Doyle 1987) modified in our laboratory to isolate high quality, clean DNA. A total of 10-20mg of frozen tissue were ground by hand using liquid nitrogen and transferred to a 2.0mL tube, and 700µL of 2% CTAB extraction buffer [20 mM EDTA, 0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, 2%CTAB] was added and mixed by inversion. The solution was then incubated at 65 °C for 45 minutes. After incubation, 400 µL of a chloroform-isoamyl alcohol (24:1) solution was added to the tubes and gently mixed by inversion. Samples were then centrifuged for 5 min at 14,000 rpm, 500 µL of the supernatant was transferred to a fresh 1.5mL tube containing 50µL of 10% CTAB buffer and mixed by inversion. 750µL of cold isopropanol (100%) was added, and then samples were incubated from 2 to 48 hours at a temperature of -20°C. Samples were gently mixed by inversion and centrifuged at 14,000 rpm for 20 min. After centrifugation, the supernatant was discarded, and the resulting pellet was air-dried for 5 minutes. The pellet was then washed with 700 µL of cold 70% ethanol to clean the DNA. The solution was then vortexed and centrifuged at 14,000 rpm for 4 minutes, the ethanol was discarded, and the pellet was air-dried for 24 hours. The DNA was then re-suspended in 100 µL TE 10:1 buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) plus 5 µL of ribonuclease (RNAse 10 mg mL–1) in each tube, and was incubated at 37 °C for 2 hours prior to storage at -20 °C. DNA was then quantified using a Nanodrop ND-1000 spectrophotometer. Samples were then diluted to 10ng/µL with deionized distilled water for simple sequence repeat (SSR) amplifications.

3- SSR Markers and Multiplex PCR Conditions

A total of 32 markers (Supplementary Materials-1) previously designed and assessed for transferability between *V. macrocarpon* and *V. oxycoccos* were used in this study (Schlautman et al. 2015; Rodriguez-Bonilla et al. 2019). Markers proven to have a consistently clear pattern of amplification across species were chosen and at least two markers per cranberry linkage group were selected. The multiplex panels were designed by developing SSR primer-pairs. These pairs were clustered into combinations of one to three markers, considering non-overlapping allele ranges and annealing temperatures based on previous studies (Rodriguez-Bonilla et al. 2019; Zhu et al. 2012; Georgi et al. 2012; Schlautman et al. 2015). To boost the potential of our combinations and maximize resources for our fragment analysis, each reaction was combined with 4 PCR multiplex reactions and a fluorescent dye (M13-FAM, HEX, NED, or PET labeled primers). The forward primers were designed with the M13 sequence (5-CACGTTGTAAAACGAC-3) for fluorescent labeling of PCR products (Schuelke 2000), and the PIG sequence (5-GTTTCTT-3) was attached to the reverse primers to promote full adenylation of SSR fragments during PCR (Brownstein et al. 1996). The development of primers with an attached M13 sequence and a fluorescently labeled M13-tail is more cost effective than using direct fluorescent primer labeling (Guichoux et al. 2011) By not using this direct fluorescence, we created any marker combinations and indirectly label them with any fluorescence needed. The PCR contained 3.5ul of 1x Jumpstart RedTaq Ready Mix (Sigma, St. Louis, MO, USA), 2.0 ul of 10 ng/ul DNA, 0.5 µL of 5 µM forward primer, 0.5 µL of 50 µM reverse primer, and 0.5 µL of 0.5 µM M13-FAM, M13-HEX, M13-NED and M13-PET primer. The 1.5 µl of forward and reverse primer was divided by the number of multiplexed SSRs (i.e. 1µL or 0.75 µL of forward and

reverse primer mix from each SSR were added when 3 or 4 markers were multiplexed together, respectively). Thermocycling conditions consisted of 94°C for 3 min, [94°C 15s, 55°C for 1 min, 72°C for 2 min] x 33, 72°C for 30 min, 4 ∞. PCR products were visualized on a 1% agarose gel and then 1 to 3 µL of multiplexed PCR product from each of the four M13 dyes were pooled using the DY632 ladder from BioVentures, Inc. A total of 25 µl in 1,500 µl of formamide (Hi-Di Formamide from Life Technologies) were added per plate of 96 wells. The poolplexed mixture was sent to the University of Wisconsin-Madison Biotechnology Center DNA sequencing facility for fragment analysis using an ABI 3730 fluorescent sequencer (Applied Biosystems, Foster City, CA, USA). Allele genotyping was performed using GeneMarker v2.63 (SoftGenetics LLC, State College, PA, USA).

4- Data Analysis

The allelic information obtained from the genotyping was formatted as a GenAlEx (Peakall and Smouse 2012) input file. This file was then converted to a geneclone object to run in the R statistical software (R Core Team 2018) package Population Genetics in R (poppr) (Kamvar et al. 2014). Using poppr, we estimated the observed number of private alleles (PA). For the PCA, the packages ade4 (Dray et al. 2007) and adegenet (Jombart 2008) were used based on the missing allele replacement method, which replaces missing data with the mean allele frequencies. The package factoextra (Kassambara and Mundt 2016) was used to visualize the PCA. The packages magrittr (Bache and Wickham 2014), pegas (Paradis 2010) and ape (Paradis et al. 2004) were also used to visualize the data. For the genetic distance based PCA's the package polysat was used to calculate a distance matrix based on Bruvo's genetic distance (Bruvo et al. 2004; Clark and Jasieniuk 2011) package ggfortify (Tang et al. 2016) was used to visualize the PCA.

Calculations of genetic diversity statistics such as number of alleles, HO, Gst, G'st(Nei), G'st(Hed),D_est and Gis were obtained from the software GenoDive (Meirmans and Van Tienderen 2004). For the dendrograms, the R stats package (R Core Team 2018) was used to obtain Euclidean genetic distance and clustering. The dendrograms were then visualized using the packages dendextend (Galili 2015) and circlize (Gu et al. 2014).

5- Tissue Culture

Plant material collected was genotyped and selected for in-vitro culture. Specifically, runners were pruned from greenhouse plants. Runners were chosen because they were fast growing, vegetative stems. Tissue culture media was prepared by dissolving 20 g of sucrose and 2.41 g of Lloyd & McCown Woody Plant Basal Medium with Vitamins in 400 mL of DI water (Lloyd and McCown 1980). The pH of the solution was then adjusted by gradually adding 1M KOH to a pH of 5.58. A second solution was prepared simultaneously by dissolving 6 g of agar in 500 mL of boiling deionized (DI) water. Once both solutions were prepared they were combined, and the volume of the final solution was brought to 1L with DI water. 5 mL of the media solution was then dispensed into borosilicate glass tissue culture test tubes 20 x 150 mm with a round bottom and capped. The tubes of media were autoclaved for 20 minutes and then allowed to cool and jellify before tissue culture. A laminar flow hood was sterilized previous to the tissue culture process by spraying 90% then 70% ethanol. The runner tissue collected was trimmed into smaller pieces to fit into capped test tubes for sterilization and rinsed in a solution containing 2 drops of molecular biology non-ionic Tween 20 soap and DI water for two minutes while gently mixed by inversion. This solution was then poured out of the tubes inside of the sterile laminar flow hood, and the test tubes were then filled with a 20% bleach solution and

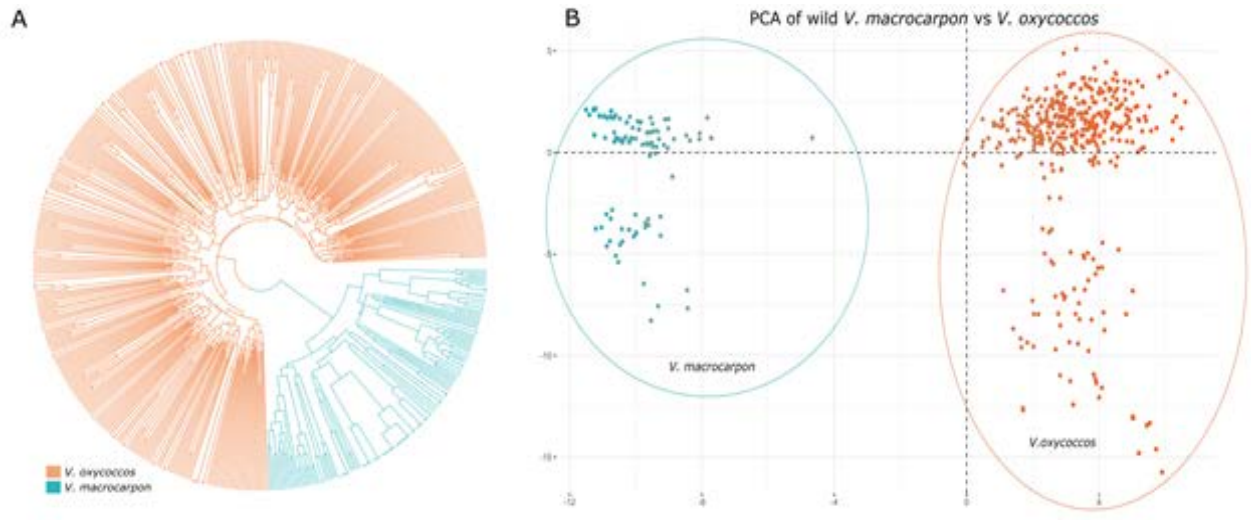
rinsed for 5 minutes while gently mixed by inversion. The bleach solution was then poured out, and runners are rinsed 3 times with sterile water, filling the tube with the water, inverting it, and pouring out the rinsed water. Sterile procedure was maintained, and any tool that left the work area was sterilized with 70% ethanol before it could enter the area again. Each piece of tissue was trimmed on both ends to remove dead tissue cause by exposure to the bleach. The healthy tissue was then cut into pieces using the sterilized scissors in the laminar flow hood, each about an inch long with at least two nodes. Explant was then placed in tissue culture tubes containing 5ml of media, proximal end down. The tubes were then sealed with parafilm to prevent water loss and limit contamination. Plants were stored at 20 °C on a 16 hr photoperiod.

Results

Genetic relationships of wild populations of cranberries

V. macrocarpon and *V. oxycoccos* are sympatric and can occur at the same time in the same space (Vander Kloet 1983). Therefore, we found nine populations in which both species were present throughout our sampling. While all samples were putatively classified into species in the field based on leaf morphology, we determined species membership, as *V. macrocarpon* and as *V. oxycoccos*, using genetic relationships based on 32 SSR markers. A dendrogram based on Euclidean genetic distance was constructed (Fig.1-A), and we observed a clear differentiation between samples identified as *V. macrocarpon* and samples identified as *V. oxycoccos*. To further confirm our results, we performed a PCA and again observed a clear differentiation between the two species. Based on these results, we divided our data into two main groups (*V. macrocarpon* and *V. oxycoccos* individuals) to further study and obtain diversity statistics by species.

Fig 1. Euclidean distance dendrogram and PCA cranberry (*Vaccinium macrocarpon* and *V. oxycoccos*) wild accessions



Overall genetic diversity of wild cranberries

When comparing the two species (Table 2), we observed that *V. oxycoccos* had the highest number of mean alleles per locus (28.18), private alleles (583) and heterozygosity indexes (H_O, H_S, H_T) with values close to 0.81. *V. macrocarpon* had a mean number of alleles per locus of 8.87, 25 private alleles and heterozygosity values (H_O, H_S, H_T) ranging from 0.50 to 0.64. Both groups had low levels of inbreeding demonstrated by the low inbreeding coefficient values (G_{is}) of 0.00 for *V. oxycoccos* and -0.03 for *V. macrocarpon*. In terms of the levels of fixation, which measures population differentiation caused by genetic structure, both groups had low values, with *V. macrocarpon* having slightly higher values for Gst (0.22), G'st (Nei) (0.24), G'st (Hed) (0.48) and D_est (0.32).

Table 2. Overall genetic diversity statistics for two cranberry (*Vaccinium macrocarpon* and *V. oxycoccos*) species

	V. oxycoccos	*V. macrocarpon*	
N	433	139	Total individuals per species
NA	902	284	Total number of alleles
Num	28.18	8.87	Mean number of alleles
PA	583	25	Private alleles
H_O	0.81	0.51	Observed Heterozygosity
H_S	0.81	0.49	Heterozygosity Within Populations
H_T	0.83	0.64	Total Heterozygosity
G_{is}	0.00	-0.03	Inbreeding coefficient
G_{st}	0.02	0.22	Fixation index
G'_{st}(Nei)	0.02	0.24	Nei, corrected fixation index
G'_{st}(Hed)	0.12	0.48	Hedrick, standardized fixation index
$D_{\_est}$	0.10	0.32	Jost, differentiation

Genetic diversity in wild *V. macrocarpon*

In *V. macrocarpon* the total number of alleles was 284. All the markers were polymorphic, and the number of alleles per locus ranged from 2 to 18 with a mean value of 8.87 (Fig. 2-A). The

observed heterozygosity per locus ranged from 0.08 to 0.97 with a mean value of 0.51 (Fig. 2-B). The total heterozygosity per locus values ranged from 0.08 to 0.89 with a mean value of 0.64 (Fig. 2-C). The inbreeding per locus coefficient ranged from -0.42 to 0.60 with a mean value of -0.01 (Fig. 2-D). The values of heterozygosity within populations per locus ranged from 0.05 to 0.77 and a mean value of 0.50 (data not shown).

Fig 2. Genetic diversity statistics per locus for *Vaccinium macrocarpon*

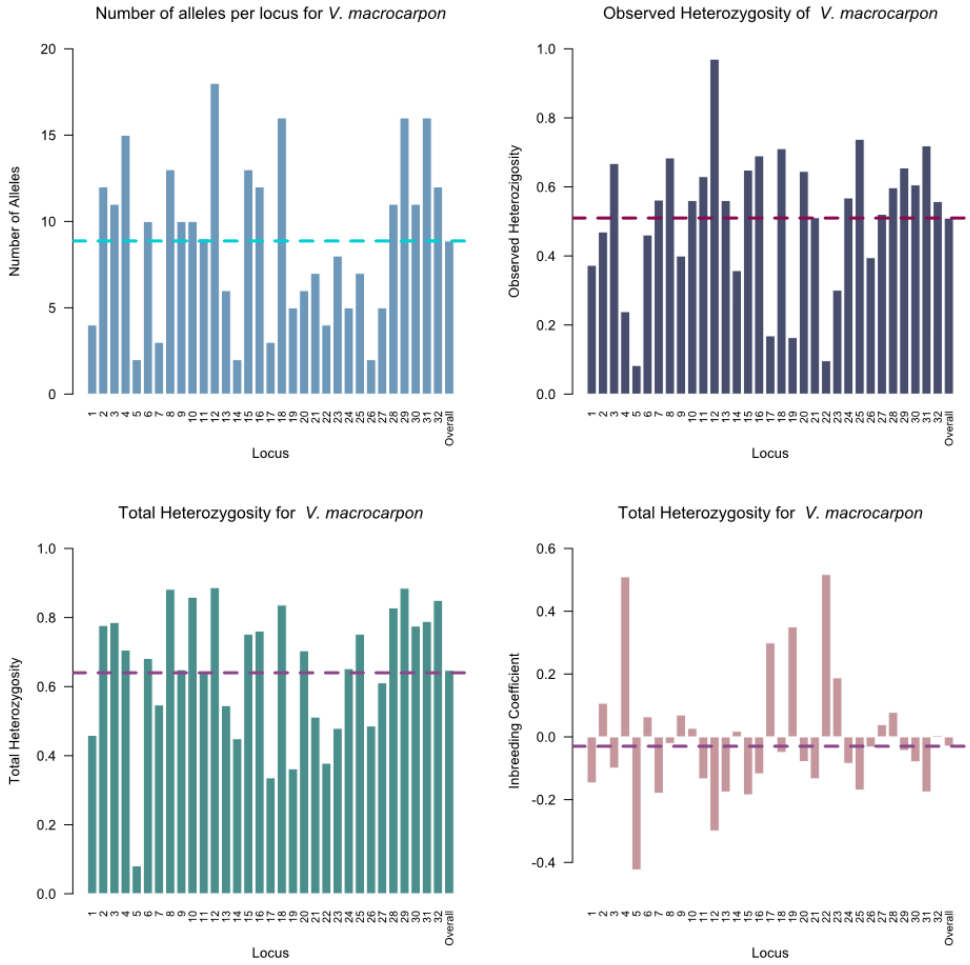


Table 3. Genetic diversity statistics per population of *Vaccinium macrocarpon*

Population	N	Num	Ho	Hs	Ht	Gis
BL	13	2.28	0.30	0.29	0.29	-0.01
Chip	20	2.50	0.51	0.34	0.34	-0.49
FRLK	3	1.65	0.58	0.37	0.37	-0.55
GYPL	13	3.25	0.58	0.52	0.52	-0.10
HW	1	NA*	NA*	NA*	NA*	NA*
LL	7	3.41	0.55	0.59	0.59	0.07
LOLM	20	2.38	0.43	0.39	0.39	-0.09
LRL	17	3.13	0.48	0.52	0.52	0.07
PMW	6	2.25	0.58	0.49	0.49	-0.18
SEB	1	NA*	NA*	NA*	NA*	NA*
SHPL	1	NA*	NA*	NA*	NA*	NA*
WABL	37	3.31	0.55	0.53	0.53	-0.05

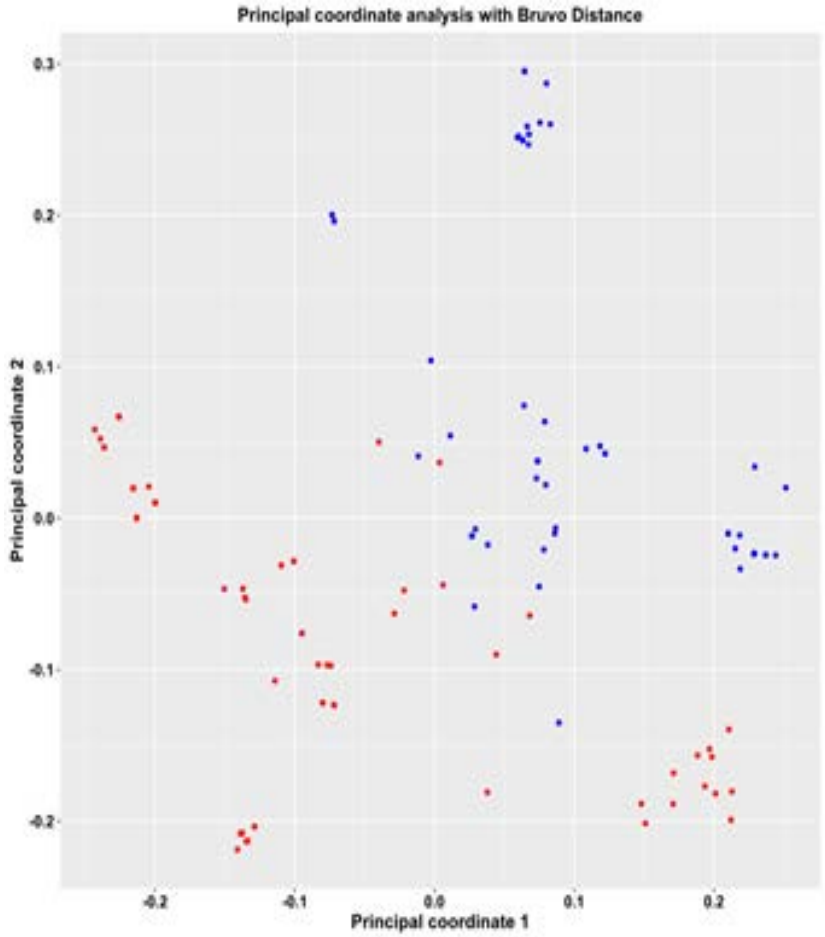
1- Number of samples, 2- Mean number of alleles, 3- Observed Heterozygosity, 4- Heterozygosity within populations, 5- Total Heterozygosity, 6- Inbreeding Coefficient, *- Low sample number, not calculated.

The populations with the highest levels of genetic diversity were FRLK, GYPL, and PMW with values of 0.58 (Table 3). Populations with only 1 to 2 samples after individuals were sorted into species were not analyzed.

A PCA based on Bruvo genetic distance (Fig 3) showed a clear separation between *V. macrocarpon* individuals from Minnesota (blue) and Wisconsin (red). In both populations, we

saw a wide genetic spread of the individuals as suggested by the levels of heterozygosity in Table 3.

Fig 3. PCA of *Vaccinium macrocarpon* based on Bruvo genetic distance



Genetic diversity in wild *V. oxycoccos*

In *V. oxycoccos* the total number of alleles was 902. All the markers were polymorphic, and the number of alleles per locus ranged from 3 to 56 with a mean value of 28.18 (Fig. 4-A). The observed heterozygosity (H_O) per locus ranged from 0.41 to 0.98 with a mean value of 0.81 (Fig. 4-B). The total heterozygosity (HT) values ranged from 0.45 to 0.95 with a mean value of 0.83 (Fig. 4-C). The inbreeding coefficient (G_{IS}) ranged from -0.34 to 0.40 with a mean value of 0.08 (Fig. 4-D). The values of heterozygosity within populations ranged from 0.48 to 0.94 and a mean

value of 0.81 (data not shown). When looking at the genetic diversity by population in *V. oxycoccos*, most exhibited very high levels of genetic diversity averaging 0.80 when compared to *V. macrocarpon*. In the PCA of *V. oxycoccos* accessions (Figure 5), we observed some spatial separation by state, we it was less population than in *V. macrocarpon*.

Fig 4. Genetic diversity statistics per locus for *Vaccinium oxycoccos*

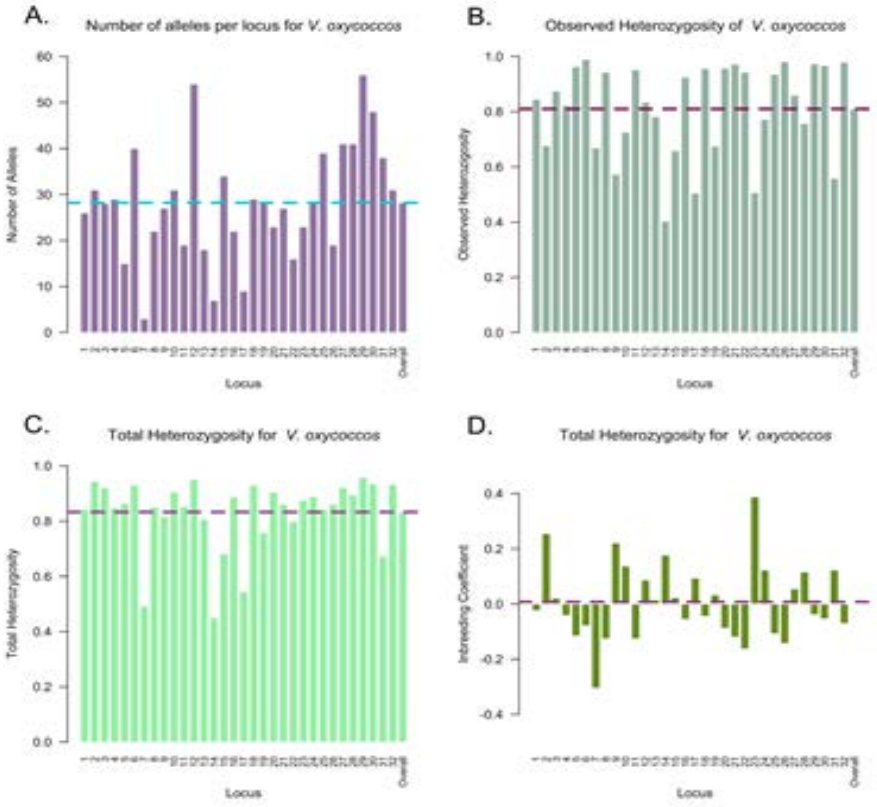


Table 4. Genetic diversity statistics per population of *Vaccinium oxycoccos*

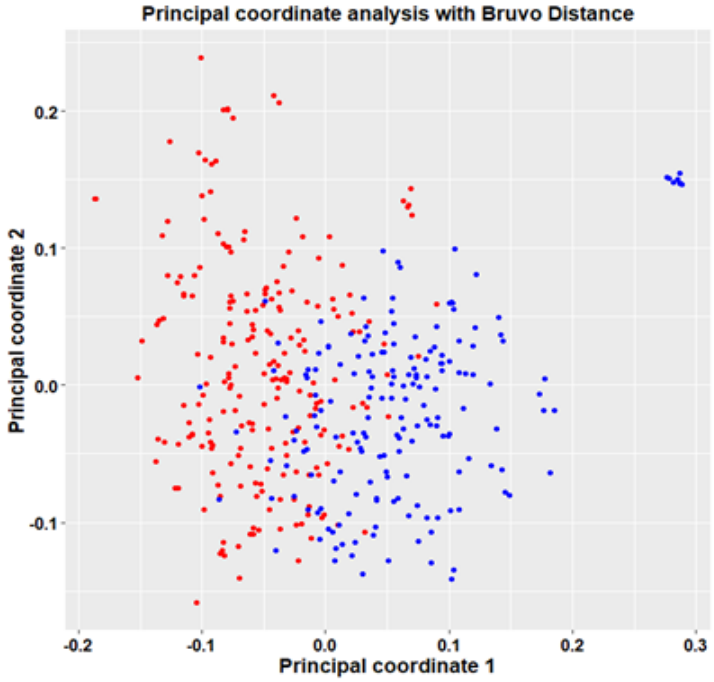
Population	N[1]	Num[2]	H_O[3]	H_S[4]	H_T[5]	G_{IS}[6]
BL	5	6.06	0.86	0.82	0.82	-0.04
FBF	7	6.18	0.81	0.75	0.75	-0.08
GL	18	11.3	0.82	0.82	0.82	0
GYPL	20	10.56	0.86	0.81	0.81	-0.05

JL	12	11.25	0.77	0.85	0.85	0.09
KLB	21	10.78	0.83	0.81	0.81	-0.02
KMP	12	6.53	0.83	0.78	0.78	-0.06
Locp	30	10.4	0.8	0.8	0.8	0
MWB	16	10	0.81	0.82	0.82	0.01
PMS	20	11.87	0.84	0.83	0.83	0
PMW	17	9.78	0.8	0.81	0.81	0
SEB	16	7.65	0.8	0.76	0.76	-0.05
SHPL	10	9	0.77	0.81	0.81	0.04
SR	17	9.75	0.77	0.79	0.79	0.02
WABL	2	NA*	NA*	NA*	NA*	NA*
NB	1	NA*	NA*	NA*	NA*	NA*
CHIPR	1	NA*	NA*	NA*	NA*	NA*
LOL	3	4.78	0.77	0.81	0.81	0.04
LU	3	3.96	0.8	0.8	0.8	0
WIN	2	NA*	NA*	NA*	NA*	NA*
BB	20	12	0.83	0.83	0.83	-0.01
BLM	13	8.93	0.85	0.8	0.8	-0.05
BRR	20	9.5	0.82	0.79	0.79	-0.03
BRRM	20	9.06	0.82	0.77	0.77	-0.06
HB	19	11.56	0.8	0.81	0.81	0.01
ISB	3	NA*	NA*	NA*	NA*	NA*
LL	8	8.56	0.79	0.83	0.83	0.05
LOLB	20	11.59	0.81	0.81	0.81	-0.01

LRL	3	5.43	0.85	0.85	0.85	0
PB	20	10.25	0.8	0.8	0.8	0
PRF	20	11.75	0.82	0.83	0.83	0.01
TR	21	12.09	0.79	0.82	0.82	0.03
FRLK	13	7.15	0.85	0.77	0.77	-0.11

1- Number of samples, 2- Mean number of alleles, 3- Observed Heterozygosity, 4- Heterozygosity within populations, 5- Total Heterozygosity, 6- Inbreeding Coefficient, *- Low sample number, not calculated.

Fig 5. PCA of *Vaccinium oxycoccos* based on Bruvo genetic distance



Comparing the relatedness between wild vs cultivated *V. macrocarpon*

When comparing the diversity present in the wild *V. macrocarpon* population samples in Wisconsin and Minnesota versus a collection of cultivars and wild selections from the US, we

found high levels of heterozygosity and mean allele numbers (Table 5). We found higher levels of observed heterozygosity and lower inbreeding index for wild populations (H_O=0.99, G_{IS}=0.69) than for cultivated cranberries (H_O= 0.69, G_{IS}=0.15).

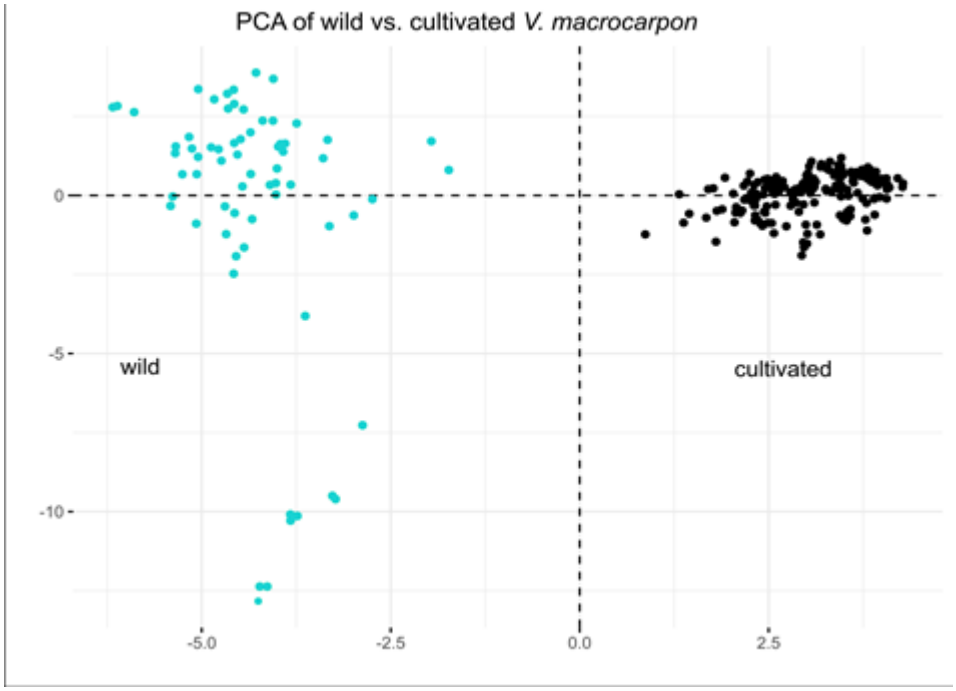
Table 5. Genetic diversity comparison among wild *V. macrocarpon* Wisconsin and Minnesota and cranberry cultivars.

Population	N[1]	Num[2]	H_O[3]	H_S[4]	H_T[5]	G_{IS}[6]
Wild	139	10.87	0.99	0.76	0.76	-0.31
Cultivated	197	19.25	0.69	0.82	0.82	0.15

1- Number of samples, 2- Mean number of alleles, 3- Observed Heterozygosity, 4- Heterozygosity within populations, 5- Total Heterozygosity, 6- Inbreeding Coefficient

We performed a PCA using eight SSR markers to compare existing cranberry cultivars and breeding selections diversity to wild *V. macrocarpon* diversity identified in the current study (Fig. 6). Interestingly, our results showed a clear differentiation between our Wisconsin and Minnesota wild accessions and cultivars such as all the major cultivars, wild accessions (mostly eastern wild germplasm), and breeding selections (Fajardo et al. 2013; Zalapa et al. 2015; Schlautman et al. 2018). We observed higher heterozygosity and more differentiation and spread in the PCA in the Wisconsin and Minnesota wild samples compared to the cultivated.

Fig. 6 PCA comparing cultivated vs wild individuals of *Vaccinium macrocarpon*



Development of an ex-situ wild cranberry collection

We successfully introduced to tissue culture a total of 359 genetically unique genotypes from our collection sites in Wisconsin and Minnesota. At least 2-3 copies per genotype have been kept as backup for a total of 1305 plants (Supplementary Materials-2)

Discussion

Although cranberry is a perennial fruit crop of great importance with economical and human health benefits, breeding has been hampered by the narrow phenotypic and genetic diversity of cultivars and breeding materials (Diaz-Garcia et al. 2019). Since the majority of cultivars used in breeding were developed from a few native selections more than half a century ago, there is great need to incorporate new traits to enhance current materials (Eck 1990). Because of this,

we studied several unexplored wild cranberry populations in Wisconsin and Minnesota to understand the genetic diversity for future conservation purposes, which will serve to increase the availability of germplasm for cranberry breeding programs.

Overview of the genetic relationship and diversity of wild *V. macrocarpon* and *V. oxycoccos* populations in Wisconsin and Minnesota

V. macrocarpon and *V. oxycoccos* share many morphological traits, which make identification in the field challenging. Therefore, we designated field cuttings as *V. macrocarpon* and *V. oxycoccos* to each of the species based on leaf size and shape (Smith et al. 2015) and then corroborated these classifications using genetic information based on 32 SSR markers. We constructed a Euclidean distance-based dendrogram and a PCA of all the accessions collected (Fig. 1 A-B). In both, we clearly saw a differentiation between the two species. This clear differentiation between *V. macrocarpon* and *V. oxycoccos* has been previously reported using AFLPs (Smith et al. 2015) and SSRs (Zalapa et al. 2015). The striking differentiation among the two species could be due to their different, but interconnected, evolutionary histories, where *V. macrocarpon* could be an ancestral diploid species while *V. oxycoccos* is a more recently derived, mostly allotetraploid species. We looked at the number of private alleles present in both species and found that *V. oxycoccos* had the highest number of private alleles (598) compared with *V. macrocarpon* with only 25. The high number of private alleles in *V. oxycoccos* could be explained by its putative allopolyploid origin resulting from the hybridization of the two know ancestral species, *V. macrocarpon* and *V. microcarpum*, or from the polyploidization with a 3rd species that could now be extinct (Darrow and Camp 1944; Diaz-Garcia et al. 2019). The involvement of *V. macrocarpon* in the speciation of *V. oxycoccos* is further demonstrated

by the number of private alleles present when compared with *V. oxycoccos*. Similar results were observed using allozymes in which they found that *V. oxycoccos* shared predominant alleles with *V. macrocarpon* (Mahy et al. 2000) (Table2).

In terms of overall genetic diversity for both species, we observed that *V. oxycoccos*, as expected due to its polyploid (4x) and outcrossing nature, was highly diverse with high heterozygosity (H_O, H_T, H_S) levels. Such high levels of heterozygosity have been observed in other crops such as sweet potato and cassava (Fregene et al. 2003; Rodriguez-Bonilla et al. 2014). *V. macrocarpon* populations were quite heterozygous for a diploid plant that can self-pollinate and reproduce (Bruederle et al. 1996), although highly heterozygous *V. macrocarpon* (H_O=0.5) exhibited lower levels of heterozygosity than those observed for *V. oxycoccos* (H_O=0.8). The high diversity observed in wild populations of cranberries may be the result of different mechanisms allowing cranberries to maintain and enrich their genetic diversity. Cranberries provide pollen and nectar to pollinators such as bumblebees, which are able to transfer it between cranberry flowers. This pollen movement can result in new genetic combinations (Ortwine-Boes and Silbernagel 2003). Aside from pollinators potentially increasing diversity in undisrupted natural areas, open spaces are inviting for bird species such as cranes as well as other animals. These animals that consume wild cranberry fruits and then move or migrate to other locations can disperse the cranberry seeds (Barzen et al. 2018). Cranberries are often found near large and interconnected bodies of water, and when fruit become detached from a cranberry plant, they float due to their locular cavities and travel long distances, which can result in chance seedlings increasing migration and diversity among populations.

***V. macrocarpon* genetic diversity**

Based on our results, we found that *V. macrocarpon* is more diverse than previously thought by Bruederle et al. (1996) and Stewart and Excoffier (1996). We found high numbers of alleles per locus ranging from 2 to 18 (Fig. 2-A) while other researchers found 1.13 to 2 alleles per locus using RAPD's (Novy and Vorsa 1995; Stewart and Excoffier 1996) and allozymes (Mahy et al. 2000). These results could be explained by the large number of hypervariable and codominant SSR markers used in this study as well as the large number of samples collected. When looking at the data by population, we also found that most populations were moderately to highly diverse, and diversity levels and alleles per loci were comparable to the six Wisconsin wild populations described by Zalapa et al. (2015). Previous research in cranberries suggested that the low levels of heterozygosity and diversity could be explained by self-pollination and inbreeding, especially after several inbreeding cycles (Bruederle et al. 1996; Zalapa et al. 2015). However, our results indicate that wild populations of *V. macrocarpon* likely outcross leading to moderate to high levels of heterozygosity (Table 2). This can be further confirmed by the low levels of inbreeding observed suggested by the G_{IS} values (-0.55-0.07).

The low levels of heterozygosity observed in some populations could be due to sampling bias, long-term evolutionary forces, and recent environmental events. For example, (Stewart and Excoffier 1996) and (Bruederle et al. 1996) believed a genetic bottleneck during the Pleistocene glaciation accounted for the low levels of diversity found in the wild populations they studied. Additionally, low levels of diversity could be the result of recent events, including environmental changes due to agriculture, urbanization, and other anthropogenic activities. For example, the declining number and health or fitness of pollinators in an area due to human

activity such as a nearby construction or principal road disrupting the area has been observed to have an effect in other plant species resulting in lower fruit set in *Halimium halimifolium* (Suárez-Esteban et al. 2014). Previous studies that found low levels of genetic variation in wild populations of cranberry were surprised by their low diversity results. Although *V. macrocarpon* can self-pollinate and can resist some levels of inbreeding, inbreeding in cranberry often results in low fertility rates and inbreeding depression, signaling that cranberry is mostly an outcrossing species (Bruederle et al. 1996). Therefore, as demonstrated by our results, natural cranberry genetic variation is actually much higher than observed in previous studies. Such diversity was not detected mostly due to the use of molecular markers with low power and clonal sampling complications (Stewart and Excoffier 1996; Mahy et al. 2000).

In the Bruvo genetic distance PCA (Fig 3), we observed that most individuals grouped together based on geographical location (Minnesota versus Wisconsin). We observed some minor clustering among some individuals in some populations that could be explained by the low fixation indexes (0.2-0.3) in those populations, indicating inbreeding.

***V. oxycoccos* genetic diversity**

V. oxycoccos is one of the wild relatives of cranberries, which are one of the few crops native to North America. Therefore, *V. oxycoccos* may be a source of genotypic traits useful for the development of new breeding lines for cranberry production. As demonstrated in Table 2 by the high number of alleles per locus (28), high levels of heterozygosity (0.8), and low levels of inbreeding (-0.019), the studied wild *V. oxycoccos* Minnesota and Wisconsin populations were highly diverse. As mentioned before, the polyploidization of multiple species and the outcrossing nature of *V. oxycoccos* has been crucial to maintain and enrich the genetic diversity

of the species (Darrow and Camp 1944; Diaz-Garcia et al. 2019). Mahy et al. in (2000) analyzed the genetic diversity of *V. macrocarpon* and *V. oxycoccos* population using allozymes and observed that for several loci *V. oxycoccos* exhibited higher levels of heterozygosity (i.e. 0.5-0.6) than *V. macrocarpon*. Our study is the first to use codominant SSR markers to investigate diversity of the species using multiple populations. The previous study only used one population consisting of 31 individuals to test cross-amplification of eight SSR markers and differentiation of the two species, but diversity statistics were not given besides similar number of alleles per locus and total number of alleles (Zalapa et al. 2015). Diversity statistics were comparable as in other polyploids species such as sweet potato (Rodriguez-Bonilla et al. 2014) and cassava (Fregene et al. 2003). Česonienė et al. (2013) analyzed the genetic diversity of *V. oxycoccos* clones in Lithuanian reserves using RAPD markers on two populations and found considerable levels of genetic diversity, suggesting that *V. oxycoccos* could be a promising crop for cultivation in different locations across central Europe. This study also demonstrates that there could be invaluable resources for breeding in *in-situ* collections and reserves as well as the wild locations surveyed in this study. In the Bruvo genetic distance PCA for *V. oxycoccos*, we observed a closer relationship among *V. oxycoccos* individuals than in *V. macrocarpon* (Figure 5). This could be explained by cranberries colonization history. It has long been believed that most wild cranberry populations resulted from a bottleneck that occurred in the Pleistocene and that they eventually populated receding glacial areas. More extensive sampling of new locations, for example, in the driftless area, should be performed to investigate the possibility of a bottleneck and possible colonization and cranberry spread (Stewart et al. 1995).

Comparing the relatedness between wild and cultivated *V. macrocarpon*

Most cranberry cultivars today come from wild selections made decades ago. Therefore, most cultivars share the same genetic background (Eck 1990). Because of this, we were interested in assessing how much of the diversity present in the wild has been harnessed for breeding. Zalapa et al. (2015) used 12 SSR markers and observed that there were considerable levels of genetic diversity present in wild populations compared to cultivars based on the number of private and unique alleles, as well as levels of observed heterozygosity. In our study, we compared 139 wild accessions collected in Wisconsin and Minnesota, with 195 cultivars, wild accessions, and breeding selections mostly from eastern origin (Fajardo et al. 2013; Zalapa et al. 2015; Schlautman et al. 2018). We found higher levels of observed heterozygosity in wild populations than in cultivars and breeding selections. As expected, we observed lower levels of inbreeding in wild populations than in cultivars. In the wild, cranberries are mostly outcrossing, which is not the case of plants used in breeding programs (Bruederle et al. 1996). As selection cycles occur more genetic diversity is lost, as has been the case of many crops, such as maize (Whitt et al. 2002).

To further compare between cultivated breeding materials and wild *V. macrocarpon* accessions, we performed a PCA in which we observed a clear separation between species on PC 1 (Fig. 6). Although all cultivated cranberries derive from wild selections made about 70 years ago, these plants have been consistently bred to have desirable traits and vigor using a small genetic pool. The reduction of diversity is an inevitable consequence of developing cultivars adapted to homogenous production environment (Zalapa et al. 2015). The wild populations, as previously mentioned, are naturally outcrossing in an open environment that allows for chance seedlings,

not in a homogeneous environment such as a cranberry bed. These results, as well as the levels of heterozygosity, highlight how little of the present diversity is currently being used in cranberry breeding.

Development of an in-vitro cranberry collection

Conservation of plants in the field or greenhouse condition present formidable challenges, many times resulting in the loss of plant genetic resources conserved (Engelmann 2011). Therefore utilizing tissue culture techniques is of great use to propagate and conserve germplasm (Engelmann 1991). We successfully introduced hundreds of unique genotypes from vastly unexplored cranberry regions of Wisconsin and Minnesota to tissue culture, and the majority of our unique genotypes and will be available for breeding in the USDA-ARS, Vegetable Crop Research Unit in Madison, WI, Cranberry Genetics and Genomics Lab. Additionally, selected accessions will be added to the National Clonal Germplasm Repository (NCGR) collection.

Conclusions

In this study, we used a set of markers capable of distinguishing between *V. macrocarpon* and *V. oxycoccos*. We demonstrated the presence of untapped and previously undiscovered cranberry diversity in natural areas of the states of Wisconsin and Minnesota. The plant material used in this study was also composed of cranberry crop wild relatives, which may possess genotypic traits useful for the development of new breeding lines for cranberry production. Particularly, since all of these populations were sampled in Minnesota and Wisconsin, they might possess unique adaptations to the largest cranberry production area in the world, central Wisconsin. Due to the lack of exploration, Wisconsin growers have been

forced to use cranberry cultivars developed for other growing areas. Therefore, the challenges faced by growers in our state are unique and include intense frost risk and cranberry fruit worm, among others. This study demonstrates the vast diversity and range of cranberry populations in two mostly unexplored and underutilized Midwest states. We believe that there could be many other states that possess wild areas with unique and unexplored populations that could be excellent targets not only to establish a germplasm collection, but also for characterization and as *in-situ* conservation areas.

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References

Aaseng NE (2003) Field guide to the native plant communities of Minnesota: the Laurentian Mixed Forest Province. Minnesota Department of Natural Resources

Anh VL, Inoue Y, Asuke S, Vy TT, Anh NT, Wang S, Chuma I, Tosa Y (2018) Rmg8 and Rmg7, wheat genes for resistance to the wheat blast fungus, recognize the same avirulence gene AVR-Rmg8: Dual recognition of a wheat blast avirulence gene. Mol Plant Pathol 19:1252–1256. doi: 10.1111/mpp.12609

Areškevičiūtė J, Paulauskas A, Česonienė L, Daubaras R (2006) Genetic characterisation of wild cranberry (Vaccinium oxycoccos) from Čepkeliai reserve by the RAPD method. Liet Tsr

Aukst Mokyklu Mokslo Darb

Bache SM, Wickham H (2014) magrittr: a forward-pipe operator for R. R package version 1

Barzen J, Thousand T, Welch J, Fitzpatrick M, Triet T (2018) Determining the diet of whooping cranes (Grus americana) through field measurements. Waterbirds 41:22–35

Brown PN, Turi CE, Shipley PR, Murch SJ (2012) Comparisons of large (Vaccinium macrocarpon Ait.) and small (Vaccinium oxycoccos L., Vaccinium vitis-idaea L.) cranberry in British Columbia by phytochemical determination, antioxidant potential, and metabolomic profiling with chemometric analysis. Planta Med 78:630–640

Brownstein MJ, Carpten JD, Smith JR (1996) Modulation of non-templated nucleotide addition by Taq DNA polymerase: primer modifications that facilitate genotyping. Biotechniques 20:1004–6, 1008–10. doi: 10.2144/96206st01

Brozynska M, Furtado A, Henry RJ (2016) Genomics of crop wild relatives: expanding the gene pool for crop improvement. Plant Biotechnol J 14:1070–1085. doi: 10.1111/pbi.12454

Bruederle LP, Hugan MS, Dignan JM, Vorsa N (1996) Genetic variation in natural populations of the large cranberry, Vaccinium macrocarpon Ait.(Ericaceae). Bull Torrey Bot Club 41–47

Bruvo R, Michiels NK, D'Souza TG, Schulenburg H (2004) A simple method for the calculation of microsatellite genotype distances irrespective of ploidy level. Mol Ecol 13:2101–2106. doi: 10.1111/j.1365-294X.2004.02209.x

Česonienė L, Daubaras R, Jasutienė I, Venclovienė J, Miliauskienė, I (2011) Evaluation of the Biochemical Components and Chromatic Properties of the Juice of Vaccinium macrocarpon Aiton and Vaccinium oxycoccos L. Plant Foods Hum Nutr 66:238–244. doi: 10.1007/s11130-011-0241-5

Česonienė L, Daubaras R, Paulauskas A, Zukauskiene J, Zych M (2013) Morphological and genetic diversity of European cranberry (Vaccinium oxycoccos L., Ericaceae) clones in Lithuanian reserves. Acta Soc Bot Pol 82:211–217. doi: 10.5586/asbp.2013.026

Chandler FB, Demoranville IE (1958) Cranberry varieties of North America. Massachusetts Agricultural Experimental Stations Bulletin. 513

Cheema AK (2018) Plant Breeding its Applications and Future Prospects. International Journal of Engineering Technology Science and Research. 5

Clark LV, Jasieniuk M (2011) POLYSAT: an R package for polyploid microsatellite analysis. Mol Ecol Resour 11:562–566. doi: 10.1111/j.1755-0998.2011.02985.x

Dana MN (1983) Cranberry cultivar list [Vaccinium macrocarpon]. Fruit Varieties Journal

Darrow GM, Camp WH (1944) Vaccinium Hybrids and the Development of New Horticultural Material. Bull Torrey Bot Club 72:1–21. doi: 10.2307/2481260

Dempewolf H, Baute G, Anderson J, Kilian B, Smith C, Guarino L (2017) Past and Future Use of Wild Relatives in Crop Breeding. Crop Sci 57:1070–1082. doi: 10.2135/cropsci2016.10.0885

Diaz-Garcia L, Rodriguez-Bonilla L, Rohde J, Smith T, Zalapa J (2019) Pacbio Sequencing Reveals Identical Organelle Genomes between American Cranberry (Vaccinium macrocarpon Ait.) and a Wild Relative. Genes 10. doi: 10.3390/genes10040291

Doyle JJ, Doyle JL (1987) CTAB DNA extraction in plants. Phytochemical Bulletin 19:11–15

Dray S, Dufour AB (2007) The ade4 package: implementing the duality diagram for ecologists. J Stat Softw 22:1–20

Eck P (1990) The american cranberry.Rutgers University Press

Engelmann F (1991) In vitro conservation of tropical plant germplasm --- a review. Euphytica 57:227–243. doi: 10.1007/BF00039669

Engelmann F (2011) Use of biotechnologies for the conservation of plant biodiversity. In Vitro Cellular & Developmental Biology - Plant 47:5–16. doi: 10.1007/s11627-010-9327-2

Fajardo D, Morales J, Zhu H, Steffan S, Harbut R, Bassil N, Hummer K, Polashock J, Vorsa N, Zalapa J (2013) Discrimination of American cranberry cultivars and assessment of clonal heterogeneity using microsatellite markers. Plant Mol Biol Rep 31:264-271

Fregene MA, Suarez M, Mkumbira J, Kulembeka H, Ndeya E, Kulaya A, Mitchel S, Gullberg U, Rosling H, Dixon AG, Dean R (2003) Simple sequence repeat marker diversity in cassava landraces: genetic diversity and differentiation in an asexually propagated crop. Theor Appl Genet 107:1083–1093. doi: 10.1007/s00122-003-1348-3

Galili T (2015) dendextend: an R package for visualizing, adjusting and comparing trees of hierarchical clustering. Bioinformatics 31:3718–3720. doi: 10.1093/bioinformatics/btv428

Gallardo RK, Klingthong P, Zhang Q, et al (2018) Breeding Trait Priorities of the Cranberry Industry in the United States and Canada. HortScience 53:1467–1474

Georgi L, Herai RH, Vidal R, Carazzolle MF, Pereira GG, Polashock J, Vorsa N (2012) Cranberry microsatellite marker development from assembled next-generation genomic sequence. Mol Breed 30:227–237. doi: 10.1007/s11032-011-9613-7

Guichoux E, Lagache L, Wagner S, Chaumeil P, Léger P, Lepais O, Lepoittevin C, Malausa T, Revardel E, Salin F, Petit RJ (2011) Current trends in microsatellite genotyping. Mol Ecol Resour 11:591–611. doi: 10.1111/j.1755-0998.2011.03014.x

Gu Z, Gu L, Eils R, Schlesner M, Brors B (2014) circlize implements and enhances circular visualization in R. Bioinformatics 30:2811–2812. doi: 10.1093/bioinformatics/btu393

Hamernik AJ, Hanneman RE Jr, Jansky SH (2009) Introgression of Wild Species Germplasm with Extreme Resistance to Cold Sweetening into the Cultivated Potato. Crop Sci 49(2):529-542. doi: 10.2135/cropsci2008.04.0209

Hummer KE, Williams KA, Bushakra JM (2019) North American Crop Wild Relatives of Temperate Berries (Fragaria L., Ribes L., Rubus L., and Vaccinium L.). In: Greene SL, Williams KA, Khoury CK, Kantar MB, Marek LF (eds) North American Crop Wild Relatives, Volume 2: Important Species. Springer International Publishing, Cham, pp 283–327

Jacquemart A-L (1997) Vaccinium Oxycoccos L. (Oxycoccus Palustris Pers.) and Vaccinium Microcarpum (Turcz. ex Rupr.) Schmalh. (Oxycoccus Microcarpus Turcz. ex Rupr.). J Ecol 85:381–396. doi: 10.2307/2960511

Jombart T (2008) adegenet: a R package for the multivariate analysis of genetic markers. Bioinformatics 24:1403–1405. doi: 10.1093/bioinformatics/btn129

Kamvar ZN, Tabima JF, Grünwald NJ (2014) Poppr: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. PeerJ 2:e281. doi: 10.7717/peerj.281

Lloyd G, McCown B (1980) Commercially-feasible micropropagation of mountain laurel, Kalmia latifolia, by use of shoot-tip culture. Combined Proceedings, International Plant Propagators' Society 30:421–427

Kassambara A, Mundt F (2016) Package "factoextra." Extract and Visualize the Results of Multivariate Data Analyses

Khoury CK, Greene S, Wiersema J, Maxted N, Jarvis A, Struik PC (2013) An inventory of crop wild relatives of the United States. Crop Sci 53(4):1496-1508

Mahy G, Bruederle LP, Connors B, Van Hofwegen M, Vorsa N (2000) Allozyme evidence for genetic autopolyploidy and high genetic diversity in tetraploid cranberry, Vaccinium oxycoccos (Ericaceae). Am J Bot 87:1882–1889

Meirmans PG, Van Tienderen PH (2004) genotype and genodive: two programs for the analysis of genetic diversity of asexual organisms. Mol Ecol Notes

National Research Council (1993) Managing Global Genetic Resources: Agricultural Crop Issues and Policies. National Academies Press

Novy RG, Vorsa N (1995) Identification of intracultivar genetic heterogeneity in cranberry using silver-stained RAPDs. HortScience 30:600–604

Ortwine-Boes C, Silbernagel J (2003) Bumblebee conservation in and around cranberry marshes. School proceedings 2003:10

Paradis E (2010) pegas: an R package for population genetics with an integrated–modular approach. Bioinformatics 26:419–420. doi: 10.1093/bioinformatics/btp696

Paradis E, Claude J, Strimmer K (2004) APE: Analyses of Phylogenetics and Evolution in R language. Bioinformatics 20:289–290

Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research--an update. Bioinformatics 28:2537–2539. doi: 10.1093/bioinformatics/bts460

R Core Team (2018) R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing

Rodriguez-Bonilla L, Cuevas HE, Montero-Rojas M, Bird-Pico F, Luciano-Rosario D, Siritunga D (2014) Assessment of genetic diversity of sweet potato in Puerto Rico. PLoS One 9:e116184.

Rodriguez-Bonilla L, Rohde J, Matusinec D, Zalapa J. (2019). Cross-transferability analysis of SSR markers developed from the American Cranberry to other *Vaccinium* species of agricultural importance. (In preparation)

Roper TR, Vorsa N (1997) Cranberry: Botany and Horticulture. In: Janick J (ed) Horticultural Reviews. John Wiley & Sons, Inc., Oxford, UK, pp 215–249

Schlautman B, Fajardo D, Bougie T, Wiesman E (2015) Development and validation of 697 novel polymorphic genomic and EST-SSR markers in the American cranberry (Vaccinium macrocarpon Ait.). Molecules 20:2001-2013

Schlautman B, Covarrubias-Pazaran G, Rodriguez-Bonilla L, Hummer K, Bassil N, Smith T, Zalapa J. 2018. Genetic diversity and cultivar variants in the National Clonal Germplasm Repository cranberry (Vaccinium macrocarpon Aiton) collection. Journal of Genetics, J Genet 97:1339-1351.

Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. Nat Biotechnol 18:233–234. doi: 10.1038/72708

Shakiba E, Edwards JD, Jodari F, Duke SE, Baldo AM, Korniliev P, McCouch SR, Eizenga GC (2017) Genetic architecture of cold tolerance in rice (Oryza sativa) determined through high resolution genome-wide analysis. PLoS One 12:e0172133. doi: 10.1371/journal.pone.0172133

Smith TW, Walinga C, Wang S, Kron P, Suda J, Zalapa J (2015) Evaluating the relationship between diploid and tetraploid Vaccinium oxycoccos (Ericaceae) in eastern Canada. Botany

93:623-636

Stewart CN, Excoffier L (1996) Assessing population genetic structure and variability with RAPD data: Application to Vaccinium macrocarpon (American Cranberry). J Evol Biol 9:153–171. doi: 10.1046/j.1420-9101.1996.9020153.x

Stewart CN, Neal Stewart C, Nilsen ET (1995) Phenotypic Plasticity and Genetic Variation of Vaccinium macrocarpon, the American Cranberry. I. Reaction Norms of Clones from Central and Marginal Populations in a Common Garden. International Journal of Plant Sciences 156:687–697

Suárez-Esteban A, Delibes M, Fedriani JM (2014) Unpaved roads disrupt the effect of herbivores and pollinators on the reproduction of a dominant shrub. Basic Appl Ecol 15:524–533. doi: 10.1016/j.baae.2014.08.001

Tang Y, Horikoshi M, Li W (2016) ggfortify: unified interface to visualize statistical results of popular R packages. R J 8:478–489

Vander Kloet SP (1988) The genus Vaccinium in North America. Agriculture Canada

Vander Kloet SP (1983) THE TAXONOMY OF VACCINIUM § OXYCOCCUS. Rhodora 85:1–43

Vorsa N, Johnson-Cicalese J, Polashock J (2008) A blueberry by cranberry hybrid derived from a Vaccinium darrowii×(V. macrocarpon× V. oxycoccos) intersectional cross. In: IX International Vaccinium Symposium 810. actahort.org, pp 187–190

Whitt SR, Wilson LM, Tenaillon MI, Gaut BS, Buckler ES (2002) Genetic diversity and selection in the maize starch pathway. Proc Natl Acad Sci USA 99:12959–12962. doi: 10.1073/pnas.202476999

Zalapa JE, Bougie TC, Bougie TA (2015) Clonal diversity and genetic differentiation revealed by SSR markers in wild Vaccinium macrocarpon and Vaccinium oxycoccos. Ann Appl Biol 166:196-207

Zhang H, Mittal N, Leamy LJ, Barazini O, Song BH (2017) Back into the wild-Apply untapped genetic diversity of wild relatives for crop improvement. Evol Appl 10:5–24. doi: 10.1111/eva.12434

Zhu H, Senalik D, McCown BH, Zeldin EL, Speers J, Hyman J, Bassil N, Hummer K, Simon PW, Zalapa JE (2012) Mining and validation of pyrosequenced simple sequence repeats (SSRs) from American cranberry (Vaccinium macrocarpon Ait.). Theor Appl Genet 124:87–96. doi: 10.1007/s00122-011-1689-2

Supplementary materials

Final Primer Pairs

Fluorescence	Primer	Range	NCBI	Source	Forward Primer	Reverse Primer
FAM	ct93137	175-229	KP279104	Schlautman et al. (2015)	AAGATTTCCGCTACAGTACCT	GCTATGGGTGTCTCAAAAAG
	ct31701	251-327	JF834268	Zhu et al. (2012)	GTCACTGGTAATGCTATTCTGA	CTTCTTTGTTTCATCTCCCTAC
	SCF162565	146-182	KP278913	Schlautman et al. (2015)	CTTCCGTGATTGTTCTTGTAG	ACACAGATGGGATGTTGTATC
	SCF125251	234-305	KP278851	Schlautman et al. (2015)	CACGACGTTGTAAAACGACTATACAGTCAGATCCAATCCAC	TGCAGATAAAGTACAAGAGTGC
	SCF139334	193-230	KP278874	Schlautman et al. (2015)	CACGACGTTGTAAAACGACGAGGGTCTAATATCTGGTTTCA	GAGAAAAGATGGAGCAAAAG
	SCF3362	320-380	KP278605	Schlautman et al. (2015)	GTACAGCAAAATTCAGCACA	GGATTTATCTACAGCCCATTAC
	ct25796	195-262	JF834263	Zhu et al. (2012)	CACTTACCTGAATCCTCTTAGC	TAGAGGAGCCAAACTGATAACT
	1trimcontig176303	181-226	KP279222	Schlautman et al. (2015)	CACGACGTTGTAAAACGACGCTGATTAGGTTCACTTTCTTC	TTTCTTCACCTCTTTCTCTCTC
HEX	SCF22477	240-271	KP278658	Schlautman et al. (2015)	CTCTCCCCTACTTTCTTCCTAT	GCCGCTAACACAATTAACTAAC
	SCF2714	189-232	KP278602	Schlautman et al. (2015)	ACAAGTCTCTGGAAGCTAACAT	GTTGATTGTTGGGTCTAAGTTC
	ct147864	275-284	KP279133	Schlautman et al. (2015)	CTCTCTTTACCCTCAATTTCTC	GGTCTAATATCAATCGATGACC

	1trimcontig209220	361-459	KP279228	Schlautman et al. (2015)	CACGACGTTGTAAAACGACGTATTTGTTCACACTCACCAGA	ACAGTTGTCGAAGCCTCAT
	ct38401	168-201	JF834272	Zhu et al. (2012)	CAATGGGAAGTACAAAGAGC	CGATGCAATCTTAGTCTTGA
	SCF40517	220-260	KP278707	Schlautman et al. (2015)	GTAGAATGGCAATAGGGTTT	GAAGAAGATGACGAAGATCAC
	SCF118468	229-281	KP278840	Schlautman et al. (2015)	ATAAGCGGAGCACAGTTACA	GATAGGATGACCTGTTTTGGT
	SCF26014	166-170	KP278667	Schlautman et al. (2015)	GGTCCCAGAATCAATGTCTA	GAAATCAGAGAAGAAACAGGTC
	1trimcontig435620	235-263	KP279244	Schlautman et al. (2015)	CACGACGTTGTAAAACGACCAACCAGCCTTACAGTGAATA	GTCCGTTCAATTTCTTTTCC
	SCF64185	316-320	KP278745	Schlautman et al. (2015)	CACCTCATTTGGTTCATTCT	CAGATACTAAAGGTTGCCGTA
NED	251788_K63	182-228	KP279166	Schlautman et al. (2015)	CACGACGTTGTAAAACGACGATCTTTACCACTCCCCACT	GGATTCTCTGTCCATTGTTG
	SCF11186	269-288	KP278636	Schlautman et al. (2015)	AGAAAGGCTAAAAGGGTATCTC	GCTCTCAACAACTCGAAAGTA
	ct04084	150-248	JF834250	Zhu et al. (2012)	GGATTCTCACTCTGATACCATT	GAACGATACACAACGAAGGT
	SCF91821	294-324	KP278792	Schlautman et al. (2015)	TTCTGTGTCTGATTCCATCTC	ACTAGCCCAACAACTTAGACTG
	ct28527	216-283	JF834266	Zhu et al. (2012)	GGACAAGTGAAATGCTAGTTG	AGATTGTTCGTAGGTAGAAGTG
PET	SCF9068	174-186	KP278624	Schlautman et al. (2015)	AAATCTAGGTAGGAGCAGGTCT	ATGGAGGAGGAGATATGTGAT

76126_K63	231-242	KP279162	Schlautman et al. (2015)	CACGACGTTGTAAAACGACTTTATTGGAGCGAAAGAGAG	AAAAGGGGAGGAGAGAGAT
418294_K63	336-348	KP279189	Schlautman et al. (2015)	CACGACGTTGTAAAACGACCAAGAACAAGAAGAAGAAGACC	AGAGACCACCCAAAAGATAAG
372875_K63	195-235	KP279174	Schlautman et al. (2015)	CACGACGTTGTAAAACGACCACACACAAATCCCAATTTC	GATGGTGTTTTCATAGTTCGAC
SCF95767	262-311	KP278796	Schlautman et al. (2015)	TGAGGAGAGGAGTATCCATAAG	CCTACAAGTCTCGCAATTCTA
418596_K63	385-413	KP279190	Schlautman et al. (2015)	CACGACGTTGTAAAACGACCGTGAGTTTGAGTGAGTAATTG	AGGACATGGTGAGTTGAGAAT
ct40600	173-247	JF834274	Zhu et al. (2012)	CAAAAGAGCCATGAAATAGG	TTGGTGAAAACTATACCTGTCC
ct52682	263-324	JF834282	Zhu et al. (2012)	CTCAGGTTATCAGGCTTATTTC	CAATTAGTGTGTTCCCAACTC
ct78806	214-274	JF834245	Zhu et al. (2012)	CAAAGAAGAGGAGGATTGAGT	GAGCGAGTATTACAAGTGTTTC

Tissue culture wild germplasm from MN and WI collection inventory

Plant ID	Count	Plant ID	Count	Plant ID	Count	Plant ID	Count
BL15	2	MWB19	4	HB02	1	TR 12	4
CHPR 07	4	MWB20	5	HB03	1	TR 13	4
CHPR 12	4	PMS01	4	HB04	2	TR 14	2
FBF02	5	PMS03	5	HB05	2	TR 15	2
FBF03	8	PMS04	4	HB06	1	TR 16	4
FBF05	2	PMS05	4	HB07	2	TR 17	3
FRLK01	3	PMS07	2	HB09	2	TR 18	2
FRLK03	2	PMS10	6	HB10	2	TR 20	2
FRLK04	3	PMS11	3	HB11	2	BL03	1
FRLK05	5	PMS13	4	HB12	2	BL04	7
FRLK08	7	PMS14	7	HB13	2	BL05	1
FRLK09	4	PMS15	2	HB14	1	BL09	5
FRLK16	4	PMS17	5	HB15	1	BL10	1
GL01	2	PMW01	1	HB16	3	BL14	1
GL03	2	PMW03	3	HB17	1	BL18	7
GL06	3	PMW06	3	HB18	2	BL19	1
GL07	5	PMW14	3	HB19	3	Chip 01	3
GL08	2	PMW15	4	HB20	3	Chip 03	2
GL09	5	PMW17	2	ISB01	3	Chip 07	1
GL11	4	PMW19	3	ISB02	4	Chip 08	3
GL12	4	SEB05	3	ISB03	3	Chip 10	1
GL15	6	SEB09	4	LL01	4	Chip 17	2
GL16	5	SEB10	3	LL02	4	FRLK12	1
GL17	2	SEB12	2	LL03	1	GYPL17	4
GL18	4	SEB13	3	LL04	1	GYPL19	3
GL19	5	SEB19	4	LL05	3	GYPL28	2
GL20	6	SEB20	4	LL06	2	GYPL32	2
GYPL01	4	SHPL06	4	LL10	4	HAWL05	4
GYPL02	2	SHPL08	4	LOLB01	2	HW05	7
GYPL06	5	SHPL16	5	LOLB02	4	HW12	5
GYPL07	7	SHPL20	5	LOLB03	3	KLB17	5
GYPL08	4	SHPL24	3	LOLB04	2	LL07	6
GYPL10	4	SHPL27	4	LOLB05	3	LL08	6
GYPL12	4	SHPL30	4	LOLB06	2	LL12	2
GYPL14	7	SR01	3	LOLB07	3	LL13	4
GYPL15	5	SR04	5	LOLB08	4	LL14	4
GYPL16	5	SR05	4	LOLB09	3	LL15	4
GYPL23	6	SR07	6	LOLB10	4	LOLM01	5
GYPL24	4	SR08	5	LOLB11	4	LOLM03	4

GYPL25	2	SR09	5	LOLB12	1	LOLM07	4
GYPL26	2	SR11	4	LOLB13	2	LOLM11	5
GYPL27	7	SR13	4	LOLB14	4	LOLM12	4
GYPL30	6	SR14	4	LOLB15	3	LRL01	4
JL02	7	SR17	4	LOLB16	4	LRL02	5
JL04	7	WABL02	4	LOLB17	4	LRL03	4
JL11	6	WIN 05	4	LOLB18	4	LRL04	5
JL12	6	WIN 17	7	LOLB19	4	LRL05	4
JL14	7	BB01	4	LOLB20	2	LRL10	4
JL16	9	BB02	4	LRL12	4	LRL11	4
JL17	6	BB03	4	LRL14	4	LRL15	4
JL19	7	BB04	4	PB01	1	LRL18	4
JL20	5	BB05	5	PB02	4	PMW16	4
KLB01	4	BB06	5	PB03	2	SEB15	4
KLB05	4	BB07	3	PB04	4	SHPL13	4
KLB07	6	BB08	3	PB06	4	WABL04	4
KLB08	4	BB09	6	PB07	3	WABL06	4
KLB09	5	BB10	4	PB08	2	WABL08	4
KLB10	6	BB11	6	PB09	3	LRL08	2
KLB11	5	BB12	4	PB10	1		
KLB12	6	BB13	4	PB12	2		
KLB13	5	BB14	3	PB13	2		
KLB14	4	BB15	3	PB14	2		
KLB18	6	BB16	4	PB15	2		
KMP01	6	BB17	3	PB16	4		
KMP02	6	BB18	4	PB17	3		
KMP08	5	BB19	6	PB18	1		
KMP13	5	BB20	4	PB19	4		
KMP14	8	BLM02	3	PB20	1		
KMP18	4	BLM03	4	PRF 01	3		
LocP02	4	BLM04	4	PRF 02	5		
LocP04	4	BLM06	4	PRF 03	2		
LocP05	4	BLM08	4	PRF 04	4		
LocP08	4	BLM10	3	PRF 05	2		
LocP08R	4	BLM11	4	PRF 06	1		
LocP09	4	BLM12	4	PRF 07	3		
LocP10	5	BRR 01	4	PRF 08	4		
LocP11	4	BRR 02	5	PRF 09	4		
LocP16	5	BRR 03	4	PRF 10	3		
LocP19	5	BRR 07	3	PRF 11	2		
LocP21	6	BRR 08	3	PRF 12	3		

LocP24	4	BRR 09	3	PRF 13	4
LocP28	6	BRR 11	6	PRF 14	5
LocP30	5	BRR 12	4	PRF 15	1
LOL15	6	BRR 13	8	PRF 16	4
LOL18	4	BRR 15	1	PRF 17	0
LOL19	4	BRRM 01	4	PRF 18	3
LU02	4	BRRM 02	5	PRF 19	3
LU07	3	BRRM 03	4	PRF 20	4
LU28	5	BRRM 06	3	TR 01	3
MWB02	1	BRRM 07	3	TR 03	3
MWB03	4	BRRM 09	3	TR 04	2
MWB06	4	BRRM 13	2	TR 05	1
MWB09	4	BRRM 14	2	TR 06	3
MWB11	4	BRRM 15	3	TR 07	2
MWB12	4	BRRM 16	4	TR 08	3
MWB13	3	BRRM 17	3	TR 09	4
MWB16	7	BRRM 18	1	TR 10	2
MWB18	6	BRRM 20	3	TR 11	4

Chapter III

The genetic diversity and *in-situ* conservation of cranberries in the wild.

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Abstract

Human activities such as urbanization have resulted in many environmental challenges including the loss of biodiversity. This loss can affect the distribution, diversity, and availability of crop wild relatives. Many of these wild relatives display more genetic variability than cultivated varieties, and have been used as a source of useful genes to confer resistance to biotic and abiotic stress. *Vaccinium macrocarpon* is one of the few species native to the United States and Canada with wild relatives still available in nature. Because of all the qualities exhibited by wild relatives, we studied 35 wild populations of *Vaccinium macrocarpon* and *V. oxycoccos* across their native range using 32 simple sequence repeat (SSR) markers. We found high levels of heterozygosity for both species across populations and private alleles ranging from 0 to 16. In *V. macrocarpon*, we found a total number of 613 alleles and high levels of heterozygosity (H_O=0.99, H_T=0.75). We also observed high numbers of alleles (881) and levels of heterozygosity (H_O=0.71, H_T=0.80) in *V. oxycoccos* (4x). The known range of *V. macrocarpon* extends west to Minnesota, however, we found a wild *V. macrocarpon* population in Washington, indicating there could be more unknown populations in other areas in the western US. Therefore, exploring new locations and better understanding their genetic diversity and population structure can help us prioritize conservation. Our results will inform conservation efforts of the United States Forest Service and aid in the development of policy to protect and designate diverse and unique areas as *in-situ* reserves for wild cranberries.

Keywords: cranberry breeding, crop wild relatives, SSR, genetic diversity

Introduction

Historically, anthropogenic activities such as urbanization and the use of natural landscapes for cultivation have produced great environmental challenges (Munshi-South et al. 2016). Examples of these include an unprecedented loss of biodiversity, the eradication of wild relatives of important food crops, and the reduced abilities of natural populations to endure stressors, in both short and long terms (Griffin et al. 2009; Engelhardt et al. 2014). The destruction of the environments that house Crop Wild Relatives (CWR) can result in genetic erosion or loss of genetic diversity, leading to irreparable damage to the wild genetic resources that will be necessary to ensure food security (Debnath 2016). Estimators of genetic diversity, such as number of private alleles; heterozygosity, and population differentiation, can be used to inform conservation decisions, such as prioritizing protection (Jost et al. 2018). Therefore, understanding the genetic diversity of wild populations is vital for the future of agricultural production.

Crop Wild Relatives, aside from being an important part of the ecosystems, can serve as a resource for crop improvement programs. These wild relatives often display more genetic variability than their cultivated counterparts, and their evolution in the wild is dynamic, driven by their interactions with both biotic and abiotic stressors (Greene et al. 2018). Cultivated plants become more homogenous after several cycles of selection, in an effect known as “domestication bottleneck,” and in a controlled environment, the ability of cultivated plants to evolve could be reduced, leaving them vulnerable to emerging threats (Olsen and Gross 2008). When problems arise in cultivated environments, CWR can be a source of useful genes to cope

with new challenges, as has been the case of potato wild relatives used to introduce pathogen resistance into cultivated varieties (Marfil et al. 2015). The use of these resources is constantly growing in breeding programs, which are using molecular biology to characterize and incorporate CWR (Khoury et al. 2013).

To protect natural diversity, two main strategies have been used. *In-situ* conservation (Li and Pritchard 2009), refers to the preservation of reproducing organisms in nature, specifically in their native environment (Wang and Zhang 2011). It allows populations to evolve and adapt to different biotic and abiotic stresses. In this strategy, the whole population is preserved, rather than individuals. The second strategy, *ex-situ* conservation, refers to the preservation of germplasm (i.e., seeds and living plants) in human-made sites, such as gene banks (Wang and Zhang 2011). It acts as a "backup" for individuals that represent the genetic diversity found in nature, and helps provide easy access of germplasm for researchers. Some of the constraints of *ex-situ* conservation are the cost, time, and difficulty of maintaining the material. Also, plants collected represent only genetic adaptation exclusive to the location and the point in time it was collected, and in vitro plants are not able to evolve under natural conditions (Greene et al. 2018). The United States and Canada contain only about 7% of the world's flora. A few of these plants are wild relatives to cultivated crops, including sunflowers, blueberries, and cranberries (Khoury et al. 2013).

The American cranberry (*Vaccinium macrocarpon*) is a diploid perennial species with a natural distribution ranging from Newfoundland to the Appalachian Mountains and extending to Minnesota (Song and Hancock 2011). In the US, the cranberry industry is valued at around $3.5

billion. This value increases each year due to the high demand for cranberry products, particularly innovative products such as pills, sweetened and dried cranberries, wine, and sparkling water (Alston et al. 2014). This increase in demand is in part due to high nutritional and antioxidant properties of cranberry, including the highest antioxidant profile among other fruits such as grapes and blueberries (Yan et al. 2002; McKay and Blumberg 2007; Brown et al. 2012). Thus, breeding programs are actively searching for large collections of wild cranberries and closely-related species to serve as gene banks, or "libraries," of desirable traits. Cranberry breeding programs aim to develop new and improved cultivars with increased fruit quality, nutrition, and sugar content, coupled with enhanced environmental adaptations such as cold tolerance.

Vaccinium oxycoccos is morphologically very similar to cultivated cranberry and grows as a small, evergreen, perennial vine. The species is mostly tetraploid, but can also be diploid, which is known as *V. microcarpum*, or hexaploid, which is known as *V. hagerupii* (Porsild, 1938; Hagerup, 1940; Bruederle et al., 1996; Mahy et al., 2000). The species produces small, over-wintering berries that have a similar flavor to cultivated cranberry, but exhibit superior antioxidant profile and cold hardiness (Vander Kloet 1988; Jacquemart 1997; Brown et al. 2012). As the closest relative to the cultivated cranberry, *V. oxycoccos* has great potential for interspecific crosses to transfer desirable traits. Particularly, since *V. oxycoccos* has a circumboreal distribution and is adapted to growing in higher latitudes and enduring harsher winters than *V. macrocarpon* (Areškevičiūtė et al. 2006), the species could be an excellent source of cold hardiness genes, as is the case of wild relatives in rice (Mao et al. 2015; Shakiba et al. 2017) and potato (Hamernik et al. 2009).

In the US, little has been done to analyze and understand the wild genetic diversity of native crops such as cranberry species, i.e., *V. oxycoccos* and *V. macrocarpon*. Knowledge about the state and distribution of genetic diversity in the wild will allow the development of efficient conservation strategies to protect and utilize wild genetic resources that may harbor valuable pockets of diversity. For this reason, in collaboration with the USDA and the Forest Service, this project provides the most comprehensive analysis of the state of the genetic diversity of wild cranberries ever conducted using Simple Sequence Repeats (SSR) markers. SSR markers have been identified across many plant species, and some have been used to analyze diversity in different crops, such as apple (Hokanson et al. 2001), melon (Fukino et al. 2007), papaya (Asudi et al. 2013), cranberry (Zalapa et al. 2015), and sweet potato (Rodriguez-Bonilla et al. 2014). Results from genetic diversity analysis have provided sufficient information to aid conservation efforts in several species, such as *Borderea chouardii*, an endangered plant from Spain (Segarra-Moragues et al. 2005), as well as wild accessions of corn in Mexico (Louette et al. 1997) and wild potatoes in Argentina (Marfil et al. 2015).

In cranberry, there is a need to understand the genetic diversity of wild populations in their native range. Our aim is to gain knowledge about the genetic diversity and population structure of these species to help guide and stimulate the conservation of natural sources of wild cranberries. To best conserve the full range of genetic variation of wild cranberries, we have identified several populations in national forests across the species' native range in order to assess the amount of diversity present and then implement long-term management plans to designate *in-situ* reserves in National Forests, as well as *ex-situ* conservation in the US National Germplasm System.

Materials and Methods

1. Plant Materials

Online herbarium records were searched for known populations of *V. macrocarpon* and *V. oxycoccos* on United States Forest Service National Forests throughout the United States. Scientific staff at the National Forests provided additional information on locations of populations. The collection sites ranged from a small seep (14 m^2) to large bogs and fens covering hundreds of thousands of square meters. In the eastern US, *V. macrocarpon* and *V. oxycoccos* were often found growing together on the same site. In the western US, all populations were *V. oxycoccos*, except one that was determined to be *V. macrocarpon*. Samples were collected by Agricultural Research Service employees, usually accompanied by USFS personnel, or in a few cases by USFS personnel working alone.

Multiple samples of leaf tissue were collected at each site. Each sample was an upright (i.e., reproductive branch) or a runner (i.e., vegetative branch) containing at least 20 leaves from a single plant. When found together at the same site, *V. macrocarpon* and *V. oxycoccos* were sampled separately. Following the technique utilized by Zalapa et al. (2015), samples for each species were collected at least 5 m apart to reduce the likelihood of collecting the same clone more than once. As much as possible the entire population was covered by taking samples in a grid pattern. Latitude and longitude were determined for each sample and recorded on the paper coin envelope. All samples for each site were placed in large paper envelope.

Each collection site was documented with location, physical characteristics, associated species, and descriptions of the populations and plants being sampled (available upon request).

Latitude, longitude and altitude were recorded using a Garmin GPSMAP 64st or other global positioning unit and /or altimeter (Table 1).

Herbarium vouchers were deposited in the USDA National Arboretum in Washington, D.C. and the USDA National Germplasm Repository in Corvallis, Oregon.

2. DNA Extraction

Genomic DNA extractions were performed using leaf tissue per a CTAB method (Doyle and Doyle 1987) modified in our laboratory to isolate high quality, clean DNA. A total of, 10-20mg of frozen tissue were ground by hand using liquid nitrogen and transferred to a 2.0mL tube, and 700µL of 2% CTAB extraction buffer [20 mM EDTA, 0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, 2%CTAB] was added and mixed by inversion. The solution was then incubated at 65 °C for 45 min. After incubation, 400 µL of a chloroform-isoamyl alcohol (24:1) solution was added to the tubes and gently mixed by inversion. Samples were then centrifuged for 5 min at 14,000 rpm, 500 µL of the supernatant was transferred to a fresh 1.5mL tube containing 50µL of 10% CTAB buffer and mixed by inversion. 750µL of cold isopropanol (100%) was added, and then samples were incubated from 2 to 48 hours at a temperature of -20°C. Samples were gently mixed by inversion and centrifuged at 14,000 rpm for 20 min. After centrifugation, the supernatant was discarded, and the resulting pellet was air-dried for 5 minutes. The pellet was then washed with 700 µL of cold 70% ethanol to clean the DNA. The solution was then vortexed and centrifuged at 14,000 rpm for 4 min, the ethanol was discarded, and the pellet was air-dried for 24 hours. The DNA was then re-suspended in 100 µL TE 10:1 buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) plus 5 µL of ribonuclease (RNAse 10 mg mL–1) in each tube, and was incubated at 37 °C

for 2 hours prior to storage at -20°C. DNA was then quantified using a Nanodrop ND-1000 spectrophotometer. Samples were then diluted to 10ng/µL with deionized distilled water for simple sequence repeat (SSR) amplifications.

3. SSR Markers and Multiplex PCR Conditions

A total of 32 markers previously designed and assessed for transferability between *V. macrocarpon* and *V. oxycoccos* were used in this study (Schlautman et al. 2015; Rodriguez-Bonilla et al. 2019; Supplementary Materials). Markers proven to have a consistently clear pattern of amplification across species were chosen, and at least two markers per cranberry linkage group were selected.

The multiplex panels were designed by developing SSR primer-pairs. These pairs were clustered into combinations of one to three markers, considering non-overlapping allele ranges and annealing temperatures based on previous studies (Rodriguez-Bonilla et al. 2019; Schlautman et al. 2015a; Zhu et al. 2012; Georgi et al. 2012; Schlautman et al. 2017).

To boost the potential of our combinations and maximize resources for our fragment analysis, each reaction was combined with four PCR multiplex reactions and a fluorescent dye (M13-FAM, HEX, NED, or PET labeled primers). The forward primers were designed with the M13 sequence (5-CACGTTGTAAAACGAC-3) for fluorescent labeling of PCR products (Schuelke 2000), and the PIG sequence (5-GTTTCTT-3) was attached to the reverse primers to promote full adenylation of SSR fragments during PCR (Brownstein et al. 1996). The development of primers with an attached M13 sequence and a fluorescently labeled M13-tail is more cost-effective than using direct fluorescent primer labeling (Guichoux et al. 2011) By not using this direct

fluorescence, we are able to create any marker combinations and indirectly label them with any fluorescence needed. PCR Reactions contained 3.5ul of 1x Jumpstart RedTaq Ready Mix (Sigma, St. Louis, MO, USA), 2.0 ul of 10 ng/ul DNA, 0.5 µL of 5 µM forward primer, 0.5 µL of 50 µM reverse primer, and 0.5 µL of 0.5 µM M13-FAM, M13-HEX, M13-NED and M13-PET primer. The 1.5 ul of forward and reverse primer was divided by the number of multiplexed SSRs (i.e. 1µL or 0.75 µL of forward and reverse primer mix from each SSR were added when 3 or 4 markers were multiplexed together, respectively). Thermocycling conditions consisted of 94°C for 3 min, [94°C 15s, 55°C for 1 min, 72°C for 2 min] x 33, 72°C for 30 min, 4 ∞. PCR products were visualized on a 1% agarose gel, and then 1 to 3 µL of multiplexed PCR product from each of the four M13 dyes were pooled using the DY632 ladder from BioVentures, Inc. A total of 25 µl in 1,500 µl of formamide (Hi-Di Formamide from Life Technologies) were added per plate of 96 wells. The poolplexed mixture was sent to the University of Wisconsin-Madison Biotechnology Center DNA sequencing facility for fragment analysis using an ABI 3730 fluorescent sequencer (Applied Biosystems, Foster City, CA, USA). Allele genotyping was performed using GeneMarker v2.63 (SoftGenetics LLC, State College, PA, USA).

4-Data Analysis

The allelic information obtained from the genotyping was formatted as a GenAlEx (Peakall and Smouse 2012) input file. This file was then converted to a geneclone object to run in the R statistical software (R Core Team 2018) package Population Genetics in R (poppr) (Kamvar et al. 2014) to estimate the observed number of private alleles (PA). Calculations of genetic diversity statistics, such as number of alleles, H_O, G'st(Nei), and G_{IS}, as well as a Principal Component

Analysis (PCA) per species, were obtained from the software GenoDive (Meirmans and Van Tienderen 2004). To visualize the PCAs, the package ggplot2 was used (Wickham 2011). For the dendrograms, the R stats package (R Core Team 2018) was used to obtain Euclidean genetic distance and clustering. The dendrograms were then visualized using the packages dendextend (Galili 2015) and circlize (Gu et al. 2014). Population structure analysis was performed using the Structure 2.3.4 with K's 1 to 36, 150,000 MCMC (Markov Chain Monte Carlo), and 50,000 burn-in period (Pritchard et al. 2000). The estimations of deltaK were obtained with Structure Harvester (Evanno et al. 2005; Earl and vonHoldt 2012). Data obtained from structure was processed and visualized using the R package pophelper 2.2.9 (Francis 2017). To geographically visualize our forest locations we used the package maps (Becker et al. 2013).

Fig1. Map depicting the locations of the national forests used as collection sites for wild cranberries (*Vaccinium macrocarpon* and *V. oxycoccos)*

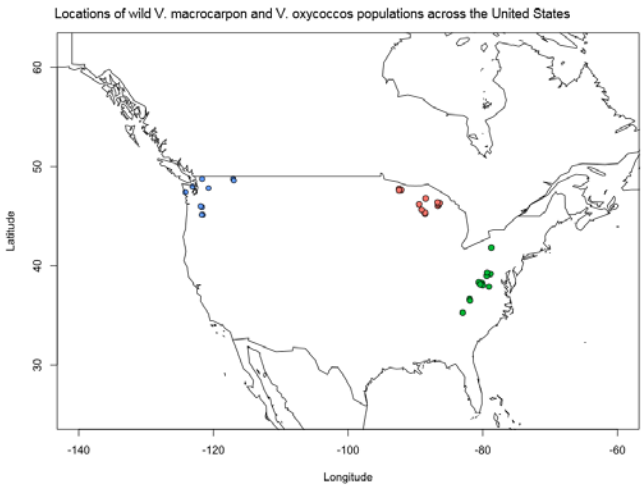


Table 1. Description of sample collection locations for cranberries (*Vaccinium macrocarpon* and *V. oxycoccos)*

Pop #	Code	Species	State	Area	Location	Latitude	Longitude	Regional Group
2	GPVA	*V. macrocarpon*	Virginia	George Washington National Forest	Green Pond	37.94087	-79.0526	East
3	JBCTN	*V. macrocarpon*	Tennessee	Cherokee National Forest	John's Bog	36.52908	-81.964	East
4	OBCTN	*V. macrocarpon*	Tennessee	Cherokee National Forest	Osborne Bog	36.48826	-81.9652	East
5	BBKPNC	*V. macrocarpon*	North Carolina	Pisgah National Forest	Black Balsam Knob	35.32943	-82.8782	East
6	IGSPNC	*V. macrocarpon*	North Carolina	Pisgah National Forest	Investor Gap Seep	35.34345	-82.8697	East
7	BRBWV	*V. macrocarpon* & *V. oxycoccos*	West Virginia	Monongahela National Forest	Big Run Bog	39.11805	-79.5845	East
8	RRBWV	*V. macrocarpon* & *V. oxycoccos*	West Virginia	Monongahela National Forest	Red Run Bog	39.07227	-79.4784	East
9	BeRBWV	*V. macrocarpon* & *V. oxycoccos*	West Virginia	Monongahela National Forest	Bear Rocks Bog	39.06535	-79.3051	East
10	LRLSMN	*V. macrocarpon* & *V. oxycoccos*	Minnesota	Superior National Forest	Little Rice Lake	47.70847	-92.4411	Central
11	SPBMI	*V. macrocarpon* & *V. oxycoccos*	Michigan	Keweenaw Bay Indian Community	Sand Point Bog	46.79143	-88.4669	Central
12	ALCNWI	*V. macrocarpon* & *V. oxycoccos*	Wisconsin	Chequamegon-Nicolet National Forest	Atkins Lake	45.64951	-89.0397	Central
13	ANFP	*V. macrocarpon*	Pennsylvania	Allegheny National Forest	Allegheny National	41.81581	-78.7341	East

					Forest			
14	RLSNMN	*V. oxycoccos*	Minnesota	Superior National Forest	Rice Lake	47.56709	-92.3689	Central
15	CLSNMN	*V. macrocarpon*	Minnesota	Superior National Forest	Cranberry Lake	47.51161	-92.0166	Central
16	GLCNWI	*V. macrocarpon* & *V. oxycoccos*	Wisconsin	Chequamegon-Nicolet National Forest	Glocke Lake	45.33362	-88.5702	Central
17	PERLOMI	*V. macrocarpon* & *V. oxycoccos*	Michigan	Ottawa National Forest	Pond east of Raven Lake	46.22302	-89.4389	Central
18	SCHMI	*V. macrocarpon* & *V. oxycoccos*	Michigan	Hiawatha National Forest	South side FR2268 to east of H94, 0. miles south of Stutts Creek crossing	46.29175	-86.455	Central
19	NHHMI	*V. macrocarpon*	Michigan	Hiawatha National Forest	North of Haywire Trail, unmarked two track West of Highway 94	46.29177	-86.454	Central
20	WFRHMI	*V. oxycoccos*	Michigan	Hiawatha National Forest	West Side of FR13, north of FR2447	46.1807	-86.4242	Central
21	ESHHMI	*V. oxycoccos*	Michigan	Hiawatha National Forest	East Side of Highway 13, north of FR2020	46.06647	-86.6438	Central
22	CG1MWV	*V. macrocarpon* & *V. oxycoccos*	West Virginia	Monongahela National Forest	Cranberry Glades 1	38.19939	-80.272	East
23	CG7MWV	*V. oxycoccos*	West Virginia	Monongahela National Forest	Cranberry Glades 7	38.20603	-80.2773	East

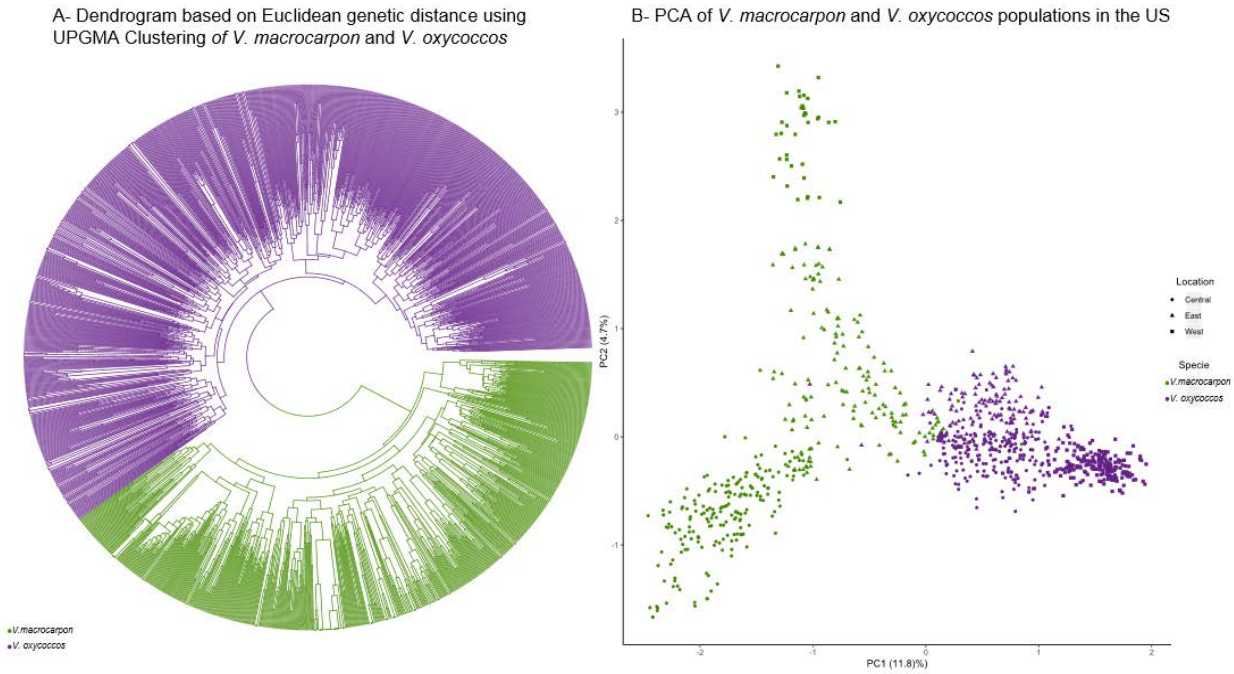
24	CG5MWV	*V. macrocarpon*	West Virginia	Monongahela National Forest	Cranberry Glades 5	38.19943	-80.2654	East
25	CG4MWV	*V. oxycoccos*	West Virginia	Monongahela National Forest	Cranberry Glades 4	38.20012	-80.2651	East
26	UILCWWI	*V. macrocarpon*	Wisconsin	Chequamegon-Nicolet National Forest	Upper Island Lake	45.25023	-88.5576	Central
27	CPMHWOR	*V. oxycoccos*	Oregon	Mount Hood National Forest	Camas Prairie	45.1382	-121.566	West
28	QIROPWA	*V. oxycoccos*	Washington	Quinault Indian Reservation	Otook Prairie	47.41137	-124.155	West
29	GPSPWA	*V. oxycoccos*	Washington	Gifford Pinchot National Forest	South Prairie	45.90969	-121.699	West
30	MHLCMOR	*V. oxycoccos*	Oregon	Mount Hood National Forest	Little Crater Meadow	45.14545	-121.741	West
31	GPMMWA	*V. oxycoccos*	Washington	Gifford Pinchot National Forest	McClellan Meadows	45.99633	-121.89	West
32	OCBVWA	*V. oxycoccos*	Washington	Olympic National Forest	Cranberry Bog Botanical Area	47.98635	-123.114	West
33	IPHLWA	*V. oxycoccos*	Idaho	Idaho Panhandle National Forest	Huff Lake	48.74059	-117.063	West
34	OWFLBWA	*V. macrocarpon*	Washington	Okanogan-Wenatchee	Fish Lake Bog	47.8253	-120.723	West
35	IPHLID	*V. oxycoccos*	Idaho	Idaho Panhandle National Forest	Hager Lake	48.59713	-116.97	West
36	MBSWA	*V. oxycoccos*	Washington	Mt. Baker-Snoqualmie	Morovitz Wetland Complex	48.74092	-121.674	West

Results

Exploring the genetic relationships of wild cranberries across their native range

Species of the section *Oxycoccos* of the genus *Vaccinium*, cranberries (*V. macrocarpon*) and small cranberries (*V. oxycoccos*), can colonize some of the same environments, although *V. oxycoccos* also has a circumboreal distribution that extends to Canada and across Northern Europe and Asia. Since these plants can coexist together in our collection sites in the US, and it sometimes can be difficult to differentiate the two species, we explored their genetic relationships using a dendrogram based on Euclidean genetic distance using a UPGMA clustering method. We found both species separate clearly into two main groups, and all the filed classifications made by botanist collectors were correct (Fig.2 A). The UPGMA tree contained one branch with all the accessions identified in the field as *V. oxycoccos*, and another branch with all the *V. macrocarpon* individuals. Interestingly, no wild *V. macrocarpon* populations have ever been reported west of Minnesota, but our field classification identified a population in Washington state as *V. macrocarpon*, which was corroborated by the genetic analysis. To confirm our findings, a PCA of all the populations was produced, in which we also saw a clear separation between the two species (Fig. 2 B). In the PCA, we saw that in the *V. macrocarpon* accessions there is a clearer spatial grouping among species from east (TN, NC, PA, WV), central (WI, MI, MN), and west (OR, WA, ID).

Fig2. Wild cranberry (*Vaccinium macrocarpon* and *V. oxycoccos*) species separation based on genetic distance



An overall comparison of genetic diversity estimators among all the individuals of the two species demonstrated high levels of heterozygosity. Observed heterozygosity levels ranged from 0.71 to 0.99 (H_O), total heterozygosity (H_T) values ranged from 0.75 to 0.80, and heterozygosity within populations (H<) ranging from 0.51 to 0.72 between species (Table 2). *V. oxycoccos* had the highest number of alleles with a total of 881 alleles (n = 539) and a mean value of alleles per locus of 27.51. Also, *V. oxycoccos* had low levels of inbreeding, as demonstrated by the G_{IS} value of 0.02, as well as a low fixation index (0.09). Overall, *V. macrocarpon* had a mean value of 19.15 alleles per locus and a total of number of alleles of 613 (n = 388). *V. macrocarpon* also exhibited low levels of inbreeding (G_{IS} -0.09), but higher values of the fixation index (0.33) than *V. oxycoccos* (Table 2).

Table 2. Genetic diversity statistics per cranberry species (*Vaccinium macrocarpon* and *V. oxycoccos)*

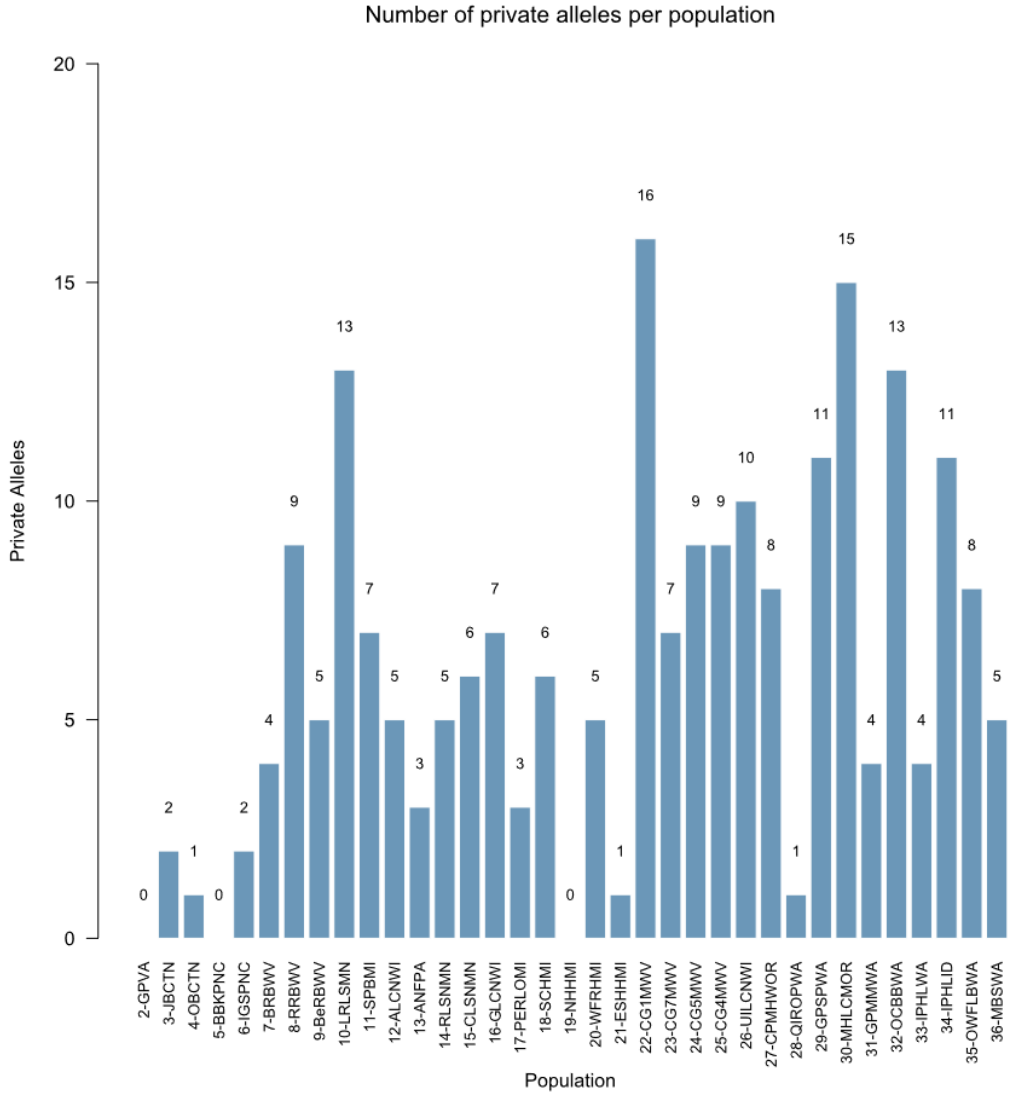
	V. oxycoccos	***V. macrocarpon***
Total alleles	**881**	**613**
N[1]	**539**	**388**
Num[2]	**27.51**	**19.15**
Ho[3]	**0.71**	**0.99**
Hs[4]	**0.72**	**0.51**
Ht[5]	**0.80**	**0.75**
Gis[6]	**0.02**	**-0.95**
G'st(Nei)[7]	**0.09**	**0.33**

1- Number of samples, 2- Mean number of alleles, 3- Observed heterozygosity, 4- Heterozygosity within populations, 5- Total heterozygosity, 6- Inbreeding Coefficient, 7- Nei's fixation index.

When analyzing the number of private alleles per population, we found that the value ranged from 0 to 16. Populations such as Little Rice Lake in Superior National Forest in MN (13),

Cranberry Glades Site 1 in Monongahela National Forest in West Virginia (16), Little Crater Meadow in Mount Hood National Forest in Oregon (15), and Fish Lake Bog in Okanogan-Wenatchee National Forest in Washington (13) possessed the highest numbers of private alleles among the populations analyzed (Fig. 3).

Fig 3. Number of private alleles per population of wild cranberries (*Vaccinium macrocarpon* and *V. oxycoccos*)



Genetic diversity estimators and population structure of *V. macrocarpon*

We found that the number of alleles per population ranged from 2 to 6.4 in *V. macrocarpon*. Also, we observed high levels of all genetic diversity estimators (H_O, H_S, H_T), ranging from 0.21 to 1. Meanwhile, levels of inbreeding were very low, varying from -1 to -0.46 (Table 3). The majority of the populations exhibited large values of observed heterozygosity, but low levels of within population heterozygosity.

Table 3. Genetic diversity statistics per population of *Vaccinium macrocarpon*

Population	N[1]	Num[2]	Ho[3]	Hs[4]	Ht[5]	Gis[6]
2 - GP VA	11	3.25	1	0.43	0.43	-1.28
3 - JBC TN	16	2.31	1	0.23	0.23	-3.20
4 - OBC TN	7	2.4	1	0.33	0.33	-2.01
5 - BBKP NC	6	2	1	0.21	0.21	-3.58
6 - IGSP NC	15	2.2	1	0.33	0.33	-2.03
7 - BRB WV	18	4.10	0.99	0.55	0.55	-0.79
8 - RRB WV	24	5.73	0.99	0.66	0.66	-0.48

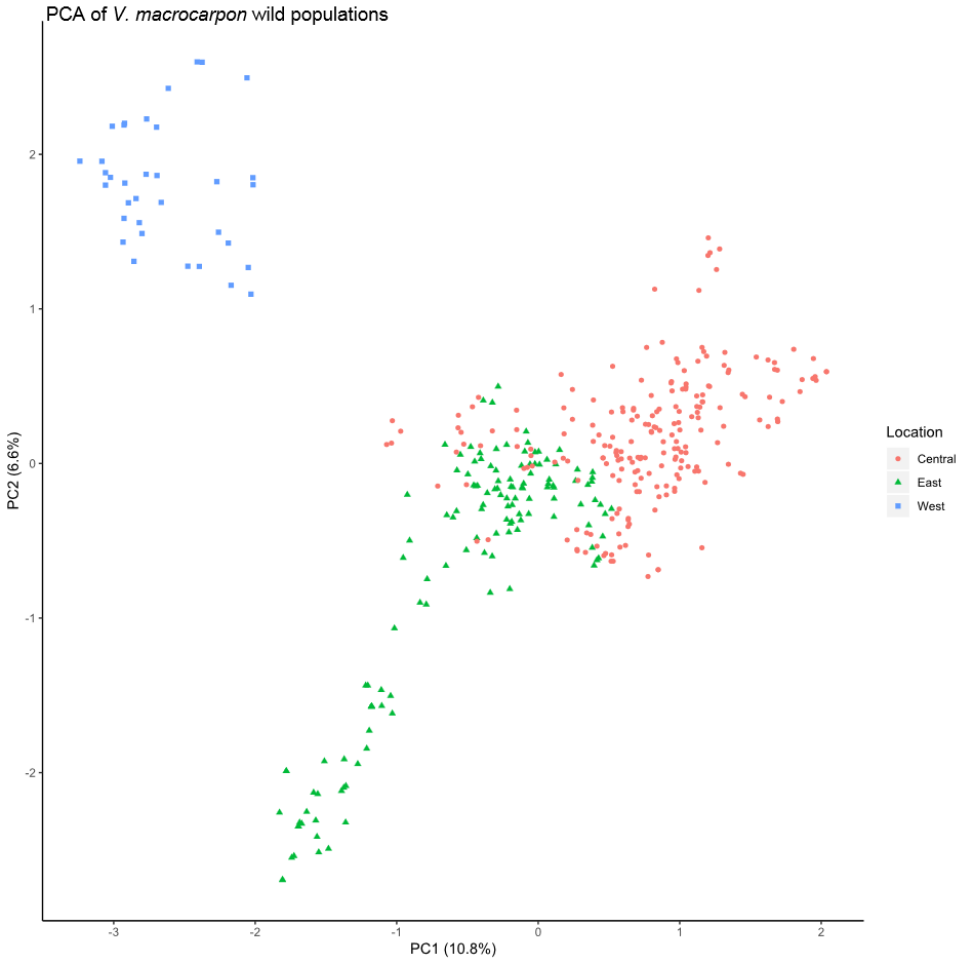
9 - BeRB WV	2	NA	NA	NA	NA	NA
10 - LRLS MN	20	4.46	1	0.55	0.55	-0.81
11 - SPB MI	31	7.156	1	0.68	0.68	-0.46
12 - ALCN WI	21	5.31	1	0.62	0.62	-0.60
13 - ANF PA	38	3.45	1	0.52	0.52	-0.89
15 - CLSN MN	25	4.64	1	0.60	0.60	-0.65
16 - GLCN WI	21	5.37	1	0.60	0.60	-0.66
17 - PERLO MI	17	4.75	1	0.51	0.51	-0.93
18 - SCH MI	31	6.40	1	0.63	0.63	-0.56
19 - NHH MI	15	3.96	1	0.51	0.51	-0.94
22 - CG1M WV	4	NA	NA	NA	NA	NA
24 - CG5M WV	16	5.34	0.99	0.62	0.62	-0.58
26 - UILCN WI	14	6.06	1	0.66	0.66	-0.49

35	-	OWFLB WA	35	3.12	0.97	0.48	0.48	-1

1- Number of samples, 2- Mean number of alleles, 3- Observed heterozygosity, 4- Heterozygosity within populations, 5- Total heterozygosity, 6- Inbreeding Coefficient.

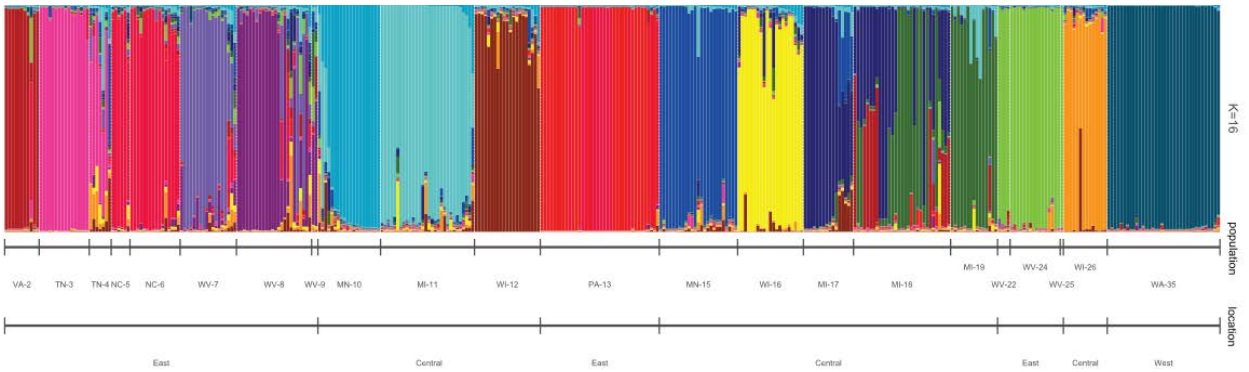
A PCA was performed to understand the genetic relationships of the populations (Fig. 4). We saw a clear differentiation between the samples from Fish Lake Bog in Okanogan-Wenatchee National Forest in WA (34-OWFLBWA), which were genetically identified as *V. macrocarpon* in the Euclidean distance dendrogram, and the other locations. We observed a separation between central and eastern locations. We also observed a large clustering spread in the PCA among the eastern populations, which have been historically considered the most ancient populations of *V. macrocarpon* (Stewart et al. 1995).

Fig 4. PCA of *Vaccinium macrocarpon* wild populations



Structure analysis was performed to visualize population clustering (Fig.5). Based on Evanno's deltaK, we found that K=16 was the most likely number of populations based on our data (Evanno et al. 2005; Earl and vonHoldt 2012), with the majority of the clusters forming based on geographical locations. Populations from West Virginia formed distinct clusters (WV-7, WV-8, and WV-(22-25)), and we observed similar patterns for populations from Minnesota (MN-10, MN-15), Wisconsin (WI-12, WI-16, WI-26), and Michigan (MI-11, MI-17,MI-18), with population MI-18 (Hiawatha National Forest) being highly admixed (Table1, Fig 5.).

Fig 5. Structure analysis of *Vaccinium macrocarpon* populations (K=16)



Genetic diversity estimators and population structure of *V. oxycoccos*

The number of alleles per population for *V. oxycoccos* ranged from 2 to 11. The levels of heterozygosity for all three indexes (H_O, H_S, H_T) ranged from 0.45 to 1 while the levels of inbreeding (G_{IS}) ranged from -0.31 to 0.23 (Table 4). The populations with the highest diversity indexes for *V. oxycoccos* were Pond East of Raven Lake (17 - PERLO MI) located in Ottawa National Forest in Michigan, and Glocke Lake in Chequamegon-Nicolet National Forest (16) (Table 1).

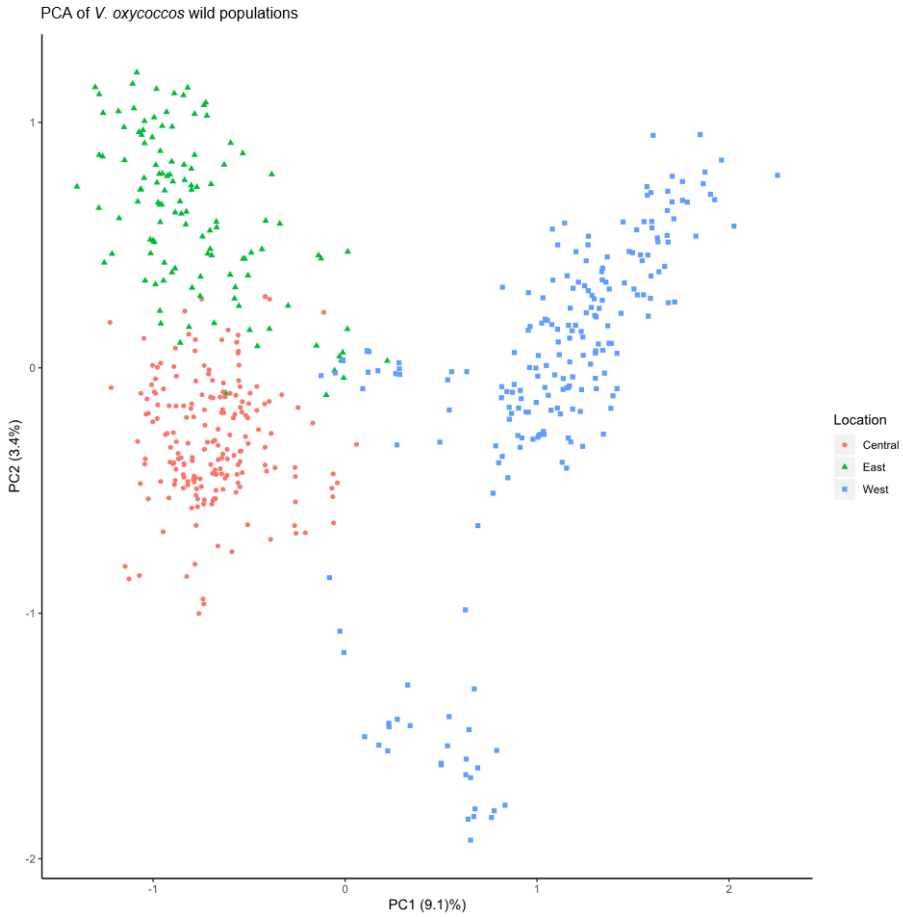
Table 4. Genetic diversity statistics per population of *Vaccinium oxycoccos*

Population	N[1]	Num[2]	Ho[3]	Hs[4]	Ht[5]	Gis[6]
7 - BRB WV	19	6.43	0.75	0.73	0.73	-0.02
8 - RRB WV	10	4.75	0.72	0.83	0.83	0.12
9 - BeRB WV	26	8.12	0.64	0.74	0.74	0.12
10 - LRLS MN	20	8.81	0.69	0.74	0.74	0.06
11 - SPB MI	32	11.32	0.69	0.77	0.77	0.10

12 - ALCN WI	23	11	0.70	0.77	0.77	0.09
14 - RLSN MN	19	9.16	0.70	0.78	0.78	0.10
16 - GLCN WI	20	9.81	0.79	0.79	0.79	-0.00
17 - PERLO MI	16	9.06	0.80	0.77	0.77	-0.03
18 - SCH MI	16	6.5	0.73	0.70	0.70	-0.03
20 - WFRH MI	24	9.65	0.74	0.77	0.77	0.03
21 - ESHH MI	25	9.65	0.75	0.76	0.76	0.01
22 - CG1M WV	30	10.32	0.74	0.76	0.76	0.01
23 - CG7M WV	16	8.78	0.77	0.76	0.76	-0.01
25 - CG4M WV	20	9.93	0.75	0.77	0.77	0.02
27-CPMHW OR	49	7.25	0.68	0.65	0.65	-0.05
28 - QIROP WA	24	4.66	0.45	0.59	0.59	0.23
29 - GPSP WA	49	9.45	0.75	0.69	0.69	-0.08
30-MHLCM OR	16	6.25	0.70	0.66	0.66	-0.07
31 - GPMM WA	7	5.06	0.76	0.69	0.69	-0.11
32-OCBVWA	29	9.54	0.71	0.68	0.68	-0.03
33 - IPHL WA	13	2.71	0.67	0.51	0.51	-0.31
35 - IPHL ID	19	4.78	0.72	0.64	0.64	-0.11
36 - MBS WA	15	4.96	0.71	0.65	0.65	-0.10

1- Number of samples, 2- Mean number of alleles, 3- Observed heterozygosity, 4- Heterozygosity within populations, 5- Total heterozygosity, 6- Inbreeding coefficient.

Fig 6. PCA of *Vaccinium oxycoccos* wild populations

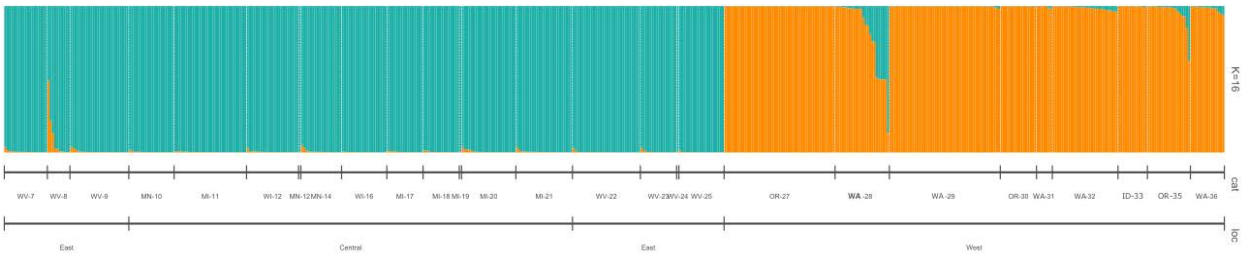


A PCA was constructed to observe any groupings among *V. oxycoccos* samples (Fig. 6). As in the *V. macrocarpon* PCA (Fig. 4), we observed a clear geographical separation among the samples from the west, central and eastern locations. The distribution of the PCA was closer than the one observed for *V. macrocarpon*, suggesting a closer relationship among *V. oxycoccos* individuals.

We performed a population structure test of the *V. oxycoccos* populations to analyze if any other grouping was occurring and found that K=2 is the best clustering representation for this group (Fig. 7). The two clusters consisted of all the accession collected in eastern and central US

and those collected in the Western US, with populations Quinault Indian Reservation (28-QIROP WA) and Idaho Panhandle National Forest (35-IPHLID) showing some levels of admixture.

Fig 7. Structure analysis of *Vaccinium oxycoccos* populations (K=2)



Discussion

Historically, the use of CWR for breeding has been crucial to advance crop production. Wild relatives of corn, wheat, cassava, banana, and other crops have contributed multiple traits, such as disease and abiotic stress tolerance for cultivar development (Hajjar and Hodgkin 2007).. Yet, very little is known about the state of the genetic diversity of wild cranberry populations across their native range. For example, in cranberry, problems such as frost risk (Roper and Vorsa 1997) and cranberry fruit rot (*Physalospora vaccinii*) can severely affect production (Gallardo et al. 2018). In order to confront climate change and other emerging challenges while increasing yield and producing quality fruit, we need to truly understand and safeguard cranberry genetic resources, both *ex-situ* and *in-situ*, which may hold genetic variation to help overcome challenges faced by the industry.

Exploring the genetic relationships of wild cranberries across their native range

In nature, *V. macrocarpon* can be found growing sympatrically with *V. oxycoccos*, its wild relative (Vander Kloet 1983). Therefore, we investigated how samples from wild populations grouped based on genetic distance and morphological identification, which was of interest as some populations had both species present at the same location (Table 1). To observe these relationships, we performed a dendrogram using Euclidean distance with UPGMA clustering. In Fig. 2 A, we observed two main clusters, one which contained all *V. oxycoccos* individuals and one that encompassed all *V. macrocarpon* samples. To further corroborate our results, a PCA was developed and we observed similar results depicting species separation as well as some geographical clustering (Fig. 2 B). Clear separation between the two species has been previously observed using AFLP (Smith et al. 2015) and SSR markers (Zalapa et al. 2015).

Genetic parameter estimates for *V. macrocarpon* and *V. oxycoccos* showed very high levels of heterozygosity (H_O, H_T, H_S), ranging from 0.5 to 0.9 (Table 2). These findings are comparable to those found by Zalapa et al. (2015) and Rodriguez-Bonilla et al. (2019), who found high levels of heterozygosity ranging, from 0.5 to 0.8, in wild populations in Wisconsin and Minnesota. Furthermore, these results are supported by cranberry biology, which favors outcrossing through dichogamy and inbreeding fertility reduction (Bruederele et al., 1996). Moreover, natural hybridization can result in new genetic combinations due to chance seedlings (Llácer et al. 2003). Polyploidy offers another source of diversity for *V. oxycoccos*, as in other polyploid crops including cassava and sweet potato (Montero-Rojas et al. 2011; Rodriguez-Bonilla et al. 2014). We also found that *V. macrocarpon* exhibited a much higher

fixation index (0.4) and population structure compared to *V. oxycoccos* (0.09), which was highly panmictic (Balloux and Lugon-Moulin 2002). Many authors believe that some populations of *V. macrocarpon* are relics of the Pleistocene Ice Age, and therefore more differentiation among populations is expected (Stewart 1993; Stewart et al. 1995; Bruederele et al., 1996).

Insight into the genetic diversity of wild populations of *V. macrocarpon*

Our results demonstrate that wild populations of *V. macrocarpon* harbor greater diversity than previously observed in other studies using markers such as RAPDs and allozymes (Novy and Vorsa 1995; Stewart and Excoffier 1996; Mahy et al. 2000). Codominant markers such as SSRs have proven to provide great insight into the genetic diversity of several species including rice (Kalyan Chakravarthi and Naravaneni 2006), lima bean (Montero-Rojas et al. 2013), and sweet potato (Rodriguez-Bonilla et al. 2014).

In our study, most populations of *V. macrocarpon* exhibited high levels of genetic diversity, low levels of inbreeding, and moderate levels of within-population heterozygosity (Table 3). Zalapa et al. (2015), Schlautman et al. (2018) and Rodriguez- Bonilla et al. (2019) also found considerably high levels of heterozygosity and low levels of inbreeding in wild populations found in Wisconsin and Minnesota. Other diploid species such as roses have also been found to be highly heterozygous for a diploid organism (Raymond et al. 2018). In cranberries, like in other crops such as cassava, the outcrossing preference and the production of chance seedlings in nature likely contributes to the preservation of high levels of genetic diversity in wild populations (Bruederle et al. 1996; Fregene et al. 2003).

We identified three main PCA clusters: one containing the accessions from western US, one containing accessions from eastern US, and one composed of all central US accessions (Fig 4). To further explore the structure of these populations, we performed a cluster analysis, for which K=16 was our best K based on Evanno's deltaK method (Evanno et al. 2005) (Figure 5). These results were expected based on the result obtained from the fixation index (0.3). Structure results showed a more in-depth differentiation of populations than observed in the PCA. We identified several populations from the same state that clustered separately, as was the case of populations found in WV, WI, MI, and MN (Fig. 5). These results suggest that these locations, although still genetically similar, have undergone genetic differentiation, potentially caused by isolation by distance (Van Strien et al. 2015).

V. macrocarpon is thought to be distributed mostly in the eastern US and Canada and found only as far west as Minnesota. However, we found a population in the Okanogan-Wenatchee National Forest in Washington (35 - OWFLBWA), which upon field visual inspection, appeared to be *V. macrocarpon* (Vander Kloet 1988). We genetically confirmed that the 35 - OWFLB population was *V. macrocarpon.* The population clearly clustered away from *V. oxycoccos* and with *V. macrocarpon* in the combined analysis (Fig. 2 A and B). Clustering in the *V. macrocarpon* analysis separated the 35 - OWFLB population almost completely from the rest of the populations. Cranberry cultivation is very localized in Washington, and the 35 - OWFLB population was very isolated and not near any commercial or cultivated marshes. Additionally, the 35 - OWFLB population was well-differentiated with vast diversity and unique alleles. Therefore, the 35 - OWFLB population is likely not derived from cultivated cranberries derived from east or central wild populations. In terms of the origin of 35 - OWFLB, a recent organellar

genome sequencing study of *V. macrocarpon* and its wild relative *V. microcarpum* (the diploid form of *V. oxycoccos*) demonstrated that an Alaskan accession of *V. microcarpum* shared near identical organellar genomes with the cultivated cranberry (Diaz-Garcia et al. 2019). One theory to explain this finding is that *V. microcarpum* from Alaska is the progenitor of cultivated cranberries. According to this idea, the expansion of *V. microcarpum* to the North East happened through the North American ice-free corridor during the deglaciation periods. This expansion possibly involved human, wildlife, or even water-mediated movement of plants and seeds through the ice-free corridor (Heintzman et al. 2016).

This is the first report of a unique wild *V. macrocarpon* population west of Minnesota in the US. The existence of a *V. macrocarpon* population outside of the known native range of the species indicates that there could be other populations in the western bogs of Oregon, Washington, Idaho, and other locations. If *V. macrocarpon* expanded from northern areas such as Alaska through the North American ice-free corridor, the range of *V. macrocarpon* could have been widespread at one point. As the glaciers receded and environmental changes ensued, making the West arid and uninhabitable for *V. macrocarpon*, populations in eastern and central US and Canada could have found desirable habitats to colonize and expand in. In conclusion, regardless of origin, our findings indicate that *V. macrocarpon* populations are more widespread than previously believed and could be present in western locations of the US and Canada, and higher latitudes than previously believed.

Insight into the genetic diversity of wild populations of *V. oxycoccos*

Wild populations of *V. oxycoccos* exhibit high levels of heterozygosity and alleles per population and low levels of inbreeding. In comparison to *V. macrocarpon*, *V. oxycoccos* had higher levels of within-population heterozygosity and total heterozygosity (Table 4). The PCA obtained showed good separation among accessions from the different geographical areas, although the grouping seemed closer together than for *V. macrocarpon* (Fig. 6). Similar results were obtained in a study of European *V. oxycoccos*, in which they observed great genetic variability among two populations. Studies of wild populations of *V. oxycoccos* in Wisconsin and Minnesota found comparable levels of heterozygosity, ranging from 0.7 to 1, and low levels of inbreeding (Rodriguez-Bonilla et al. 2019).

To better understand the population structure present in *V. oxycoccos*, we performed a structure analysis with K=2 based on Evanno's deltaK (Evanno et al. 2005) (Fig. 7). This analysis showed a clear geographical separation between the east and central populations, and the western populations, but further differentiation was not observed as expected from the obtained fixation index (0.09). Similar structure has been observed before in switchgrass ecotypes from different regions clustered based on geographical distribution (Zhang et al. 2011). We observed some levels of admixture in individuals from populations located in Quinault Indian Reservation (28-QIROP WA) and Idaho Panhandle National Forest (35-IPHLID). These results could be due to potential human plant exchange or migrating birds spreading seeds west.

Implications for the conservation and designation of *in-situ* reserves for wild cranberries

Plants provide humans with fuel, medicine, and food, but excessive habitat destruction constantly threatens their environments (Khoury et al. 2019). Because of this, science-based policy to protect crop wild relatives is needed moving forward. With that in mind, our goal was to understand the genetic variation of wild cranberries in National Forests across their native range. This would provide the USDA-ARS and USDA Forest Service with the genetic information needed to designate *in-situ* genetic resources reserves for two species of wild cranberries.

We looked at three diversity and uniqueness indicators: private alleles, heterozygosity, and population structure, since most populations exhibited low levels of inbreeding. Private alleles are those found in one population among a group of populations, and are a great measurement of uniqueness, and therefore important for us to base our recommendations (Szpiech and Rosenberg 2011). We looked at the populations with the highest number of private alleles and found that Little Rice Lake in Superior National Forest in Minnesota (10-LRLSMN), all Cranberry Glades sites in Monongahela National Forest in West Virginia (22-CG1MWV, 23-CG7MWV, 24-CG5MWV, 25-CG4MWV), Little Crater Meadow in Mount Hood National Forest in Oregon (30-MHLCMOR), Fish Lake Bog in Okanogan-Wenatchee National Forest in Washington (34-OWFLBWA), Upper Island Lake in Chequamegon-Nicolet National Forest in Wisconsin (26-UILCWWI), and South Prairie in Gifford Pinchot National Forest in Washington (29-GPSPWA) had the highest numbers (Fig. 3). These populations also demonstrated high levels of genetic variation based on their heterozygosity values for one or both species (Table 3, Table 4).

However, for populations like Cranberry Glades (22-CG1MWV, 23-CG7MWV, 24-CG5MWV, 25-CG4MWV), a more thorough collection of *V. macrocarpon* individuals would be desirable, as we had low numbers of samples (Fuerst and Maruyama 1986). In the structure analysis, we found that these populations also separated into their own clusters independently from other groups of the same geographical location. This is especially true for the populations containing *V. macrocarpon*, since for *V. oxycoccos* we were only able to discriminated among east and central populations compared to west populations (Fig. 5, Fig. 7).

Based on these results, we propose that the aforementioned populations be included in the designation of *in-situ* genetic resources for cranberries in National Forests. They possess interesting genetic profiles, are distributed across the US, and the high levels of genetic variation could be translated into potentially useful traits for breeding (Steiner et al. 2019).

Conclusion

Our study elucidated the genetic diversity and population structure of wild populations of cranberries located in National Forests. Most of these accessions are distributed across the US and exhibit unique allelic combinations that could be useful for breeding. Therefore, based on their genetic diversity and structure, we provided recommendations for the establishment of certain populations as *in-situ* genetic resources reserves in National Forests.

References

Alston JM, Medellín-Azuara J, Saitone TL (2014) Economic impact of the North American cranberry industry. University of California: Davis, CA, USA

Areškevičiūtė J, Paulauskas A, Česonienė L, Daubaras R (2006) Genetic characterisation of wild

cranberry (Vaccinium oxycoccos) from Čepkeliai reserve by the RAPD method. Liet Tsr Aukst Mokyklu Mokslo Darb

Asudi GO, Ombwara FK, Rimberia FK, Nyende AB, Ateka EM, Wamocho LS (2013) Evaluating diversity among Kenyan papaya germplasm using simple sequence repeat markers. Afr J Food Agric Nutr Dev 13:7307–7324

Balloux F, Lugon-Moulin N (2002) The estimation of population differentiation with microsatellite markers. Mol Ecol 11:155–165

Becker RA, Wilks AR, Brownrigg R, et al (2013) maps: Draw geographical maps. R package version 2

Brown PN, Turi CE, Shipley PR, Murch SJ (2012) Comparisons of large (Vaccinium macrocarpon Ait.) and small (Vaccinium oxycoccos L., Vaccinium vitis-idaea L.) cranberry in British Columbia by phytochemical determination, antioxidant potential, and metabolomic profiling with chemometric analysis. Planta Med 78:630–640

Brownstein MJ, Carpten JD, Smith JR (1996) Modulation of non-templated nucleotide addition by Taq DNA polymerase: primer modifications that facilitate genotyping. Biotechniques 20:1004–6, 1008–10. doi: 10.2144/96206st01

Bruederle LP, Hugan MS, Dignan JM, Vorsa N (1996) Genetic variation in natural populations of the large cranberry, Vaccinium macrocarpon Ait.(Ericaceae). Bull Torrey Bot Club 41–47

Debnath SC (2016) Genetic Diversity and Erosion in Berries. In: Ahuja MR, Jain SM (eds) Genetic Diversity and Erosion in Plants: Case Histories. Springer International Publishing, Cham, pp 75–129

Diaz-Garcia L, Rodriguez-Bonilla L, Rohde J, Smith T, Zalapa J (2019) Pacbio Sequencing Reveals Identical Organelle Genomes between American Cranberry (Vaccinium macrocarpon Ait.) and a Wild Relative. Genes 10.: doi: 10.3390/genes10040291

Doyle JJ, Doyle JL (1987) CTAB DNA extraction in plants. Phytochemical Bulletin 19:11–15

Earl DA, vonHoldt BM (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv Genet Resour 4:359–361. doi: 10.1007/s12686-011-9548-7

Engelhardt KAM, Lloyd MW, Neel MC (2014) Effects of genetic diversity on conservation and restoration potential at individual, population, and regional scales. Biol Conserv 179:6–16. doi: 10.1016/j.biocon.2014.08.011

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol 14:2611–2620. doi: 10.1111/j.1365-294X.2005.02553.x

Francis RM (2017) pophelper: an R package and web app to analyse and visualize population structure. Mol Ecol Resour 17:27–32. doi: 10.1111/1755-0998.12509

Fregene MA, Suarez M, Mkumbira J, et al (2003) Simple sequence repeat marker diversity in cassava landraces: genetic diversity and differentiation in an asexually propagated crop. Theor Appl Genet 107:1083–1093. doi: 10.1007/s00122-003-1348-3

Fuerst PA, Maruyama T (1986) Considerations on the conservation of alleles and of genic heterozygosity in small managed populations. Zoo Biol 5:171–179. doi: 10.1002/zoo.1430050211

Fukino N, Sakata Y, Kunihisa M, Matsumoto S (2007) Characterisation of novel simple sequence repeat (SSR) markers for melon (Cucumis melo L.) and their use for genotype identification. J Hortic Sci Biotechnol 82:330–334. doi: 10.1080/14620316.2007.11512237

Galili T (2015) dendextend: an R package for visualizing, adjusting and comparing trees of hierarchical clustering. Bioinformatics 31:3718–3720. doi: 10.1093/bioinformatics/btv428

Gallardo RK, Klingthong P, Zhang Q, et al (2018) Breeding Trait Priorities of the Cranberry Industry in the United States and Canada. HortScience 53:1467–1474

Georgi L, Herai RH, Vidal R, Carazzolle MF, Pereira GG, Polashock J, Vorsa N (2012) Cranberry microsatellite marker development from assembled next-generation genomic sequence. Mol Breed 30:227–237. doi: 10.1007/s11032-011-9613-7

Greene SL, Khoury CK, Williams KA (2018) Wild Plant Genetic Resources in North America: An Overview. In: Greene SL, Williams KA, Khoury CK, et al. (eds) North American Crop Wild Relatives, Volume 1: Conservation Strategies. Springer International Publishing, Cham, pp 3–31

Griffin JN, O'Gorman EJ, Emmerson MC, Jenkins SR, Klein AM, Loreau M, Symstad A (2009) Biodiversity and the stability of ecosystem functioning. Biodiversity, Ecosystem Functioning, and Human Wellbeing--an Ecological and Economic Perspective, pp. 78–93

Gu Z, Gu L, Eils R, Schlesner M, Brors B (2014) circlize implements and enhances circular visualization in R. Bioinformatics 30:2811–2812. doi:

Guichoux E, Lagache L, Wagner S, Chaumeil P, Léger P, Lepais O, Lepoittevin C, Malausa T, Revardel E, Salin F, Petit RJ (2011) Current trends in microsatellite genotyping. Mol Ecol Resour 11:591–611. doi: 10.1111/j.1755-0998.2011.03014.x

Hajjar R, Hodgkin T (2007) The use of wild relatives in crop improvement: a survey of developments over the last 20 years. Euphytica 156:1–13. doi: 10.1007/s10681-007-9363-0

Hagerup O (1940) STUDIES ON THE SIGNIFICANCE OF POLYPLOIDY. Hereditas 26:399–410. doi:

10.1111/j.1601-5223.1940.tb03244.x

Hamernik AJ, Hanneman RE Jr, Jansky SH (2009) Introgression of Wild Species Germplasm with Extreme Resistance to Cold Sweetening into the Cultivated Potato. Crop Sci 49. doi: 10.2135/cropsci2008.04.0209

Heintzman PD, Froese D, Ives JW, Soares AE, Zazula GD, Letts B, Andrews TD, Driver JC, Hall E, Hare PG, Jass CN (2016) Bison phylogeography constrains dispersal and viability of the Ice Free Corridor in western Canada. Proc Natl Acad Sci U S A 113:8057–8063. doi: 10.1073/pnas.1601077113

Hokanson SC, Lamboy WF, Szewc-McFadden AK, McFerson JR (2001) Microsatellite (SSR) variation in a collection of Malus (apple) species and hybrids. Euphytica 118:281–294. doi: 10.1023/A:1017591202215

Jacquemart A-L (1997) Vaccinium Oxycoccos L. (Oxycoccus Palustris Pers.) and Vaccinium Microcarpum (Turcz. ex Rupr.) Schmalh. (Oxycoccus Microcarpus Turcz. ex Rupr.). J Ecol 85:381–396. doi: 10.2307/2960511

Jost L, Archer F, Flanagan S, Gaggiotti O, Hoban S, Latch E (2018) Differentiation measures for conservation genetics. Evol Appl 11:1139–1148. doi: 10.1111/eva.12590

Kalyan Chakravarthi B, Naravaneni R (2006) SSR marker based DNA fingerprinting and diversity study in rice (Oryza sativa. L). Afr J Biotechnol 5

Kamvar ZN, Tabima JF, Grünwald NJ (2014) Poppr: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. PeerJ 2:e281. doi: 10.7717/peerj.281

Khoury CK, Amariles D, Soto JS, Diaz MV, Sotelo S, Sosa CC, Ramírez-Villegas J, Achicanoy HA, Velásquez-Tibatá J, Guarino L, León B (2019) Comprehensiveness of conservation of useful wild plants: An operational indicator for biodiversity and sustainable development targets. Ecol Indic 98:420–429. doi: 10.1016/j.ecolind.2018.11.016

Khoury CK, Greene S, Wiersema J, Maxted N, Jarvis A, Struik PC (2013) An inventory of crop wild relatives of the United States. Crop Sci 53:1496-1508

Llácer G, Badenes ML, Calvo JM (2003) Plant material of loquat in Mediterranean countries. In: Proc. First Int. Loquat Symp. Options Méditerranéennes : Série A. Séminaires Méditerranéens 58: 45–52

Li D-Z, Pritchard HW (2009) The science and economics of ex situ plant conservation. Trends Plant Sci 14:614–621. doi: 10.1016/j.tplants.2009.09.005

Louette D, Charrier A, Berthaud J (1997) In Situ conservation of maize in Mexico: Genetic diversity and Maize seed management in a traditional community. Econ Bot 51:20–38. doi:

10.1007/BF02910401

Mahy G, Bruederle LP, Connors B, Van Hofwegen M, Vorsa N (2000) Allozyme evidence for genetic autopolyploidy and high genetic diversity in tetraploid cranberry, Vaccinium oxycoccos (Ericaceae). Am J Bot 87:1882–1889

Mao D, Yu L, Chen D, Chen D, Li L, Zhu Y, Xiao Y, Zhang D, Chen C (2015) Multiple cold resistance loci confer the high cold tolerance adaptation of Dongxiang wild rice (Oryza rufipogon) to its high-latitude habitat. Theor Appl Genet 128:1359–1371. doi: 10.1007/s00122-015-2511-3

Marfil CF, Hidalgo V, Masuelli RW (2015) In situ conservation of wild potato germplasm in Argentina: Example and possibilities. Global Ecology and Conservation 3:461–476. doi: 10.1016/j.gecco.2015.01.009

McKay DL, Blumberg JB (2007) Cranberries (Vaccinium macrocarpon) and cardiovascular disease risk factors. Nutr Rev 65:490–502

Montero-Rojas M, Correa AM, Siritunga D (2011) Molecular differentiation and diversity of cassava (Manihot esculenta) taken from 162 locations across Puerto Rico and assessed with microsatellite markers. AoB Plants 2011. doi: 10.1093/aobpla/plr010

Montero-Rojas M, Ortiz M, Beaver JS, Siritunga D (2013) Genetic, morphological and cyanogen content evaluation of a new collection of Caribbean Lima bean (Phaseolus lunatus L.) landraces. Genet Resour Crop Evol 60:2241–2252. doi: 10.1007/s10722-013-9989-9

Munshi-South J, Zolnik CP, Harris SE (2016) Population genomics of the Anthropocene: urbanization is negatively associated with genome-wide variation in white-footed mouse populations. Evol Appl 9:546–564. doi: 10.1111/eva.12357

Novy RG, Vorsa N (1995) Identification of intracultivar genetic heterogeneity in cranberry using silver-stained RAPDs. HortScience 30:600–604

Olsen KM, Gross BL (2008) Detecting multiple origins of domesticated crops. Proc Natl Acad Sci USA 105:13701–13702. doi: 10.1073/pnas.0807439105

Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research--an update. Bioinformatics 28:2537–2539. doi: 10.1093/bioinformatics/bts460

Porsild AE (1938) The cranberry in Canada

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959

Raymond O, Gouzy J, Just J, Badouin H, Verdenaud M, Lemainque A, Vergne P, Moja S, Choisne

N, Pont C, Carrere S (2018) The Rosa genome provides new insights into the domestication of modern roses. Nat Genet 50:772–777. doi: 10.1038/s41588-018-0110-3

R Core Team (2018) R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing

Rodriguez-Bonilla L, Cuevas HE, Montero-Rojas M, Bird-Pico F, Luciano-Rosario D, Siritunga D (2014) Assessment of genetic diversity of sweet potato in Puerto Rico. PLoS One 9:e116184. doi: 10.1371/journal.pone.0116184

Rodriguez-Bonilla L, Rohde J, Matusinec D, Zalapa J (2019). Cross-transferability analysis of SSR markers developed from the American Cranberry to other Vaccinium species of agricultural importance. (In preparation)

Rodriguez-Bonilla L, Rodriguez Bonilla F, Matusinec D, Maule A, Wiesman E, Atucha A, Zalapa J (2019). Unraveling the genetic diversity and breeding implications of wild cranberry populations in Wisconsin and Minnesota. (In preparation)

Roper TR, Vorsa N (1997) Cranberry: Botany and Horticulture. In: Janick J (ed) Horticultural Reviews. John Wiley & Sons, Inc., Oxford, UK, pp 215–249

Schlautman B, Fajardo D, Bougie T, Wiesman E (2015) Development and validation of 697 novel polymorphic genomic and EST-SSR markers in the American cranberry (Vaccinium macrocarpon Ait.). Molecules 20(2):2001-2013

Schlautman B, Covarrubias-Pazaran G, Rodriguez-Bonilla L, Hummer K, Bassil N, Smith T, Zalapa J. (2018) Genetic diversity and cultivar variants in the National Clonal Germplasm Repository cranberry (Vaccinium macrocarpon Aiton) collection. Journal of Genetics, J Genet 97(5):1339-1351.

Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. Nat Biotechnol 18:233–234. doi: 10.1038/72708

Segarra-Moragues JG, Palop-Esteban M, González-Candelas F, Catalán P (2005) On the verge of extinction: genetics of the critically endangered Iberian plant species, Borderea chouardii (Dioscoreaceae) and implications for conservation management. Mol Ecol 14:969–982

Shakiba E, Edwards JD, Jodari F, Duke SE, Baldo AM, Korniliev P, McCouch SR, Eizenga GC (2017) Genetic architecture of cold tolerance in rice (Oryza sativa) determined through high resolution genome-wide analysis. PLoS One 12:e0172133. doi: 10.1371/journal.pone.0172133

Song G-Q, Hancock JF (2011) Vaccinium. In: Kole C (ed) Wild Crop Relatives: Genomic and Breeding Resources: Temperate Fruits. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 197–221

Steiner B, Michel S, Maccaferri M, Lemmens M, Tuberosa R, Buerstmayr H (2019) Exploring and exploiting the genetic variation of Fusarium head blight resistance for genomic-assisted breeding in the elite durum wheat gene pool. Theor Appl Genet 132:969–988. doi: 10.1007/s00122-018-3253-9

Stewart CN (1993) The molecular ecology of Vaccinium macrocarpon Aiton, the American cranberry. Doctoral dissertation, Virginia Tech

Stewart CN, Excoffier L (1996) Assessing population genetic structure and variability with RAPD data: Application to Vaccinium macrocarpon (American Cranberry). J Evol Biol 9:153–171. doi: 10.1046/j.1420-9101.1996.9020153.x

Stewart CN, Neal Stewart C, Nilsen ET (1995) Phenotypic Plasticity and Genetic Variation of Vaccinium macrocarpon, the American Cranberry. I. Reaction Norms of Clones from Central and Marginal Populations in a Common Garden. Int J Plant Sci 156:687–697

Szpiech ZA, Rosenberg NA (2011) On the size distribution of private microsatellite alleles. Theor Popul Biol 80:100–113. doi: 10.1016/j.tpb.2011.03.006

Vander Kloet SP (1988) The genus Vaccinium in North America. Agriculture Canada

Vander Kloet SP (1983) THE TAXONOMY OF VACCINIUM § OXYCOCCUS. Rhodora 85:1–43

Van Strien MJ, Holderegger R, Van Heck HJ (2015) Isolation-by-distance in landscapes: considerations for landscape genetics. Heredity 114:27–37. doi: 10.1038/hdy.2014.62

Wang SM, Zhang ZW (2011) The state of the world's plant genetic resources for food and agriculture. J Plant Genet Resour 12:325–338

Wickham H (2011) ggplot2. WIREs Comp Stat 3:180–185. doi: 10.1002/wics.147

Yan X, Murphy BT, Hammond GB, Vinson JA, Neto CC (2002) Antioxidant activities and antitumor screening of extracts from cranberry fruit (Vaccinium macrocarpon). J Agric Food Chem 50:5844–5849

Zalapa JE, Bougie TC, Bougie TA (2015) Clonal diversity and genetic differentiation revealed by SSR markers in wild Vaccinium macrocarpon and Vaccinium oxycoccos. Ann Appl Biol 166:196-207

Zhang Y, Zalapa J, Jakubowski AR, Price DL (2011) Natural hybrids and gene flow between upland and lowland switchgrass. Crop Sci 51:2626-2641

Zhu H, Senalik D, McCown BH, Zeldin EL, Speers J, Hyman J, Bassil N, Hummer K, Simon PW, Zalapa JE (2012) Mining and validation of pyrosequenced simple sequence repeats (SSRs) from American cranberry (Vaccinium macrocarpon Ait.). Theor Appl Genet 124:87–96. doi: 10.1007/s00122-011-1689-2

Supplementary materials

Final Primer Pairs

Fluorescence	Primer	Range	NCBI	Source	Forward Primer	Reverse Primer
FAM	ct93137	175-229	KP279104	Schlautman et al. (2015)	AAGATTTCCGCTACAGTACCT	GCTATGGGTGTCTCAAAAAG
	ct31701	251-327	JF834268	Zhu et al. (2012)	GTCACTGGTAATGCTATTCTGA	CTTCTTTGTTTCATCTCCCTAC
	SCF162565	146-182	KP278913	Schlautman et al. (2015)	CTTCCGTGATTGTTCTTGTAG	ACACAGATGGGATGTTGTATC
	SCF125251	234-305	KP278851	Schlautman et al. (2015)	CACGACGTTGTAAAACGACTATACAGTCAGATCCAATCCAC	TGCAGATAAAGTACAAGAGTGC
	SCF139334	193-230	KP278874	Schlautman et al. (2015)	CACGACGTTGTAAAACGACGAGGGTCTAATATCTGGTTTCA	GAGAAAAGATGGAGCAAAAG
	SCF3362	320-380	KP278605	Schlautman et al. (2015)	GTACAGCAAAATTCAGCACA	GGATTTATCTACAGCCCATTAC
	ct25796	195-262	JF834263	Zhu et al. (2012)	CACTTACCTGAATCCTCTTAGC	TAGAGGAGCCAAACTGATAACT
	1trimcontig176303	181-226	KP279222	Schlautman et al. (2015)	CACGACGTTGTAAAACGACGCTGATTAGGTTCACTTTCTTC	TTTCTTCACCTCTTTCTCTCTC
HEX	SCF22477	240-271	KP278658	Schlautman et al. (2015)	CTCTCCCCTACTTTCTTCCTAT	GCCGCTAACACAATTAACTAAC
	SCF2714	189-232	KP278602	Schlautman et al. (2015)	ACAAGTCTCTGGAAGCTAACAT	GTTGATTGTTGGGTCTAAGTTC
	ct147864	275-284	KP279133	Schlautman et al. (2015)	CTCTCTTTACCCTCAATTTCTC	GGTCTAATATCAATCGATGACC
	1trimconti	361-459	KP279228	Schlautman et	CACGACGTTGTAAAACGACGTATTTGTTCACACT	ACAGTTGTCGAAGCCTCAT

	g209220			al. (2015)	CACCAGA	
	ct38401	168-201	JF834272	Zhu et al. (2012)	CAATGGGAAGTACAAAGAGC	CGATGCAATCTTAGTCTTGA
	SCF40517	220-260	KP278707	Schlautman et al. (2015)	GTAGAATGGCAATAGGGTTT	GAAGAAGATGACGAAGATCAC
	SCF118468	229-281	KP278840	Schlautman et al. (2015)	ATAAGCGGAGCACAGTTACA	GATAGGATGACCTGTTTTGGT
	SCF26014	166-170	KP278667	Schlautman et al. (2015)	GGTCCCAGAATCAATGTCTA	GAAATCAGAGAAGAAACAGGTC
	1trimcontig435620	235-263	KP279244	Schlautman et al. (2015)	CACGACGTTGTAAAACGACCAACCAGCCTTACAGTGAATA	GTCCGTTCAATTTCTTTTCC
	SCF64185	316-320	KP278745	Schlautman et al. (2015)	CACCTCATTTGGTTCATTCT	CAGATACTAAAGGTTGCCGTA
NED	251788_K63	182-228	KP279166	Schlautman et al. (2015)	CACGACGTTGTAAAACGACGATCTTTACCACTCCCCACT	GGATTCTCTGTCCATTGTTG
	SCF11186	269-288	KP278636	Schlautman et al. (2015)	AGAAAGGCTAAAAGGGTATCTC	GCTCTCAACAACTCGAAAGTA
	ct04084	150-248	JF834250	Zhu et al. (2012)	GGATTCTCACTCTGATACCATT	GAACGATACACAACGAAGGT
	SCF91821	294-324	KP278792	Schlautman et al. (2015)	TTCTGTGTCTGATTCCATCTC	ACTAGCCCAACAACTTAGACTG
	ct28527	216-283	JF834266	Zhu et al. (2012)	GGACAAGTGAAATGCTAGTTG	AGATTGTTCGTAGGTAGAAGTG
PET	SCF9068	174-186	KP278624	Schlautman et al. (2015)	AAATCTAGGTAGGAGCAGGTCT	ATGGAGGAGGAGATATGTGAT
	76126_K6	231-242	KP279162	Schlautman et	CACGACGTTGTAAAACGACTTTATTGGAGCGAA	AAAAGGGGAGGAGAGAGA

3			al. (2015)	AGAGAG	T
418294_K63	336-348	KP279189	Schlautman et al. (2015)	CACGACGTTGTAAAACGACCAAGAACAAGAAGAAGAAGACC	AGAGACCACCCAAAAGATAAG
372875_K63	195-235	KP279174	Schlautman et al. (2015)	CACGACGTTGTAAAACGACCACACACAAATCCCAATTTC	GATGGTGTTTTCATAGTTCGAC
SCF95767	262-311	KP278796	Schlautman et al. (2015)	TGAGGAGAGGAGTATCCATAAG	CCTACAAGTCTCGCAATTCTA
418596_K63	385-413	KP279190	Schlautman et al. (2015)	CACGACGTTGTAAAACGACCGTGAGTTTGAGTGAGTAATTG	AGGACATGGTGAGTTGAGAAT
ct40600	173-247	JF834274	Zhu et al. (2012)	CAAAAGAGCCATGAAATAGG	TTGGTGAAAACTATACCTGTCC
ct52682	263-324	JF834282	Zhu et al. (2012)	CTCAGGTTATCAGGCTTATTTC	CAATTAGTGTGTTCCCAACTC
ct78806	214-274	JF834245	Zhu et al. (2012)	CAAAGAAGAGGAGGATTGAGT	GAGCGAGTATTACAAGTGTTTC

Chapter IV

The development of cranberry/blueberry interspecific hybrids and *V. microcarpum* mapping populations.

Abstract

The genus *Vaccinium* houses over 450 individual species with rich and diverse traits with cranberries and blueberries leading *Vaccinium* production. Therefore, using *Vaccinium* resources to develop interspecific hybrids in newer crops such as cranberry can be useful in moving the industry forward. To date, several interspecific hybrids have been developed between blueberry species. Crosses between *V. macrocarpon* and *V. microcarpum* have also been successful and further used as a bridge to cross with *V. darrowii*. However there are no documented successful *V. macrocarpon* x blueberry hybrids to date. Therefore, we tested the cross-ability of a wild cranberry collection with blueberry species that possess interesting traits. We successfully crossed *V. macrocarpon* and *V. oxycoccos* against a mix of blueberry pollen (*V. darrowii* (2n=2x), *V. corymbosum* [2n=2x and (2)2n=4x], *V. stamineum* (2n=2x), *V. crassifolium* (2n=2x), and *V. elliottii* (2n=2x). We obtained fruit and viable seeds for both species, although the *V. oxycoccos* x blueberry cross produced a much larger amount of viable seeds that were successfully transferred to soil. Potential hybrids of this cross exhibited an intermediate phenotype between *V. oxycoccos*, *V. elliottii*, and a *V. corymbosum* (4x) accession that has *V. elliottii* in its background. Our next steps include testing for paternity using molecular markers that successfully transfer among *Vaccinium* species. Also, developed a self F_1 population of *V. microcarpum*, a sister species of *V. macrocarpon*, to develop a linkage map and perform comparative genetics studies.

Keywords: Interspecific hybrids, mapping population, cranberry breeding

Introduction

The genus *Vaccinium* houses over 450 individual species with rich and diverse traits. (Vander Kloet 1988). The majority of the berry production in this genus comes from sections *Cyanococcus* and *Oxycoccus,* which includes all blueberries, cranberries, and their wild relatives (Galletta and Ballington 1996). The use of wild relatives, secondary, and tertiary gene pools can provide breeders with new and interesting traits, such as cold tolerance and resistance to biotic stress (Dwivedi *et al.* 2008). Therefore, using such resources to develop interspecific hybrids in newer crops such as cranberry can be useful in moving the industry forward.

In *Vaccinium*, there have been many successful instances in which interspecific hybrids have been developed. This has been possible because of the presence of 2n gametes in *Vaccinium* species. These gametes have been observed in 2x to 6x pollen, and serve as a way to help overcome ploidy barriers (Ortiz *et al.* 1991). Examples of these interspecific hybrids include blueberry Northern Highbush x Darrow's Evergreen (Sharpe *et al.* 1960), *V. ashei* (6x) and *V. tenellum* (2x) to produce the Beltsville 17 (Darrow *et al.* 1949), and southern highbush varieties, which include *V. darrowii*, *V. elliottii*, *V. corymbosum*, and *V. virgatum* in their background (Ballington 2008).

Crosses between cranberries and blueberries have not been as successful as blueberry interspecific hybrids have been. Crosses of *V. macrocarpon* and *V. corymbosum* have failed to produce offspring (Vorsa *et al.* 2008). Vorsa *et al.* in 2008 successfully developed a *V. macrocarpon* x *V. oxycoccos* hybrid which was then used as a bridge to cross with *V. darrowii*. The offspring of this cross resulted in an intermediate phenotype with flowers with three to five

petals, some of them partially fused. Our lab has previously attempted and successfully made crosses between cranberries and blueberries (Covarrubias-Pazaran, 2016). However, the offspring of these crosses have been very few and weak and all have died very easily, which points to the need of trying different crossing combinations that may allow vigorous seedlings.

Very little is known about the true genetic relationship and taxonomy of *V. macrocarpon* (2x) and its sister species *V. oxycoccos*. There has been much debate about whether the species is one of two distinct species, 2x as *V. microcarpum* (Turcz.) and 4x as *V. oxycoccos* L (Mahy *et al.* 2000), or a single species with different ploidy levels, *V. oxycoccos* (2x, 4x) (Vander Kloet 1983). Smith *et al.* 2015 found morphological and phenological differences between *V. microcarpum* (2x) and *V. oxycoccos*, such as flowering time, petal length, anther length, and style length, and suggested the plants be treated as separate species.

Whether *V. oxycoccos* and *V. microcarpum* are different species is still under debate as are in general the evolutionary relationships among section *Oxycoccus*. Authors such as Vander Kloet (1983) and (Darrow and Camp 1945) suggest that *V. macrocarpon* is the most primitive species. Recent studies seeking to sequence the organellar genomes of *V. macrocarpon* and *V. microcarpum* found identical organelles in both species (Diaz-Garcia *et al.* 2019). These findings further confirm how little we know about the evolution and domestication of cranberries.

In order to better understand the origin and evolution of these species, we developed *V. microcarpum* mapping populations for linkage mapping and comparative genomics. A *V. macrocarpon* linkage map developed by (Schlautman *et al.* 2015) can be used as a base for comparison between the two species to test collinearity, candidate gene selection, and QTL mapping as has been suggested for other plants, such as *Arabidopsis* (Schlautman and Diaz-

Garcia 2018, Kuittinen 2004). Finally, based on previous cranberry x blueberry hybridization attempts in our lab (Covarrubias-Pazaran, 2016), we tested several crosses using the germplasm collected in Wisconsin and Minnesota as mothers to blueberry fathers to develop our own populations of interspecific hybrids. Cross-transferability studies in this thesis of simple sequence repeat (SSR) markers among species of *Vaccinium* species and a recent comparative genetic mapping revealing synteny and collinearity between the cranberry and diploid blueberry genomes (Schlautman et al. 2018) both point to the possibility of successfully developing cranberry/blueberry hybrids.

Materials and Methods

Plant Materials for the development of interspecific hybrids

Wild *V. macrocarpon* and *V. oxycoccos* germplasm collected in Wisconsin and Minnesota were used as mothers to develop interspecific hybrids. Blueberry species were either wild or free cultivars, including *V. darrowii* (2n=2x), *V. corymbosum* [2n=2x and (2)2n=4x], *V. stamineum* (2n=2x), *V. crassifolium* (2n=2x), and *V. elliottii* (2n=2x). Pollinations were performed on 2 to 3-year-old plants (mothers), and pollen was collected from 4 to 5-year-old blueberry species. Blueberry pollen was harvested, and a pollen mixture was developed using the aforementioned 6 blueberry species and stored at 4° C in microcentrifuge tubes. Flowers were emasculated 2 to 3 days before anthesis, and the stigmas were pollinated with the pollen mix. Fruits that were completely developed and colored were collected in bags and stored at 4°C for 3 months to fulfill the vernalization period required prior to germination.

Seed germination and sterilization for interspecific hybrid seeds

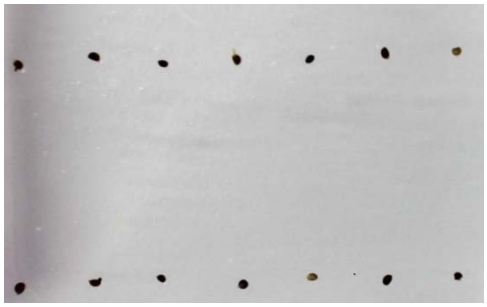
Media Preparation

To prepare 20 100-mm square petri plates, 1 L of WPM seed germination media was prepared [0.60 g woody plant basal medium (WPBM), 6 g of agar, pH 5.58]. Media was then sterilized 30 minutes and allowed to cool to 60°C before pouring into plates. To obtain the seeds, fruits were placed on a flat surface and using a razor blade, shallow cuts around the equator of the fruits were performed. Seeds were removed using forceps and were separated from any traces of fruit flesh.

Seed sterilization

Six-well microtiter plates with filters (previously sterilized) were sprayed with 95% ethanol and allowed to dry. Plates were then aligned in the laminar flow hood so that there were 4 columns and 3 rows of wells. The left-most column was 2/3 full with a 20% dilution of commercial bleach and the remaining wells were 2/3 full with sterile water. Before moving to the laminar flow hood, seeds were separately placed in labeled filters. Filters were then moved into the laminar flow hood, and placed into separate bleach wells. Seeds were placed in the bleach solution for 10 minutes, agitating every 2 minutes. The filters were then transferred from the bleach solution to the first column of water wells. Seeds were then rinsed for 1 minute before moving to the next columns with sterile water. The process was repeated for the second and third water rinses. After the third water rinse, each filter was set on sterile filter paper and allowed to dry briefly. Using sterile forceps, the seed were places in horizontal rows, 3 cm from the bottom of the plate, and 1 cm apart from each other and the edges of the plate. Each seed was slightly pressed into the gel, and the plates were sealed with parafilm and labeled (Fig.1). Plates were then placed upside down in an incubator set to 27-29° C for 3 days.

Fig 1. Example of seed and seedlings in media plates



Seed germination

Plates were then stored in a room at 20°C (24-h photoperiod, 350 mol m-2 s-1, daylight fluorescent tubes). After germination plants were then transferred into small square pots and kept in a room at 20°C (16-h photoperiod, 350 mol m-2 s-1, daylight fluorescent tubes)

Plant Materials for *V. microcarpum* mapping populations

The *V. microcarpum* material used for this work was provided by Nicholi Vorsa from the Philip E. Marucci Center for Blueberry and Cranberry Research and Extension in Chatsworth, NJ. Cuttings from *V. microcarpum* were planted in fall 2014 in 96 plant starting cells and transferred into a tub after their root systems were developed (Fig.2). Three-year-old plants from *V. microcarpum* were self-pollinated during spring of the years 2017 and 2018. Fruits that were completely developed and colored were collected in bags and stored at 4°C for 3 months to fulfill the vernalization period required prior to germination.

Seed germination and sterilization for *V. microcarpum* mapping populations

Fruits were placed on a flat surface and using a razor blade, shallow cuts around the equator of the fruits were performed. Seeds were removed using forceps and separated from any traces of fruit flesh. Seeds were then placed in 1.5 mL micro-centrifuge tubes and stored at 4°C until

ready to sterilize. The seeds were placed in a metal mesh leaf tea infuser and dipped into a 20% concentrated commercial bleach solution for 3 minutes, stirring every 30 seconds. Seeds were then rinsed 3 times with sterile water.

Fig 2. *Vaccinium microcarpum* plant materials and fruit

After sterilization, seeds were placed in 60 x 15 mm plastic petri dishes containing a circle of 7.0 cm P5 filter paper. A total of 10 seeds were placed per petri dish with 7 mL of sterile water. All seeds were floating in the water and not caught on the side of the dish or clumped together with other seeds. This ensured that seeds were moist, and that once germinated, the seedlings would be easier to plant by preventing root entanglement. The petri dishes were then closed and sealed with two layers of parafilm around the edge of the dish.

Growing Conditions for *V. microcarpum* mapping populations

Plants were then transferred into 96 plant starting cells and kept in a room at 20°C (16-h photoperiod, 350 mol m-2 s-1, daylight fluorescent tubes)

Results

Development of interspecific hybrids with *V. macrocarpon*

After several rounds of pollinations using our blueberry pollen mix on several plants from *V. macrocarpon*, we obtained putative hybrid seeds. The majority of the crosses yielded non-viable seeds, and from those that were viable, only five genotypes germinated, and 8 seedlings out of the 10 were successfully transferred to soil (Table 1). From the ones that germinated, only one of the plants was a cultivar, HyRed, while the rest of the plants were wild germplasm (Fig.3).

Fig. 3 *Vaccinium macrocarpon*/blueberry potential hybrids

Table 1. *Vaccinium macrocarpon*-blueberry potential hybrid seeds

Mother	Fruit number	Viable seed number	Non-viable seed number	Number of germinated seeds	Number of plants
BL05	4	4	6	3	3
Chip 01	4	0	6	0	0
Chip 05	5	2	8	0	0
HW05	9	0	19	0	0
LL08	1	0	3	0	0
Ben Lear	1	1	0	0	0
WC12-055	6	2	21	0	0
WC12-101	22	3	27	3	1
WC12-104	8	3	13	0	0
WC12-110	14	3	17	0	0
WC12-114	2	1	8	0	0
WC12-221	2	0	5	0	0
WC12-280	2	2	6	2	2
WC12-288	4	0	66	0	0
WC12-306	4	0	45	0	0
WC12-357	4	1	9	1	1
WC16-007	2	0	5	0	0
WC16-010	3	0	8	0	0
WC16-011	3	2	6	0	0
WC16-013	3	0	3	0	0
WC16-016	3	11	1	0	0
WC16-018	15	5	25	0	0

WC16-023	1	0	3	0	0
WC16-032	3	0	7	0	0
WC16-043	2	0	2	0	0
WC16-055	1	0	3	0	0
WC16-061	5	5	7	0	0
WC16-062	1	2	3	0	0
WC16-070	1	0	3	0	0
WC16-072	1	0	3	0	0
WC17-001	18	0	29	0	0
WC17-002	1	0	8	0	0
WC17-003	2	0	1	0	0
WC17-004	6	7	6	0	0
WC12-112 CC	8	3	18	0	0
WC12-131 CC	10	10	18	0	0
Yellow Bell CC	1	5	2	0	0
J-21	1	8	0	0	0
Tetraploid 17	1	6	1	0	0
MG07	2	3	2	0	0
MG12	2	1	4	0	0
YBS20	2	0	4	0	0
08-233-1	1	0	4	0	0
08-233-32	1	1	5	0	0
08-233-41	2	0	8	0	0
12-58-33	1	1	0	0	0
WC16-018	5	1	8	0	0
HyRed	2	1	33	1	1

Total	**202**	**94**	**489**	**10**	**8**

Development of interspecific hybrids with *V. oxycoccos*

The same blueberry pollen mix used on *V. macrocarpon* plants was used on several wild *V. oxycoccos* plants. From the *V. oxycoccos* x blueberry crosses, we also obtained a large number of non-viable seeds (Table 2). However, we had higher germination rates using *V. oxycoccos* as a pollen recipient than *V. macrocarpon*. A total of 92 seeds germinated, and from those 49 have been successfully transferred to soil (Fig. 4) and exhibit an intermediate phenotype between *V. oxycoccos* and a potential parent (*V. elliottii* and a *V. corymbosum* 4x which has *V. elliottii* in its background). The rest of the seeds are still growing on media and will be transferred to soil in the next few weeks.

Fig.4 *Vaccinium oxycoccos*/blueberry hybrids

Table 2. *Vaccinium oxycoccos*-blueberry hybrid seeds

Mother	Fruit number	Viable seed number	Non-viable seed number	Number of germinated seeds	Number of plants
BLM10	1	4	1	2	1
BLM11	2	3	3	1	1

BRR 08	1	0	2	0	0
BRRM 07	9	9	31	8	7
BRRM 17	3	1	4	0	0
LL11	3	4	0	3	3
LOLB19	1	2	4	2	2
LOLB20	1	2	1	0	0
PB02	1	0	3	0	0
PB09	5	4	7	3	0
PB10	30	60	102	28	10
PB14	1	2	9	1	0
PB15	5	5	10	4	1
PB16	6	19	18	13	7
PB17	13	37	42	31	17
Total	**82**	**152**	**237**	**96**	**49**

Table does not include seeds from PB18, PB20, TR 01, Oxy 2x-1, WC12-165, GL01, GL03, GL06, GL09, GL15, GL18, GL19, KLB09, KLB11, LocP04, LocP05, LocP11, LocP28, PMS03, PMS14, PMS15, SHPL08, SR05, SR09 and SR13, as germination has not yet begun.

Development of *V. microcarpum* mapping populations

V. microcarpum plants flowered and were self-pollinated, producing a total of 465 fruit. From those, we obtained a total of 1426 seeds, and a total of 1059 germinated and were successfully transferred to soil (Table 3, Fig. 5-6).

Fig.5 *Vaccinium microcarpum* self-fruits

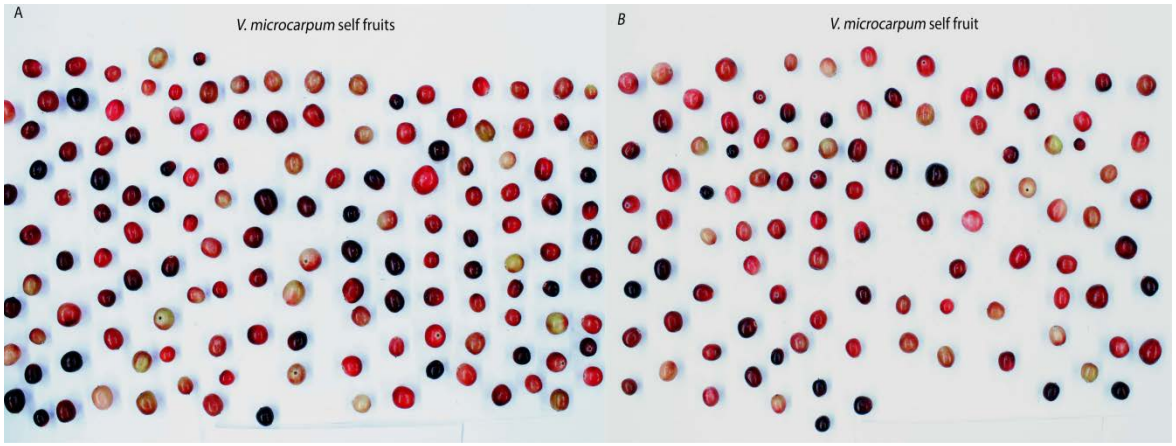


Fig.6 *Vaccinium microcarpum* F1 self-individuals

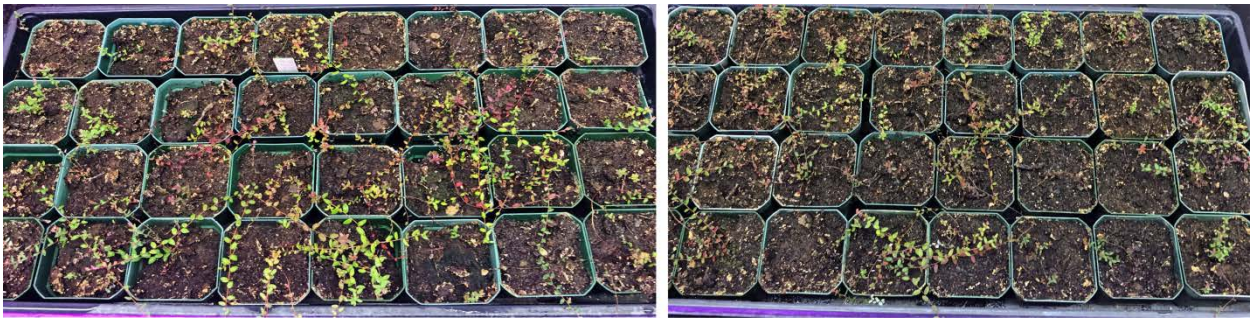


Table 3. *Vaccinium microcarpum* self-fruits

Mother	Number of fruit harvested	Number of seeds collected	Mean number of seeds per small fruit	Mean number of seeds per large fruit	Number of germinations
V. microcarpum	465	1426	2.8	3.9	1059

Discussion

Species in the genus *Vaccinium* are becoming more popular around the world. The increased interest is also increasing the demand for new products. Therefore, there is a need to breed

new and improved varieties. Historically, crosses between species of the section *Cyanococcus*, which houses all blueberries, have been successful. This is in part due to the presence of 2n gametes in the diploid parents (Ortiz *et al.* 1991). However, there has not been any documented successful *V. macrocarpon* x blueberry crosses to date. *V. oxycoccos* x blueberry have been successfully developed before (Covarrubias-Pazaran, 2016), but plants suffered from outbreeding depression and died (Wiesman, personal communication, Lynch 1991; Covarrubias-Pazaran 2016). A considerable number of our crosses among *Vaccinium* species, including *V. darrowii* (2n=2x), *V. corymbosum* (2n=2x, (2)2n=4x), *V. stamineum* (2n=2x), *V. crassifolium* (2n=2x), and *V. elliottii* (2n=2x) with *V. macrocarpon* and *V. oxycoccos* were compatible and resulted in multiple viable seeds and seedlings.

Development of interspecific hybrids

To date, several interspecific hybrids have been developed between blueberry species. Crosses between *V. macrocarpon* and *V. microcarpum* have also been successful and further used as a bridge to cross with *V. darrowii* (Vorsa *et al.* 2008). As mentioned, there are no documented *V. macrocarpon* x blueberry successful hybrids (Covarrubias-Pazaran, 2016). We observed that our crosses yielded mostly non-viable seeds (Table 1). From a total of 92 viable seeds only 10 germinated and 8 were successfully transferred to soil (Fig. 3-7). Plants growing in soil exhibit mostly a *V. macrocarpon* phenotype. However, individuals BL05 and WC12-280 show differences in leaf morphology, instead of having *V. macrocarpon* oval shape they exhibit a more orbicular shape (Fig. 7).

Fig. 7 Examples of the leaf morphology in potential *Vaccinium macrocarpon* x blueberry hybrid

Our *V. oxycoccos* x blueberry crosses were highly successful in comparison to the *V. macrocarpon* crosses. We observed high germination rates, with 49 already successfully transferred to soil. For the plants growing in soil, at early stages, we were able to observe a phenotype not present in *V. oxycoccos*. All of the offspring of the *V. oxycoccos* x blueberry crosses have trichomes surrounding the leaf margin (Fig. 4). Although trichomes are not found on *V. oxycoccos*, they are present in two of the blueberries included in the pollen mix (*V. elliottii* and a *V. corymbosum* 4x with *V. elliottii* in its background) (Figure 8).

In our assessment of the cross-transferability of SSR markers among species of *Vaccinium*, we identified multiple markers (and their genetic fingerprint) that transferred among the species used in these crosses (Chapter 1). Once plants are more mature, leaf material will be used to extract DNA and perform genetic fingerprinting to accurately identify the parents.

Fig. 8 Leaf margin of potential blueberry parents of *Vaccinium oxycoccos* x blueberry offspring

Development of *V. microcarpum* mapping populations

We successfully developed an F_1 self-population of 1095 *V. microcarpum* individuals (Fig. 5). Such high numbers were expected due to the tendency of species in the section *Oxycoccos* to self-pollinate (Fröborg *et al.* 1996). The amount of individuals obtained will allow us to develop a linkage map using the SSR markers that were successfully transferred between *V. macrocarpon* and *V. microcarpum* (Chapter 1). Linkage maps have been used in comparative genetic studies to understand the evolutionary and genetic relationships among species. Schlautman *et al.* 2018 constructed a *V. macrocarpon* and *V. darrowii* x *V. corymbosum* map and observed high synteny and collinearity in the maps. These results suggested that the species are very similar and have not undergone significant divergence. In 2019, Diaz-Garcia

found identical organelles between *V. macrocarpon* and *V. microcarpum*, suggesting a closer evolutionary relationship than previously thought for the sister species. Because of this, we are interested in constructing a linkage map that could allow us to better explore and understand the genetic relationships and evolution of *V. macrocarpon* and its wild relatives.

Conclusions

The increase in the demand for *Vaccinium* products is pushing breeders to explore new breeding combinations. Here, we presented results from intersectional crosses made with *V. macrocarpon*, *V. oxycoccos*, and several blueberry species. We successfully crossed, germinate, and grew various putative hybrids of both *V. macrocarpon* x blueberry and *V. oxycoccos* x blueberry. Future work includes using markers transferable among species for paternity testing.

References

Ballington JR (2009) The role of interspecific hybridization in blueberry improvement. Acta Hortic 810:49–60. doi: 10.17660/ActaHortic.2009.810.2

Pazaran, Giovanny Eduardo Covarrubias. "Breeding the American Cranberry (Vaccinium macrocarpon Ait.) with Quantitative Genetic Tools." The University of Wisconsin - Madison, 2016. Ann Arbor: ProQuest. Web. 5 May 2019.

Darrow GM, Camp WH (1945) Vaccinium Hybrids and the Development of New Horticultural Material. Bull Torrey Bot Club 72:1–21

Darrow GM, Dermen H, Scott DH (1949) A TETRAPLOID BLUEBERRY: From a Cross of Diploid and Hexaploid Species. J Hered 40:304–306. doi: 10.1093/oxfordjournals.jhered.a105965

Diaz-Garcia L, Rodriguez-Bonilla L, Rohde J, et al (2019) Pacbio Sequencing Reveals Identical Organelle Genomes between American Cranberry (Vaccinium macrocarpon Ait.) and a Wild Relative. Genes 10.: doi: 10.3390/genes10040291

Dwivedi SL, Upadhyaya HD, Stalker HT, et al (2008) Enhancing crop gene pools with beneficial traits using wild relatives. Plant Breed Rev 30:179

Fröborg H (1996) Pollination and seed production in five boreal species of Vaccnium and Andromeda (Ericaceae). Can J Bot 74:1363–1368. doi: 10.1139/b96-165

Galletta GJ, Ballington JR (1996) Blueberries, cranberries and lingonberries. Fruit breeding 2:107

Kuittinen H, de Haan AA, Vogl C, et al (2004) Comparing the linkage maps of the close relatives Arabidopsis lyrata and A. thaliana. Genetics 168:1575–1584. doi: 10.1534/genetics.103.022343

Lynch M (1991) THE GENETIC INTERPRETATION OF INBREEDING DEPRESSION AND OUTBREEDING DEPRESSION. Evolution 45:622–629. doi: 10.1111/j.1558-5646.1991.tb04333.x

Mahy G, Bruederle LP, Connors B, et al (2000) Allozyme evidence for genetic autopolyploidy and high genetic diversity in tetraploid cranberry, Vaccinium oxycoccos (Ericaceae). Am J Bot 87:1882–1889

Ortiz R, Bruederle LP, Laverty T, Vorsa N (1991) The origin of polyploids via 2n gametes in Vaccinium section Cyanococcus. Euphytica 61:241–246. doi: 10.1007/BF00039664

Schlautman B., L. Diaz-Garcia, G. Covarrubias-Pazaran, N. Schlautman, N. Vorsa, J. Polashock, E. Ogden, A. Brown, Y-C Lin, N. Bassil, E. Buck, C. Wiedow, S. McCallum, J. Graham, M. Iorizzo, L. Rowland, J. Zalapa. 2018. Comparative genetic mapping reveals synteny and collinearity between the American cranberry and diploid blueberry genomes. Molecular Breeding 38: 9.

Schlautman B, Diaz-Garcia L, Covarrubias-Pazaran G, et al (2017) Comparative genetic mapping reveals synteny and collinearity between the American cranberry and diploid blueberry genomes. Mol Breed 38:9. doi: 10.1007/s11032-017-0765-y

Smith TW, Walinga C, Wang S, et al (2015) Evaluating the relationship between diploid and tetraploid Vaccinium oxycoccos (Ericaceae) in eastern Canada. Botany 93(10): 623-636 doi: https://doi.org/10.1139/cjb-2014-0223

Sharpe RH, Darrow GM, Others (1960) Breeding blueberries for the Florida climate. Proceedings of the Florida State Horticultural Society, 1959 72:308–311

Vander Kloet SP (1988) The genus Vaccinium in North America. Agriculture Canada

Vander Kloet SP (1983) THE TAXONOMY OF VACCINIUM § OXYCOCCUS. Rhodora 85:1–43

Vorsa N, Johnson-Cicalese J, Polashock J (2008) A blueberry by cranberry hybrid derived from a Vaccinium darrowii×(V. macrocarpon× V. oxycoccos) intersectional cross. In: IX International Vaccinium Symposium 810. actahort.org, pp 187–190

General Conclusion

The genus *Vaccinium* has a complex evolutionary history that is yet to be understood. Many *Vaccinium* species produce edible berries, with cranberries being one the most popular commercially produced. *V. macrocarpon* was a staple in Native American diets and passed on to colonizers in the early 1600s (Eck 1990). The first selections were made in Massachusetts in the early 1800s, and cultivation has grown ever since giving rise to an important America industry. Cranberries have become the quintessential and iconic American fruit, its presence demanded for holiday dinners, social gatherings, and in everyday life. Cranberries contain multiple biochemical compounds such as vitamins and antioxidants, making it a great target for breeding and nutraceutical production (Skrovankova et al. 2015). To supply the production and breeding demands, the development of genetic resources such as sequences (Fajardo et al. 2014; Polashock et al. 2014; Diaz-Garcia et al. 2019), markers (Zhu et al. 2012; Georgi et al. 2012; Schlautman et al. 2017), and maps (Schlautman et al. 2015, 2017; Covarrubias-Pazaran et al. 2016) was crucial. Although all these resources are available to facilitate breeding in cranberries, the availability of germplasm for breeding was narrow. As a result, it was necessary to identify both new sources of germplasm and the genetic diversity of wild populations in order to continue developing breeding programs in Wisconsin.

Our research was the first to identify, collect, genetically analyze, and conserve *ex-situ* a germplasm collection of wild cranberries from Wisconsin and Minnesota. We found a considerable amount of genetic diversity in wild populations of both *V. macrocarpon* and one of its sister species, *V. oxycoccos*. Our results demonstrate the vast diversity present in the wild, especially when compared to cultivars and breeding selections. These resources will allow our

breeding program to grow and test new genetic combinations to accelerate breeding in cranberry.

As one of the few crops native to North America, there is an interest in understanding and protecting wild cranberry resources across the US. In collaboration with the USDA, USFS, and ARS, we embarked on a quest to understand the genetic diversity and population structure of wild populations on cranberries and wild relatives across their native range. Our results demonstrated the magnitude of the genetic diversity present in the wild. The majority of the accessions analyzed are distributed across the US and exhibit unique allelic combinations that could be useful for breeding. One of these *V. macrocarpon* populations found outside of its native range was the Okanogan-Wenatchee population. There are multiple genetically unique locations, and based on their genetic diversity and structure, we provided recommendations for the establishment of certain populations as *in-situ* genetic resources reserves in National Forests.

Understanding the genetic diversity and incorporating germplasm into cranberry breeding programs is one of the first steps to move breeding forward. Additionally, the incorporation of secondary and tertiary genetic pools can create new and interesting combinations while maximizing available resources. We, therefore, used markers developed in *V. macrocarpon*, and tested their transferability among other non-commercial species of the genus *Vaccinium* which exhibit unique and useful traits, as is the case of blueberries. Our results demonstrated the ability of SSR markers to be transferrable among closely related species. We provided a core set of markers shared among all species tested to facilitate genetic diversity

studies and genetic fingerprinting. Having a suitable set of markers that transfer among species can be very useful when breeding with poorly understood species such as *V. elliottii*.

The availability of plant genetic resources and genetic information can help to develop crosses between *V. macrocarpon* x blueberry and *V. oxycoccos* x blueberry to generate intersectional hybrids. Although not always successful, we obtained fruit with viable seeds, and germinated and plant dozens of potential hybrids, the majority of which exhibited intermediate phenotypes. Not only were we able to develop potential hybrids, but we also developed an F_1 selfed population of *V. microcarpum*, another one of *V. macrocarpon* sister species. Both species are closely related, share similar morphological traits and have identical organelles. This population will make linkage mapping possible and could allow us to better understand the genetic and evolutionary relationship between these two closely related diploid species.

Future work

The research presented in this dissertation gives us a basis upon which multiple other research ideas could be built. Some of our recommendations for future work include:

- Expanding the area of collection to historically interesting areas, such as the driftless area located in Wisconsin. This location is one of the few areas left unglaciated in Wisconsin during the Pleistocene and could contain unique genotypes that help us understand migration and evolution of cranberries.
- Exploring locations in the western US to search for new *V. macrocarpon* populations that might exist outside of their native range.

- Sequencing individuals from all over the US to perform comparative genomic analysis to try to elucidate the evolutionary history of species in section *Oxycoccus*.
- Discussing our findings with the USFS and ARS scientists for the designation of locations as *in-situ* reserves.
- Phenotyping all the plants previously collected to detect any interesting morphological traits that could be useful for breeding.
- Germinating more potential hybrid seeds and using the markers analyzed in this dissertation to test for paternity of the offspring.
- Developing a *V. microcarpum* linkage map and performing comparative genetic studies.

References

Covarrubias-Pazaran G, Diaz-Garcia L, Schlautman B, et al (2016) Exploiting genotyping by sequencing to characterize the genomic structure of the American cranberry through high-density linkage mapping. BMC Genomics 17:451. doi: 10.1186/s12864-016-2802-3

Diaz-Garcia L, Rodriguez-Bonilla L, Rohde J, et al (2019) Pacbio Sequencing Reveals Identical Organelle Genomes between American Cranberry (*Vaccinium* macrocarpon Ait.) and a Wild Relative. Genes 10.: doi: 10.3390/genes10040291

Eck P (1990) The American cranberry. Rutgers University Press.

Fajardo D, Schlautman B, Steffan S, Polashock J (2014) The American cranberry mitochondrial genome reveals the presence of selenocysteine (tRNA-Sec and SECIS) insertion machinery in land plants. Gene

Georgi L, Herai RH, Vidal R, et al (2012) Cranberry microsatellite marker development from assembled next-generation genomic sequence. Mol Breed 30:227–237. doi: 10.1007/s11032-011-9613-7

Polashock J, Zelzion E, Fajardo D, et al (2014) The American cranberry: first insights into the whole genome of a species adapted to bog habitat. BMC Plant Biol 14:165. doi: 10.1186/1471-2229-14-165

Schlautman B, Covarrubias-Pazaran G, Diaz-Garcia LA, et al (2015) Development of a high-density cranberry SSR linkage map for comparative genetic analysis and trait detection. Mol Breed 35:177. doi: 10.1007/s11032-015-0367-5

Schlautman B, Diaz-Garcia L, Covarrubias-Pazaran G, et al (2017) Comparative genetic mapping reveals synteny and collinearity between the American cranberry and diploid blueberry genomes. Mol Breed 38:9. doi: 10.1007/s11032-017-0765-y

Skrovankova S, Sumczynski D, Mlcek J, et al (2015) Bioactive Compounds and Antioxidant Activity in Different Types of Berries. Int J Mol Sci 16:24673–24706. doi: 10.3390/ijms161024673

Zhu H, Senalik D, McCown BH, et al (2012) Mining and validation of pyrosequenced simple sequence repeats (SSRs) from American cranberry (*Vaccinium* macrocarpon Ait.). Theor Appl Genet 124:87–96. doi: 10.1007/s00122-011-1689-2