NICHE VARIATION THROUGH POLYPLOID FORMATION IN HIGHBUSH BLUEBERRY (*VACCINIUM CORYMBOSUM*)

By

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A dissertation submitted to the

School or Graduate Studies

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate program in Ecology and Evolution

Written under the direction of

Steven N. Handel

And approved by

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New Brunswick, New Jersey

October 2018

ABSTRACT OF THE DISSERTATION

NICHE VARIATION THROUGH POLYPLOID FORMATION IN HIGHBUSH BLUEBERRY (*VACCINIUM CORYMBOSUM*)

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Dissertation Director:

Steven N. Handel

Chapter 1

Highbush blueberries (*Vaccinium* sect. *Cyanococcus*, family Ericaceae) are an important agricultural crop in the United States, as well as ecologically significant native shrubs in eastern hardwood forests and wetlands. The New York Metropolitan Flora Project has found that nearly all members of the heath family (Ericaceae) in the New York metropolitan area have been decreasing in range size over the past 100 years, with a corresponding decrease in abundance as well (Clemants & Moore, 2005). Additionally, morphological, ecological, and genetic differences within the genus complicate the taxonomy of *Vaccinium*, so the status of highbush blueberry, in particular, is not very clear. Highbush blueberry has recently been considered one species, Vaccinium corymbosum (Flora of North America; Vander Kloet 1980), but the highbush blueberries have been separated into as many as 12 different species over the years (Camp 1945). Some of this taxonomic confusion is due to the mix of diploids, tetraploids, and hexaploids within the genus, all showing different morphological and ecological characteristics (triploids do not generally survive (Vorsa & Ballington 2001). With

estimates of polyploidy occurring in 30-80% of angiosperm species (Soltis & Soltis 2000), this is not an uncommon problem in botany today.

Stebbins (1971) suggested that parental diploid strains are more fit than their tetraploid descendants (WHY). The opposite hypothesis has also been presented, that polyploids may be more fit than diploid parents (Ortiz et al. 1992, Soltis & Soltis 2000) WHY. I hypothesize that tetraploid highbush blueberries will show higher fitness in a wider range of ecological conditions than diploid highbush blueberries.

I am currently investigating differences between native varieties of diploid and tetraploid highbush blueberry to (1) help define variation and establish whether there is gene flow between these two cytotypes; (2) explore ecological tolerances to determine fitness and evolutionary advantages and/or disadvantages of polyploidy; (3) determine the causes of the decrease in range and abundance of the native highbush blueberry (Vaccinium corymbosum); and (4) establish restoration protocols for highbush blueberry that can help preserve, improve, and restore the native populations of this economically and ecologically important native shrub.

Applications of these findings are critical to the ecological restoration community and industry, as appropriate genotypes for improving the nation's natural resources are needed. The concern for appropriate genotypes to use in different ecological settings is an active area of research (Handel et al. 1994, Hufford & Mazer 2003). These investigations will assist in determining the cytotypes most useful for sustainable and resilient restored populations of this important understory species, as well as inform commercial and home growers of the best cytotypes for local conditions. These studies

will add to our understanding of the putative ecological advantages of polyploidy in plants.

Chapter 2

Due to its ubiquitous presence in all green plant lineages, polyploidy (or whole-genome duplication) is being studied as an important factor in ecological and evolutionary trajectories in plants. The highbush blueberry (*Vaccinium corymbosum* L.) species complex contains diploids, tetraploids, and hexaploids, although it has been historically debated whether these cytotypes represent separate species or collectively constitute a single polymorphic taxon. Diploid and tetraploid cytotypes are sympatric in several New Jersey locales, but the ecological effects of mixed ploidy are unknown. Here, we conducted a greenhouse experiment testing whether diploid and tetraploid seedlings from natural populations respond similarly to different levels of soil pH, soil moisture, and additional soil mycorrhizae. Size measurements for stem length, numbers of leaves, and total biomass were recorded prior to the experiment, and then again after 6-7 weeks of treatment exposure.

Plants in the low pH (5.0) soil were significantly larger than those in the high pH (6.8) soil, regardless of ploidy. Under low pH conditions, tetraploids grew slightly larger than diploids, but under high pH conditions, diploids had significantly larger biomass. Neither ploidy nor the addition of mycorrhizae had any effect on plant size, but wet treatments produced plants with longer stems than did dry treatments. Wet treatments also had significantly more mycorrhizal colonization than dry treatments, though there were no significant differences in mycorrhizal

colonization between treatments with liquid ericoid mycorrhizae added and control treatments. Diploids may have an ecological advantage under high soil pH conditions, and therefore may be better suited for restoration purposes in non-acidic soils.

Chapter 3

Polyploidy is a common phenomenon in plants that affects genetic variation and can lead to speciation. *Vaccinium corymbosum* (highbush blueberry) taxonomy is confounded by sympatric populations of diploid and tetraploid cytotypes. Although prezygotic isolating barriers are known to exist, the rate of interploidal mating in sympatric populations is unknown. Here, we use hand-pollinated interploidal crosses and comparisons of fruiting rate, seeds per berry, and seedling emergence rate to test the reproductive consequences of polyploidy in this species. Ploidal levels were confirmed with flow cytometry and crosses were confirmed with PCR analysis using 9 SSR markers. Diploid plants at two field sites in New Jersey were pollinated with tetraploid pollen and tetraploid plants in a greenhouse were pollinated with diploid pollen collected from the field sites. Homoploid crosses were used as controls.

Homoploid crosses produced viable seed, confirming successful pollination techniques both in the field and in the greenhouse. Homoploid crosses had significantly higher average fruiting rate, average seeds per berry, and average seedling emergence rate than heteroploid crosses. Tetraploid homoploid crosses had significantly more average seeds per berry than diploid homoploid crosses.

Pollen ploidy was a significant factor in fruiting rate, seeds per berry, and emergence rate, although maternal ploidy was only significant in seeds per berry. Heteroploid crosses on diploid maternal plants had significantly higher fruiting rate than heteroploid crosses on tetraploid maternal plants, but had significantly lower average number of seeds. Heteroploid crosses were conducted on 734 flowers, 42 berries were produced, and three seedlings developed from these crosses, two of which were triploid, and one tetraploid. Due to a high rate of unreduced gamete production in diploid individuals, heteroploid crosses produce fruit with viable seed that contribute to the population structure and gene flow of this species complex.

ACKNOWLEDGEMENTS

Chapter 1

The authors thank Dr. Nicholi Vorsa and the Philip E. Marucci Center for Blueberry and Cranberry Research and Extension at Rutgers University for providing invaluable information, resources, and access to the field site; Steven Henry and Michael Horne, and the Great Swamp National Wildlife Refuge for information and access to the field site; Glenn Rogers and the Pinelands Nursery and Supply for providing propagation information and supplies; and Kristine Rogers, Rachel Rodriguez, and Michelle Segovia for their help with fieldwork. The authors also thank two anonymous reviewers and the editors at Botany for the helpful comments to improve the manuscript. LSP was supported by the Bayard Long Award from the Philadelphia Botanical Club, the Rogers McVaugh Graduate Student Research Grant from the American Society of Plant Taxonomists, and a Small Grant for Graduate Student Research in Ecology and Evolution from Rutgers University; SNH was supported by funding from a National Institute of Food and Agriculture Hatch / Multistate grant NJ17205; PES was supported by USDA/NJAES-17160.

This chapter has been published with the following citation:

Poster, L. S., Handel, S. N., and Smouse, P. E. 2017. Corolla size and temporal displacement of flowering times among sympatric diploid and tetraploid highbush blueberry (*Vaccinium corymbosum*). *Botany*, *404*(March), 1–10. doi:10.1139/cjb-2016-0137

Chapter 2

The authors thank the Greenbelt Native Plant Center, and especially Camille Joseph and Catherine Molanphy, for providing greenhouse space and constant plant care and patience as this experiment evolved, failed, repeated, and succeeded. The authors thank Nicholi Vorsa and James Polashock at the Philip E. Marucci Center for Blueberry and Cranberry Research and Extension at Rutgers University for providing information, resources, and access to the field site; Richard Lear, Eric Gehrig, and Scott Meyler at the Jamesburg Park Conservation Area for access to the field site; and Great Swamp National Wildlife Refuge for access to their field site. We thank Michelle Segovia, Rachel Rodriguez, and David Kung for assistance in the field, greenhouse, and microscopy lab. Funding was provided by a Rutgers Teaching Assistant Professional Development Fund grant, the Hutcheson Memorial Forest Summer Research Grant, and the USDA National Institute of Food and Agriculture Hatch/Multistate grant 233357.

Chapter 3

The authors thank the Greenbelt Native Plant Center, and especially Camille Joseph and Catherine Molanphy, for providing greenhouse space and constant plant care and patience. The authors thank the Philip E. Marucci Center for Blueberry and Cranberry Research and Extension at Rutgers University for providing information, resources, and access to the field site, and Great Swamp National Wildlife Refuge for access to their field site. We thank Michelle Segovia and Rachel Rodriguez for assistance in the field and greenhouse. Funding was provided by a Rutgers

Teaching Assistant Professional Development Fund grant, the Hutcheson Memorial Forest Summer Research Grant, and the USDA National Institute of Food and Agriculture Hatch/Multistate grant 233357.

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CHAPTER 1

Corolla size and temporal displacement of flowering times among sympatric diploid and tetraploid highbush blueberry (*Vaccinium corymbosum* L.)[1]

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Abstract

Polyploidy (whole-genome duplication) is common in vascular plants, but the modes of establishment and persistence, as well as the ecological consequences, of polyploidy remain vague. Highbush blueberry (*Vaccinium corymbosum* L.) is an ecologically and economically important understory shrub with an unclear species definition, coexisting in sympatric populations of diploid and tetraploid cytotypes. This study analyzes differences in bloom time between sympatric diploid and tetraploid *V. corymbosum* in natural populations, testing the potential for these cytotypes to interbreed and contributing to the formation and continuity of ploidy-level diversification within this species.

Ploidal level was confirmed through DNA flow cytometry of sympatric plants from two populations in New Jersey, USA. Flower bloom date and corolla size were

recorded over a three-year period. Diploid corollas were 32% smaller than tetraploid corollas, making them easily identifiable in the field. Ploidy accounted for 55-69% of the variation in bloom date, with diploids flowering about one week before tetraploids, and the remaining variation distributed among plants, among branches, and within branches. Notwithstanding these differences, there was modest overlap in flowering time between cytotypes, suggesting that cross-pollination is possible. This contributes evidence to the most current species definition of *V. corymbosum* as a single (mixed ploidy) species.

This chapter has been published with the following citation:

Poster, L. S., Handel, S. N., and Smouse, P. E. 2017. Corolla size and temporal displacement of flowering times among sympatric diploid and tetraploid highbush blueberry (*Vaccinium corymbosum*). *Botany*, *404*(March), 1–10. doi:10.1139/cjb-2016-0137

Introduction

Although previously thought to be an evolutionary dead-end (Stebbins, 1950; Levin, 2002; Mayrose et al. 2011) polyploidy, or whole genome duplication, is now understood to be ubiquitous among angiosperms and found extensively among green plants (Soltis and Soltis, 2000; Husband et al., 2013; Soltis et al., 2014;). Polyploid formation is generally recurrent (Soltis and Soltis, 1993), yet the factors for establishment, persistence, and success of polyploids are still poorly understood and must be evaluated on a case-by-case basis (Stebbins, 1980; Soltis and Soltis,

2009; Visger et al. 2016). Polyploids can only persist if they can outcompete their diploid progenitors or can occupy new niches, via morphological and physiological changes. These changes in ecological adaption, known as the niche shift hypothesis (Levin, 1975; Husband and Schemske, 2000; Maherali et al. 2009; Ramsey, 2011; Levin, 2011; Glennon et al., 2014), contrast with the traditional null hypothesis of niche conservatism, where polyploids and their diploid progenitors are thought to have similar ecological requirements because they are close relatives (Godsoe et al., 2013; Glennon et al., 2014).

Polyploidy is known to affect plant cell size (Stebbins, 1971; Masterson, 1994), genetic variation (Stebbins, 1950; Husband and Schemske, 1997; Soltis and Soltis, 2000; Levin, 2002; Soltis et al., 2014), and physiology (Stebbins, 1985; Li et al., 1996; Soltis and Soltis, 2000; Levin, 2002; Maherali et al., 2009). It also affects morphology (Giles, 1942; Stebbins, 1950; Vander Kloet, 1980; Stebbins, 1985; Segraves and Thompson, 1999; Levin, 2002), phenology (Garbutt and Bazzaz, 1983; Segraves and Thompson, 1999; Husband and Schemske, 2000; Thompson and Merg, 2008), and geographic distribution (Giles, 1942; Fowler and Levin, 1984; Stebbins, 1985; Thompson and Lumaret, 1992; Levin, 2002; Treier et al, 2009; Levin, 2011). Any combination of those effects might contribute to the ubiquity and success of polyploids in nature.

Some studies have shown that such changes have pushed polyploids into different ecological niches than their diploid progenitors, supporting the niche shift hypothesis (Giles, 1942; Treier et al., 2009; Levin, 2011; Visger et al., 2016). However, other recent studies have shown no shift in ecological niche between

diploids and their polyploid counterparts (Godsoe et al., 2013; Glennon et al., 2014.) Many polyploid lines are thought to become extinct during the initial establishment phase (Stebbins, 1985; Levin, 2002; Mayrose et al. 2011; Godsoe et al. 2013). Levin (1975) offered a minority cytotype exclusion theory (henceforth, MCE), the central feature being that interbreeding between common diploids and rare polyploids produces infertile inter-cytotype hybrids; thus, diploid pollen swamping would impede the establishment of the minority (polyploidy) cytotype.

Mechanisms that limit inter-cytotype matings, such as shifts in flowering phenology (Segraves and Thompson, 1999; Husband and Schemske, 2000; Godsoe et al. 2013), changes in floral traits that impact pollination and pollinator preference (Segraves and Thompson, 1999; Husband and Schemske, 2000), or spatial segregation of cytotypes (Fowler and Levin, 1984; Thompson and Lumaret, 1992; Ramsey, 2011; Visger et al. 2016), could potentially contribute to polyploid establishment and success. If polyploids can escape MCE by mating within their own niche, separated from diploid gametes, genome-doubling events can lead to speciation events, either instantaneously or over time via natural selection (Segraves and Thompson, 1999; Maherali et al., 2009; Ramsey, 2011).

This study examines pre-zygotic mating barriers in natural sympatric populations of diploid and tetraploid highbush blueberries (*Vaccinium corymbosum* L., family Ericaceae). These polyploids have avoided MCE and established themselves as persistent populations by means other than spatial segregation. We specifically focus on floral traits and flowering phenology and how these affect sterile inter-ploidal mating.

Blueberries are an important agricultural crop worldwide (Eck and Childers, 1966; Eck, 1988), but they are also ecologically important native shrubs in habitats along the eastern and southern coast of the United States, inland to Texas and Wisconsin, and as far north as Ontario and eastern Canada. Morphological, ecological, and genetic differences within the genus complicate the taxonomy of *Vaccinium*, and the status of highbush blueberry, in particular, is unclear. Highbush blueberry has most recently been considered one polymorphic species, *Vaccinium corymbosum* L. (Vander Kloet, 1980; eFloras, 2008) containing diploids, tetraploids, and hexaploids, but the complex has been separated into as many as 12 different species over the years (Camp, 1945). Triploids and pentaploids are generally inviable (Vorsa and Ballington, 1991).

Vander Kloet (1980) reported no gross morphological characters that would separate these cytotypic gene pools, but anecdotal information provided by blueberry researchers and growers suggest that subtle distinctions do exist (Nicholi Vorsa, personal communication; Gary Pavlis, personal communication). In particular, tetraploid highbush blueberry flowers are larger than diploid flowers and tetraploids bloom later than diploids. Additionally, all cultivated varieties of *V. corymbosum* are tetraploid, producing larger and more abundant fruit (Ortiz et al., 1992a). There have been no formal studies of ploidal level differences associated with ecology, morphology, or phenology, either in the greenhouse or in the wild. If there are, in fact, differences among the cytotypes that strongly affect gene flow, then taxonomic separation may yet be in order.

If Vander Kloet's (1980) classification of *V. corymbosum* as a single, polymorphic species is apt, however, there must be at least episodic gene flow among (2x, 4x and 6x) cytotypes. Ortiz et al. (1992b) found that a substantial amount of unreduced (2n) pollen was produced by diploid *Vaccinium* spp. If tetraploids are produced by sexual means, i.e., by combining unreduced (2n) diploid gametes, these (2n) gametes might well continue providing (unidirectional) gene flow from the diploid into the tetraploid population. In seven diploid species examined, an average of 13.5% of individuals produced unreduced pollen grains, and as much as 30% of the pollen produced by diploid individuals was itself diploid (Ortiz et al., 1992a), suggesting that there might be at least a modicum of gene flow between sympatric diploid and tetraploid individuals.

Here, we will test: (1) whether corolla size varies between cytotypes, which could influence pollinator preference; (2) whether there is a phenological offset in bloom time between sympatric diploid and tetraploid highbush blueberries in New Jersey populations; and (3) whether there is sufficient overlap in bloom time to allow for genetic exchange between sympatric cytotypes. This information will help define the *Vaccinium corymbosum* species complex, provide some insight into the likely future of this important species, and will contribute to our understanding of polyploid establishment and persistence in general.

Methods

Vaccinium corymbosum L. (Ericaceae) is a perennial, crown-forming, multi-stemmed shrub (2-4 meters tall) with leaves that are deciduous, alternate, simple,

elliptic or ovate, 3.8 -8.2 cm long, glaborous or glaucous beneath, with margins that are entire, subserrate, or sharply serrate. The corolla is urn-shaped and white, and berries are dull black or blue, 4-12mm in diameter. Flowering occurs between February and June, while fruiting occurs between April and October, depending on the location. The most common native habitats are in open swamps and bogs of moderate to high acidity, around marshes, swamps, and lakes, pine barrens, upland woods and ravines, and pine-oak woods. The species complex is widespread in eastern North America, ranging from Nova Scotia to Ontario, Maine to Wisconsin, and south to Texas and Florida (Vander Kloet, 1980; USDA Plants Database, 2016). Diploids (2n = 2x = 24), tetraploids (2n = 4x = 48), and hexaploids (2n = 6x = 72) occur sympatrically throughout the range, although diploids tend to be more common in the southeastern United States, and tetraploids, being more cold tolerant, in the northeastern United States and Canada. Hexaploids are found in a few isolated populations on mountain summits along the Blue Ridge in the southeastern United States (Vander Kloet, 1980). *Vaccinium corymbosum* is mainly bee-pollinated, with seeds that are widely dispersed by birds and mammals. Highbush blueberry is the main blueberry used for cultivation, which began 100 years ago in New Jersey (Coville, 1910; Coville 1916). Blueberries are now extensively cultivated in New Jersey, Michigan, North Carolina, Florida, Massachusetts, and Washington (USDA Plants Database, 2016).

First year of study – In April 2013, branch samples were collected from wild diploid and tetraploid *Vaccinium corymbosum* plants, growing in a mixed population along the perimeter of the Philip E. Marucci Center for Blueberry and Cranberry

Research and Extension, a substation of the New Jersey Agricultural Experiment Station of Rutgers University in Chatsworth, Burlington County, New Jersey (39.71543°N, -74.51046°W). Ploidy was initially estimated using morphological floristic characters (larger corollas were considered polyploids). Three branches from each plant were cut and immediately put into buckets of water. Branch samples were selected based on having at least 10 flower buds, which were generally found in clusters of 4-5 (rarely 2-10) flowers each. The branches were immediately transported to a local greenhouse and kept outside in full sun in buckets of water.

Eight diploid and eight tetraploid plants were studied. Ten flower buds were marked on each of the three branches per plant, and bloom date was recorded for each flower. We selected the first 10 flower buds from the tip on the branch below the leader branch. The number of open flowers was recorded 2-3 times per week until all flowers had opened and senesced. Flowers were considered "open" when the tips of the corolla lobes were recurved. We randomly selected five diploid branches and seven tetraploid branches, each from a different plant. The first five open flowers closest to the base of the branch were measured and recorded for corolla size.

Second and third years of study – A natural population of mixed diploid and tetraploid *V. corymbosum* was flagged at the Great Swamp National Wildlife Refuge (Basking Ridge, Morris County, New Jersey; 40.70421°N, -74.49526°W) in the spring of 2013. This site has not been disturbed for at least the past 100 years. Twenty diploid plants and twenty tetraploid plants were used in this part of the study.

Voucher specimens were deposited at the Chrysler Herbarium of Rutgers University (Poster, s.n., 7 May 2014; CHRB145201 – CHRB145208).

In April 2014 and 2015, flower buds were marked on each plant, following the same protocol as used for the 2013 study, except that the branches were not cut off, and only two branches per plant were marked. The number of open flowers was recorded 2-3 times per week until all flowers had opened and senesced. Duration of bloom time for each flower was also recorded. Flowers were considered to be finished blooming when their corollas abscised. The same plants were used in both 2014 and 2015, but different branches and clusters of buds were used because almost none of the branches used in 2014 had flower buds on them in 2015.

Ploidy analysis – Ploidal levels were confirmed through DNA flow cytometry (Costich et al., 1993) performed by Plant Cytometry Services (Laageinde 6, 4016 CV Kapel Avezaath Buren, The Netherlands). Young leaf samples of at least 3cm^2 in total area were collected from at least 2 different branches on each plant used in the study (57 plants from the Great Swamp site and 27 plants from the Marucci site; not all plants analyzed for ploidal level were used in the final analysis).

Leaf material was chopped with a sharp razor blade in an ice-cold DNA (DAPI) buffer, containing Cystain UV precise P from Partec with 0.1% DTT (Dithiothreitol) and 1% PVP 10, in a plastic petri dish. When an internal standard was used, fresh material of the standard was chopped together with the other leaf material. After chopping, the solution was passed through a 50 μm nylon mesh filter to isolate the nuclei. The stained nuclei were sent through the flow cytometer (CyFlow Space with a UV high power LED, Partec GmbH, Otto Hahnstrasse 32, D-

4400 Munster, Germany) where fluorescence was measured by a photomultiplier, converted into voltage pulses, and processed by a computer to yield integral and peak signals (Flomax version 2.8, Partec).

Statistical analysis – The Welch *t*-test was used to compare diploid and tetraploid averages of corolla length and width. We used ANOVA to determine variation in bloom date within diploids and within tetraploids, apportioning the variance among contributing factors. We estimated the variation among flowers (V_{AF}), among branches (V_{AB}), and among plants (V_{AP}) for each of the cytotypes, as well as among cytotypes (V_{AC}), starting from a matrix of pairwise flowering time differences among all pairs of flowers in the sample, using the AMOVA Routine in *GenAlEx 6.503* (http://biology.anu.edu.au/GenAlEx/); Peakall and Smouse, 2006, 2012). This program is typically used for analysis of molecular variance of genetic markers, but any character set presented as a matrix of squared inter-pair distances (as computed from our flowering time dates here) can be used. We used a non-parametric (permutational) analogue of Bartlett's test (Snedecor and Cochran, 1989) of variance homogeneity to compare flowering-time variances of diploid and tetraploid plants, separately for the collections of 2013, 2014, and 2015.

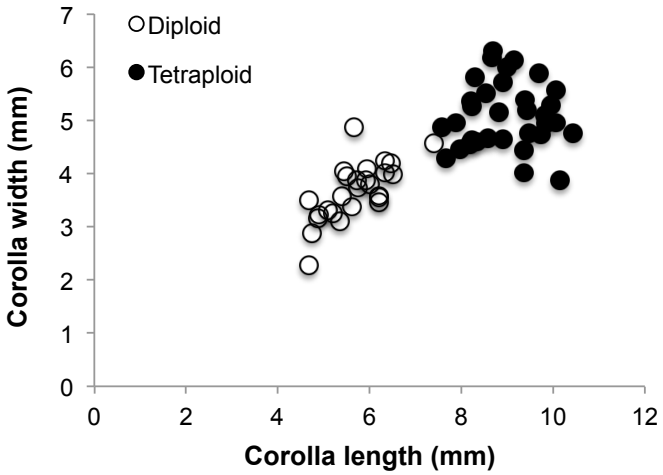
Results

Floral morphology – Combining the two field sites, a total of 45 diploid plants and 41 tetraploid plants were marked. Based on a random sample of flowers (26 diploid and 33 tetraploid), we found that diploid flowers had a corolla length ranging from 4.7 mm – 7.4 mm (mean = 5.7 mm ± 0.13 mm) and a corolla width of

2.3 mm – 4.9 mm (mean = 3.7mm ± 0.11 mm), and tetraploid flowers had a corolla length ranging from 7.6 mm – 10.4 mm (mean = 9.0 mm ± 0.14 mm) and a corolla width of 3.9 mm – 6.3 mm (mean = 5.1 mm ± 0.11 mm) (Fig. 1 and 2). Welch *t*-tests of corolla length and widths differences between diploids and tetraploids were significant ($P < 0.0001$). Of the 86 plants we classified based on corolla size, only one was identified incorrectly according to DNA flow cytometry.

Fig. 1. A. Photo of diploid flowers (left) and tetraploid flowers (right). B. Photo of diploid flowering branch (left) and tetraploid flowering branch (right).

Fig. 2. Corolla size of diploid and tetraploid *Vaccinium corymbosum*. Tetraploid flowers (dark circles) are generally larger than diploid flowers (open circles), which may affect pollinator preference and a niche shift between the cytotypes.

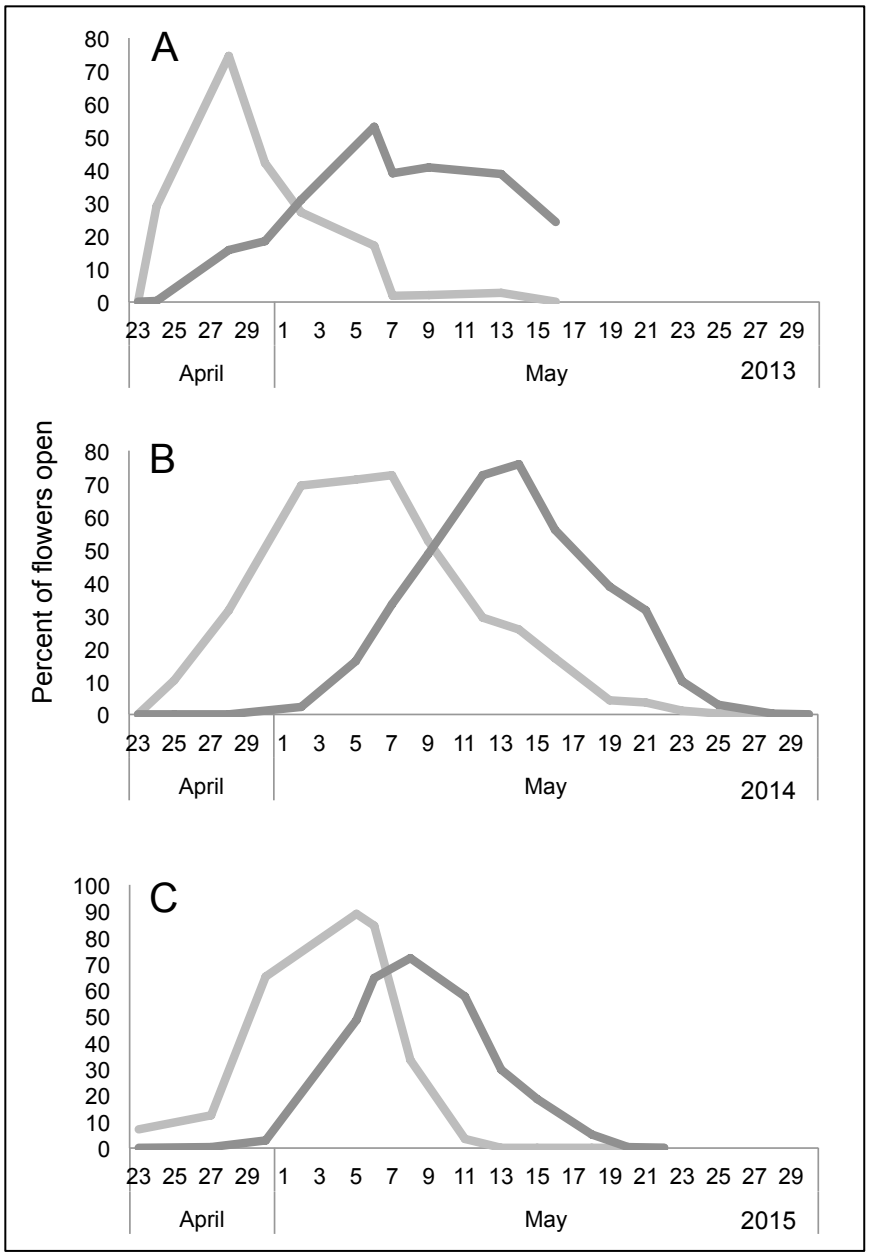


Floral phenology – In all three years, diploid flowers began blooming between 23 April and 25 April; peak diploid bloom was between 28 April and 7 May; and final dates of bloom ranged from 11 May to 25 May (Fig. 3). Tetraploid flowers first bloomed between 24 April and 2 May; peak bloom was between 6 May and 20 May; and final bloom was between 16 May and 28 May. In 2013, there was an 8-day lag between peak diploid and peak tetraploid bloom dates. In 2014, there was a 7-day lag. In 2015, there was only a 3-day lag between peak diploid and peak tetraploid bloom dates, but we may have missed the actual peak bloom for the diploids (there was a 5-day gap between sampling dates, 30 April to 5 May, due to logistical and weather constraints; see "2015 2x" in Fig. 3).

Peak bloom dates were about one week later in 2014 than in 2013 (Fig. 3). This was most likely due to an extremely cold winter in 2014 lasting well into the spring season. Both diploid and tetraploid flowers also stayed open for the longest

period of time in 2014 (30 days for diploids and 26 days for tetraploids, versus 19 and 22 days, respectively, in 2013, and 18 and 23 days, respectively, in 2015, Fig. 3). This probably reflects the long winter and subsequent cooler spring in 2014. Peak bloom dates in 2015 fell in between the peak bloom dates of 2013 and 2014. While there was yearly climatic variation, the patterns for all three years were similar.

Fig. 3. Temporal differences in bloom date of sympatric 2x and 4x *Vaccinium corymbosum*. Percent of total flowers in each cytotype open on a given date. Tetraploid plants have a peak bloom date that is about one week later than diploid plants. This niche shift may help the tetraploids avoid minority cytotype exclusion and become an established population by flowering when there is more tetraploid pollen available, and less diploid pollen, since triploids are mostly inviable. Light gray lines = diploid; dark grey lines = tetraploid. (A) 2013. (B) 2014. (C) 2015.



ANOVA showed, for both diploids and tetraploids, that temporal variation among flowers on the same plant accounted for 41-61% of the total variation within diploids and tetraploids across all three years (Fig. 4). Among branch variation accounted for 8-20% of the total variation, and among plant variation accounted for 23-42% of the total variation. The distribution of variation within diploids and within tetraploids was similar in all three years (Fig. 4).

Fig. 4. Percentage of total variation within diploid and within tetraploid *Vaccinium corymbosum* bloom time in New Jersey, USA. Diploids and tetraploids show a similar distribution of variation among themselves and among years. White = Among flower variation (V_{AF}); Light gray = Among branch variation (V_{AB}); Dark grey = Among plant variation (V_{AP}). (See Table 1 for ANOVA analysis.)

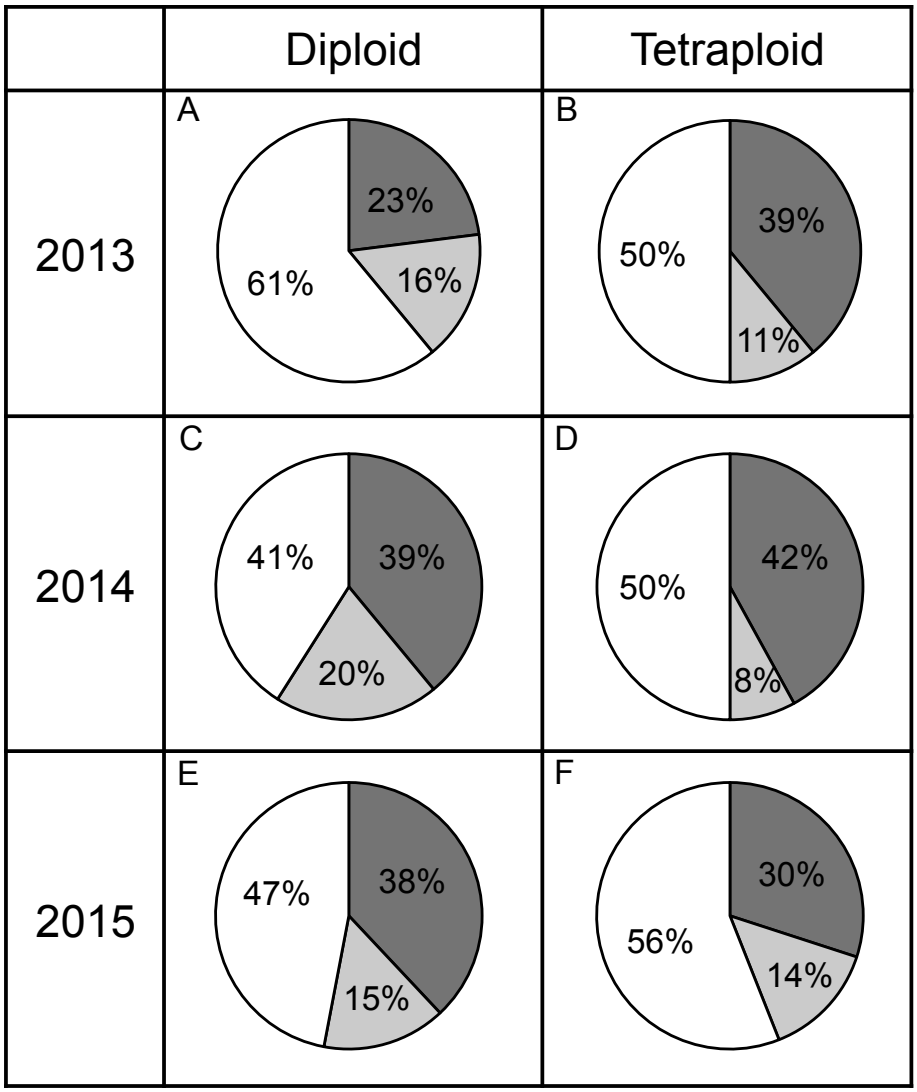


Table 1. ANOVA results of bloom time within diploid and tetraploid *Vaccinium corymbosum* bloom dates. V_{AP} = Variation among plants; V_{AB} = Variation among branches, within plants; V_{AF} = Variation among flowers, within branches.

Year	Ploidy	Source of Variation	Degrees of Freedom	Sum of Squares (SS)	Mean Squares (MS)	Estimated Variance
2013	2x	V_{AP}	7	575.183	82.169	2.06
		V_{AB}	16	326.667	20.417	1.48
		V_{AF}	216	1206.800	5.587	5.59
		Total	239	2108.650	--	9.13 = V_{2n}
	4x	V_{AP}	7	3043.063	434.723	12.72
		V_{AB}	16	851.533	53.221	3.71
		V_{AF}	216	3482.300	16.122	16.12
		Total	239	7376.896	--	32.55 = V_{4n}
2014	2x	V_{AP}	19	2981.748	156.934	5.98
		V_{AB}	20	747.350	37.368	3.11
		V_{AF}	360	2250.500	6.251	6.25
		Total	399	5979.598	--	15.34 = V_{2n}
	4x	V_{AP}	19	3006.248	158.224	6.85
		V_{AB}	20	426.150	21.308	1.33
		V_{AF}	360	2880.700	8.002	8.00
		Total	399	6313.098	--	16.18 = V_{4n}
2015	2x	V_{AP}	19	2184.988	114.999	4.54
		V_{AB}	20	485.850	24.293	1.87
		V_{AF}	360	2017.100	5.603	5.60
		Total	399	4687.938	--	12.00 = V_{2n}
	4x	V_{AP}	19	1142.148	60.113	2.28
		V_{AB}	20	290.650	14.533	1.03
		V_{AF}	360	1520.500	4.224	4.22
		Total	399	2953.298	--	7.53 = V_{4n}

Bartlett's test of homogeneity of variance showed that the total variances within diploids and within tetraploids were significantly different ($P < 0.001$) in both 2013 and 2015, but in 2014, the total variances were not significantly different ($P = 0.588$). In 2013, the tetraploids exhibited greater variance than the diploids, but that was reversed in 2015, and in 2014, the variances were similar.

$$\left[\left(\frac{V_{2n}}{V_{4n}}\right)_{2013} = 0.28, P < 0.001\right] < \left[\left(\frac{V_{2n}}{V_{4n}}\right)_{2014} = 0.95, P = 0.588\right] < \left[\left(\frac{V_{2n}}{V_{4n}}\right)_{2015} = 1.59, P < 0.01\right]$$

These results show that the variation in seasonal bloom period was different for the two cytotypes, but the size and the direction of that difference changed profoundly from year to year, despite being in the same location and experiencing the same climate.

When comparing bloom dates within any single year, combining diploids and tetraploids, ploidy/cytotype (V_{AC}) accounted for most of the variation, 55-69% (Fig. 5; Table 2). If sampling of the tetraploids had continued in the first year (2013) until all flowers were finished blooming, the calculated mean would have accounted for more than 55% of the total variance. Variation among plants (V_{AP}), within cytotypes, accounted for 15-20%, among branch variation (V_{AB}) accounted for 12-18%, and among flower variation (V_{AF}) accounted for 4-7% of the total variation. Within each year, the variation was distributed similarly (Fig. 5), as we found in the previous analysis comparing within diploids to within tetraploids (Fig. 4). Year to year estimates of total variation were significantly different (Bartlett's test, $P < 0.001$; Table 2), probably due to yearly climatic variation.

Fig. 5. Percentage of total variation of combined diploid and tetraploid bloom time in New Jersey, USA. All three years show a similar distribution of variance, and the majority of the variation each year is found among cytotypes. White = Among flower variation (V_{AF}); Light grey = Among branch variation (V_{AB}); Medium grey = Among plant variation (V_{AP}); Dark grey = Among cytotype variation (V_{AC}) . (A) 2013. (B) 2014. (C) 2015. (See Table 2 for ANOVA analysis.)

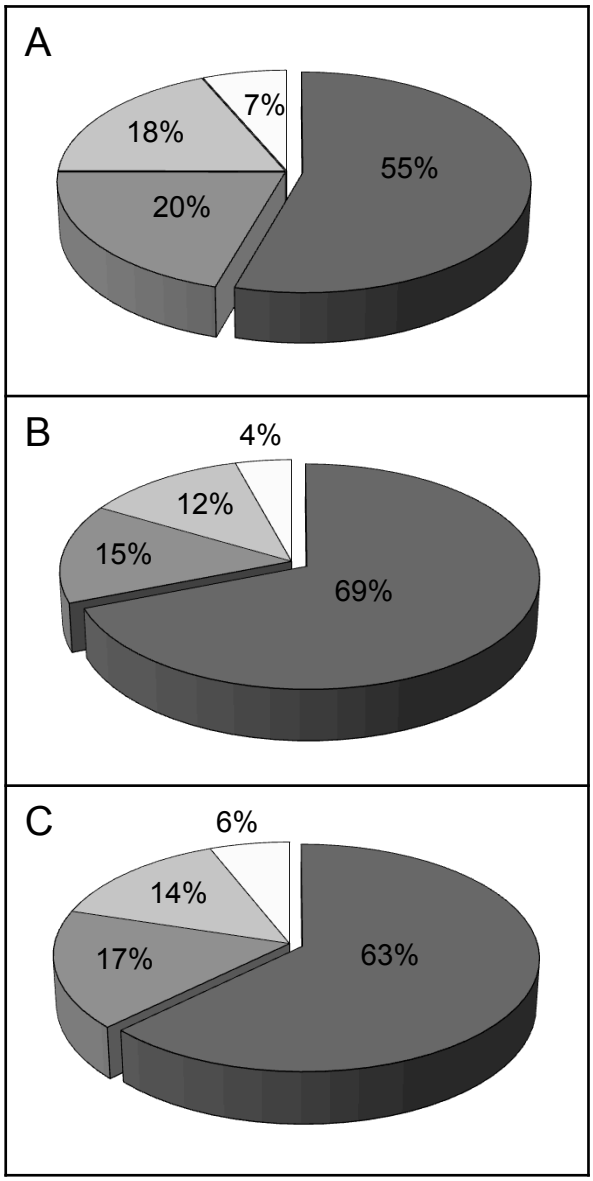


Table 2. ANOVA results of bloom time of combined sympatric diploid and tetraploid *Vaccinium corymbosum* from Figure 5. V_{AC} = Variation among cytotypes; V_{AP} = Variation among plants, within cytotypes; V_{AB} = Variation among branches, within plants; V_{AF} = Variation among flowers, within branches.

Year	Source of Variation	Degrees of Freedom	Sum of Squares (SS)	Mean Squares (MS)	Estimated Variance
2013	V_{AC}	1	5490.727	5490.727	21.80
	V_{AP}	14	3618.246	258.446	8.19
	V_{AB}	32	1178.200	36.819	7.39
	V_{AF}	432	4689.100	10.854	2.60
	Total	479	14976.273	--	39.98
2014	V_{AC}	1	14519.605	14519.605	35.91
	V_{AP}	38	5987.995	157.579	7.46
	V_{AB}	40	1173.500	29.338	6.41
	V_{AF}	720	5131.200	7.127	2.22
	Total	799	26812.300	--	52.00
2015	V_{AC}	1	6127.245	6127.245	15.10
	V_{AP}	38	3327.135	87.556	4.09
	V_{AB}	40	776.500	19.413	3.41
	V_{AF}	720	3537.600	4.913	1.45
	Total	799	13768.480	--	24.05

Discussion

Corolla morphology and ploidy – Significant and consistent differences in corolla size were found between diploid and tetraploid highbush blueberry flowers. Tetraploid flowers were larger overall than diploids flowers. This is consistent with previous studies that found differences in morphological floral traits between polyploids and their diploid progenitors, with tetraploid flowers being larger than diploids (Giles, 1942; Garbutt and Bazzaz, 1983; Segraves and Thompson, 1999; Husband and Schemske, 2000; Maherali et al. 2009). This difference can easily be used to separate diploid and tetraploid *V. corymbosum* visually in mixed populations. Larger organ size is thought to be a direct effect of chromosome doubling, as polyploids generally have larger cell size due to higher DNA content (Stebbins, 1971; Masterson, 1994).

Flower morphology may affect other pre-zygotic isolating factors that limit gene exchange between cytotypes, particularly pollinator preference (Garbutt and Bazzaz, 1983; Segraves and Thompson, 1999; Husband and Schemske, 2000). For example, Blarer et al. (2002) found that larger flowers often contained larger nectar rewards and received more pollinator visits than smaller flowers. Galen and Newport (1987) found that bumblebees visited larger flowers more often, but deposited fewer outcross pollen grains per visit (i.e., lower pollination efficiency) on *Polemonium viscosum* Nutt.. Thompson and Merg (2008) found that some pollinators preferentially visited one cytotype over the other in mixed sympatric populations of diploid and polyploid *Heuchera grossulariifolia* Rydb. Future studies on the ecological impacts of flower morphology, particularly pollinator preference,

would be useful in further assessing gene flow and potential reproductive isolation between sympatric diploid and tetraploid populations of *V. corymbosum*.

Flowering separation and overlap – Despite significantly different levels of phenological variation from year to year, the two cytotypes showed remarkably similar internal distributions of the total variation, within and among plants, within and among branches, for all three years ($V_{AF} > V_{AP} > V_{AB}$, Fig. 4; and $V_{AC} > V_{AP} > V_{AB} > V_{AF}$; Fig. 5). This implies that the phenological process is generally conserved among cytotypes, regardless of chromosome doubling.

Although the 2015 data only showed a 3-day difference in peak bloom date (Fig. 3), we may have missed the peak diploid bloom date that year due to a 5-day gap in sampling. Overall, our data suggest an average difference in peak bloom date between diploids and tetraploids of approximately one week. Tetraploids have also been found to flower approximately one week later than diploids in *Chamerion angustifolium* (L.) Holub (Onagraceae) (Husband and Schemske, 2000; Maherali et al., 2009). However, Segraves and Thompson (1999) found that tetraploids bloomed earlier than diploids in *Heuchera grossulariifolia*. A shift in timing of flowering phenology, whether earlier or later, is nonetheless a shift in niche utilization, which most likely contributes to the success of polyploids in these situations. Yet, the inconsistency of the direction of the shift illustrates the difficulty in predicting physiological and morphological effects of chromosome doubling.

Despite this difference in peak bloom date, we observed modest overlap between diploid and tetraploid flowering dates. Coinciding flowering time, combined with high rates of unreduced gametes (Ortiz et al., 1992a and 1992b),

could provide, at least, an opportunity for genetic exchange between cytotypes. Gene flow, if occurring, would be unidirectional, from diploids to tetraploids via the 2n unreduced gametes produced by the diploid plants. Intercytotype crossings in blueberries performed in the greenhouse have resulted in few viable plants (Ballington and Galletta, 1976, recovered 3 seedlings per 1000 pollinations; Vander Kloet, 1980, recovered 13 plants after four years of crossing trials), however, heteroploid crossings have not been tested in the field. If genetic exchange is occurring consistently across populations, this would certainly support the most recent taxonomic treatment of the highbush blueberries (eFloras, 2008; Vander Kloet, 1980), in which *Vaccinium corymbosum* is treated as one polymorphic species.

Nonetheless, co-flowering is not evidence of cross-breeding. There could be other pre-zygotic and/or post-zygotic mating barriers preventing gene flow between the cytotypes, such as pollinator preference or triploid block (Husband and Schemske, 2000), which could reinforce cytotypic subdivision, and even speciation. The proven overlap in flowering times among sympatric populations of mixed cytotypes found in this study calls for further examination of direct evidence for or against genetic exchange before being able to fully support the single polymorphic species definition of this group.

It is unclear how the cytotypes of *Vaccinium corymbosum* are performing in the wild. Vander Kloet (1980; p. 1191) suggests that, "these gene pools, at the utmost, might be considered species *in statu nascendi.*" We may be at a point in blueberry evolutionary history where these cytotypes are beginning to differentiate themselves into separate species. Although other ecological differences remain to

be documented, the differences found in the reproductive characters studied here support the conclusion that ploidy variation within an interbreeding polyploid series will almost surely influence microevolutionary processes, and possibly lead to speciation events.

These reproductive differences may have applied consequences. Highbush blueberries are commonly used in habitat restoration throughout their range, but the importance of ploidy variation in developing new economic products and populations most useful for ecological restoration initiatives remains vague (Handel et al., 1994; Hufford and Mazer, 2003; Handel et al., 2013.). Planting success is a necessary contractual part of restoration projects, and effects of chromosome doubling, such as phenology and corolla size as it relates to pollinator preference, may play an important role in plant survival. The New York Metropolitan Flora Project has found that nearly all members of the heath family (Ericaceae), including *V. corymbosum*, in its 25 county study area have experienced decreasing range sizes over the past 100 years, with a corresponding decrease in abundance (Clemants and Moore, 2005). More precision in restoration ecology initiatives may counter this regional decline. Knowledge of additional ecological traits between the cytotypes may advance these economic uses.

Acknowledgements

The authors thank Dr. Nicholi Vorsa and the Philip E. Marucci Center for Blueberry and Cranberry Research and Extension at Rutgers University for providing invaluable information, resources, and access to the field site; Steven

Henry and Michael Horne, and the Great Swamp National Wildlife Refuge for information and access to the field site; Glenn Rogers and the Pinelands Nursery and Supply for providing propagation information and supplies; and Kristine Rogers, Rachel Rodriguez, and Michelle Segovia for their help with fieldwork. The authors also thank two anonymous reviewers and the editors at Botany for the helpful comments to improve the manuscript. LSP was supported by the Bayard Long Award from the Philadelphia Botanical Club, the Rogers McVaugh Graduate Student Research Grant from the American Society of Plant Taxonomists, and a Small Grant for Graduate Student Research in Ecology and Evolution from Rutgers University; SNH was supported by funding from a National Institute of Food and Agriculture Hatch / Multistate grant NJ17205; PES was supported by USDA/NJAES-17160.

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CHAPTER 2

Comparative performance of diploid and tetraploid highbush blueberries (*Vaccinium corymbosum* L.) in variable soil and mycorrhizal experimental treatments

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Abstract

Due to its ubiquitous presence in all green plant lineages, polyploidy (or whole-genome duplication) is being studied as an important factor in ecological and evolutionary trajectories in plants. The highbush blueberry (*Vaccinium corymbosum* L.) species complex contains diploids, tetraploids, and hexaploids, although it has been historically debated whether these cytotypes represent separate species or collectively constitute a single polymorphic taxon. Diploid and tetraploid cytotypes are sympatric in several New Jersey locales, but the ecological effects of mixed ploidy are unknown. Here, we conducted a greenhouse experiment testing whether diploid and tetraploid seedlings from natural populations respond similarly to different levels of soil pH, soil moisture, and additional soil mycorrhizae. Size

measurements for stem length, numbers of leaves, and total biomass were recorded prior to the experiment, and then again after 6-7 weeks of treatment exposure. Plants in the low pH (5.0) soil were significantly larger than those in the high pH (6.8) soil, regardless of ploidy. Under low pH conditions, tetraploids grew slightly larger than diploids, but under high pH conditions, diploids had significantly larger biomass. Neither ploidy nor the addition of mycorrhizae had any effect on plant size, but wet treatments produced plants with longer stems than did dry treatments. Wet treatments also had significantly more mycorrhizal colonization than dry treatments, though there were no significant differences in mycorrhizal colonization between treatments with ericoid mycorrhizae added and control treatments. Diploids may have an ecological advantage under high soil pH conditions, and therefore may be better suited for restoration purposes in non-acidic soils.

INTRODUCTION

Modern studies suggest that polyploidy (whole genome duplication) is widespread and occurs in all green plant lineages (Soltis and Soltis 2000; Soltis and Soltis 2009; Li et al. 2016). In the past few decades, as technologies for genetic analysis have become more sophisticated and affordable (Soltis et al. 2014b; Barker et al. 2016), research on the ecological and evolutionary impacts of such polyploidy has become an important and influential area of research. Ecological niche modeling has also helped to understand the impact of polyploidy under natural environmental circumstances (Godsoe et al. 2013; Glennon et al. 2014; Blaine Marchant et al. 2016).

Polyploid formation is generally recurrent (Soltis et al. 2014b) and many polyploid derivatives go extinct during the initial establishment phase (Stebbins 1985; Levin 2002; Mayrose et al. 2011; Godsoe et al. 2013) due to a lack of available gametes for production of fertile offspring (minority cytotype exclusion theory, Levin 1975). In spite of these difficulties, polyploids in myriad lineages have established themselves, flourished, and had profound influences on evolution and diversification (Soltis et al. 2014a). Given the ubiquitous nature of ploidal increase in green plant evolution, it is obvious that other factors must be in play. Newly formed polyploid individuals must either outcompete their diploid progenitors or occupy novel niches through some combination of morphological, reproductive, and/or physiological changes (niche shift hypothesis, Levin 1975, 2011).

If polyploids become separated from their diploid progenitors through altered niche requirements, polyploidy can lead to speciation events either instantaneously or over time via natural selection (Segraves and Thompson 1999; Maherali et al. 2009; Ramsey 2011). Otto and Whitton (2000) found that polyploidization is one of the more prominent mechanisms of sympatric speciation in plants.

The highbush blueberry species complex (*Vaccinium corymbosum* L.) has historically been taxonomically difficult, due to the existence of diploids, tetraploids, and hexaploids (Camp 1945, Vander Kloet 1980, eFloras 2008). Triploids and pentapoids are generally inviable (Vorsa and Ballington 1991). Vander Kloet (1980) reported no gross morphological characters separating the cytotypes, but it has recently been shown that diploids have smaller corolla size and bloom about a week

earlier than tetraploids (Poster et al. 2017). There have been no studies to date of ploidal level differences associated with ecological niche requirements.

Highbush blueberries are ecologically important native understory shrubs, an economically important agricultural crop, and commonly used in ecological restoration to increase urban and suburban biodiversity, provide a food source for wildlife, increase pollinator diversity, and protect wetlands. The species is a facultative wetland species, preferring wet, acidic soils (pH < 5), and forms strong associations with ericoid mycorrhizal fungi (Coville 1910; Eck and Childers 1966; Vander Kloet 1980; Eck 1988; Heckman et al. 2002; Cairney and Meharg 2003; Trehane 2004; Lichvar et al. 2016). Ericaceous plant species (and particularly *Vaccinium* spp.) show elevated plant growth in the presence of ericoid mycorrhizae, due to increased nitrogen and phosphorous uptake (Read and Stribley 1973), increased vigor (Haselwandter 1979), and increased fruit yield (Powell and Bates 1981). These associations allow ericaceous plants to tolerate stressful soil environments, such as wetlands and bogs, that exhibit poor nutrient status, low pH, and poor drainage (Cairney and Meharg 2003).

Many wetland areas in the New York metropolitan area have been lost to urbanization. In New Jersey, 39% of the wetlands found in 1870 had been lost by 1970. Urban and suburban soils are often more basic in pH, drier, and devoid of ericoid mycorrhizae (Pouyat et al 2007; Karpati et al. 2011), relative to undisturbed soils. Due to the presence of alkalizing products, urban soils can often have (7 < pH < 8) in the top 25 cm, and have been found to be more basic (9 < pH) at 110-135 cm depth (De Kimpe and Morel 2000). This loss of moist, acidic habitat and the

depletion of mycorrhizae in soil communities could have caused the dramatic decline in Ericaceous species found throughout the New York metropolitan region by Clemants and Moore (2005).

Here, we focus on greenhouse growth performance in altered levels of soil hydrology, soil pH, and mycorrhizal inoculation, to evaluate whether diploid and tetraploid plants are exploiting different ecological niches in sympatry, and whether one cytotype may be better for ecological restoration of urban and suburban sites. This greenhouse experiment tests the relative performance of (2N) versus (4N) plants: (1) whether soil pH affects seedling growth differently in (2N) and (4N) plants; (2) whether soil hydrology levels affect seedling growth differently in (2N) and (4N) plants; (3) whether adding ericoid mycorrhizae to the soil increases colonization of root cells with hyphae; and (4) whether adding ericoid mycorrhizae to the soil affects seedling growth differently. We compare these growth responses to different ecological stressors in seedlings from sympatric populations of diploid and tetraploid highbush blueberries from New Jersey, USA. This information will not only contribute to our understanding of the *V. corymbosum* species complex, but will also provide valuable information for ecological restoration initiatives, native plant nurseries, and blueberry agriculture.

Methods

Two greenhouse experiments were set up simultaneously, one to measure whether mycorrhizae and hydrology affect growth pattern differences between (2N) versus (4N) plants, and the second to determine whether soil pH affects growth for the same comparison. Both experiments were set up in a complete

randomized block design. Seedlings about two months old were used to test the effects of these factors on early life history stage. Seedlings were grown from wild collected seed from two field sites, Marucci Blueberry Cranberry Research Center (hereafter "MBC"), Chatsworth, Burlington County, New Jersey (39.71543°N, 74.51046°W) and Jamesburg Park Conservation Area (hereafter "JA"), East Brunswick, Middlesex County, New Jersey (40.38876°N, 74.42807°W). Plants at both field sites were classified as either diploid or tetraploid in the spring of 2015 using DNA flow cytometry (Costich et al. 1993) by Plant Cytometry Services (Berkenhof 37, 6941 ZR, Didam, The Netherlands). Diploid and tetraploid mother plants were used from the MBC site, but only tetraploid mother plants were used from the JA site, because not enough diploid seedlings were produced from this site to allow a balanced design for the experiment.

In July and August 2015, berries were collected from marked plants. The berries were refrigerated until 17 August 2015, then all seeds from the berries were sowed into a mix containing equal parts of Fafard® Super-Fine Germinating Mix, peat moss, and sterilized sand. The germinating mix was sterilized using steam above 93°C. The sowed seed trays were covered in plastic and refrigerated (1.6 – 4.4°F). The trays of seeds were removed on 4 January 2016 and placed on greenhouse benches in full sun. On 28 March 2016 (12 weeks later), seedlings were transplanted into individual 8.25 cm^2 pots with 450 ml of air-dried soil, and with treatments described below.

As seedlings were being transplanted, the following size characteristics were measured and recorded: number of stems, total stem length (cm), number of leaves,

height (cm), average diameter (cm), and root length (cm). These initial measurements were used to calculate growth (changes in size) at the end of the experiment.

pH Experiment – Soil pH was measured with a handheld pH meter (Fieldscout SoilStik pH Meter, Spectrum Technologies, Inc., Aurora, IL, USA), using a 1:1 soil:water mixture. A substrate containing equal parts germinating mix, peat moss, and sand was created. Before transplanting seedlings, this mix had measured (pH = 5.0), which is within the range of blueberry preference (pH 4.0 – 5.2; Eck and Childers 1966; Eck; 1988; Trehane 2004). This mix, without amendments, was used as the control ("Low pH") soil. Twenty-five grams of Bakers Broadcast Turf Lime ($CaCO_3$) was added to 15 liters of air-dried base mix and allowed to incubate for 60 days with alternating wet and dry cycles (Heckman et al. 2002). The soil was mixed 2-3 times per week. Final testing resulted in a soil (pH = 6.8), which was then used for the "High pH" soil treatment. This elevated soil pH level is more typical of a soil in urban, suburban, or otherwise disturbed conditions (DeKimpe and Morel 2000).

The experiment was set up in a randomized block design on a greenhouse bench, with 10 replicates for each of the pH treatments (low pH = 5.0; high pH = 6.8) and each of seedling groups (MBC diploid, MBC tetraploid, and JA tetraploid). Greenhouse temperatures ranged from 17.1 - 29.7°C (62.8 – 85.5 °F) with 20% - 63% humidity, and plants were watered as needed on a daily basis. Plants were harvested eight (8) weeks after transplanting. Leaves, stems, and roots were separated, root systems were washed free of adhering soil, and all plant parts were air-dried. Biomass was recorded once all plant parts were completely dry, using an

Ohaus Pioneer scale with a readability of 0.0001g (Ohaus Corp., Pine Brook, NJ, USA).

Mycorrhizae/Hydrology Experiment - A complete factorial randomized block design was set up with the following treatments: (1) "wet" with mycorrhizae; (2) "dry" with mycorrhizae; (3) "wet" without mycorrhizae; and (4) "dry" without mycorrhizae. These four treatments were repeated for MBC diploid seedlings, MBC tetraploid seedlings, and JA tetraploid seedlings, with 10 replicates for each treatment and for each ploidy group. The "wet" watering treatment was maintained at 80% total water capacity of the soil, while the "dry" watering treatment was maintained at 40% total water capacity of the soil. All pots were checked three times per week and water was added by weighing each pot to maintain water capacity treatments.

The associations between ericaceous plant species and ericoid mycorrhizae have been well documented (Coville 1910; Read and Stribley 1973; Haselwandter, K. 1979; Powell and Bates 1981; Cairney and Meharg 2003), thus motivating the addition of ericoid mycorrhizae as an additional treatment condition. Bioradis-Ericoid mycorrhizal solution, purchased from Horticultural Alliance (https://horticulturalalliance.com), is a liquid suspension of hyphal fragments, containing *Oidiodendron* spp., *Rhizoscyphus ericae* (isolate type 1), and *Rhizoscyphus ericae* (isolate type 2) in a semisolid gel solution.

Half of the solution was autoclaved to kill the mycorrhizae, while maintaining nutrients, as a control (the "no mycorrhizae" treatment). It was autoclaved for 30 minutes at 121°C at 21 PSI, and after 24 hours, it was re-autoclaved at the same

settings. After cooling to room temperature, a small amount of the solution was placed on a plate of MEA+LA to check for possible contamination. No growth was recorded after 48 hours on autoclaved media, demonstrating sterilization of the solution.

At the time of transplant, half of the pots were treated with 5 ml of the ericoid mycorrhizal inoculum, applied directly onto the roots before soil was filled in around the base of the plant. The other half of the plants received 5 ml of the autoclaved mycorrhizal inoculum in the same manner. Plants were arranged in a randomized block design on a greenhouse bench. Root samples (10cm in length) were randomly collected from 24 seedlings (8 from each seedling origin: MBC diploid, MBC tetraploid, and JA tetraploid) before mycorrhizal/non-mycorrhizal treatments, to check for initial fungal colonization. There was less than 1% colonization.

Plants were harvested after seven weeks. Leaves, stems, and roots were separated. Leaves and stems were placed into paper bags for air-drying. Root systems were washed free of adhering soil, and 10cm samples were collected from each seedling to measure mycorrhizal colonization. The remaining roots were then also air-dried and biomass was recorded, once all plant parts were completely dry.

Initial and final root samples collected for mycorrhizal analysis were washed clean of adhering soil, packed in plastic bags with damp paper towels, and refrigerated. The roots were subsequently cleared in 10% KOH for 3-4 days at room temperature and then rinsed 3-4 times with water. They were acidified in 1% HCl for a few minutes and then put into Trypan Blue stain for 1-2 days at room

temperature. Roots were then removed from the stain and placed in lactoglycerol and storage. One wet mount was prepared for each sample, using lactoglycerol. Fifty random 20X fields of view of each slide were analyzed for the presence or absence of intracellular hyphal coils.

Statistical Analysis - Data were first tested for homoscedasticity with Levene's test. If heteroscedasticity was non-significant ($P > 0.05$), then analysis of variance was used to gauge differences in mean growth measurements between populations of seedlings. The Tukey test was used to correct *P*-values for multiple comparisons. If the data did not show homogenous variances, then the non-parametric Mann-Whitney/Wilcoxon test was used to determine significant differences in mean growth measurements, as this test does not assume normality. All statistical analyses were performed in R 3.3.1 (2016).

Results

We focused analysis on total stem length, number of leaves, and total biomass of the seedlings, because these are the most ecologically responsive characters of those measured. Other recorded size measurements (seedling height, seedling diameter, and number of stems) were highly correlated with this trio of variables, and were not tested further. To analyze growth results at the conclusion of the experiment, the stem lengths and number of leaves were log-transformed $\Delta = \log(\text{final}/\text{initial})$, providing more homogeneous variances and more symmetrically distributed data. Initial measurements of stem length and number of leaves were log-transformed ($\log = (x)$) for accurate comparison with the final measurements.

Final biomass data were also log-transformed (log = (x)) because initial data were not collected.

Tetraploid plants in this experiment were comprised of seedlings from two different field sites (MBC and JA), but diploids were all from MBC. All statistical tests performed on 2x versus 4x plants were also performed on MBC versus JA tetraploids, to ensure that combining the 4x populations did not obscure the results. The two 4x populations showed a significant difference only once (biomass, see Fig. 3E), but there were no significant differences between either 4x population and the 2x population, so they remained combined for the results presented here (see Appendix 1 for ANOVA results comparing the 4x populations).

Using analysis of variance, neither the log of the initial mean stem length or the number of leaves differed significantly between treatment groups in the pH experiment, nor did they in the Myc/Hyd experiment (Fig. 1; Table 1). *P*-values were corrected for multiple comparisons using the Tukey test. All seedlings (regardless of origin or ploidy level) were similar in size prior to the beginning of the experiment.

Fig. 1. A) Log of initial mean stem length (cm) and B) log of initial mean number of leaves did not differ among seedling origin in the pH experiment (left) or the mycorrhizae / hydrology experiment (right).

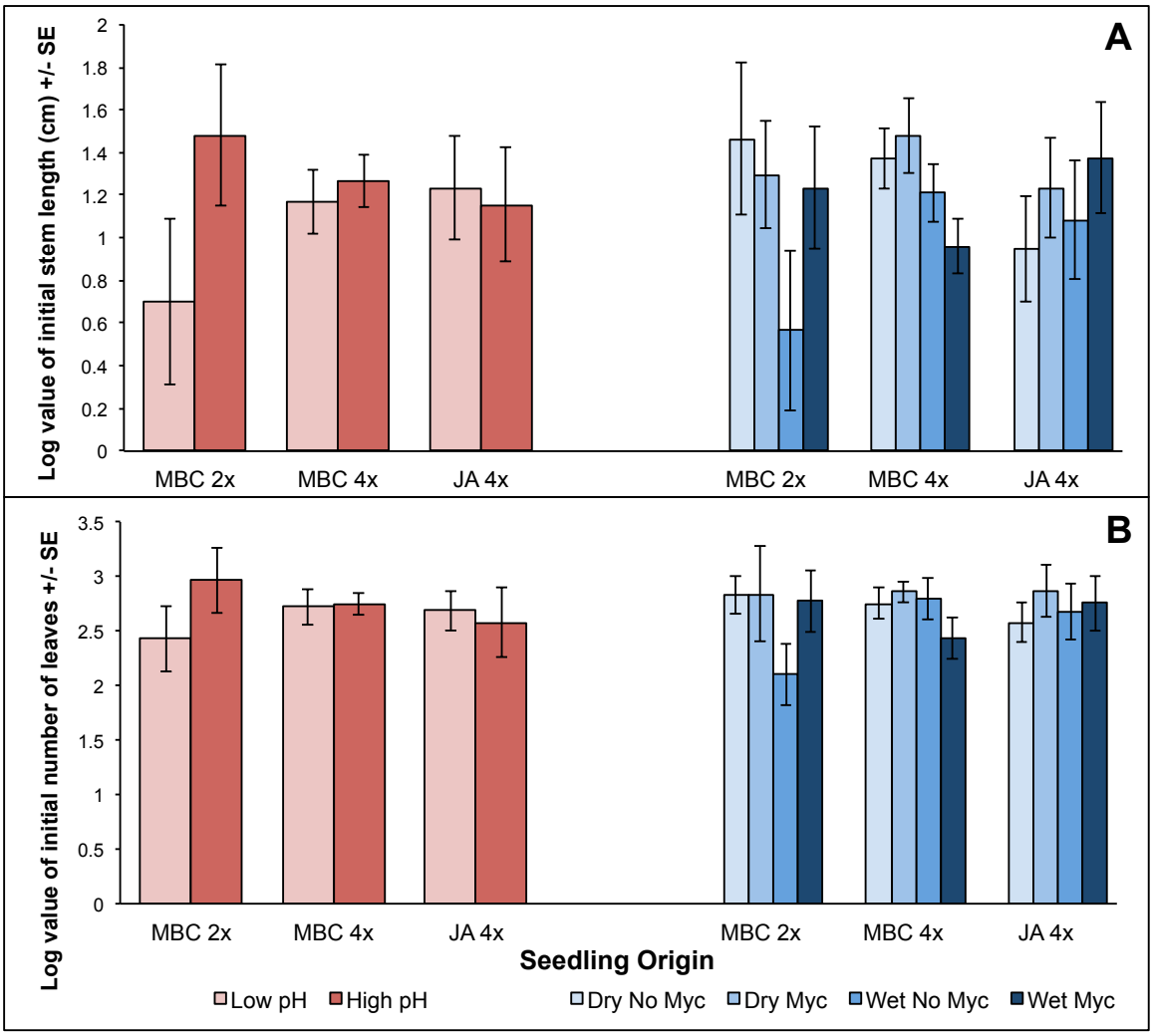


Table 1. ANOVA results of the effects of assigned treatment group on (A) initial stem length (cm) and (B) initial number of leaves in the pH experiment and the Myc/Hyd experiment. All data were log-transformed. Equal numbers of 2x MBC, 4x MBC, and 4x JA seedlings were assigned to each treatment group.

	Experiment	Factor	df	SS	MS	*F*	*P*
(A) Stem Length	pH	Treatment group	5	3.28	0.66	0.92	0.48
		Error	54	38.62	0.72		
	Myc/Hyd	Treatment group	11	7.54	0.69	1.08	0.38
		Error	108	68.28	0.63		
(B) Number of Leaves	pH	Treatment group	5	1.58	0.32	0.54	0.74
		Error	54	31.60	0.59		
	Myc/Hyd	Treatment group	11	5.48	0.50	0.85	0.59
		Error	108	63.09	0.58		

pH Experiment - The final measurements show that plants in the low pH treatments (pH = 5.0) had significantly longer total stem lengths, more leaves, and larger total biomass ($P < 0.001$ for all three), with homogenous variances (Levene's test, $P > 0.05$ for all three) than did plants in the high pH treatments (pH = 6.8) (Fig. 2A). The pH level was the most important factor in determining all of the outcome variables under consideration (Table 2), in agreement with literature, which states that *V. corymbosum*, as well as other Ericaceous species, perform better in acidic (low pH) soils (Coville 1910; Eck and Childers 1966; Vander Kloet 1980; Eck 1988; Heckman et al. 2002; Trehane 2004). Biomass has negative log values because the raw values were less than one.

Fig. 2. **A)** Low pH treatments compared to high pH treatments with all seedlings (diploid and tetraploid) combined. Low pH treatments had significantly higher final

log mean stem length (cm), number of leaves, and total biomass (g). **B)** Diploid performance compared to tetraploid performance with all treatments combined. There were no significant differences. **C)** Low pH final measurements of log stem length (cm), number of leaves, and total biomass (g) for diploids compared to tetraploids. Tetraploid seedlings had longer stem length, higher number of leaves, and larger biomass. **D)** High pH treatment final measurement of raw (not log-transformed, for Mann-Whitney / Wilcoxon test) mean total, stem, leaf, and root biomass (g) for diploids and tetraploids. Diploids had larger mean biomass in all categories, and were significant ($P < 0.05$) for total and stem biomass.

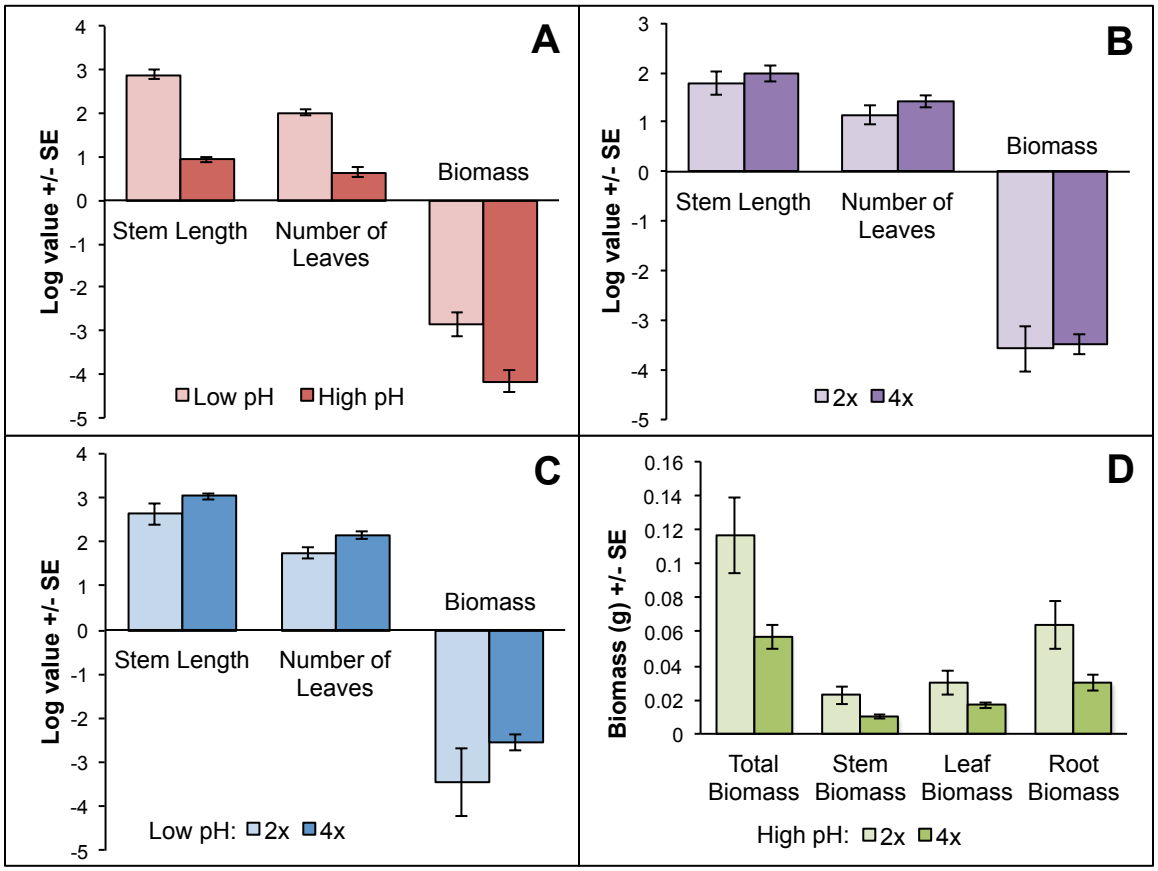


Table 2. ANOVA results for the effects of pH treatment, ploidy, block, and all interactions on stem length (cm), number of leaves, and total biomass (g). Seedling origin was never a significant factor and was therefore excluded from the analysis.

	Factor	**df**	**MS**	***F***	***P***
Stem length (cm)	Ploidy	1	0.51	2.60	0.11
	pH treatment	1	56.85	288.71	< 0.001
	Block	1	0.17	0.87	0.36
	Ploidy × pH treatment	1	0.60	3.03	0.09
	Ploidy × block	1	0.97	4.93	0.03
	pH treatment × block	1	0.05	0.25	0.62
	Error	53	0.20		
Number of leaves	Ploidy	1	0.96	3.55	0.07
	pH treatment	1	28.23	104.42	< 0.001
	Block	1	0.21	0.76	0.39
	Ploidy × pH treatment	1	0.20	0.73	0.40
	Ploidy × block	1	0.63	2.3	0.13
	pH treatment × block	1	0.07	0.25	0.62
	Error	53	0.27		
Total biomass (g)	Ploidy	1	0.12	0.06	0.81
	pH treatment	1	25.80	12.38	< 0.001
	Block	1	1.65	0.79	0.38
	Ploidy × pH treatment	1	8.96	4.30	0.04
	Ploidy × block	1	0.80	0.39	0.54
	pH treatment × block	1	2.63	1.26	0.27
	Error	53	2.09		

With all pH treatments combined, 2x and 4x seedlings did not differ significantly in stem length, number of leaves, or biomass (Fig. 2B). Within the low pH treatments, tetraploids had longer stems (P = 0.06), significantly more leaves (P = 0.02), and greater biomass than did diploids (Fig. 2C), although not significantly so (P = 0.13). Tetraploids have historically been found to be larger and more robust than their diploid progenitors when grown under ideal conditions (Stebbins 1950, 1971; Levin 2002). All pairwise comparisons had homogenous variances (Levene's test, P > 0.05), except biomass for the (2x) vs. (4x) comparison (P = 0.04) and for

stem length of the (MBC 4x) vs. (JA 4x) comparison (P = 0.02). Since the average values were not significantly different, the data were not run in the Mann-Whitney/Wilcoxon non-parametric test.

Because high pH is stressful for *V. corymbosum*, we were interested in whether tetraploids performed better than diploids by virtue of their duplicated genome. There were no significant differences between ploidies for stem length or number of leaves in the high pH treatment. However, the diploid seedlings had significantly larger changes in total biomass by the end of the experiment than did the tetraploid seedlings (P = 0.02; Fig. 2D, Appendix 2). To determine where this performance difference originated, total biomass was analyzed separately for stem, leaf, and root biomass (Fig. 2D). All biomass measurements failed Levene's test for homoscedasticity in raw form, although they showed significant differences between 2x and 4x seedlings. When log-transformed, they passed the Levene's test, but the P-values were no longer significant. (See Appendix 3 for ANOVA results.) In order to further examine the data, the non-parametric Mann-Whitney/Wilcoxon test was used to determine significant differences in mean values, since this test assumes neither normality nor variance homogeneity. In the high pH treatments, diploids were significantly larger than tetraploids in total biomass (P = 0.02) and stem biomass (P = 0.03). For leaf and root biomass, the mean of the diploids was larger than that of the tetraploids, but the difference was not significant (P = 0.13 and P = 0.08, respectively). The two tetraploid populations were not significantly different (P > 0.45 for all; Appendix 1).

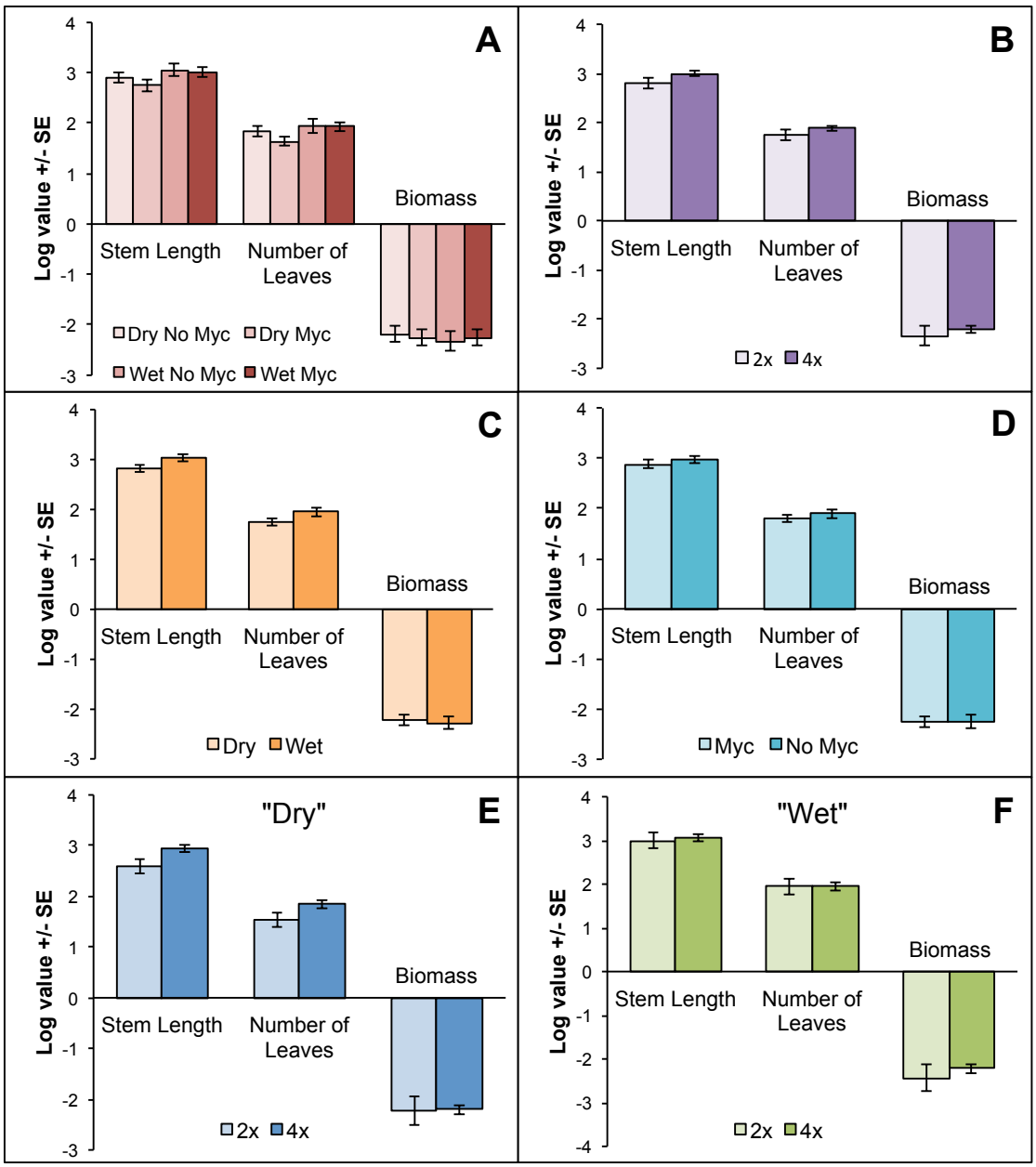
Mycorrhizae/Hydrology Experiment - At the conclusion of the experiment, with measurements from all seedling origins combined, there were no significant differences after correcting for multiple comparisons in stem length, number of leaves, and total biomass (P = 0.19, 0.18, 0.94 respectively) between the treatments in this experiment: (1) dry without mycorrhizae; (2) dry with mycorrhizae; (3) wet without mycorrhizae; (4) wet with mycorrhizae (Fig. 3A). There were also no significant differences in stem length, number of leaves, or total biomass between the ploidies with all treatments combined (P = 0.09, 0.23, 0.49 respectively) (Fig. 3B).

In the final measurements, with all treatments combined, tetraploids had longer stem lengths than did diploid plants, although not significantly so (P = 0.09), and the number of leaves and total biomass did not differ significantly (P = 0.23 and 0.77 respectively; Fig. 3B). Levene's test showed homogenous variances for stem length and number of leaves, when comparing diploids with tetraploids ($P > 0.1$ for both), but total biomass failed the Levene's test for variance homogeneity ($P < 0.001$). Therefore, the non-parametric Mann-Whitney/Wilcoxon test was used to determine the significance of difference between the biomass diploid and tetraploid measurements (P = 0.77).

Blueberries typically prefer hydric soils (Eck 1988), and we found that seedlings in "Wet" treatment groups, where 80% soil moisture was maintained, had larger stem length (P = 0.05) and number of leaves than did seedlings in "Dry" treatment groups with 40% soil moisture, though not significantly so (P = 0.07) (Fig 3C). Total biomass was not significantly different (P = 0.66). There were no

significant differences in the log of mean stem length (P = 0.40), number of leaves (P = 0.36), or total biomass (P = 0.99) between treatments with and without added mycorrhizae (Fig. 3D).

Fig. 3. Mean and standard error (SE) of the log value of final stem length (cm), number of leaves, and total biomass (g) for different comparisons. **(A)** Between all treatment combinations, all ploidies combined, with no significant differences. **(B)** Between diploids and tetraploids, all treatments combined, with no significant differences. **(C)** Between the "dry" and "wet" treatments, both ploidies combined. Seedlings in the "Wet" treatment had significantly larger stem lengths. **(D)** Between the treatments with mycorrhizae added ("Myc") and the control groups ("No Myc"), both ploidies combined, with no significant differences. **(E)** Between diploids and tetraploids only within the "dry" treatment. Tetraploids had significantly larger stem length and number of leaves. **(F)** Between diploids and tetraploids only within the "wet" treatment, with no significant differences.



Because hydrology had significant effects on at least some of the growth measurements, further analysis was conducted between 2x and 4x plants within each hydrological treatment. Mycorrhizae did not have a significant effect, and therefore these treatments were not investigated further. Within the "dry" treatments, tetraploids had significantly larger stem length ($P = 0.02$) and number of

leaves (P = 0.05). In the "wet" treatment, there were no significant differences between ploidies.

Table 3. ANOVA results for Fig. 3.

		Factor	df	SS	MS	*F*	*P*
A) By treatment	Stem length	Treatment group	3	1.78	0.59	1.60	0.19
		Error	116	43.07	0.37		
	Number of leaves	Treatment group	3	1.83	0.61	1.66	0.18
		Error	116	42.63	0.37		
	Biomass	Treatment group	3	0.34	0.11	0.13	0.94
		Error	116	100.25	0.86		

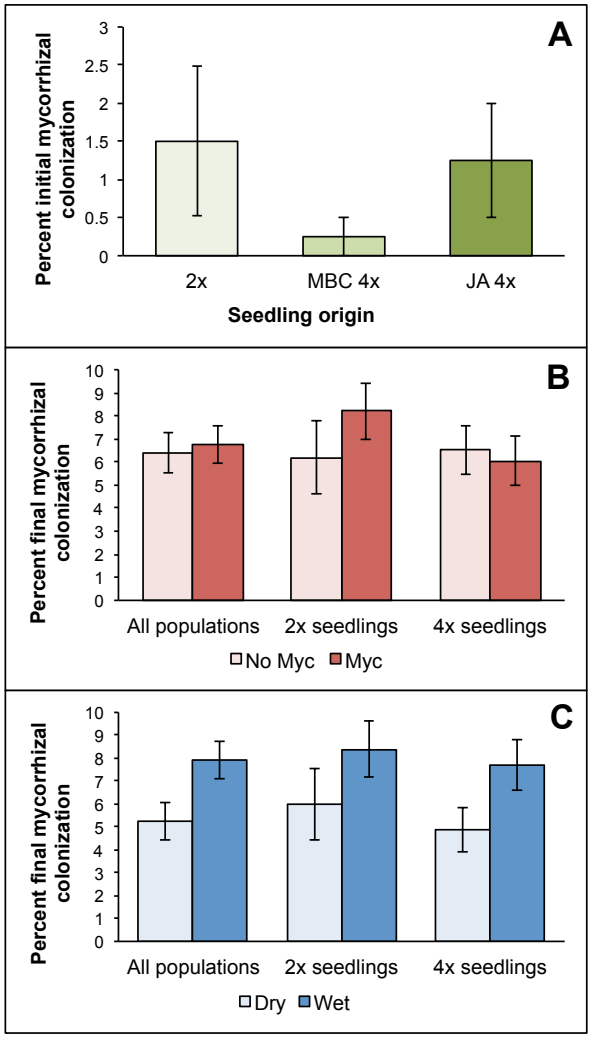
		Factor	df	MS	*F*	*P*
B) By ploidy	Stem length	Ploidy	1	1.09	2.94	0.09
		Error	118	0.37		
	Number of leaves	Ploidy	1	0.54	1.46	0.23
		Error	118	0.37		
	Biomass	Ploidy	1	0.40	0.48	0.49
		Error	118	0.85		
C) Dry vs. Wet	Stem length	Treatment group	1	1.39	3.77	0.05
		Error	118	0.37		
	Number of leaves	Treatment group	1	1.27	3.47	0.07
		Error	118	0.37		
	Biomass	Treatment group	1	0.17	0.20	0.66
		Error	118	0.85		
D) Myc vs. No Myc	Stem length	Treatment group	1	0.27	0.70	0.40
		Error	118	0.38		
	Number of leaves	Treatment group	1	0.31	0.84	0.36
		Error	118	0.37		
	Biomass	Treatment group	1	0.00	0.00	1.00
		Error	118	0.85		
E) By ploidy (dry only)	Stem length	Ploidy	1	1.56	5.23	0.03
		Error	58	0.30		
	Number of leaves	Ploidy	1	1.15	4.03	0.05
		Error	58	0.27		
	Biomass	Ploidy	1	0.01	0.01	0.91
		Error	58	0.75		
F) By ploidy (wet only)	Stem length	Ploidy	1	0.05	0.12	0.73
		Error	58	0.42		
	Number of leaves	Ploidy	1	0.00	0.00	0.96
		Error	58	0.44		
	Biomass	Ploidy	1	0.63	0.65	0.42
		Error	58	0.97		

Mycorrhizal Colonization - Mycorrhizal colonization in the seedling roots, prior to the experiment ("initial") were negligible, with an average of 0.25 – 1.5%, and did not differ among seedling origin (P = 0.45; Fig. 4A). At the end of the experiment ("final"), there were no significant differences between diploid and tetraploid seedlings in percent colonization of intracellular hyphae between any of the treatment groups ("Dry Myc", "Dry No Myc", "Wet Myc", "Wet No Myc"; $P > 0.3$ for all). There were no significant differences in mycorrhizal colonization between treatments where mycorrhizae were added ("Myc") versus those that were not added ("No Myc") (P = 0.76; Fig. 4B). However, treatments with high soil moisture ("Wet") had significantly greater colonization than did treatments with low soil moisture ("Dry") (P = 0.02; Fig. 4C). In the diploid population, the difference in colonization between "Dry" and "Wet" treatments was not significant (P = 0.25), but it was in tetraploid populations (P = 0.05).

Table 4. ANOVA results of the effects of ploidy, hydrology treatment, mycorrhizae treatment, and all interactions on final percent mycorrhizal colonization.

Factor	**df**	**MS**	***F***	***P***
Ploidy	1	36.82	0.86	0.36
Hydrology treatment	1	218.70	5.12	0.03
Mycorrhizae treatment	1	4.03	0.09	0.76
Ploidy × hydrology treatment	1	0.15	<0.01	0.95
Ploidy × mycorrhizae treatment	1	0.82	0.02	0.89
Hydrology treatment × mycorrhizae treatment	1	0.83	0.02	0.89
Error	113	42.74		

Fig. 4. **(A)** Percent mycorrhizal colonization in root cells at the start of the experiment. **(B)** Percent mycorrhizal colonization in root cells at the end of the experiment between treatments with and without added mycorrhizae. **(C)** Percent mycorrhizal colonization in root cells at the end of the experiment between dry and wet treatments. ANOVA results in Appendix 4.



Discussion

Pre-treatment measurements (stem length and number of leaves) showed little or no differentiation between diploids and tetraploids within the first three months after germination, when grown under ideal greenhouse conditions.

pH Experiment - Our results (Fig. 3) confirm reports showing that all blueberries, regardless of ploidy, thrive and grow significantly larger in acidic rather than in basic soil (Eck and Childers 1966; Eck 1988). This trait is typical of many Ericaceous species, and both diploids and tetraploids exhibited this preference in this greenhouse experiment.

In ideal low pH soil conditions, tetraploids grew larger than diploids in this experiment, which confirms other studies that polyploids generally grow larger than their diploid progenitors (Stebbins 1950, 1971, 1985; Levin 2002). Tetraploids have been shown to produce larger fruits with more seeds per berry and have higher germination rates under ideal conditions, and this has also been shown in blueberries (Ortiz et al. 1992).

Morphological and physiological changes in polyploids may increase their tolerance to stressful, more extreme environments (Stebbins 1985; Brochmann et al 2004; Hijmans et al 2007), or they may not (Baack and Stanton 2005; Buggs and Pannell 2007; Raabova and Munzbergova 2008; Godsoe et al 2013). Either way, it is unknown why diploid populations continue to exist and thrive, especially when in direct competition with established tetraploid populations that can grow larger. In one common garden experiment, no differences were found in competitiveness

between diploid and polyploidy *Aster amellus* (Asteraceae), however, polyploid plants did produce significantly more leaves in competition (Munzbergova 2007).

Here, we found that in the stressful high pH treatment, diploid seedlings had larger biomass than the tetraploid seedlings (Fig. 4A). This implies that diploid blueberries may perform better than tetraploid blueberries under these conditions, particularly in non-agricultural soils. Further exploration of this potential ecological advantage should be pursued, and experiments should be extended to highbush blueberries from their entire range, including populations of single ploidy.

Hydrology - In the Mycorrhizae/Hydrology experiment, major differences in growth were not observed between the different treatments (Fig. 8) or between the ploidies. The experiment confirmed reports showing that blueberries prefer hydric soils because seedlings generally had larger stem lengths and numbers of leaves in the "Wet" treatments (Fig. 9). Preferences for soil moisture conditions and differences in physiology related to water stress and drought tolerance have been observed in other diploid / polyploid species complexes. Levin (2011) found that hexaploid *Achillea borealis* (Asteraceae) were better adapted to drier, more xeric dune sites than were tetraploids. Maherali et al. (2009) found that tetraploid *Chamerion angustifolium* (Onagraceae) plants were physiologically more tolerant of water stress than diploids. Both of these species complexes are allopatric, not sympatric like the blueberry populations studied here, which could affect evolutionary processes. Garbutt and Bazzaz (1983) found that tetraploid *Phlox drummondii* plants did not produce flowers as well as diploids in hydric environments. These three studies all found that plants with higher ploidy levels

had higher fitness in dry conditions. However, Visger et al. (2016) found that diploid *Tolmiea* sp. were better suited to dry habitats than their tetraploid counterparts. Blueberries are known to prefer hydric soils (Eck and Childers 1966; Trehane 2004), but based on our study, ploidy does not affect water stress tolerance in blueberry seedlings.

Mycorrhizae - The addition of ericoid mycorrhizae did not make a difference in growth of seedlings (Fig. 10), nor did it increase percent colonization of root cells by intracellular hyphae (Table 14 "Myc vs. No Myc"). Mycorrhizal colonization did increase in the treatments with high soil moisture (Fig. 13), confirming that ericoid mycorrhizae are more successful in damp environments. However, because there were no differences in hyphae colonization of root cells, the observed fungus must have been present in the soil medium prior to treatment with the ericoid mycorrhizal inoculum. Three theories could explain the results here: the inoculation technique was ineffective; not enough of the mycorrhizae inoculation was added to make a difference; or the inoculum was somehow contaminated, mislabeled, or not viable prior to inoculation. A colleague also observed no differences from treating blueberry plants with this same Bioradis-Ericoid mycorrhizal inoculum product purchased from the same vendor, Horticultural Alliance (James Polashock, personal communication), leading us to conclude that the product was not viable as purchased.

We were particularly interested in the "Dry No Myc" treatment, since this is the most stressful condition for blueberries. Blueberries generally thrive in moist soils with ericoid mycorrhizae, so maintaining a dry soil with no addition of ericoid

mycorrhizae should theoretically produce a very stressful environment. Although no significant differences were seen between diploids and tetraploids with the log-transformed data, we did find that diploids had larger total biomass and root biomass when using the raw data (Table 10). However, this raw data set failed the Levene's test. Non-parametric tests were also performed and resulted in no significant differences. Although average diploid total biomass, stem biomass, leaf biomass, and root biomass were all larger than the average tetraploid measurements, these differences were subtle and non-significant.

Even though not all results were significant, the average biomasses were larger for diploids rather than tetraploids in the most stressful treatments, "Dry No Myc" and "High pH". Generally, tetraploids are thought to be able to withstand a wider range of environmental conditions than diploids (Soltis and Soltis 2000; Soltis et al. 2014b). The evidence here suggests that, at worst, the reverse may be true. Diploids may be performing better under stressful conditions, and perhaps this ecological advantage is responsible for keeping the diploid populations alive in the presence of their more competitive tetraploid neighbors. This should be explored in future studies.

Overall, the two tetraploid populations performed similarly in the Mycorrhizae/Hydrology experiment, just as they did in the pH experiment. However, tetraploids were larger than diploids with all treatments combined in the Mycorrhizae/Hydrology experiment, with significantly larger stem lengths, and larger (although not significantly) number of leaves and biomass (Fig. 11A). Ploidy is therefore more influential in growth than location of seed origin.

The ecological effects of polyploidy are still poorly understood and even in situations where ecological factors have been studied in the presence of polyploid events, the outcomes are proving to be species-specific. The fact that diploids proved to perform better in stressful conditions in these experiments warrants further investigation. These results would be of particular interest for restoration ecologists and native plant nurseries in the mid-Atlantic zone, given that highbush blueberries are a common native plant used in restoration projects. If the soil at a particular restoration site is known to be particularly basic, dry, or devoid of mycorrhizae, a better decision might be to plant diploid blueberries, rather than tetraploids, even though the latter are more commonly available from nurseries. It might be beneficial to ensure that diploid highbush blueberries are available in plant nurseries providing native plants for ecological restoration and remediation projects, because these plants may actually perform better than tetraploids under stressful soil conditions. Provenance of seeds is often specified for restoration projects; ploidy may also be a criterion of value.

Acknowledgements

The authors thank the Greenbelt Native Plant Center, and especially Camille Joseph and Catherine Molanphy, for providing greenhouse space and constant plant care and patience as this experiment evolved, failed, repeated, and succeeded. The authors thank Nicholi Vorsa and James Polashock at the Philip E. Marucci Center for Blueberry and Cranberry Research and Extension at Rutgers University for providing information, resources, and access to the field site; Richard Lear, Eric Gehrig, and Scott Meyler at the Jamesburg Park Conservation Area for access to the field site; and Great Swamp National Wildlife Refuge for access to their field site. We thank Michelle Segovia, Rachel Rodriguez, and David Kung for assistance in the field, greenhouse, and microscopy lab. Funding was provided by a Rutgers Teaching Assistant Professional Development Fund grant, the Hutcheson Memorial Forest Summer Research Grant, and the USDA National Institute of Food and Agriculture Hatch/Multistate grant 233357.

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Appendices

Appendix 1. JA4x seedling growth measurements compared to MBC4x seedling growth measurements for Fig. 2B - D, 3B, 3E, 3F, 4A – D. The only significant difference found between the 4x populations was in biomass of the dry treatments.

		Factor	**df**	**MS**	***F***	***P***
(Fig. 2B) All treatments combined - pH	Stem length	Site	1	0.002	0.001	0.97
		Error	38	1.28		
	Number of leaves	Site	1	0.02	0.03	0.87
		Error	38	0.76		
	Total biomass	Site	1	0.48	0.26	0.61
		Error	38	1.80		
(Fig. 2C) Low pH treatments only	Stem length	Site	1	0.09	0.60	0.45
		Error	18	0.16		
	Number of leaves	Site	1	0.05	0.36	0.56
		Error	18	0.14		
	Total biomass	Site	1	0.07	0.10	0.75
		Error	18	0.71		
(Fig. 2D) High pH treatments only	Stem length	Site	1	0.06	0.55	0.47
		Error	18	0.11		
	Number of leaves	Site	1	0.19	0.68	0.42
		Error	18	0.28		
	Biomass (Mann-Whitney / Wilcoxon test)		Var1	Var2	*U*	*P*
		Total biomass	MBC4x	JA4x	54	0.80
		Stem biomass	MBC4x	JA4x	45	0.74
		Leaf biomass	MBC4x	JA4x	46	0.79
		Root biomass	MBC4x	JA4x	60.5	0.45
(Fig. 3B) All treatments combined – Myc/Hyd	Stem length	Site	1	0.26	0.90	0.35
		Error	78	0.29		
	Number of leaves	Site	1	0.10	0.38	0.54
		Error	78	0.26		
	Total biomass	Site	1	1.34	3.38	0.07
		Error	78	0.40		
(Fig. 3E) Dry treatments only	Stem length	Site	1	0.70	3.07	0.09
		Error	38	0.23		
	Number of leaves	Site	1	0.01	0.06	0.80
		Error	38	0.21		
	Total biomass	Site	1	1.56	4.91	0.03
		Error	38	0.32		
(Fig. 3F)	Stem length	Site	1	0.01	0.04	0.84

Wet treatments only		Error	38	0.34		
	Number of leaves	Site	1	0.11	0.35	0.56
		Error	38	0.32		
	Total biomass	Site	1	0.15	0.31	0.58
		Error	38	0.49		
(Fig. 4A) Dry No Myc	Stem length (cm)	Site	1	0.34	1.88	0.19
		Error	18	0.18		
	Number of leaves	Site	1	0.01	0.06	0.80
		Error	18	0.15		
	Biomass (g)	Site	1	1.25	3.71	0.07
		Error	18	0.34		
(Fig. 4B) Dry Myc	Stem length (cm)	Site	1	0.36	1.24	0.28
		Error	18	0.29		
	Number of leaves	Site	1	0.07	0.23	0.64
		Error	18	0.30		
	Biomass (g)	Site	1	0.42	1.31	0.27
		Error	18	0.32		
(Fig. 4C) Wet No Myc	Stem length (cm)	Site	1	<0.01	<0.01	0.99
		Error	18	0.43		
	Number of leaves	Site	1	<0.01	0.01	0.92
		Error	18	0.37		
	Biomass (g)	Site	1	0.13	0.19	0.67
		Error	18	0.69		
(Fig. 4D) Wet Myc	Stem length (cm)	Site	1	0.03	0.11	0.75
		Error	18	0.29		
	Number of leaves	Site	1	0.16	0.62	0.44
		Error	18	0.26		
	Biomass (g)	Site	1	0.03	0.10	0.75
		Error	18	0.34		

Appendix 2. ANOVA results for Fig. 2 and Mann-Whitney / Wilcoxon non-parametric test comparing 2x and 4x biomass in the high pH treatment. Diploids had larger average biomass in all biomass categories.

		Factor	**df**	**MS**	***F***	***P***
(A) Low pH vs. High pH (all ploidies combined)	Stem length	pH treatment	1	56.85	258.88	<0.001
		Error	58	0.220		
	Number of leaves	pH treatment	1	28.23	99.92	<0.001
		Error	58	0.28		
	Total biomass	pH treatment	1	25.80	12.01	0.001
		Error	58	2.15		
(B) 2x vs. 4x (all treatments combined)	Stem length	Ploidy	1	0.51	0.43	0.51
		Error	58	1.19		
	Number of leaves	pH treatment	1	0.96	1.28	0.26
		Error	58	0.75		
	Total biomass	Ploidy	1	0.12	0.05	0.83
		Error	58	2.59		
(C) 2x vs. 4x (low pH treatment only)	Stem length	Ploidy	1	1.11	3.98	0.06
		Error	28	0.28		
	Number of leaves	Ploidy	1	1.01	6.52	0.02
		Error	28	0.16		
	Total biomass	pH treatment	1	5.56	2.38	0.13
		Error	28	2.34		
(D) 2x vs. 4x (high pH treatment only)	Stem length (not shown in Fig. 2)	Ploidy	1	0.002	0.01	0.91
		Error	28	0.14		
	Number of leaves (not shown in Fig. 2)	Ploidy	1	0.14	0.37	0.55
		Error	28	0.39		

		Var1	Var2	*U*	*P*
Mann-Whitney / Wilcoxon test	Total biomass	2x	4x	151	0.02
	Stem biomass	2x	4x	149	0.03
	Leaf biomass	2x	4x	135	0.13
	Root biomass	2x	4x	140	0.08

Appendix 3. Biomass issues in the high pH treatment. Diploid raw biomass was significantly larger than tetraploid raw biomass, but failed the Levene's test for homoscedasticity. Log-transformed data (log = (x)) passed the Levene's test, but no longer showed significantly different values. Data from the two 4x sites (GS and MBC) were never significantly different.

		Levene's test *P*	Factor	df	MS	*F*	*P*
2x vs. 4x (high pH treatment) Raw Biomass	Total biomass	0.02	Ploidy	1	0.02	9.88	0.004
			Error	28	0.002		
	Stem biomass	0.002	Ploidy	1	0.001	9.43	0.005
			Error	28	<0.001		
	Leaf biomass	0.002	Ploidy	1	0.001	5.55	0.03
			Error	28	<0.001		
	Root biomass	0.002	Ploidy	1	0.008	8.13	0.008
			Error	28	<0.001		
2x vs. 4x (high pH treatment) Log Biomass	Total biomass	0.32	Ploidy	1	3.52	1.97	0.17
			Error	28	1.79		
	Stem biomass	0.10	Ploidy	1	4.21	2.82	0.10
			Error	28	1.49		
	Leaf biomass	0.15	Ploidy	1	0.82	0.42	0.52
			Error	28	1.94		
	Root biomass	0.23	Ploidy	1	3.59	1.56	0.22
			Error	28	2.30		
MBC4x vs. JA4x (high pH treatment) Raw Biomass	Total biomass	0.37	Site	1	<0.001	<0.001	0.98
			Error	18	0.001		
	Stem biomass	0.19	Site	1	<0.001	0.35	0.56
			Error	18	<0.001		
	Leaf biomass	0.17	Site	1	<0.001	0.03	0.87
			Error	18	<0.001		
	Root biomass	0.44	Site	1	<0.001	004	0.85
			Error	18	<0.001		
MBC4x vs. JA4x (high pH treatment) Log Biomass	Total biomass	0.26	Site	1	0.50	0.43	0.52
			Error	18	1.17		
	Stem biomass	0.13	Site	1	0.007	0.008	0.93
			Error	18	0.85		
	Leaf biomass	0.25	Site	1	0.24	0.20	0.66
			Error	18	1.22		
	Root biomass	0.19	Site	1	1.57	1.00	0.33
			Error	18	1.56		

Appendix 4. ANOVA results for Fig. 4. Mycorrhizae treatments are "Myc" or "No Myc". Hydrology treatments are "Wet" or "Dry".

		Factor	**df**	**MS**	***F***	***P***
A) Initial colonization		Seedling origin	2	<0.01	0.83	0.45
		Error	21	<0.01		
B) Final colonization	All seedlings	Mycorrhizae treatment	1	4.03	0.09	0.76
		Error	118	43.11		
	2x	Mycorrhizae treatment	1	3.60	0.07	0.79
		Error	38	50.39		
	4x	Mycorrhizae treatment	1	1.25	0.03	0.86
		Error	78	40.19		
C) Final colonization	All seedlings	Hydrology treatment	1	218.70	5.30	0.02
		Error	118	41.29		
	2x	Hydrology treatment	1	<0.01	1.39	0.25
		Error	38	<0.01		
	4x	Hydrology treatment	1	0.02	3.95	0.05
		Error	78	<0.01		

CHAPTER 3

Pre-zygotic isolating mechanisms do not prevent unidirectional gene flow among cytotypes in *Vaccinium corymbosum* L.

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Abstract

Polyploidy is a common phenomenon in plants that affects genetic variation and can lead to speciation. *Vaccinium corymbosum* (highbush blueberry) taxonomy is confounded by sympatric populations of diploid and tetraploid cytotypes. Although prezygotic isolating barriers are known to exist, the rate of interploidal mating in sympatric populations is unknown. Here, we use hand-pollinated interploidal crosses and comparisons of fruiting rate, seeds per berry, and seedling emergence rate to test the reproductive consequences of polyploidy in this species. Ploidal levels were confirmed with flow cytometry and crosses were confirmed with PCR analysis using 9 SSR markers. Diploid plants at two field sites in New Jersey

were pollinated with tetraploid pollen and tetraploid plants in a greenhouse were pollinated with diploid pollen collected from the field sites. Homoploid crosses were used as controls. Homoploid crosses produced viable seed, confirming successful pollination techniques both in the field and in the greenhouse. Homoploid crosses had significantly higher average fruiting rate, average seeds per berry, and average seedling emergence rate than heteroploid crosses. Tetraploid homoploid crosses had significantly more average seeds per berry than diploid homoploid crosses. Pollen ploidy was a significant factor in fruiting rate, seeds per berry, and emergence rate, although maternal ploidy was only significant in seeds per berry. Heteroploid crosses on diploid maternal plants had significantly higher fruiting rate than heteroploid crosses on tetraploid maternal plants, but had significantly lower average number of seeds. Heteroploid crosses were conducted on 734 flowers, 42 berries were produced, and three seedlings developed from these crosses, two of which were triploid, and one tetraploid. Due to a high rate of unreduced gamete production in diploid individuals, heteroploid crosses produce fruit with viable seed that contribute to the population structure and gene flow of this species complex.

Introduction

Polyploidy, or whole genome duplication, is known to occur in all angiosperm families (Soltis and Soltis 2000; Husband et al. 2013). Polyploidy affects genetic variation and can lead to speciation, as the gametes change ploidal level and different cytotypes become potentially incompatible (Stebbins 1950; Husband and Schemske 2000; Levin 2002). Polyploid speciation is estimated to influence 30-70% of plant diversity (Stebbins 1971; Masterson 1994). In many species, polyploid formation is recurrent (Soltis and Soltis 1993), and can result in strong postzygotic barriers between parental populations and neopolyploid plants, due to inviability and infertility of triploid plants ("triploid block"). Additionally, polyploid population establishment should be difficult because of the minority cytotype exclusion principle, in which the minority cytotype (the polyploids) are unable to mate with compatible gametes due to pollen-swamping from the common (diploid) cytotype (Levin 1975).

However, prezygotic isolating mechanisms may prevent neopolyploids from mating with parental populations, allowing polyploid population establishment and possible speciation. Four factors that could influence this are: (1) tetraploid ability to self-pollinate (Mable 2004); (2) phenological differences and/or pollinator preference (Segraves and Thompson 1999; Husband and Schemske 2000; Poster et al. 2017); (3) ecological differentiation leading to spatial segregation (Maherali et al. 2009; Levin 2011; Visger et al. 2016; Poster et al. 2018, *in preparation*); or (4) high levels of 2N gamete production by diploid plants, providing a constant input of

tetraploid seedlings into the population (Bretagnolle and Thompson 1996; Sutherland and Galloway 2017).

Diploid and tetraploid highbush blueberries (*Vaccinium corymbosum* L.) exist sympatrically throughout their range along the eastern and southern coasts of the United States, inland from Texas to Wisconsin, and north into Ontario and eastern Canada. They are ecologically important understory shrubs and valuable agriculture crops that are cultivated worldwide, particularly in places such as New Jersey, Wisconsin, California, Florida, Georgia, Oregon, British Colombia (Canada), and Chile (South America) (US Highbush Blueberry Council). The taxonomy of the genus has been examined several times, due to recurring complications involving the existence of diploids, tetraploids, and hexaploids in sympatric locations. Occasional interploidal crosses occur, but both triploids and pentaploids are generally inviable (Vorsa and Ballington 1991). *V. corymbosum* has been both divided into 12 species (Camp 1945), and grouped into one polymorphic species (Vander Kloet 1980; eFloras 2008).

The four prezygotic isolating mechanisms listed above have been reported in *V. corymbosum*. Although self-pollination has been shown to decrease reproductive success in all ploidy levels of *V. corymbosum* (Vander Kloet and Lyrene 1985), tetraploids are more self-compatible than diploids (Ballington and Galletta 1978). Phenological differences occur between the cytotypes; diploids bloom about a week earlier than tetraploids, and morphological differences in corolla size may affect pollinator preference, although this has not been tested (Poster et al. 2017). Ecological differentiation between the cytotypes does not appear to be strong,

although diploid seedlings prove to be more tolerant of high pH soils (Poster et al. 2018, *in preparation*). Finally, diploid *Vaccinium* spp. have been reported to produce high numbers of unreduced (2N) gametes (Ortiz et al. 1992). In spite of these isolating mechanisms, the rate of interploidal mating in sympatric populations of diploid and polyploid *V. corymbosum* remains unknown. If no interploidy offspring are successful, this could contribute to a speciation event. If and how these interploidy offspring contribute to population growth also remains untested.

This study tests reproductive compatibility between diploid and tetraploid *Vaccinium corymbosum* in New Jersey, USA, using a series of hand-pollinated heteroploid crosses, followed by genetic analyses to confirm parentage. Fruiting rate, seeds per berry, and emergence rate are compared between cytotypes. By using diploid mother plants in the field, and standard 4x pollen, we can test the rate of unreduced (2N) gamete production in these natural populations. Similarly, in the greenhouse, we tested performance of wild-collected diploid pollen on tetraploid plants. Results from this experiment will help understand the nature and level of interploidal gene flow within the species complex, the rate of recurrent polyploidy formation, intrinsic barriers to triploid seed formation, and how interploidal pollination affects seed set and germination.

Methods

Pollen collection - In March 2016, pollen was collected from both diploid and tetraploid *V. corymbosum* plants and stored until selected flowers were ready for pollination. Diploid pollen was collected from plants at two field sites, Marucci Blueberry Cranberry Research Center (hereafter "MBC"), Chatsworth, Burlington

County, New Jersey (39.71543°N, 74.51046°W) and Great Swamp National Wildlife Refuge (hereafter "GS"), Basking Ridge, Morris County, New Jersey (40.70421°N, 74.49526°W). Budwood (branches containing premature flower buds) was collected from 26 known diploid plants (Poster et al. 2017), and brought into greenhouses at the Greenbelt Native Plant Center (NYC Parks and Recreation, Staten Island, New York, 40.49724°N, 74.1812°W). Once the flowers on the budwood opened, pollen was emptied from each flower and stored on deep well slides in a refrigerator at (1.7 – 4.4°C).

Tetraploid pollen was collected from cut branches of the cultivar 'Bluecrop' from the MBC greenhouses. Once flowers opened, pollen was emptied from each flower and stored in the same way as the diploid pollen.

Field pollination – Bud clusters containing 5-10 flowers were bagged on 27 diploid highbush blueberry plants (12 from MBC site; 15 from GS site) using mesh organza bags in March 2016, before flowers opened. After flowers opened in April 2016, flower clusters were pollinated with one of three treatments: (1) control – no pollen, flowers were agitated to simulate pollination; (2) pollinated with 2x pollen; or (3) pollinated with 4x pollen. The mesh organza bags were replaced immediately after pollination. The eraser side from pencils was used to transfer pollen, and a new pencil was used for each flower cluster. Each of the three treatments was established once each per plant.

Greenhouse pollination – Eighteen tetraploid highbush blueberry plants were obtained from Pinelands Nursery (Columbus, Burlington County, NJ) and transported to the greenhouses at Greenbelt Native Plant Center. When the flower

buds were fully formed, but not yet open, with stigmas still non-receptive, 622 flowers among the 18 4x plants were emasculated by removing anthers with tweezers, so as to prevent selfing. Stigmas became receptive 5-7 days after emasculation. Once stigmas were receptive, we pollinated 497 flowers with 2x pollen from 26 individuals (from GS and MBC field sites; 17-21 flowers per pollen type), and 204 flowers with 4x pollen, as a control.

Berry collection, Sowing seeds, and Germination – All berries were collected from inside the organza bags at the GS and MBC field sites, as well as from all 4x plants in the greenhouse. Seeds were extracted by crushing the berries by hand in a 500ml glass beaker filled halfway with water. Viable seeds sink to the bottom, while unviable seeds float to the top (Nicholi Vorsa, pers. comm., 2016). Water and unviable seeds were poured off, and the viable seeds per berry were counted and saved. All seeds were sown into a mix containing equal parts of Fafard® Super-Fine Germinating Mix, peat moss, and sterilized sand. The sown seed trays were covered in plastic and stored in a refrigerator (1.7 – 4.4°C) for 3 months. The trays of seeds were then removed and placed on greenhouse benches in full sun. Seedling emergence was recorded for 6 months after removal from refrigeration.

Ploidal Cytometry – DNA flow cytometry (Costich et al. 1993) was performed on all parent plants and seedlings that came from heteroploid crosses to confirm seedling ploidal levels. Flow cytometry was performed by Plant Cytometry Services (Berkenhof 37, 6941 ZR, Didam, The Netherlands).

Parentage Analysis - To confirm parentage, 10 SSR markers were used to compare seedling alleles with parental (pollen and ovule) alleles. Plant genomic

DNA was extracted from young leaves of the seedlings that arose from interploidal pollinations, as well as from all mother plants (field and greenhouse) and all diploid pollen donors. The leaves from the tetraploid pollen parents were no longer available, so DNA was extracted from the 4x pollen mix used for pollination.

DNA extraction followed the CTAB protocol (Stewart and Via 1993), with modifications using steel beads (Georgi et al. 2012). Two 5mm steel beads and approximately 50mg fresh leaf tissue were placed into Eppendorf Safe-Lock tubes for each sample. For the 4x pollen sample, 0.5 ml of 0.5 mm zirconia/silica beads (BioSpec Products, Bartlesville, OK) were used instead of the steel beads. A total of 800 µL of CTAB extraction buffer was added, consisting of 50 ml of 100 mM Tris-HCl, 41.1 g of 1.4M NaCl, 20 ml of 20mM EDTA, 10 g of 2% w/v CTAB, 10 g of 2% PVP-40, and 5 µL / ml β-mercaptoethanol (BME), resulting in a final volume of 500 mL. Samples were ground in a TissueLyser II (Qiagen, Germantown, MD) placed in a 65 °C water bath for 15 minutes, and then combined with 700 µL of chloroform, mixed with a vortex, and centrifuged. Approximately 600 µL of supernatant were extracted and placed in a fresh tube with 2 µL of RNAse, then incubated at 37 °C for 30 minutes. Approximately 500 µL (0.7 volumes) of isopropanol were added and gently mixed, then placed on ice for at least 10 minutes. After centrifuging the samples and pouring off the supernatant, 500 µL of 70% ETOH were added and gently mixed. Samples were again centrifuged and ETOH was removed with a pipette. After allowing the samples to air dry, the pellet was resuspended in 50 µL sterile dH_2O. DNA was quantified using a NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE).

Ten newly developed SSR markers for *Vaccinium* spp. were used (Bassil et al. 2018, *in preparation*), only 9 of which proved to be usefully polymorphic. PCR for genotyping was performed following the procedure in Honig et al. (2016). It was performed in 96-well plates with a total reaction volume of 13 µL per sample, using approximately 5 ng of genomic DNA, 1 x ImmoBuffer (Bioline, Tauton, MA, USA), 2mM $MgCl_2$, 0.25 mM each dNTP (Bioline), 0.5 U 1 x Immolase DNA polymerase (Bioline), 0.5 pmol forward primer with M13(-21) addition, 1 pmol reverse primer with "PIG-tailing" addition, and 1 pmol forward M13(-21) primer with FAM, NED, PET, or VIC fluorescent labels in each reaction. Thermal cycling conditions were an initial denaturation at 94°C for 5 minutes, 30 cycles of 94°C for 30 seconds, 55°C for 45 seconds, 72°C for 45 seconds, followed by 20 cycles of 94°C for 30 seconds, 53°C for 45 seconds, 72°C for 45 seconds, and a final extension of 72°C for 10 minutes. PCR products were analyzed using capillary electrophoresis (Applied Biosystems 3500xl Genetic Analyzer), sized with a LIZ 600 size standard v2.0 (Applied Biosystems), and Genemapper 4.1 software (Applied Biosystems) (Honig et al. 2016). Seedling alleles were then compared with all parental alleles.

Statistical Analysis - Data were first tested for homoscedasticity with Levene's test. If heteroscedasticity was non-significant ($P > 0.05$), then analysis of variance was used to gauge differences in mean reproductive measurements between ploidal populations. The Tukey test was used to correct *P*-values for multiple comparisons. All statistical analyses were performed in R 3.3.1 (2016).

Results

Field sites – In the field, the organza bags proved to be effective in eliminating pollinators from reaching the flowers. At the GS site, no berries formed inside organza bags that were not hand-pollinated. At the MBC site, three berries were collected from inside bags that had no pollination. These berries had an average of 10.3 seeds per berry, but they did not appear to be viable, as they produced no emergent seedlings. This was most likely an example of parthenocarpy, which has been shown to occur and be highly variable in this species (MacKenzie 1997).

Homoploid Crosses

Diploid (♀) by Diploid (♂) Pollination – All plants at MBC and GS field sites used in this experiment were diploids, and the control treatment involved pollinating these flowers with pollen from other diploid plants. This treatment is identified here as diploid x diploid (2x/2x), where the ploidy of the mother plant is described first, followed by the ploidy of the pollen parent (Fig. 1). In total, 124 diploid flowers were pollinated with diploid pollen at GS, and 121 at MBC, for a total of 245 flowers (Table 1). From these crosses, 96 berries were collected, 54 from GS and 42 from MBC. This gave an average fruiting rate (number of berries per number of flowers pollinated) of 40.4% for all diploid x diploid crosses (43.8% at GS; 36.6% at MBC, Fig. 1A). From the total 96 diploid x diploid berries, 1,639 seeds were extracted, with 2 – 44 (mean 14.4, Fig. 1B) seeds per berry, yielding 317 seedlings grown within 6 months (21% average emergence rate; Fig. 1C). Differences between GS and MBC sites were non-significant for average fruiting rate, average seeds per berry, and average emergence rate ($P > 0.05$ for all).

Table 1. Heteroploid and homoploid (control) crosses were made in the field and in the greenhouse. Crosses recorded as "Ploidy of mother plant ← ploidy of father plant" to indicate pollination. Fruiting rate = 100 * (berries collected / flowers pollinated). Emergence rate = 100 * (seedlings grown / seeds planted). GS = Great Swamp field site; MBC = Marucci Center field site; GH = greenhouse.

	Cross	Site	Flowers pollinated	Berries collected	Average fruiting rate (%)	Seeds per berry			Total seeds planted	Emergent seedlings	Average emergence rate (%)
						Min.	Mean	Max.			
Homoploid	**2x (♀) ← 2x (♂)**	GS	124	54	43.8	2	12.6	44	682	151	24.1
		MBC	121	42	36.6	2	16.0	36	957	166	18.5
	4x (♀) ← 4x (♂)	GH	204	108	53.7	3	35.5	63	85	35	30.6
	Total homoploid		449	204	46.1	2	17.8	63	1724	352	21.5
Heteroploid	**2x (♀) ← 4x (♂)**	GS	128	21	14.0	1	5.6	18	118	3	1.9
		MBC	109	16	13.6	2	8.9	25	177	0	0
	4x (♀) ← 2x (♂)	GH	497	5	1.1	1	14.0	33	70	0	0
	Total heteroploid		734	42	7.2	1	7.9	33	365	3	0.9

Tetraploid (♀) x Tetraploid (♂) Pollination – Only tetraploid mother plants were used in the greenhouse, therefore the control treatment involved pollinating flowers from these plants with the same tetraploid pollen that was used in the heteroploid crosses in the field. In this control group, 204 flowers were pollinated and produced 108 berries (46.1% average fruiting rate; Table 1). Seeds per berry ranged from 2 – 63 (mean 17.8) and 782 seeds were collected in total. Due to logistical constraints, only 85 randomly selected seeds were sowed. Thirty-five seedlings emerged (30.6% average emergence rate).

Tetraploid homoploid crosses had significantly more seeds per berry than diploid homoploid crosses ($P < 0.001$), but did not differ significantly in fruiting or emergence rates ($P = 0.15$ and $P = 0.35$, respectively).

Heteroploid Crosses

Diploid (♀) x Tetraploid (♂) Pollination – The interploidal crosses in the field are identified as diploid x tetraploid (2x/4x) (Fig. 1). In total, 237 diploid flowers were pollinated with pollen from tetraploid plants, 128 flowers from GS, and 109 flowers from MBC (Table 1). Twenty-one berries were collected from GS and 16 were collected from MBC, resulting in a 14.0% average fruiting rate at GS and 13.6% average fruiting rate at MBC, for a combined average fruiting rate of 13.8% of 2x/4x crosses in the field. From the total of 37 2x/4x berries collected, 295 seeds were extracted (118 from GS and 177 from MBC), ranging from 1 – 25 (mean 7.2) seeds per berry. The GS plants had an average of 5.6 seeds per berry,

and this was significantly less than the MBC average of 8.9 seeds per berry (P = 0.04).

Seven seedlings grew from these cross-ploidy pollinations in the field, 6 from GS and 1 from MBC, resulting in an average 4.0% emergence rate for all 2x/4x crosses. Emergence and fruiting rates were not significantly different between GS and MBC sites for 2x/4x crosses ($P > 0.1$ for both).

Tetraploid (♀) x Diploid (♂) Pollination – On the tetraploid mother plants in the greenhouse, 497 flowers were pollinated with diploid pollen for the experimental heteroploid treatment group (Table 1). Five berries resulted from these pollinations, for an average fruiting rate of 1.1%. The five berries produced 70 seeds, ranging from 1 – 33 (mean 14.0) seeds per berry. Four seedlings grew from these seeds, resulting in a 3.9% average emergence rate.

Combining the (2x) ♀ ← (4x) ♂ treatments in the field with the (4x) ♀ ← (2x) ♂ treatments in the greenhouse, 734 flowers were pollinated with alternate-ploidy pollen, 42 berries were collected (7.2% average fruiting rate), and 365 seeds were extracted (1 – 33 seeds per berry, mean 7.9). Eleven seedlings germinated and grew, resulting in an average emergence rate of 4.0%.

The (2x) ♀ ← (4x) ♂ crosses had significantly higher average fruiting rate than the (4x) ♀ ← (2x) ♂ crosses (P = 0.001), significantly lower average number of seeds per berry (P = 0.02), and no difference in emergence rate.

When multiple factors were combined in the ANOVA analysis (Table 2), cross type, either heteroploid or homoploid, was significant in all effects (average fruiting rate, average number of seeds per berry, and average emergence rate). Maternal ploidy was

only significant in number of seeds per berry, as tetraploids have more than diploids. Pollen ploidy, however, was significant in all three outcomes ($P < 0.03$).

Figure 1. (A) Average fruiting rate, (B) average seeds per berry, and (C) average emergence rate of homoploid (2x/2x) and heteroploid (2x/4x) crosses at the GS and MBC field sites, as well as homoploid (4x/4x) and heteroploid (4x/2x) crosses in the greenhouse. Error bars represent standard error (SE).

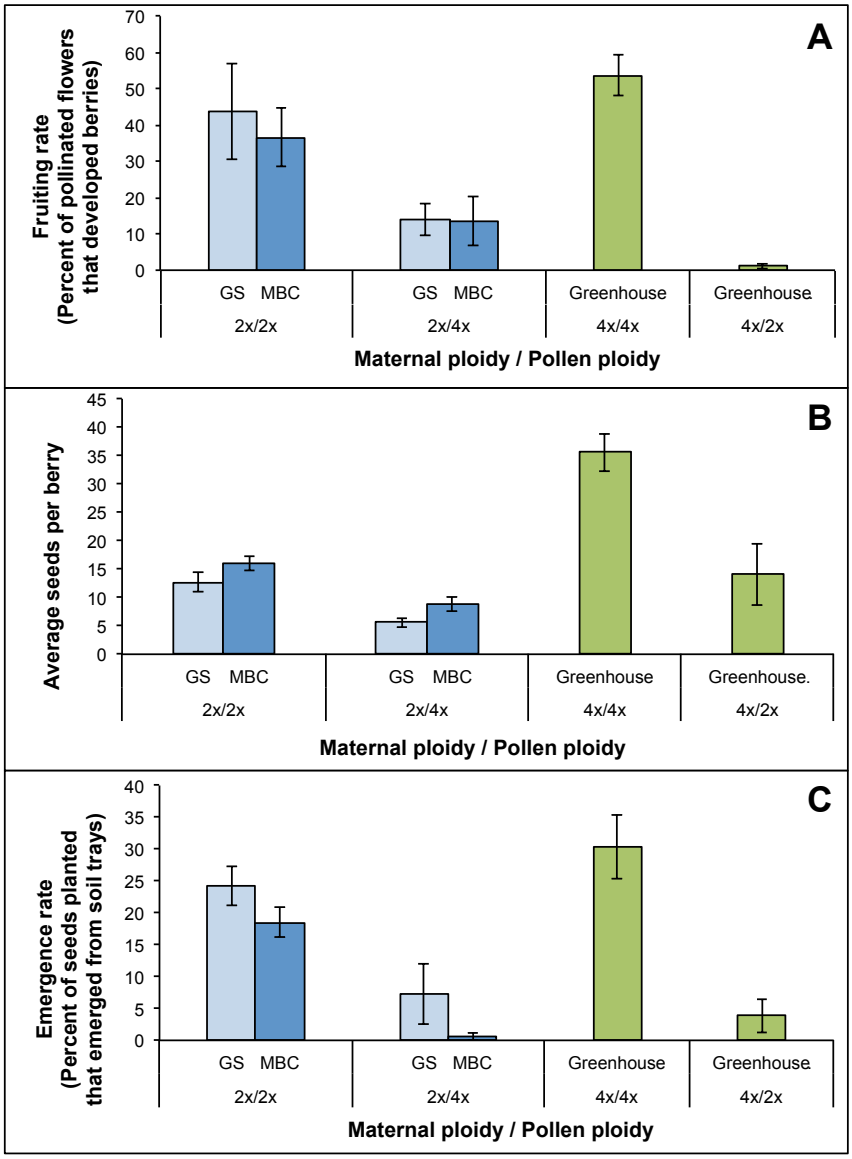

Table 2. ANOVA results of the effects of maternal ploidy (2x or 4x), pollen ploidy (2x or 4x), cross (homoploid or heteroploid), site, and the interaction between pollen ploidy by site on (A) average fruiting rate; (B) seeds per berry; and (C) average emergence rate. The interaction of maternal ploidy by site could not be analyzed because only one maternal ploidy (2x) was used at GS and MBC, and the other maternal ploidy (4x) was only used in the GH.

	Factor	**df**	**MS**	***F***	***P***
Average fruiting rate	Maternal ploidy	1	563	1.33	0.25
	Pollen ploidy	1	2358	5.59	0.02
	Cross	1	32946	78.05	<0.001
	Site	1	142	0.34	0.56
	Pollen ploidy × site	1	126	0.30	0.59
	Error	81	422		
Seeds per berry	Maternal ploidy	1	8369.4	83.31	<0.001
	Pollen ploidy	1	484.6	4.82	0.03
	Cross	1	2962.0	29.49	<0.001
	Site	1	420.2	4.18	0.04
	Pollen ploidy × site	1	0.1	<0.001	0.98
	Error	176	100.5		
Average emergence rate	Maternal ploidy	1	5.1	0.01	0.91
	Pollen ploidy	1	6495.7	17.91	<0.001
	Cross	1	4037.8	11.13	0.001
	Site	1	1324.9	3.65	0.06
	Pollen ploidy × site	1	7.4	0.02	0.89
	Error	158	362.8		

Genetic analysis – DNA extraction and PCR analyses were performed on leaves from the 11 interploidal seedlings, leaves from the parents, and the 4x pollen to confirm parentage. DNA extraction was successful for all samples, including the

4x pollen, resulting in DNA concentrations of at least 120 ng / μL per sample. PCR was performed twice to confirm results.

Of the 11 seedlings that grew from interploidal hand pollinations, only three proved to be successful crosses, with all alleles from the seedling matching up with the parental alleles. Three seedlings appear to be a result of the tetraploid mother selfing, which is known to occur in tetraploids (MacKenzie 1997). Five of the seedlings had alleles that did not match the parental alleles at all of the markers, which could be due to contamination during the pollination process, unsuccessful hand-pollination, followed by or preceded by uncontrolled pollination from an unknown source, genetic mutation, or homeologous recombination.

The three successful interploidal pollinations were from two diploid mother plants at the GS site. Two of the seedlings were triploid and one was tetraploid.

Three tetraploid seedlings from 4x mother plants in the greenhouse appear to be results of self-pollination by the mother plant. In all of these seedlings, only maternal alleles were found and they do not show any alleles that are unique to the pollen parent. All seedlings are tetraploid, but they were not genetically identical to the mother plants. At 1-3 loci per seedling, not all alleles from the mother plant were seen, confirming that these seedlings most likely grew out from a self-pollinated flower, as opposed to being formed from an unreduced meiotic tetraploid product of the mother plant, in which all alleles from the mother would be found in the seedling.

Five seedlings did not match the alleles of their parents at all markers tested. One is a tetraploid seedling that came from a tetraploid mother in the greenhouse,

pollinated with diploid pollen. PCR for one of the markers did not work on the seedling sample. Of the eight remaining markers, four of them showed alleles in the seedling that were not found in either of the parents. The seed was probably mislabeled or represents some other logistical error.

Two seedlings from diploid mother plants at the GS site were pollinated with 4x pollen, but produced 2x seedlings, suggesting that these were a result of diploid pollen contamination. Both of the seedlings had alleles at two (different) loci that were not found in either parent, although they did match parental alleles at the other seven loci. The ploidy of the seedlings is enough to confirm that pollen contamination occurred here, but possibly seed / parental mislabeling as well.

The remaining two seedlings that did not match parental alleles were both triploid seedlings that came from diploid mother plants in the field, one at the GS site, one at the MBC site, pollinated with 4x pollen. The seedling from the GS site matched the parental alleles at six out of nine loci, but had 1-2 alleles that were not found in either parent at the other three loci. The seedling from the MBC site only matched parental alleles at five loci, and had 1-2 alleles not found in the parents at the remaining four loci. The fact that these seedlings are triploid implies that they must have been pollinated by 4x pollen, but it may have been different 4x pollen than used in this experiment. Both sites contain a mixed-ploidal population of *V. corymbosum* plants, so pollen contamination is possible, and interploidal pollination events may be occurring at a high rate.

Discussion

Greenhouse and Field Sites – Berries with viable seeds were produced both in the field sites and in the greenhouse, showing that the pollination techniques used in this study were successful and that greenhouse conditions were appropriate.

The two field sites of diploid mother plants did not significantly differ from each other in any observational categories, except GS had slightly less seeds per berry than MBC in the 2x/4x heteroploid crosses. This confirms that the 2 sites are generally behaving the same way in regards to the characteristics studied here.

Tetraploids of many species are often larger, more robust, and more prolific than their diploid progenitors (Stebbins 1950, 1971; Levin 2002), and this has also been shown in blueberries (Ortiz et al. 1992; Poster et al. 2018, *in preparation*). Although this study did not uncover any differences between diploids and tetraploids in fruiting rate or emergence, the tetraploids did have a significantly larger number of average seeds per berry, which supports previous research.

Interestingly, the heteroploid crosses seemed to behave differently depending on the direction of the pollination event. Both heteroploid crosses had significantly reduced average fruiting rate, seeds per berry, and emergence rate compared to homoploid crosses ($P < 0.001$). However diploid mother plants pollinated with tetraploid pollen (2x/4x crosses) had significantly higher average fruiting rates than 4x/2x crosses. However, 4x/2x crosses had significantly higher average number of seeds per berries. The two types of heteroploid crosses did not differ in emergence rate. More importantly, only polyploid seedlings from diploid mothers and tetraploid pollen were confirmed as heteroploid crosses through the

genetic analysis. None of the seedlings from diploid pollen crossed onto tetraploid mother plants proved to match the proposed parental alleles. This may suggest a preference for unreduced gamete production in the ovules as opposed to pollen in *V. corymbosum,* which can be studied in future tests. It is unclear why pollen ploidy was significant for all three dependent variables, but maternal ploidy was only significant for number of seeds. This, too, could be examined in future work.

Although the heteroploid crosses had lower reproductive performances, the observation reported here in which heteroploid crosses did produce berries with viable seeds that emerged upon sowing, combined with the overlap of flowering times observed by Poster et al. (2017), proves that gene flow from the diploid to the tetraploid populations is possible. The sample size in this study was not large enough to estimate rates of polyploidy formation, but the phenomenon was observed as seeds collected from berries on diploid mother plants produced two triploid and one tetraploid seedling, proving that polyploidy formation is indeed recurrent in this species complex.

Genetic Analysis – PCR reactions generally provided consistent results, despite differences in the thermocycler program used here compared to the one used to develop these markers (Bassil et al. 2018, *in preparation*). In particular, the annealing temperature (55°C for 45 seconds) used here was different from that (65°C for 1 minute and 30 seconds) used to develop the markers. Repeating the PCR helped to confirm any questionable results from the first round.

Of the 11 seedlings grown from interploidal crosses, only one cross showed unreduced gamete production in the diploid population by producing a 4x

individual from a 2x (♀) x 4x (♂) mating. Two other seedlings with confirmed parentage of an interploidal cross grew, but they were triploid (3x), and will probably not be able to reproduce further.

Ortiz et al. (1992a; 1992b) tested the occurrence of unreduced (2N) gamete production in diploid, tetraploid, and hexaploid natural populations of several *Vaccinium* species. They found that the average frequency of (2N) pollen-producing plants was 7.4% for diploid plants and 10.5% for tetraploid plants, not significantly different. They also found that in diploid species, the average amount of (2N) pollen production per plant ranged from ≤1% to 28.6%, and that from tetraploid plants was similar, ranging from 1% to 37.4%. The frequency of (2N) pollen-producing individuals, as well as the amount of (2N) pollen produced per plant, varied significantly, both within and among populations, as well as within clonal replicates, from year to year. This shows that unreduced gamete production is highly variable in the *Vaccinium* genus, but that it does consistently occur. This implies that more polyploid genotypes are regularly being added to natural populations, upon which natural selection can act. The tetraploid fraction of populations is charging as well as the diploid component of these stands.

Three tetraploid seedlings appear to be grown from tetraploid mother plants that selfed. Most likely, the stigmas on these flowers were already receptive at the time of emasculation, and the emasculation process accidentally pollinated the flower. Selfing has been shown to occur in tetraploid *V. corybosum*, while diploid *V. corymbosum* is obligately outcrossing (Ballington and Galletta 1978).

Only five seedlings did not match parental alleles. It is possible that during sowing, the seeds were mislabeled. The seeds are very small, measuring about 1mm in length and width (Garcia and Ligarreto 2014). If logistical error occurred in recording the crosses, it most likely occurred in the collected and sowing of seeds because they are so small.

The cytotypes regularly coexist in natural populations but differ in aspects of their ecological tolerance. They overlap in flowering time allowing potential gene flow between cytotypes. This studies show that diploids and tetraploids can cross and produce viable seeds, despite the isolating mechanisms that have been recorded. Consequently, natural populations have a dynamic cytotypic foundation, with diploid, tetraploid, and occasional intercross seedlings emerging to enrich the genetic diversity in natural stands. Triploids were uncommonly formed, but their success in future reproductive events is unlikely. Together these findings confirm that *V. corymbosum* populations continue to generate genetic variants at a high rate, upon which natural selection can act. In a field setting of rapid climate and habitat change, this rich genetic diversity may give this species greater tolerance for ecological stress than plant species with less labile genetic mechanisms.

Acknowledgements

The authors thank the Greenbelt Native Plant Center, and especially Camille Joseph and Catherine Molanphy, for providing greenhouse space and constant plant care and patience. The authors thank the Philip E. Marucci Center for Blueberry and Cranberry Research and Extension at Rutgers University for providing information, resources, and access to the field site, and Great Swamp National Wildlife Refuge for access to their field site. We thank Michelle Segovia and Rachel Rodriguez for assistance in the field and greenhouse. Funding was provided by a Rutgers Teaching Assistant Professional Development Fund grant, the Hutcheson Memorial Forest Summer Research Grant, and the USDA National Institute of Food and Agriculture Hatch/Multistate grant 233357.

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