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THE NATURE OF INFERTILITY AND RESPONSE TO INBREEDING IN
VACCINIUM (BLUEBERRY) SPECIES

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THE NATURE OF INFERTILITY AND RESPONSE TO INBREEDING IN VACCINIUM (BLUEBERRY) SPECIES

By

Gary William Schott

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ABSTRACT

THE NATURE OF INFERTILITY AND RESPONSE TO INBREEDING IN VACCINIUM (BLUEBERRY) SPECIES

By

Gary William Schott

The study of plant mating systems is a very active area of research. In particular, determining the nature of infertility is important. The two predominant schools of thought on why plants have low or no self-fertility are self-incompatibility (SI) and inbreeding depression (ID). With SI, self pollinated flowers set no seeds. Arrest of the reproductive process can occur at any stage between pollen germination and ovule development. In late-acting SI, otherwise known as ovular self-incompatibility (OSI), self-fertilized ovules are aborted at a relatively uniform stage of development. With ID, inbred progeny have lower fitness, which can manifest itself at any stage of development. Whether the abortion of self-pollinated ovules is due to OSI or early-acting ID will impact the mating system, which will impact genetic variation within and between populations, and influence the evolution of plant populations and species. I examined self-infertility and the response to inbreeding in several blueberry species (Vaccinium).

The response to inbreeding was studied by performing self and outcross pollinations on 7-13 plants in each of two populations of Vaccinium myrtilloides (diploid) and V. corymbosum (tetraploid). I monitored fruit development, seed set, seed germination and seedling growth for evidence of

ID, which is calculated as $\delta = 1 - w_s/w_o$, with w_s and w_o representing the fitness of selfed and outcross progeny respectively. The effect of outcrossing distance on fertility was studied in the same two species by performing outcross pollinations of increasing distance from experimental plants. The pollination treatments consisted of self, close (w/in 10 m), distant (>100 m) and other (other population) pollinations. The nature of infertility was studied in V. corymbosum and V. angustifolium (tetraploid) by monitoring the development of self and outcross ovules. Self and outcross fruits were sampled at 1, 2, 3, and 4 weeks following pollination and at maturity. Seed length was measured and germinability was tested.

I found that both V. myrtilloides and V. corymbosum suffer ID at most life-history stages examined with ID being greater at early stages in the diploid and consistent across stage in the tetraploid. Cumulative ID was comparable in the two species. The diploid shows evidence of outbreeding depression while the tetraploid does not. Seed development and size was variable with V. corymbosum being more self-fertile than V. angustifolium. The data suggest that seed abortion and low self-fertility in blueberries is due to early-acting ID, not OSI. The phenomena of pseudo-self-fertility is invoked to explain how blueberries respond to inbreeding. It is thought that relatively high self-fertility in V. corymbosum is an artifact of polyploidy formation.

DEDICATION

I would like to dedicate this dissertation to my wife Carolyn and my children Sara, Cole and Jacob. They help me keep everything in the proper perspective.

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I would like to thank Mike Green, the head ranger at Cheyboygan State Park. Mike provided assistance in my field work at the state park and would notify me when the plants were doing their thing, saving me the time and effort of going up to find out myself.

Finally, I would like to thank my family. My wife Carolyn and children Sara, Cole and Jacob were very patient during these many years. They offered unwavering support and kept my spirits up during those times when I didn't think all of this effort would amount to a hill of beans. Carolyn's ability to support the family and stroke my fragile ego has helped tremendously. My parents, Bill and Jeannine Schott, and Carolyn's parents Fred and Marilyn Wenzel, have been indispensable. They have been very generous with financial support and took care of the kids and our home during those times when I was very busy. Extended family member often wondered what I was up to these many years. Although I am sure they often wondered about me at times, they were always supportive and kept a straight face when I told them that I studied reproduction in blueberries.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 3

LIST OF FIGURES

Figure Page

CHAPTER 1

CHAPTER 2

CHAPTER 3

INTRODUCTION

Understanding the various aspects of plant reproduction is very important for conservation biology and agriculture. Human population growth contributes to the loss of natural habitats and arable land. Maintaining viable wild populations requires knowledge of the reproductive dynamics of plant populations. Maintaining adequate food supplies requires maximizing the reproductive output of crops. A study that examines the nature of infertility and the performance of plants under different pollination regimes will add to our knowledge base and aid in conservation efforts and agriculture. This study is unique because it tests theories on the nature of infertility, and response of different ploidy levels to inbreeding, in a wild species that is the direct progenitor, and same species, as a widely cultivated species, the highbush blueberry (Vaccinium corymbosum). This species is of increasing importance due to its widely recognized health benefits (Prior et al., 1998). Studying wild populations of V. corymbosum species, and closely related species, will help us to develop better cultivation practices and assist in the conservation of wild populations that can serve as reservoirs of genetic information.

Inbreeding, Infertility and Optimal Outcrossing

Inbreeding is, of course, the mating of closely related individuals. It often results in inbreeding depression, a decrease in the fitness of resulting progeny. There are several theories that can explain the phenomenon of ID (Charlesworth and Charlesworth, 1987). In the partial dominance model, deleterious recessive alleles are preferentially expressed in the homozygous state after inbreeding. In the overdominance model, heterozygotes have higher fitness than either homozygote, and their frequency decreases with inbreeding. The higher fitness of heterozygotes presumably comes from positive interactions between different alleles. Overdominance can also result from the heterozygote having more diverse gene products (enzymes) which provides a multiplicity of metabolic pathways for enzymatic reactions (Richards, 1997). Empirical data suggests that partial dominance plays a greater role in inbreeding depression (Charlesworth and Charlesworth, 1987).

Since the advent of plant cultivation and animal husbandry, humans have recognized the negative consequences of inbreeding. Darwin (1876) was the first person to systematically study inbreeding in plants. He found that 24 out of the 40 species he studied exhibited inbreeding depression. He also found that some species thought to be outbreeders showed no evidence of inbreeding depression and other species thought to be inbreeders suffered from inbreeding depression (Darwin, 1876). Our knowledge of the impact of inbreeding depression has increased a great deal over the past century and this has led to the recognition that inbreeding depression can occur at many stages in plant development. Inbreeding is particularly detrimental in small populations. However, inbreeding can be a valuable tool for the plant breeder by allowing them to create true breeding lines, that are then used to create hybrids that are often of superior quality. This phenomenon of hybrid vigor, or heterosis, has been used extensively in agriculture.

Many mechanisms have evolved in natural populations to reduce or avoid inbreeding. In animals, sex-biased dispersal and kin recognition reduce the likelihood that closely related individuals will mate. Plants have many morphological and biochemical mechanisms to avoid inbreeding. Dioecy assures outcrossing while monoecy can favor outcrossing. Plants with hermaphroditic flowers often spatially separate stigma(s) and anthers (herkogamy) or mature the stigma(s) and anthers at different times (dichogamy). However, dioecy cannot prevent biparental inbreeding and herkogamy, dichogamy and spatial separation of male and female flowers cannot completely prevent the pollination of one flower, by another, on the same plant (geitonogamy). As a result, many species are self-incompatible to prevent the production of inbred progeny. The introduction of chapter 3 provides a detailed description of the forms of self-incompatibility found in plants.

Outbreeding depression can also be detrimental in plant populations. Progeny of a mating between individuals that are too distantly related and/or widely spaced may have decreased fitness, relative to progeny resulting from a mating of closer relatedness and/or distance. When a species exhibits both inbreeding depression and outbreeding depression, there may be an intermediate, optimal outcrossing distance that results in progeny with the highest fitness. Outbreeding depression may arise in situations where mating with a too dissimilar individual breaks up epistatic, coadapted gene complexes. An intermediate optimal outcrossing distance can help explain the phenomenon of mixed mating systems, where species or populations have crossing rates that are somewhere between predominate selfing and predominate outcrossing (Waser, 1993).

Blueberries

The true blueberries are in the family Ericaceae, section Cyanococcus, genus *Vaccinium*. Other species in this genus that are not considered true blueberries include cranberries. The term huckleberries is also used when referring to blueberries. While huckleberries are Ericaceous, they are in the genus *Gaylussacia*. The genus *Vaccinium* occurs primarily in North America, but there are a few European and Asiatic species (Camp, 1945). Three species of *Vaccinium* are included in this study and are described below.

Vaccinium corymbosum is known as the highbush blueberry. It is a North American species found from Florida to southern Canada, across to eastern Texas in the south and Michigan in the north. It is found in open swamps, bogs and along the margins of lakes and ponds in acidic sandy soil. Vander Kloet (1978) provides a detailed description of the morphology and ecology of *V. corymbosum*. Camp (1945) recognized 12 highbush blueberry species. He divided these species between northern and southern complexes with different diploid progenitors. The putative diploid progenitors of the northern complex are *V. pallidum*, *V. ceasariense*, *V. atrococcum* and *V. angustifolium*. Camp mistakenly thought that *V. angustifolium* was a diploid, it is tetraploid. For the southern complex they are *V. ceasariense*, *V. atrococcum*, *V. darrowii* and *V. tenellum*. Vander Kloet (1980, 1988) combined Camp's 12 species into one species, Vaccinium corymbosum, with infraspecific taxa at the diploid, tetraploid and hexaploid levels. Vander Kloet (1980) believes that V. tenellum, V. darrowii and V. pallidum are the diploid progenitors of all highbush blueberries. This argument suggests that V. corymbosum has multiple origins and a diverse genetic background. Hybridization among local Vaccinium taxa is creating genetically distinct groups. In the southeastern U.S., tetraploid (4n) V. corymbosum hybridizes with 4n V. myrisinites (Cockerham and Galletta 1976) and diploid (2n) V. corymbosum hybridizes with V. darrowii, V. tenellum and V. pallidum (Darrow and Camp, 1945; Vander Kloet, 1977; Lyrene and Sherman, 1981; Ballington and Galletta. 1978; Ballington et al., 1979; 1982; 1985). In northern areas, 4n V. corymbosum commonly hybridizes with 4n V. angustifolium (Vander Kloet, 1976).

Vaccinium angustifolium, commonly called the low sweet blueberry, is a North American species partial to dry upland habitats. It is found in oak and pine barrens, rock outcrops and burned areas. It requires acidic soil. It is found primarily in the north eastern U.S. and eastern Canada, as far south as Virginia and west to Minnesota. Camp (1945) recognized V. angustifolium as a unique species but mistakenly classified it as a diploid. More recent studies clearly demonstrate that this species is a tetraploid (Hall and Aalders, 1961; Vander Kloet, 1978). The putative progenitors of V. angustifolium are the diploids V. boreale and V. pallidum (Vander Kloet, 1977).

Vaccinium myrtilloides, commonly called the velvet-leaf blueberry, is a diploid that was also recognized by Camp (1945) as a unique species. Vander Kloet and Hall (1981) maintain that status and provide a detailed description of the species. Preferred habitats are boreal forest, bogs, barrens and rocky outcrops. It is found across North America as far south as Michigan and Pennsylvania and as far north as the mid-latitudes of Hudson Bay. In Michigan, V. myrtilloides is often found in sympatry with V. angustifolium, and is sometimes sympatric with V. corymbosum.

Blueberries are monoecious with bisexual flowers. Inflorescences consist of approximately 5-12 flowers and are usually clustered towards the ends of year-old branches. There is spatial separation of stamens and the stigma. The stamens are attached to the base of the fused corolla and extend half way up the corolla. The style extends to the opening of the corolla and the stigma surface is located just inside the corolla or extends beyond the corolla. The anther sacks are poricidal and pollen is released with the ultrasonic vibrations of buzz pollinators or from physical manipulation. They are generally acknowledged to be highly outcrossed. However, many geitenogamous pollinations (within plant) do occur (Vander Kloet and Lyrene, 1987).

The broad geographic ranges of blueberries in North America, and variation in ploidy among species, make them ideal for studying the relationship between inbreeding and polyploidy. Not only do individuals from different regions have divergent evolutionary histories, but the species also have variant life-history strategies. The lowbush types, such as V. angustifolium and V. myrtilloides, are predominantly rhizomatous and form large colonies of single genotypes. In contrast, the highbush types are crown formers that exist in more finely-grained patches. The rhizomatous growth pattern in the lowbush blueberry potentially favors more self pollinations. As clones expand in lateral extent, pollinators move between shoots of plants in the same clone. Relatively speaking, a greater proportion of outcross pollinations should occur in the highbush blueberry because of the closer proximity of different clones. However, it is also possible that the extensive lowbush clones are interdigitated, and a greater number of outcross pollinations are occurring than otherwise might be expected.

The experiments described in this dissertation address questions concerning the reproductive biology of blueberries focusing on the responses to inbreeding, the nature of infertility and the effect of outbreeding. In Chapter 1, I examine how diploid (V. myrtilloides) and tetraploid (V. corymbosum) blueberries respond to inbreeding. I examine ID at several life history stages and reevaluate notions of inbreeding from previous work. In Chapter 2, I examine outbreeding depression in V. myrtilloides and V. corymbosum. In Chapter 3, I address the nature of self-infertility in blueberries, following seed development and viability in the tetraploid species V. angustifolium and V. corymbosum. I review previous studies (Krebs and Hancock, 1990; Hokanson and Hancock, 2000) to conclude that low self-fertility in blueberries is due to inbreeding depression, not self-incompatibility as previously suggested (Vander Kloet and Lyrene, 1987).

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CHAPTER 1

THE RESPONSE OF THE BLUEBERRIES *VACCINIUM MYRTILLOIDES* AND *V. CORYMBOSUM* TO INBREEDING UNDER NATURAL CONDITIONS

ABSTRACT

Low self fertility can result from various forms of self incompatibility or inbreeding depression (ID). The nature of self-fertility in blueberries (Vaccinium) is in question with some studies suggesting self-incompatibility and others suggesting ID. This study examined the response of Vaccinium corymbosum (tetraploid) and V. myrtilloides (diploid) blueberries to inbreeding. In both species, there was significant ID for fruit set, seed set and growth rate. V. myrtilloides had significantly greater ID for fruit set relative to V. corymbosum. There was significant ID for germination rate in V. corymbosum but not V. myrtilloides. The ability of self and outcross pollen to fertilize ovules was comparable, especially in V. corymbosum. Vaccinium corymbosum had a more consistent response to inbreeding across life history stage than V. myrtilloides. Inbreeding depression in V. myrtilloides was more severe for early traits and decline more dramatically at later stages. Estimates of lethal equivalents were higher in V. corymbosum. These data suggest that ID in the diploid, V. myrtilloides, is caused by strong deleterious alleles that are readily purged in early reproductive stages, while self-infertility in the tetraploid, V. corymbosum, results from the more gradual loss of higher order allelic interactions. The results are consistent with earlier studies that have shown that decreased self fertility in blueberries is caused by early-acting inbreeding depression, not late-acting self incompatibility.

INTRODUCTION

In plants, a reduction in fitness due to inbreeding results from one of three phenomena: pre-zygotic self-incompatibility (Proctor et al., 1996), post-zygotic self-incompatibility (ovular self-incompatibility or OSI) (Seavey and Bawa, 1986; Sage et al., 1994) or inbreeding depression (Charlesworth and Charlesworth, 1987). The theoretical nature of these phenomena has been discussed in detail in the literature and the citations above provide recent reviews. Elucidating the nature of self-sterility is important when one is interested in the evolution of mating systems as genetic system and ecological conditions interact to optimize the level of outcrossing in successful species.

Inbreeding depression (ID) is defined as a reduction in fitness from self pollination or a cross between closely related individuals relative to outcrossed individuals. Inbreeding depression is a quantitative trait, and individuals express differing levels of self-fertility depending on the number of deleterious alleles they carry. Inbreeding depression can manifest itself at any stage of the life cycle, affecting early to later traits such as fruit set, seed set, seed weight, germination rate, seedling development, growth and survival, flower production and fruit production.

The degree to which ID is manifest can depend on environmental conditions (Pray et al., 1994). Poor or unpredictable conditions can favor the survival of outcrossed, highly heterozygous individuals. In contrast, variation between habitats can favor local adaptation and the maintenance of coadapted gene complexes, thus favoring inbred individuals (Schmitt and Gamble, 1990).

Ideally, one should carry out a study of ID under field conditions where resource levels, herbivory and other environmental factors may be of importance, relative to the greenhouse.

Many studies have documented the presence of inbreeding depression (Rathcke and Real, 1993; Mayer et al., 1996; Daehler, 1999). Hokanson and Hancock (2000) found evidence of inbreeding depression in native blueberry species of the genus Vaccinium. In a greenhouse study, they demonstrated that selfing reduced fruit and seed set. For both traits, the lowered fertility of self crosses was attributed to early acting inbreeding depression, as opposed to self-incompatibility. Evidence for this assertion included a range in self-fertility among parents and the presence of fertilized, yet apparently aborted ovules in fruits (Figure 1). These aborted ovules varied in size and shape, suggesting that they were aborted at different stages. However, this assumption was not directly tested, except to show that self-pollen can sometimes accomplish fertilization (Krebs and Hancock, 1990; Hokanson and Hancock, 2000). Hokanson and Hancock (2000) suggested that differences in ploidy level among Vaccinium species may influence the expression of ID, with selfing accelerating the purging of deleterious alleles in diploids and polyploidy slowing this process down.

Polyploidy is a common phenomenon in plants which can influence patterns of genetic variation and the response to inbreeding (Grant, 1981; Bever and Felber, 1992). In general, polyploids are more heterozygous than diploids and therefore can potentially harbor more deleterious alleles than diploids. A tetraploid can have up to three deleterious recessive alleles without reducing its fitness, if only one "good" allele is needed. For a given level of heterozygosity, progeny of diploids are more likely to have been the result of outcrossing than those of tetraploids, because diploids carry only two alleles at a locus and have a higher likelihood of fixing deleterious alleles through selfing.

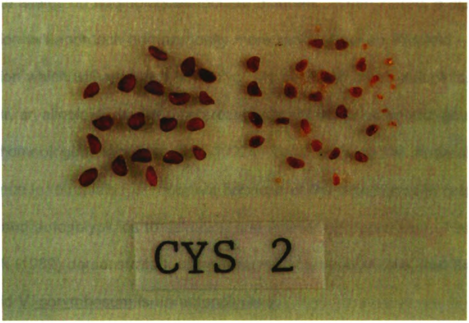


Figure 1. Range in seed sizes from a representative Vaccinium corymbosum fruit. Size and appearance in V. myrtilloides is comparable. The text in the picture is 12 point courier.

Given the same amount of outcrossing and heterozygosity, diploid species are expected to initially suffer more inbreeding depression than polyploids, but will more rapidly purge deleterious alleles after one or more generations of selfing.

Among polyploids, the mode of inheritance affects how genetic variation is distributed within and between individuals. Autopolyploids have what is termed tetrasomic inheritance and form multivalents, or a random association of bivalents during meiosis; therefore, an allele can segregate with any of the other three alleles in gametes. Allopolyploids have disomic inheritance with no pairing between homeologous loci. This can result in "fixed heterozygosity" when the alleles on homeologous loci are different. When inbred, a diploid population will approach homozygosity more rapidly than an alloploid population which will approach homozygosity quicker than an autoploid. However, an alloploid with fixed heterozygosity will never be homozygous across homeologous loci (Hancock, 1992). Thus, knowing the mode of inheritance in tetraploids is important because of the differences in response of allo- and autopolyploids to selection and inbreeding depression. Krebs and Hancock (1989) demonstrated, using isozyme segregation data, that the tetraploid V. corymbosum is an autopolyploid.

Until recently, most of the information on self-fertility in blueberries came from studies of fruit set in cultivars of narrow genetic background. These cultivars were selected for their ability to set fruit when planted in blocks of one or a few clones and were likely to be self-fertile (Morrow, 1943; Krebs and Hancock, 1989; Lang et al., 1990; Lang and Danka, 1991) . However, the reproductive success of a wild species is much more dependent on genotypic and environmental variability. Krebs and Hancock (1990) and Hokanson and Hancock (2000) have measured seed set in native clones in greenhouse studies and found considerable inbreeding depression. However, no one has studied the reproductive success of blueberries under field conditions.

One of the goals of this study was to determine the degree to which the results of Hokanson and Hancock (2000) are reproducible in the field. The number of fitness traits examined was also expanded by including germination rate and seedling growth rate, in addition to the fruit set and seed set included in their studies. These traits are expressed later in development and the expectation was that ID would be more severe under natural conditions in these presumably highly outcrossing species. In addition, the influence of ploidy level on inbreeding depression was examined with the hypothesis that diploid V. myrtilloides would have a more negative response to inbreeding than the autotetraploid V. corymbosum.

MATERIALS AND METHODS

Study System--The true blueberries in the genus Vaccinium, section Cyanococcus, are ideal for studying the relationship between inbreeding depression and ploidy. The section consists of closely related species at different ploidy levels with presumably high outcrossing rates. Camp (1942) referred to the entire section as "interlocking parts of a common polyploid complex". The diploid and tetraploid species of Vaccinium are described in detail in Vander Kloet (1980, 1981). The species used in this study were the diploid V. myrtilloides and tetraploid V. corymbosum.

Study Sites--Two populations of each species were studied (Figure 2). The two V. corymbosum populations are found in southwest Michigan. The

COL population is located in the Barry State Game Area at Otis Lake. The CCL population is located in the Allegan State Game Area at Crooked Lake. Both of these populations are found along the margins of shallow, acidic lakes with organic soils. The surrounding vegetation is primarily deciduous forest on sandy soils, with *Acer rubrum*, *Quercus alba* and *Fagus grandifolia* as the dominant overstory trees.

The *V. myrtilloides* populations are located further north. The MPR population is located at Cheboygan State Park in a mixed forest dominated by *Thuja occidentalis* and *Acer rubrum*. This habitat is characterized by low, wet areas and dry uplands which contain the bulk of the blueberry plants. *Vaccinium angustifolium* is sympatric with *V. myrtilloides* at MPR, but is much less common. The MFR population is located in Michigan's Upper Peninsula, in the Superior State Forest. The habitat here is a matrix of clear-cut and secondary growth with *V. angustifolium* common in cut areas and *V. myrtilloides* more often found under forest canopy. The forests are dominated by *Pinus banksiana*, *Pinus resinosa*, *Populus tremuloides* and *Betula papyrifera*.

Procedures--Pollinations were performed on a total of 76 experimental plants over two years, including 44 tetraploid and 32 diploid plants. There were 7-13 experimental plants in each population. Unique plants of *V. myrtilloides* were used each year, while several of the same *V. corymbosum* plants were used both years, one in the COL population and seven in the CCL population.

To examine the response to inbreeding, I performed a series of outcross and self-pollinations in the field. Pollen was collected from five randomly chosen individuals in a population and pooled for all the outcrossed pollinations. These individuals were termed "pollen donors". Pollen was collected by removing several recently opened flowers from a donor plant and rolling each of them between the thumb and forefinger, which caused the pollen to dehisce from the anthers. Pollen was collected on a cover slip and kept in a small petri dish. 30-40 flowers were emasculated on each experimental plant, divided between 2-3 areas of the plant. Approximately 2/3 of the emasculated flowers were pollinated with self-pollen while the remaining third were pollinated with the outcross pollen. More flowers were self-pollinated because of the anticipated lower set of selfed fruit. Crosses were performed in mid-May on V. corymbosum and in mid-June on V. myrtilloides in 1997. Pollinations were performed approximately three weeks earlier in 1998 due, presumably, to an earlier, warmer spring which sped up flower maturation. Each bunch of emasculated, hand-pollinated flowers was covered with a nylon mesh bag to exclude pollinators and herbivores, and to prevent removal of the developing fruits. Fruit from the artificial crosses were harvested when ripe, approximately 11 weeks after pollination.

Fig. 2. The locations of study populations. COL= *V. corymbosum* at Otis Lake, CCL= *V. corymbosum* at Crooked Lake, MFR= *V. myrtilloides* at Fox River, MPR= *V. myrtilloides* at Poe Reef. See the text for detailed descriptions of the location and habitat of each population.


ONT.
ONTARIO
LAKE SUPERIOR
MFR
SCHOOLCRAFT
LUCE
CHIPPEWA
MACKINAC
MARQUETTE
DELTA
IRON
GOGEBIC
ONTONAGON
MPR
LAKE HURON
WISCONSIN
LAKE MICHIGAN
HURON
CCL
COL
MICHIGAN
ONT.
LAKE ERIE
INDIANA
OHIO

Several fitness traits were monitored including fruit set, total ovules fertilized, seed set, percent germination and seedling size. Fruit set was determined as the number of mature fruit/flowers pollinated. Total ovules fertilized was the number of seeds, either aborted or mature, in a mature fruit. Fertilized seeds were identified by sorting through the contents of a fruit with a spatula, seeing the larger seeds and feeling for the hard seed coats of smaller seeds. Both of these species can have anywhere from 100-122 total ovules in a fruit, but in no fruit are all of the ovules fertilized and fully developed (Palser, 1961). Seed set was determined by the number of plump, mature seeds/total number of fertilized ovules. Percent germination was the fraction of mature seeds that germinated. Seedling growth rate was the height of a seedling when it initiated its fourth leaf/number of days to reach that height.

Individual fruits were sorted by plant and cross type, weighed and sliced through the middle with a razor blade and the seeds were spread on a piece of filter paper. Mature seeds were identified as those that were plump and brown, while ovules that were not filled or were white or small nubbins were considered aborted (Hokanson and Hancock 2000; Figure 1). Mature seeds were transferred to filter paper in a petri dish and moistened with a water-phosphoric acid solution of pH=5.0. The seeds were placed in a growth chamber under 16 hour/25° C days and 15° C nights, and seed germination was monitored every other day from August through December taking note of the day of germination.

To compare seedling growth rate, a random subset of the germinated seeds were removed from the petri dishes and transplanted into a 50:50 mixture of Scotts Metro Mix (Scotts-Sierra Horticultural Products Co.) and peat moss. The height of seedlings at the four leaf stage, in relation to time since emergence, was quantified in the fall of 1997. It was necessary to factor in the age of a seedling when it was measured, as not all seeds germinated on the same day.

Data Analysis--Mean population and species level values were calculated for fruit set, number of seeds, seed set, percent germination and growth for self and outcross pollinations. Cumulative fitness was calculated using these mean values for each trait, from each population as follows: the mean value for each trait, for each population was divided by 100, these new values were summed for each trait, within population and this sum was multiplied by 100 to arrive at cumulative fitness for each population for self and outcross pollinations. Cumulative inbreeding depression for each population and species was calculated as $1 - w_s/w_o$ where w_s and w_o are cumulative fitness for self and outcross pollinations respectively.

Fruit set, total ovules and seed set were analyzed using a three-factor split-plot ANOVA with nesting. The sources of variation were Year, Species, Population [Species] (random), Pollination, Pollination x Species, Pollination x Population[Species] (random), Year x Species, Year x Population[Species] (random), Year x Pollination, Year x Pollination x Species and Year x Pollination x Population[Species] (random). Percent germination was analyzed using a two-factor split-plot ANOVA with year nested within species because germination data was not available for the MFR population in 1997 (Table 1). Seedling size was analyzed with a two-factor ANOVA with Species, Pollination and Pollination x Species as sources of variation. Seed set and seedling size were log transformed to normalize the data. The other three variables did not require transformation. The significance of the Pollination x Species interaction term was tested using log transformed data. This was necessary in order to compare the relative rather than absolute difference between selfing and outcrossing (Johnston and Schoen, 1994).

Inbreeding depression was also measured at the family level using relative performance (RP), where RP is $1-w_S/w_O$ when $w_O > w_S$ and RP = $w_O/w_S - 1$ when $w_S > w_O$. w_O is the mean fitness for a particular trait when outcrossed and w_S is the mean fitness when selfed. This estimator is more informative than the traditional estimator of inbreeding depression ($1 - w_S/w_O$) when one has cases where selfed pollinations perform better than outcross pollinations (Ågren and Schemske, 1993). Relative performance values that are significantly > 0 indicate that outcrossed individuals were superior to inbred ones, signaling inbreeding depression. Relative performance values significantly < 0 indicate better performance of selfed individuals and outbreeding depression. For each species, cumulative RP was calculated as the sum of individual family RP values for fruit set, number of seeds, seed set and % germination. The data for growth rate were not quantified on a per family basis and RP values are simply means for all of the self and outcross pollination data.

Relative survival was calculated for each species and compared to the relative survival values of Hokanson and Hancock (2000). Relative survival is the percent mature seed per self pollination relative to outcross pollinations. A value is calculated for each plant, and then a mean is calculated for each species.

Lethal equivalents (LE) were also calculated. Lethal equivalents estimate the mean number of deleterious genes (genetic load) carried by each member of a population multiplied by the mean probability that each gene will cause genetic death when homozygous (Morton et al., 1956). Lethal equivalents were estimated as:

$LE = 2(-1/F)\ln RS$ F = Fixation Index
$F = 1/2$ in a Diploid
$F = 1/6$ in a Tetraploid
RS = Relative Survivorship (% mature seed per self pollination relative to outcross pollinations)

Statistical analyses were conducted using JMP 3.1.6 software (SAS Institute, 1994).

For all the traits, except seedling size, standard errors for relative performance were calculated using family means The family level analysis necessitated excluding some of the data because some plants did not set fruit when self pollinated (*V. myrtilloides* - 14 of 32 plants, *V. corymbosum* - 5 of 44 plants). A fruit set of zero was considered relevant; however, families that set no fruit when selfed were not considered for subsequent traits because it is not possible to follow the fate of a seed that never existed.

RESULTS

Inbreeding depression was apparent for all the fitness traits measured in both species, except percent germination in the diploid species, V. myrtilloides (Table 1). However, there was a great deal of plant to plant variation, with some individuals showing little evidence of ID, especially several V. corymbosum clones in the CCL population. Cumulative ID was comparable between the two species (Table 1). The estimated number of lethal equivalents (LE) was 17.4 and 3.3 in the V. corymbosum and V. myrtilloides, respectively (p=0.01, t-test).

Fruit set was the only trait in which there was a significant Pollination x Species interaction (Table 2). Vaccinium myrtilloides had a more negative response to inbreeding than V. corymbosum, particularly in 1997 (Figure 4). V. corymbosum had a comparable response to selfing in both years, with reductions of 42% (1997) and 40% (1998), while V. myrtilloides was reduced by 77% (1997) and 43% (1998). Inbreeding depression measured at the family level was significantly greater in V. myrtilloides (Figure 3; p<0.05, t-test).

Table 1. Mean fitness of selfed (S) and outcrossed (OC) offspring in the blueberry species Vaccinium myrtilloides and V. corymbosum. The abbreviations represent percent fruit set (% Frt. Set), mean number of fertilized ovules per fruit (# Seeds), percentage of fertilized ovules that develop into mature seeds (Seed Set), percent germination (% Germ.), growth rate of seedlings (Growth) and cumulative fitness of the product of the five life-history stages (Cum. Fitness). Cumulative inbreeding depression (ID) is 1- cumulative self/cumulative OC. Values in parentheses are standard errors. F values are for a one-way ANOVA comparing the means of the four populations.

	% Frt. Set		# Seeds		Seed Set		% Germ.		Growth		Cum. Fitness		ID
	S	OC	S	OC	S	OC	S	OC	S	OC	S	OC	
Vaccinium myrtilloides													
MPR	18.3(5.4)	54.9(6.3)	54.8(2.5)	59.7(1.8)	8.2(2.6)	27.2(2.0)	63.3(7.0)	40.4(5.1)	34.9(3.2)	40.7(2.4)	.182	1.466	.876
MFR	15.9(5.3)	38.9(6.1)	45.3(2.3)	56.3(1.7)	16.0(2.3)	23.5(1.9)	29.7(8.4)	33.4(7.5)		40.3(4.4)	.119	.693	.828
Mean	17.1(3.8)	46.6(4.5)	49.6(1.7)	58.0(1.3)	12.6(1.8)	25.3(1.4)	49.6(5.5)	38.2(4.2)	34.9(3.2)	40.6(2.3)	.185	1.060	.745
Vaccinium corymbosum													
COL	26.9(5.6)	63.3(6.5)	33.6(1.5)	38.1(1.2)	13.7(1.5)	30.1(1.3)	41.2(3.6)	60.0(2.5)	29.0(5.9)	49.0(3.7)	.148	2.134	.931
CCL	42.6(5.4)	55.4(6.3)	40.0(1.2)	39.9(1.2)	22.3(1.2)	25.3(1.3)	57.8(2.9)	58.4(2.6)	54.0(2.8)	73.2(3.2)	1.186	2.391	.504
Mean	34.8(4.0)	59.2(4.6)	37.4(1.0)	39.0(0.8)	18.8(1.0)	27.7(0.9)	51.1(2.3)	59.2(1.8)	49.3(2.8)	62.8(2.6)	.616	2.378	.741
F	4.8**	2.7	19.1***	52.0***	11.4***	3.6**	7.5***	7.2***	13.4***	23.8***			

* $p<0.05$, ** $p<0.01$, *** $p<0.001$

Table 2. Effects of year, species and pollination treatment on fitness at five life-history stages. Pop[Species], and all interactions involving it, are treated as a random effect. Significance tests for all transformed data were conducted on log-transformed data (Johnston and Schoen 1994).

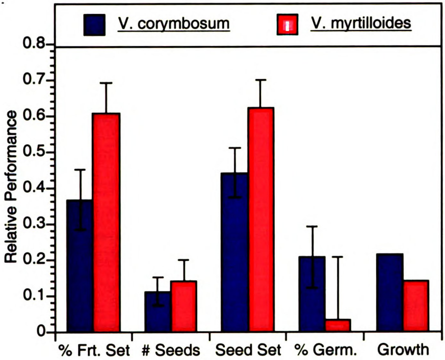
Life-History Stage	**Source**	**df**	**SS**	**F**
Fruit Set	Year	1	2528.7	4.6*
	Species	1	102.1	0.2
	Pop[Species]	2	503.0	0.2
	Pollination	1	5513.1	10.1**
	Pollination x Species	1	1696.9	6.4*
	Pollination x Pop[Species]	2	2344.0	4.1
	Year x Species	1	769.0	2.3
	Year x Pop[Species]	2	214.9	0.4
	Year x Pollination	1	1522.6	2.8
	Year x Species x Pollination	1	1546.2	3.8
	Year x Pollination x Pop[Species}	2	483.8	0.4
	error	112	61120.5	
Total Ovules	Year	1	5785.1	26.2***
	Species	1	23158.7	117.8***
	Pop[Species]	2	355.0	0.1
	Pollination	1	4705.0	21.3***
	Pollination x Species	1	3096.8	4.6
	Pollination x Pop[Species]	2	1846.9	2.3
	Year x Species	1	186.7	0.4
	Year x Pop[Species]	2	1823.7	1.9
	Year x Pollination	1	3.6	0.0
	Year x Species x Pollination	1	53.8	0.2
	Year x Pollination x Pop[Species}	2	840.0	1.9
	error	1000	220484.1	
Seed Set[1]	**Year**	1	688.0	2.2
	Species	1	2008.1	3.0
	Pop[Species]	2	3087.3	0.4
	Pollination	1	3134.0	16.1***
	Pollination x Species	1	291.3	0.4
	Pollination x Pop[Species]	2	2018.0	1.4
	Year x Species	1	2321.6	1.8
	Year x Pop[Species]	2	5829.2	3.0
	Year x Pollination	1	486.6	6.6**
	Year x Species x Pollination	1	2.2	0.0
	Year x Pollination x Pop[Species}	2	1591.5	2.5
	error	988	310979.8	
% Germination	Species	1	17937.8	11.4**
	Year[Species]	1	5956.5	1.7
	Pop[Species]	2	3461.8	0.2
	Pollination	1	162.8	0.1
	Pollination x Species	1	1007.1	0.3
	Pollination x Pop[Species]	2	14228.2	4.4
	Year x Pop[Species]	1	3579.4	1.7
	Year x Pollination	1	3372.9	1.6
	Year x Pollination x Pop[Species]	1	1947.6	1.4
	error	832	1133612.0	
Seedling Growth Rate[1]	Species	1	15736.3	36.8***
	Pollination	1	653.9	4.6*
	Pollination x Species	1	667.5	1.8
	error	198	73591.1	

***P<0.05; **P<0.01; ***P<0.001**

[1] Log transformed data

Fig. 3. Relative performance of the blueberries Vaccinium myrtilloides and V. corymbosum at several life history stages with % Frt. Set = fruit set, # Seeds = the total number of fertilized ovules, Seed Set = the fraction of fertilized ovules developing into mature fruits, % Germ. = the percentage of mature seeds that germinated, Growth = the growth rate of seedlings. Fruit set is significantly different at p=0.05 (t-test).

Figs. 4. Fruit set of selfed and outcrossed (OC) pollinations in two blueberry species in 1997 and 1998.


V. corymbosum
V. myrtilloides
Relative Performance
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
% Frt. Set
# Seeds
Seed Set
% Germ.
Growth

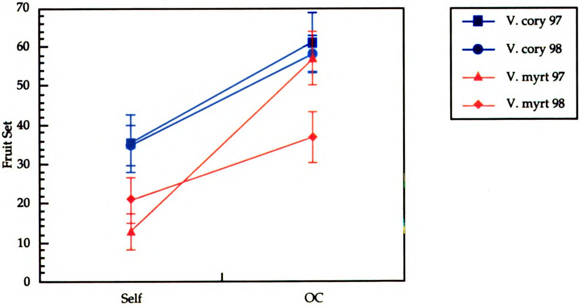
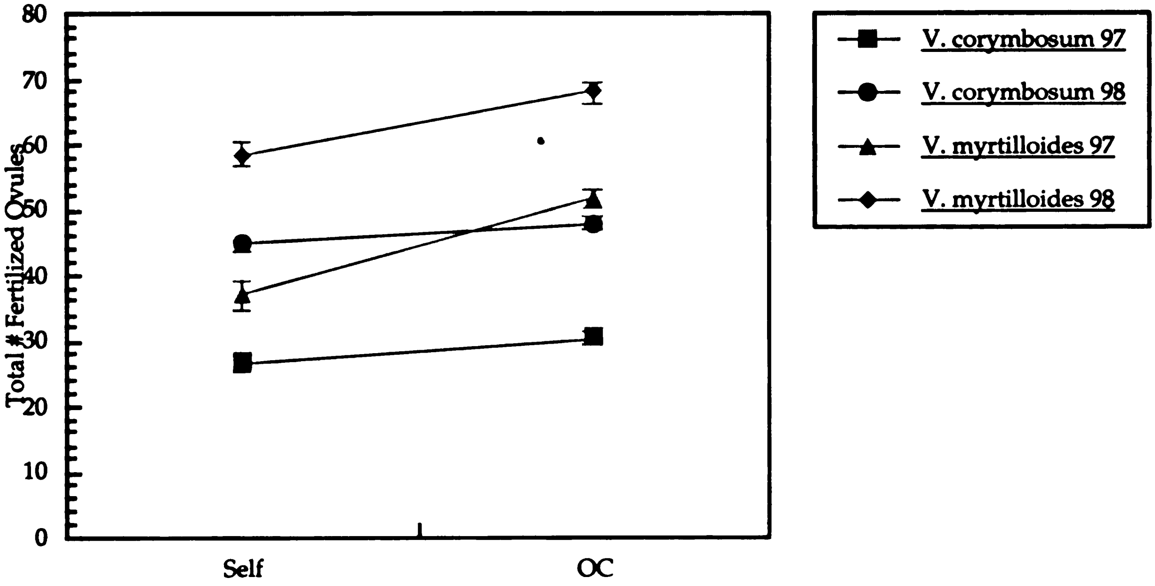

V. cory 97
V. cory 98
V. myrt 97
V. myrt 98
Fruit Set
70
60
50
40
30
20
10
0
Self
OC

Fig. 5. Total number of ovules fertilized in selfed and outcrossed (OC) pollinations in two blueberry species in 1997 and 1998.

Fig. 6. Average seed set in selfed and outcrossed (OC) blueberries in the years 1997 and 1998.


80
70
60
50
40
30
20
10
0
Total # Fertilized Ovules
Self
OC
V. corymbosum 97
V. corymbosum 98
V. myrtilloides 97
V. myrtilloides 98

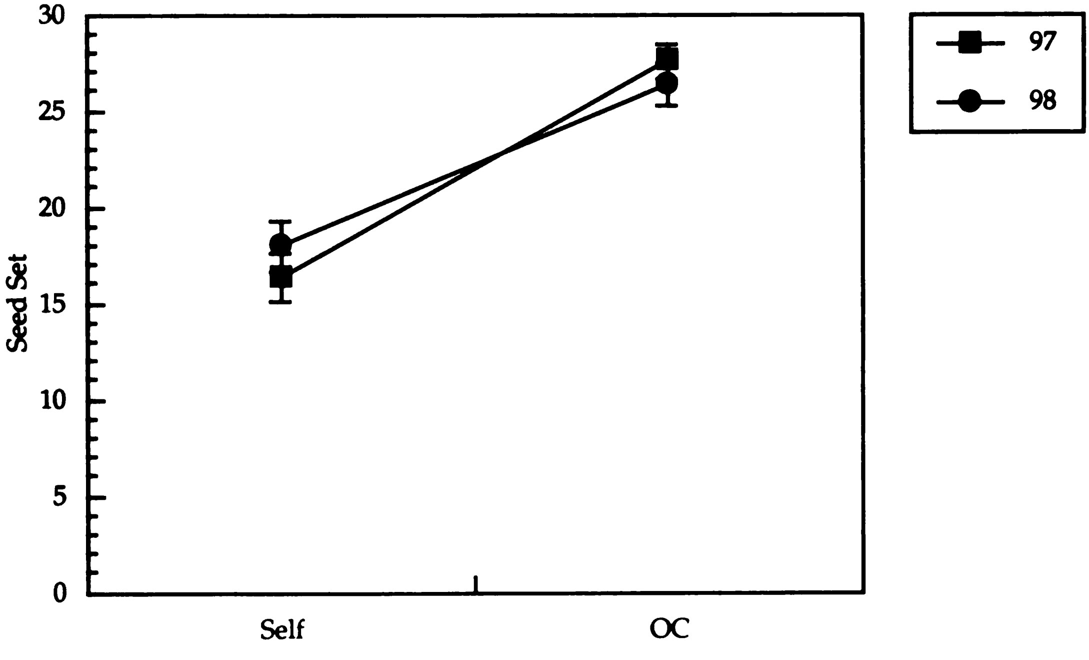
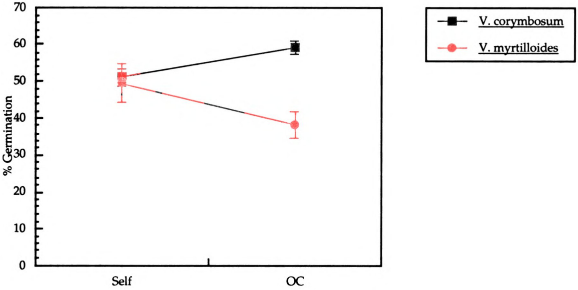
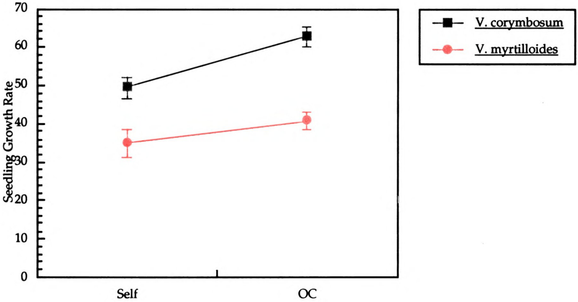

30
25
20
15
10
5
0
Seed Set
Self
OC
97
98

Fig. 7. Percent germination in two blueberry species for self and outcross (OC) pollinations.

Fig. 8. Growth rate in two blueberry species for self and outcross (OC) pollinations.


% Germination
70
60
50
40
30
20
10
0
Self
OC
V. corymbosum
V. myrtilloides


Seedling Growth Rate
70
60
50
40
30
20
10
0
Self
OC
V. corymbosum
V. myrtilloides

The two species had comparable reductions in the total number of ovules fertilized when selfed. For both species, the percent reduction was greater in 1997 than 1998, when more ovules were fertilized (Figure 5). The number of fertilized ovules was reduced 13% (1997) and 7% (1998) in V. corymbosum, and 28% (1997) and 14% (1998) in V. myrtilloides. Inbreeding depression measured at the family level was not significantly different in the two species (Figure 3).

There was significant inbreeding depression in both species for seed set, with average reductions of 50% and 32% in V. myrtilloides and V. corymbosum respectively (Figure 6). V. myrtilloides fitness was reduced 64% in 1997 vs. 39% in 1998, while V. corymbosum seed set was reduced 34% and 30% in 1997 and 1998, respectively. The degree of inbreeding depression measured at the family level was greater in V. myrtilloides (Figure 3) at the $p < 0.10$ significance level .

For percent germination, species was the only significant source of variation in the ANOVA, although there was only one year of data for V. myrtilloides (Table 2). Outcrossed progeny of V. corymbosum germinated 30% greater than V. myrtilloides; however, the decrease in germination after selfing (14% vs. 10% decrease) was not significantly different between V. corymbosum and V. myrtilloides (Figure 7).

Inbreeding depression was also apparent in seedling growth rate (Figure 8). Species was significant in the ANOVA, because V. corymbosum seedlings were, in general, taller than those of V. myrtilloides. However, the relative performance of inbred V. myrtilloides progeny was higher (Figure 3). V. corymbosum seedlings had a 22% decrease in size when selfed, while V. myrtilloides decreased 15%.

Absolute values of relative survival were higher in our study than in Hokanson and Hancock and were significantly different for V. myrtilloides (Table 3).

Table 3. A comparison of self and outcross performance between Hokanson and Hancock (2000) and the current study. Relative survival is the percent mature seed per self pollination relative to outcross pollination (standard error). Percent self is the percentage of plants that set a self pollinated fruit. Lethal equivalents is an estimate of the genetic load . See the text for a detailed description of lethal equivalents.

	Relative Survival	**n**	**Percent Self**	**Lethal Equivalents**
Hokanson				
V. myrtilloides	0.02 (.01)	4	27	15.7
V. corymbosum	0.26 (.06)	7	47	18.6
Schott				
V. myrtilloides	0.38 (.10)	14	60	3.3
V. corymbosum	0.28 (.09)	18	90	17.4

DISCUSSION

Overall, inbreeding depression was quite severe in both V. corymbosum and V. myrtilloides as was shown previously by Hokanson and Hancock (2000). This is expected in species that are presumed to have high outcrossing rates. While there have been no direct estimates of outcrossing rates in blueberries, their negative response to selfing, floral architecture and wide ranging bee pollinators would suggest that they have high realized outcrossing rates. Still, these two species of blueberries are hardly self-infertile or self-incompatible, particularly *V. corymbosum*. There is a great deal of intraspecific variation in selfing ability, with some genotypes performing as well or better when selfed and others that are relatively self-infertile (Krebs and Hancock, 1991; Hokanson and Hancock, 2000).

Vander Kloet and Lyrene (1987) suggested that geitonogamous pollinations are probably very common in blueberries and this may be one reason why a small fraction of their total ovules reach maturity. The low number of fertilized ovules and low seed set observed in this study are consistent with previous studies and support the idea that natural pollination involves many geitonogamous pollinations.

A comparison of Hokanson and Hancock (2000) to this study, indicates that relative survival and genetic load were comparable in *V. corymbosum* but differed considerably for *V. myrtilloides*. This field study observed much higher, and more diverse levels of self fertility in *V. myrtilloides* than Hokanson and Hancock's greenhouse study. As a result, estimates of genetic load are much higher in Hokanson and Hancock. My data may more accurately reflect the nature of fertility in *V. myrtilloides*, as my study had a considerably larger sample size, covered two years instead of one, and was performed in the field.

It is likely that ploidy level influences the self-fertility of diploid *V. myrtilloides* and tetraploid *V. corymbosum*. The response of diploid and polyploid species to ID can have a complex genetic basis and lead to considerable variation in response to inbreeding. Inbreeding is known to purge diploid populations of genetic load (Barrett and Charlesworth 1991), but it is less effective in polyploids. Relatively poor performance of selfed diploids is usually attributed to the expression of deleterious alleles in what is known as the partial dominance model of ID. Relatively poor performance of selfed polyploids is usually attributed to the loss of higher order allelic interactions in what is known as the overdominance model of ID (Bever and Felber, 1992). Serial inbreeding will increase levels of homozygosity and purge deleterious recessive alleles relatively quickly in diploids. However, polyploids reach homozygosity much slower than diploids, and if ID in polyploids is due to the loss of higher order allelic interactions, inbreeding and purging are less likely to yield plants and populations free of ID.

In this study, the diploid V. myrtilloides had fewer lethal equivalents than tetraploid V. corymbosum, but V. myrtilloides still carried sufficient numbers of deleterious alleles to significantly reduce its self-fertility. The highly outcrossed mating system of V. myrtilloides, and high reproductive output, may have slowed its approach to homozygosity.

The results of other studies examining the response to inbreeding in related diploid and polyploid species have varied. Polyploid complexes with lower seed and/or fruit set in diploids include wheatgrass (Dewey, 1969), maize (Alexander, 1960), clover (Townsend and Remmenga, 1968), ferns (Hedrick, 1987), and Epilobium angustifolium (Husband and Schemske, 1997). Polyploid complexes with less ID in diploids include orchardgrass (Kalton et al., 1952) and Amsinckia sp. (Johnston and Schoen, 1996).

Husband and Schemske (1997) examined several life history stages in *Epilobium angustifolium* and found consistently higher ID in diploids across stage, although selfing rates in the two ploidy levels were nearly identical, and relatively high (r=0.45). They attributed greater ID in the diploid to the expression of deleterious alleles in the homozygous state. The diploid studied by Johnston and Schoen (1996) was highly selfed and suffered significantly lower ID than the outcrossing tetraploid. Vander Kloet and Lyrene (1987) demonstrated significant, and comparable, ID in diploid, tetraploid and hexaploid *V. corymbosum* for seed set and seedling survival. Hokanson and Hancock (2000) found significantly greater ID in diploid *V. myrtilloides*, relative to tetraploid *V. corymbosum* for seed set, but the level of ID in tetraploid *V. angustifolium* was comparable to *V. myrtilloides*. The variation among these studies in the response of diploids and polyploids to inbreeding is not surprising, as the ability of a species to purge deleterious alleles depends on many factors including not only its ploidy, but also its mating system, selection pressure and population size.

The degree of ID varied across developmental stage in this study, with the highest values occurring for fruit set and seed set in both species, although the difference in the magnitude of ID between early and late stages was much greater in the diploid. These results suggest that the diploid may be more effective in purging, genetic load at these early stages than the polyploid, and the progeny that make it through the selective sieve consist of generally highly fit genotypes. Beneficial allelic combinations are probably not broken up as quickly in the tetraploid and while there is ID, there are fewer low quality individuals at the early reproductive stages amongst the tetraploids. In fact, the results for total fertilized ovules (Figure 3) show relatively low ID, suggesting that self pollen is only somewhat poorer at fertilizing ovules than outcrossed pollen.

Husband and Schemske (1996) found that in primarily outcrossing species most expressed ID at early (seed set) or late (growth and development) life history stages, while primarily selfing species expressed the majority of their ID at later stages. Their explanation was that outcrossers carry many strong recessive lethals, that are readily purged in early stages while the selfers carry only weakly deleterious mutations that are hard to purge until late stages. Husband and Schemske (1996) predicted that if the nature of ID is the same in two species, there should be consistent levels of ID across stages, but they did not consider ploidy level. The data described here My indicate that the nature of ID may differ between the diploids and tetraploids. The diploid may be expressing strong deleterious alleles in the homozygous state at early stages, while the more gradual decline in ID across stages in the tetraploid may be due to the loss of higher order allelic interactions as per the overdominance model of ID (Bever and Felber, 1992).

The higher degree of self-fertility in the polyploid may also have a simple historical basis. Vaccinium corymbosum presumably arose from diploid progenitors as a minority cytotype in a predominately diploid population. Consequently, most matings involving the new polyploid would be diploid x polyploid crosses resulting in inviable or infertile triploid hybrids. The ability of the new polyploid to persist requires a certain degree of self fertility. Tetraploid blueberries in the middle latitudes of North America are relatively young species that may have multiple origins (Vander Kloet, 1988). Relatively high self fertility in current tetraploid populations may be an historical artifact of their creation and establishment.

The difficulty Camp (1945) and Vander Kloet (1988) have had in classifying North American Vaccinium species and the intraspecific fertility that exists (Ballington and Galletta, 1978) suggests this is a young, rapidly evolving phyletic group. Indeed, the fact that morphologically indistinguishable V. corymbosum populations exist at the diploid, tetraploid and hexaploid levels (Vander Kloet, 1988) indicates that this group is in the early stages of speciation. The mating system of the group is probably evolving as well. It may be moving from a state of low self-fertility, due to ID, to self-incompatibility at the postzygotic stage. The variation in levels of self-fertility that exists in the populations studied suggest past purging of deleterious alleles in certain genotypes or segregation of self-compatibility and self-incompatibility alleles.

The response to inbreeding and nature of self-infertility is quite variable among many plant species. The response in blueberries depends on cytotype, with diploids easily purging deleterious alleles and tetraploids purging with greater difficulty. Blueberries have, in general, low self-fertility and are probably highly outcrossed. However, they are clearly not self-incompatible, especially the tetraploid V. corymbosum. Vaccinium corymbosum may have relatively high self fertility due to its recent hybrid origin from diploid progenitors.

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CHAPTER 2

THE EFFECT OF CROSSING DISTANCE ON FERTILITY IN *VACCINIUM* SPECIES

ABSTRACT

Outbreeding depression (OD) is defined as a reduction in fitness when distantly related or distantly spaced individuals are mated, compared to when more closely spaced individuals are crossed. This reduction is thought to occur when locally coadapted gene complexes are broken up in areas where there is spatial variation in selective regimes. I examined whether or not OD is important in diploid (*Vaccinium myrtilloides*) and tetraploid (*V. corymbosum*) blueberries, by performing self and outcross pollinations at differing distances in four populations (two for each species). I corroborated earlier studies demonstrating significant inbreeding depression in both of these species, but found that parental distance had no effect on outcross performance in *V. corymbosum.* In *V. myrtilloides*, there was higher fruit set and percent germination at intermediate outcrossing distances. Differences in ploidy level, and clonal growth patterns, may contribute to the differing responses of these species to crossing distance.

INTRODUCTION

Mating systems in plants evolve depending on how different types of crosses effect the fitness of individuals and progeny. Inbreeding depression (ID), outbreeding depression (OD), decreased resources and insufficient pollinators can all influence the reproductive output of individuals. In the previous chapter, I demonstrated that there is significant ID for fruit set, ovule fertilization, seed set, germination and seedling growth in two species of wild blueberries V. corymbosum and V. myrtilloides. This study was designed to examine the importance of OD in these same two species.

Outbreeding depression is defined as a decrease in any fitness trait when mating occurs between distantly related, and possibly distantly spaced individuals. It arises when there is spatial variation in selection regimes resulting in the evolution of specific locally adapted genes or gene complexes. An intermediate, optimal outcrossing distance may emerge when populations are highly differentiated and maximum fitness is associated with intermediate genetic similarities between parents (Campbell and Waser, 1987).

Studies searching for optimal outcrossing distances have both supported and refuted its existence. Vander Kloet and Lyrene (1987) found that crosses between unrelated, presumably more distantly spaced, individuals of V. corymbosum performed better than self-pollinated individuals. Species with intermediate, optimal outcrossing distance include Zea mays (Moll et al., 1965), Picea canadensis (Coles and Fowler, 1976), Delphinium nelsonii (Waser and Price, 1979 & 1994), Ipomopsis aggregata (Price and Waser, 1979), Amphicarpaea bracteata (Parker, 1992) and Agave schottii (Trame et al., 1995). Species without an optimal outcrossing distance include Clarkia tembloriensis (Holtsford, 1996), Gentiana pneumonanthe (Oostermeijer et al., 1995), and Chamaecrista fasciculata (Fenster and Sork, 1988). Clearly, numerous factors interact to determine which types of pollinations are successful in a species including: how closely related is the pollen received by a plant, levels of genetic differentiation, the genetic system that accepts or rejects pollen, the number of deleterious alleles carried, patterns of segregation, and environmental effects.

The evolution of mating systems in hermaphroditic plants is complex. The degree of pollen diversity accepted by a plant is dependent on the importance of genetic differentiation in regulating fertility. The plant is constrained by what pollinators bring to the plant and what arrives via wind and water. Several mechanisms have evolved to modify the degree to which a plant receives self or outcross pollen. Physiologically, plants can accept or reject self pollen through incompatibility systems. Morphologically, inflorescence design can influence rates of geitonogamy. Dispersal distance and plant spacing can also regulate the degree to which biparental inbreeding occurs.

The coevolution of plants and pollinators is also complex, with fitness being maximized in each to the point that does not compromise the fitness of the other. The genetic system of the plant will constrain the coevolutionary process and determine the extent to which it will accept or reject self or outcross pollen. If pollination between close related and/or spaced plants results in biparental inbreeding, and inbreeding is deleterious, then plant reproductive success will be highest with pollen from distant sources. If local adaptation is more important, then distant pollen will reduce reproductive success. Examining the effect of pollen source on fitness characters can lend insight into the evolution of a mating system and whether or not genetic differentiation is important.

Herein, a crossing experiment is described using Vaccinium myrtilloides and V. corymbosum that tests whether or not there is an optimal outcrossing distance in these diploid and tetraploid species. The data support previous work demonstrating that ID has a negative impact on the reproductive success of both these species (Hokanson and Hancock, 2000; Schott, 2000), but OD was found to be only important in V. myrtilloides.

MATERIALS AND METHODS

Four different types of crosses were made in the field: 1) self – self pollination, 2) close – pollen from five local, randomly selected donors (<10 m apart, 3) distant – pollen from five distant, randomly selected donors from the same population (100-200 m apart), 4) other – pollen from five, randomly selected donors from another population.

Fig. 1. The locations of study populations. *Vaccinium corymbosum* at Otis Lake (COL), *V. corymbosum* at Crooked Lake (CCL), *V. myrtilloides* at Fox River (MFR), *V. myrtilloides* at Poe Reef (MPR). The Black River population served as the distant pollen donor for the MPR population. See the text for detailed descriptions of the location and habitat of each population.
Pollen was pooled for all the outcrossed pollinations.


ONT.
ONTARIO
LAKE SUPERIOR
MFR
Black River
MPR
LAKE HURON
WISCONSIN
LAKE MICHIGAN
CCL
COL
MICHIGAN
INDIANA
OHIO
ONT.
LAKE ERIE

Pollen was collected by removing several recently opened flowers from a donor plant and rolling each of them between the thumb and forefinger, which caused the pollen to dehisce from the anthers. Pollen was collected on a cover slip and kept in a small petri dish. Outcross pollen was applied intraspecifically using the CCL population as the 'Other" donor for the COL population and vice versa. The MPR population served as the 'Other' donor for the MFR population; however, the reverse was not possible. The MFR population was not releasing pollen when the MPR population was ready for pollination. Instead, pollen was obtained from a more southern Upper Peninsula population as the source of 'Other' pollen for MPR. This population was located at the Black River Campground in the Lake Superior State Forest (Fig. 1). There were nine experimental plants in each of the V. corymbosum populations and four and five experimental plants in the MPR and MFR populations, respectively. Vaccinium myrtilloides populations are, in general, less robust than V. corymbosum and there are fewer plants with enough flowers on which to perform the pollinations. On each V. corymbosum plant, an average of 23 self-pollinations were performed, along with 12 of each of the three outcross pollinations. On each V. myrtilloides plant, an average of 19 self-pollinations were performed, along with 13 of each of the three outcross pollinations.

Five fitness traits were analyzed including fruit set, total number of fertilized ovules, seed set, percent germination and seedling size. Fruit set was calculated as the proportion of pollinated flowers that developed into mature fruit. Total number of fertilized ovules was averaged across fruit in each treatment combination. Blueberries have 100-122 ovules per fruit (Palser 1961), and only a subset of these ovules are fertilized (approx. 44%) and develop into mature seeds (approx. 24%) (Schott, 2000). Seed set is defined as the proportion of fertilized ovules that developed into mature, plump seed (Schott, 2000). To calculate percent germination, seeds were extracted from ripe fruit and kept moist in a pH=5 solution of water and phosphoric acid. The seeds were isolated by fruit in a petri dish and kept in a growth chamber set at 20°C, 15 hour day and 15°C night. Germination was monitored twice weekly from late August until the middle of January, when it had ceased. A subset of the germinated seeds were transferred to a 50:50 mixture of peat moss:Scots Metro Mix 360 (Scotts-Sierra Horticultural Products Co.). Seedling height was measured when a seedling had initiated its fourth set of leaves, which was, on average, 76 days after germination and 65 days after emergence from the soil surface. The seedlings were grown in the growth chamber set at 20°C, 15 hour day and 15°C night.

The data were analyzed in a two-factor split-plot ANOVA with nesting. The sources of variation were Species, Populations[Species] (random), Pollination, Pollination x Species and Pollination x Population[Species] (random). The seed set data was log transformed and the seedling size data was arcsine-square root transformed to improve normality. The Pollination by Species interaction term indicates whether the response to pollination treatment varies between species. The significance of this interaction was tested on log transformed data for all traits, in order to determine the relative difference between selfing and outcrossing treatments (Johnston and Schoen 1994). All analyses were performed using JMP 3.1.6 software for the MAC (SAS Institute 1994).

Relative performance (RP) was calculated across families as an alternative to the traditional measure of ID (1 - ws/wo). RP is 1-w_S/w_O when $w_O \geq w_S$ and w_O/w_S - 1 when $w_S > w_O$. w_O is the mean fitness for a particular trait when outcrossed and w_S is the mean fitness when selfed. RP is a more appropriate estimator of ID when you have individuals performing better when selfed (Ågren and Schemske 1993). Relative performance values significantly > 0 indicate better performance of outcrossed than selfed individuals and ID. Relative performance values significantly < 0 indicate better performance of selfed individuals and outbreeding depression. RP was calculated on all the data for fruit set, but only those plants producing selfed seed were analyzed for the other parameters.

RESULTS

Pollination treatment had a significant affect on fruit set (Table 1), but the performance of the two species and their populations were not significantly different. Selfs performed relatively poorly, while the various outcross treatments performed equally well (Figure 2). Outcross performance in V. myrtilloides peaked at the intra-populational 'Distant' treatment, suggesting an optimal outcrossing distance, but this difference was not significant. In V. corymbosum, fruit set was lower in selfed than out-crossed pollen, but the

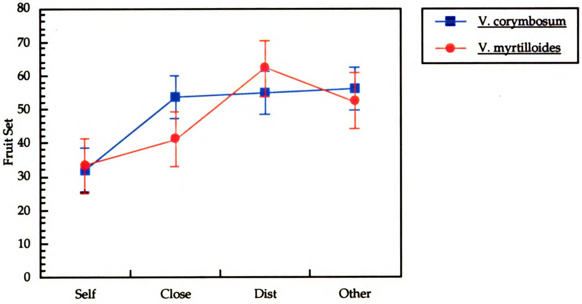
Table 1: Effects of species and pollination treatment on fitness at five life-history stages in Vaccinium myrtilloides and V. corymbosum blueberries. Pop[Species], and all interactions involving it, is treated as a random effect. The Pollination x Species interaction was tested on log transformed data for all stages as per Johnston and Schoen 1994.

Stage	Source	df	SS	F
Fruit Set	Species	1	84.7	0.07
	Pop[Species]	2	3967.0	2.9
	Pollination	**3**	**6754.2**	**3.7***
	Pollination x Species	3	2198.7	0.2
	Pollination x Pop[Species]	7	4855.7	1.1
	Error	108	66310.6	
Total Seeds/ Fruit	Species	1	24457.9	30.6
	Pop[Species]	2	1994.4	0.4
	Pollination	**3**	**22713.1**	**23.9*****
	Pollination x Species	**3**	**22695.1**	**4.4***
	Pollination x Pop[Species]	**6**	**13984.7**	**7.4*****
	Error	701	222284.3	
Seed Set[1]	Species	1	6960.4	2.0
	Pop[Species]	2	9360.3	2.4
	Pollination	**3**	**35477.9**	**32.0*****
	Pollination x Species	3	15599.6	3.5
	Pollination x Pop[Species]	**6**	**11778.9**	**6.3*****
	Error	701	307385.8	
% Germination	Species	1	14683.3	7.7
	Pop[Species]	2	4625.8	1.0
	Pollination	**3**	**29096.6**	**9.7*****
	Pollination x Species	3	15583.3	2.6
	Pollination x Pop[Species]	**6**	**13736.8**	**2.3***
	Error	708	777147.2	
Seedling Growth Rate[2]	Species	1	0.08	1.1
	Pop[Species]	2	0.1	2.6
	Pollination	**3**	**0.3**	**4.8****
	Pollination x Species	**3**	**0.2**	**3.5***
	Pollination x Pop[Species]	5	0.1	1.0
	Error	389	9.7	

1: log transformed
2: arcsine-square root transformed
*p<.05, **p<.01, ***p<.001

Fig. 2. The percent fruit set in *V. myrtilloides* and *V. corymbosum* blueberries for self, close (pollen source within 10 m), distant (pollen source 100-200 m distant) and other (reciprocal pollination between populations within species) pollinations.

Fig. 3. The relative performance (RP) for fruit set in *V. myrtilloides* and *V. corymbosum* blueberries for self, close (pollen source within 10 m), distant (pollen source 100-200 m distant) and other (reciprocal pollination between populations within species) pollinations. [RP is $1-w_S/w_O$ when $w_O \geq w_S$ and $w_O/w_S - 1$ when $w_S > w_O$, w_S and w_O are the fitness of outcross and selfed individuals respectively] An RP value > 0 represents ID while a value < than 0 represents OD.


80
70
60
50
40
30
20
10
0
Fruit Set
Self
Close
Dist
Other
V. corymbosum
V. myrtilloides

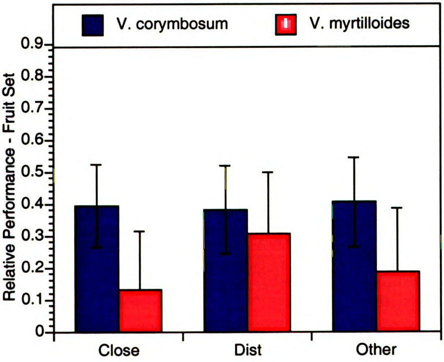
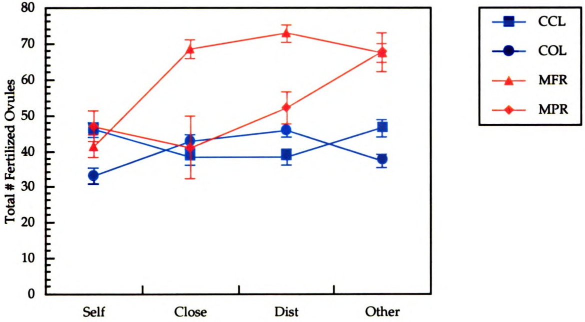

V. corymbosum
V. myrtilloides
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Relative Performance - Fruit Set
Close
Dist
Other

Fig. 4. The total number of ovules fertilized (aborted & mature) in *V. myrtilloides* and *V. corymbosum* blueberries for self, close (pollen source within 10 m), distant (pollen source 100-200 m distant) and other (reciprocal pollination between populations within species) pollinations.

Fig. 5. The relative performance (RP) for total number of ovules fertilized in *V. myrtilloides* and *V. corymbosum* blueberries for self, close (pollen source within 10 m), distant (pollen source 100-200 m distant) and other (reciprocal pollination between populations within species) pollinations. [RP is $1-w_S/w_O$ when $w_O \geq w_S$ and $w_O/w_S - 1$ when $w_S > w_O$, w_S and w_O are the fitness of outcross and selfed individuals respectively] An RP value > 0 represents ID while a value < than 0 represents OD.


80
70
60
50
40
30
20
10
0
Total # Fertilized Ovules
Self
Close
Dist
Other
CCL
COL
MFR
MPR

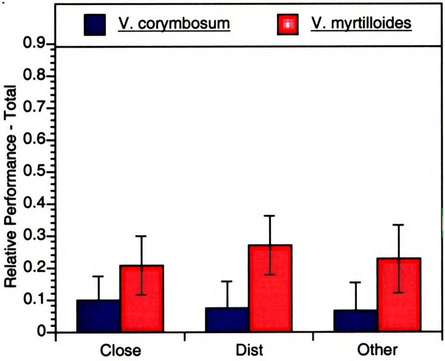
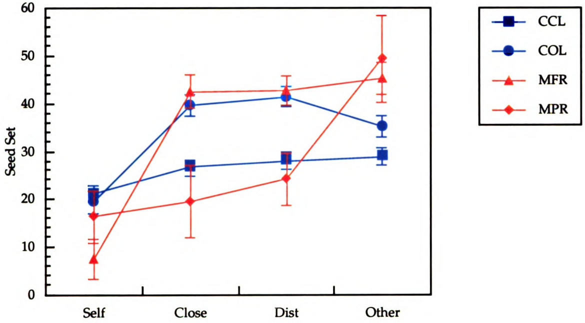

V. corymbosum
V. myrtilloides
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Relative Performance - Total
Close
Dist
Other

Fig. 6. The percentage of fertilized ovules developing into mature seeds (seed set) in *V. myrtilloides* and *V. corymbosum* blueberries for self, close (pollen source within 10 m), distant (pollen source 100-200 m distant) and other (reciprocal pollination between populations within species) pollinations.

Fig. 7. The relative performance (RP) for seed set in *V. myrtilloides* and *V. corymbosum* blueberries for self, close (pollen source within 10 m), distant (pollen source 100-200 m distant) and other (reciprocal pollination between populations within species) pollinations. [RP is $1\text{-}w_S/w_O$ when $w_O \geq w_S$ and $w_O/w_S - 1$ when $w_S > w_O$, w_S and w_O are the fitness of outcross and selfed individuals respectively] An RP value > 0 represents ID while a value < than 0 represents OD.


60
50
40
30
20
10
0
Seed Set
Self
Close
Dist
Other
CCL
COL
MFR
MPR

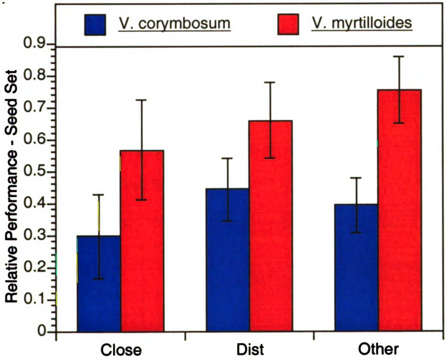
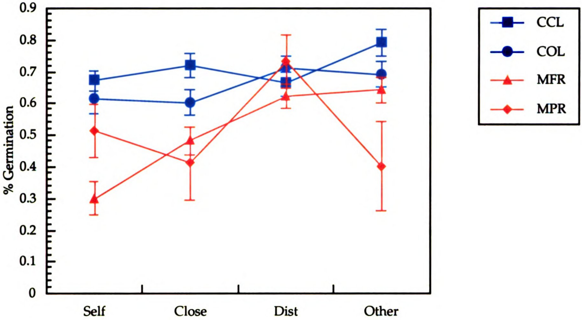

V. corymbosum
V. myrtilloides
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Relative Performance - Seed Set
Close
Dist
Other

Fig. 8. The percentage of mature seed germinating in *V. myrtilloides* and *V. corymbosum* blueberries for self, close (pollen source within 10 m), distant (pollen source 100-200 m distant) and other (reciprocal pollination between populations, within species) pollinations.

Fig. 9. The relative performance (RP) for seed germination in *V. myrtilloides* and *V. corymbosum* blueberries for self, close (pollen source within 10 m), distant (pollen source 100-200 m distant) and other (reciprocal pollination between populations within species) pollinations. [RP is $1-w_S/w_O$ when $w_O \geq w_S$ and $w_O/w_S - 1$ when $w_S > w_O$, w_S and w_O are the fitness of outcross and selfed individuals respectively] An RP value > 0 represents ID while a value < than 0 represents OD.


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
% Germination
Self
Close
Dist
Other
CCL
COL
MFR
MPR

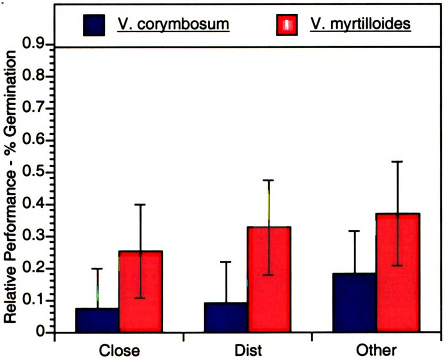
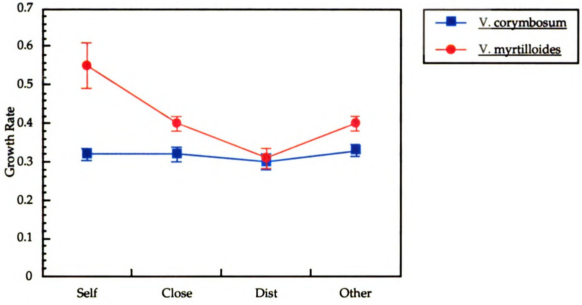
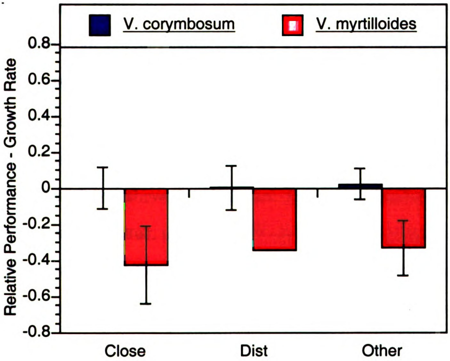

V. corymbosum
V. myrtilloides
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Relative Performance - % Germination
Close
Dist
Other

Fig. 10. Seedling growth rate in *V. myrtilloides* and *V. corymbosum* blueberries for self, close (pollen source within 10 m), distant (pollen source 100-200 m distant) and other (reciprocal pollination between populations, within species) pollinations. The value for seedling growth rate represents the height of the seedling, in mm, divided by the number of days since the seedling had emerged from the soil.

Fig. 11. The relative performance (RP) for growth rate in *V. myrtilloides* and *V. corymbosum* blueberries for self, close (pollen source within 10 m), distant (pollen source 100-200 m distant) and other (reciprocal pollination between populations within species) pollinations. [RP is $1-w_S/w_O$ when $w_O \geq w_S$ and $w_O/w_S - 1$ when $w_S > w_O$, w_S and w_O are the fitness of outcross and selfed individuals respectively] An RP value > 0 represents ID while a value < than 0 represents OD.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Growth Rate
Self
Close
Dist
Other
V. corymbosum
V. myrtilloides


V. corymbosum
V. myrtilloides
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
Relative Performance - Growth Rate
Close
Dist
Other

source distance was not important. Relative performance for fruit set was comparable between pollination treatments and species (Figure 3), although the absolute values were lower in V. myrtilloides suggesting less ID.

The total number of ovules fertilized also differed significantly between pollination treatments. The response varied depending on the population (Table 1). The COL and MFR populations showed significant ID for most pollination treatments, while in the CCL and MPR populations, selfed and outcrossed progeny had a comparable performance for all but the most distant cross in the MPR population (Figure 4). Only in the MFR population was there evidence of an optimal, intermediate, outcrossing distance (Figure 4). Inbreeding depression was consistently higher in V. myrtilloides than V. corymbosum, but only significantly for the 'Distant' treatment (Figure 5).

Pollination treatment had a significant effect on seed set. The effect depended on species and population (Table 1). The CCL, COL and MFR populations had a significant boost in performance when outcrossed. The COL population showed a significant intermediate, optimal outcrossing distance for this trait, while the MPR population again had the highest performance with the most distant pollination treatment (Figure 6). Inbreeding depression was highest for this trait in V. myrtilloides (Figure 7).

Pollination treatment and population had significant effects on germination (Table 1). Germination rate was higher in V. corymbosum populations for all treatments except 'Distant' and there was less ID in V. corymbosum. The MFR population showed a steady increase in germination as pollen distance increased while the MPR population showed an intermediate optimal outcrossing distance (Figure 8). Again, ID was greater in *V. myrtilloides* than *V. corymbosum*, and selfed and outcrossed progeny of *V. corymbosum* had similar germination rates, except for the most distant outcross treatment (Figure 9).

For seedling growth rate, the performance of *V. corymbosum* progeny was similar across treatments, while in *V. myrtilloides* there was considerable outbreeding depression across all treatments (Figure 11). While few self-pollinated progeny made it through to the seedling stage in *V. myrtilloides*, those that did had significantly higher growth rates than outcrossed seed. Many of the germinated seeds emerged from the soil, but died before they could be measured. Over 70% of the transferred seeds from the MPR population emerged from the soil surface, but none of them survived.

DISCUSSION

There was not a uniform response to crossing distance between *V. myrtilloides* and *V. corymbosum*, and between populations within species, especially for the *V. myrtilloides* populations. This result is comparable to that of Waser et al. (2000) who found variable expression of outbreeding depression among cohorts of *Ipomopsis aggregata*. *Vaccinium myrtilloides* showed evidence of an optimal outcrossing distance, or outbreeding depression for several traits; however, *V. corymbosum* had a relatively uniform response to outcrossing distance. The presence of outbreeding depression in *V. myrtilloides* suggests that there is more genetic structuring within these populations than *V. corymbosum*. This may be related to ploidy differences, as the tetraploid *V. corymbosum* has been shown to be much more heterozygous than the diploid *V. myrtilloides* (Hokanson and Hancock, 2000) and this increased heterozygosity may adapt it to a broader range of environmental conditions. There also may be selection within the polyploid genome to maintain higher order allelic associations, as per the overdominance model of inbreeding depression (Charlesworth and Charlesworth, 1987). In previous inbreeding studies done on cultivated blueberries, Krebs and Hancock (1988) found that all cultivars suffered reductions in fertility when selfed, but the use of any outcrossed parent restored fertility, regardless of relatedness. The diploid species may be more finely tuned to its environment, than the tetraploid, as the diploid condition would encourage more homozygosity and the formation of unique coadapted complexes.

The data from this study corroborates earlier reports of inbreeding depression in both species (Hokanson and Hancock. 2000; Schott, 2000). The more clonal growth pattern of *V. myrtilloides* may explain, at least a part of, the difference in the degree of outbreeding depression we observed between the two species. *Vaccinium myrtilloides* populations are composed of large clones often extending several meters, while *V. corymbosum* clones are crown formers covering much smaller, discrete surface areas. As a result, pollinations within a 10 m area have a higher probability of involving self-pollination in *V. myrtilloides*, than *V. corymbosum*. This effect may have been most pronounced in the MPR population where clones were large and continuous with undefined boundaries, compared to the MFR population which consisted of smaller, well defined clones

In this study, the pollinations were performed in the field but germination and seedling growth were monitored in environmental chambers. This may have overestimated the fitness of outcrossed progeny, as plants resulting from local pollination might perform better under local conditions. However, seedling survival was very low in the environmental chamber and greenhouse, and my anecdotal observations in the field suggest that seedling survival in the field is low as well. Any study attempting to assess seedling survival under natural conditions will likely suffer from small sample size given high putative seedling mortality.

In summary, there is no evidence for OD, or an optimal outcrossing distance, in the tetraploid *V. corymbosum*. However, in the diploid species *V. myrtilloides*, there does appear to be an optimal outcrossing distance of approximately 100 m for fruit set and percent germination. An interplay between growth pattern and ploidy probably contributed to these differential patterns. The tetrasomic inheritance of *V. corymbosum* may moderate the negative effects of inbreeding or outbreeding, and the clonal growth pattern of this particular species will favor a higher percentage of outcross pollinations. The growth pattern of the diploid *V. myrtilloides* will favor a higher proportion of self pollinations, relative to the tetraploid, and its disomic inheritance pattern will more readily manifest the negative effects of inbreeding or outbreeding. It appears that levels of inbreeding depression in natural species are regulated by a complex interaction of factors.

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CHAPTER 3

THE NATURE OF INFERTILITY IN *VACCINIUM* SPECIES

ABSTRACT

The nature of self-infertility is an active area of research in plant biology. The mode of self-infertility, inbreeding depression (ID) or post-zygotic incompatibility, otherwise known as ovular self-incompatibility (OSI), can have important consequences for the evolution of mating systems. Previous studies attempting to determine whether low self-fertility in blueberries is due to ID or OSI have been equivocal. Criteria by which one can distinguish between ID and OSI include measuring levels of inter-plant variation in self-fertility and seed development. In this study seed development was monitored in self- and cross-pollinated fruits in two species of tetraploid blueberries, Vaccinium angustifolium and V. corymbosum. It was discovered that many self-fertilized ovules begin to develop, but abort at different times. In addition, there was considerable variation in self-fertility among individual plants. These data, in conjunction with previous work, support the hypothesis that blueberries suffer from ID, not OSI.

INTRODUCTION

An active area of research in plant biology is the nature of self-fertility or, alternatively, self-infertility. Darwin (1876) was first to systematically examine self-fertility in plants. He self-pollinated 40 species for 10 consecutive generations and observed that some species showed a decline in fitness when selfed, relative to outcrossed lines. This phenomenon of inbreeding depression has subsequently been well documented.

Despite the known negative consequences of inbreeding, a common pathway in the evolution of plant mating systems is a shift from self-incompatibility (obligate outcrossing) to predominant self-fertilization (Stebbins, 1957). This shift is believed to have occurred in many plant families (Stebbins, 1974; Wyatt, 1988), including many cultivated species (Rick, 1988). The shift to self-compatibility is believed to be due to genes that modify the functioning of self-incompatibility alleles at so called S-loci (Lewis, 1979). This shift to self-compatibility, due to modifier alleles, is known as pseudo-self-fertility (de Nettancourt, 1977). Pseudo-self-fertility is differentiated from true self-fertility based on the criteria outlined in Table 1.

Table 1: Criteria for differentiating between pseudo-self-fertility and true self-fertility as specified by Levin (1996). The statements apply to a species that is subject to pseudo-self-fertility.

1. Greater seed production with cross pollination relative to self pollination.
2. Earlier germination and more rapid pollen tube growth with cross pollen.
3. A continuous distribution of self-fertility levels in progeny.
4. Self versus outcross performance depends on environmental conditions and plant age.
5. Variable levels of seed set are found in crosses between plants that share both S alleles.

The inability of self-pollinated flowers to set seed can be grouped into many classes according to the time at which the pollination/fertilization process is arrested. Self-infertility occurs most immediately when self pollen fails to germinate on the stigma. This is usually attributed to sporophytic self-incompatibility (SSI) where gene products of the diploid sporophyte, deposited on the pollen grain coat, are recognized and rejected. The next class arises when pollen tube growth is arrested in the style. This is usually attributed to gametophytic self-incompatibility (GSI) where the haploid genotype of the pollen grain nuclei is recognized and rejected (Proctor et al. 1996). The existence of SSI and GSI systems has been known for quite some time, and recognition of self is attributed to alleles at one or a few of the S-loci (Lewis, 1979). The appropriateness of using only two classes to describe pre-zygotic SI has been recently questioned by Sage et al. (1999) who believe that there are a wide array of pollen-pistil interactions. They hypothesize that in their system (Narcissus triandrus) embryo sacs degenerate due to the lack of a proper stimulus, even though pollen tubes appear to develop normally. Indeed, pre-zygotic incompatibility probably occurs on a continuum from pollen recognition, and rejection, to gamete rejection after the pollen tube penetrates the micropyle.

The next stage of reproductive arrest is post-zygotic, and can occur due to inbreeding depression (ID) or ovarian self-incompatibility (OSI). With ID, developing embryos abort at different stages of development due to genetic load or the loss of heterotic interactions, and selfing results in variable seed production among genotypes (Charlesworth and Charlesworth 1987). In OSI systems, there is a uniform rejection of self fertilized ovules in all individuals at an early stage of embryo development, and species segregate within families for self- incompatibility alleles.

Seavey and Bawa (1986) first spelled out the criteria for differentiating between ID and OSI (Table 2). A number of recent studies have documented the presence of OSI. Naaborgh and Willemse (1992) observed a uniform rejection of self-pollinated ovules approximately two days after fertilization in Gasteria verrucosa. Gibbs and Bianchi (1999) observed zero self-seed set in Dolichandria cynanchoides and Tabebuia nodosa despite penetration of the micropyle by pollen tubes. Lipow and Wyatt (2000) determined that Asclepias exaltata exhibits OSI and that the occasional self-fertile plant is due to pseudo-self-fertility alleles that alter S-locus functioning.

Table 2: Criteria for differentiating between inbreeding depression (ID) and ovular self-incompatibility (OSI) as specified by Seavey and Bawa (1986).

1. Uniform abortion of embryos at the same stage of development suggests OSI while abortion at various stages suggests ID.
2. Variability among plants in self seed set suggests ID.
3. With ID, those selfed seeds that do mature will likely express the effects of genetic load at following stages of development.
4. Embryo rescue should be possible on aborted embryos subject to OSI, not ID.

Numerous studies have documented ID at various stages of plant development including seed set in alfalfa (Holland and Bingham 1994), maize (Pinnisch and Stucker 1998) and blueberries (Harrison et al. 1994, Hokanson and Hancock 2000; Schott, 2000), forage production in alfalfa (Holland and Bingham 1994, Sain et al. 1994) and leeks (Smith and Crowther 1995), and disease resistance in winter rye (Miedaner et al. 1995). In addition, selfing has been shown to result in variable seed production among genotypes in maize (Vasal et al. 1995) and blueberries (Krebs and Hancock, 1991; Schott, 2000).

Traditionally, ID was considered the primary factor in post-zygotic infertility. However, OSI has gained credence and is considered a widespread phenomena (Sage et al. 1999). Since the nature of the infertility system can impact the evolution of the mating system, distinguishing between ID and OSI is important. Where self-fertility is minimal, early recognition of self pollen minimizes the wastage of resources into partially developed seeds, and the fouling of the style with self pollen tubes that inhibit subsequent outcross pollinations. Because fertilized ovules are rejected early and uniformly in OSI, resource wastage is decreased with OSI relative to abortion due to ID. Strict self-incompatibility allows for little or no leakage of selfed progeny through the system. However, some leakage can be beneficial when other pollen sources are unavailable or unreliable, or if there is a reasonably high probability that beneficial recessive alleles are found in the population. Such an allele has a higher probability of being expressed with self pollination, as a higher proportion of loci will be homozygous. Where ID is severe, and a premium is placed on outcrossing, one can envision a scenario where a species evolves from later to early stage self recognition and rejection. This would depend on there being a selective advantage to individuals who reject self pollen earlier in the reproductive process. Pollen discounting, the decrease in paternal fitness due to self pollination, can promote this trend. However, pollinator behavior will also need to evolve where pollen discounting is a factor. Self-compatibility is favored under the following conditions: when the fitness of selfed progeny is at least half that of outcross progeny (Fisher 1941); when autogamy can assure successful reproduction under variable pollinator activity (Kalisz et al. 1999); when pollinator behavior promotes geitonogamy, which is the movement of pollen between flowers on the same plant.

The nature of inbreeding depression in blueberries is still unfolding. Some researchers have claimed that reduced seed set is due to self-incompatibility (Vander Kloet and Lyrene 1987), while others maintain that blueberries suffer from early acting ID (Krebs and Hancock, 1990). Both of these studies demonstrated that self pollen tubes penetrate ovules in Vaccinium corymbosum. Thus, pre-zygotic incompatibility is not likely in blueberries and decreased self-fertility is due to either ID or OSI. Vander Kloet (1991) has examined ovule development in diploid V. corymbosum. He observed relatively uniform rejection of ovules and suggested that these species are subject to OSI. Several other observations on blueberry species suggest that Vaccinium suffer from inbreeding depression due to the expression of deleterious alleles. First, there exists a range of self fertility in different genotypes, thus addressing criteria #2 of Seavey and Bawa (Table 1). There is also evidence for a positive correlation between self and outcross seed set (Krebs and Hancock 1990) and a range of "seed" sizes from fully developed seeds to nubbins that have presumably developed little beyond fertilization, partially addressing criteria #1 of Seavey and Bawa (Table 1) (Hokanson and Hancock 2000; Schott, 2000). In addition, Krebs and Hancock (1990) found that V. corymbosum has an inbreeding threshold of F > 0.3 (F = Wright's inbreeding coefficient). Individuals below the threshold were facultative selfers and those above it were obligate outcrossers. In a separate study, Krebs and Hancock (1991) found that ID was more severe in those individuals with the greatest number of alleles per locus. They suggest that the polyploid nature of tetraploid V. corymbosum allows the harboring of a large genetic load resulting in low self-fertility.

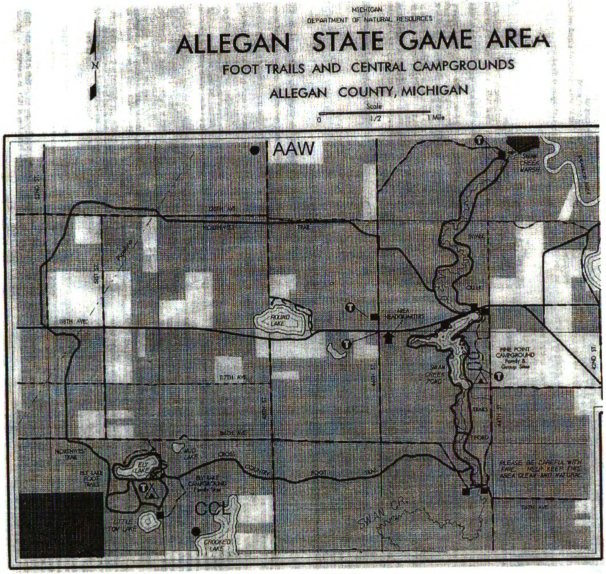
Comparing fruit and seed set between self- and out-cross pollinations alone is inadequate in differentiating between inbreeding depression and self-incompatibility (Gibbs and Bianchi 1999). One needs to examine the development of fertilized ovules to determine if self pollinated ovules are preferentially aborted and if these abortions occur at various stages over a relatively long period of time. Self-incompatibility will result in uniform abortion at an early stage of development. Abortion due to the expression of deleterious alleles (ID) will occur at different stages over an extended period of time (Seavey and Bawa 1986).

In this study, the question of ID versus OSI in blueberries is addressed by taking the work of Krebs and Hancock (1990) and Hokanson and Hancock (2000) a step further. Seed abortion is shown to occur at various stages of seed development which implicates ID. Blueberries appear to suffer from reduced self-seed set due to the manifestation of genetic load upon inbreeding.

MATERIALS AND METHODS

Study system--We used the wild, tetraploid blueberries *Vaccinium angustifolium* and *V. corymbosum* in this study. These are primarily bee-pollinated species that are subject to many geitonogamous pollinations (Vander Kloet and Lyrene, 1987). These species readily reproduce vegetatively with *V. angustifolium* spreading laterally, via rhizomes, and *V. corymbosum* producing many stems, or canes, from a central underground stem. The study populations are located in the Allegan State Game Area in Allegan County, Michigan (Figure 1). The *V. angustifolium* population is in a dry, upland habitat dominated by white oak (*Quercus alba*). The *V. corymbosum* population is on the margin of a shallow, acidic lake with red oak (*Quercus rubra*), red maple (*Acer rubrum*), beech (*Fagus grandifolia*) and white pine (*Pinus strobus*) as dominant tree species. Vander Kloet (1988) provides detailed descriptions of these blueberry species.

Figure 1. Map of the Allegan State Game Area, Allegan county Michigan, showing the locations of the study populations. AAW represents the Vaccinium angustifolium population and CCL represents the V. corymbosum population.



MICHIGAN
DEPARTMENT OF NATURAL RESOURCES
ALLEGAN STATE GAME AREA
FOOT TRAILS AND CENTRAL CAMPGROUNDS
ALLEGAN COUNTY, MICHIGAN
N
Scale
0
1/2
1 Mile
AAW
CCL
52ND ST
51ST ST
120TH AVE
NORTHWEST
TRAIL
Pipeline
118TH AVE
ROUND LAKE
117TH AVE
48TH ST
116TH AVE
NORTHWEST TRAIL
ELY LAKE FOOT TRAILS
ELY LAKE
MUD LAKE
ELY LAKE CAMPGROUND Family Sites
LITTLE TOY LAKE
CROOKED LAKE
CROSS
COUNTRY
FOOT
TRAIL
SWAN CR.
AREA HEADQUARTERS
46TH ST
SWAN CREEK POND
SWAN CREEK MARSH
KALAMAZOO RIVER
SWALE
CREEK
PINE POINT CAMPGROUND Family & Group Sites
TRAILS
FORD
44TH ST
42ND ST
PLEASE BE CAREFUL WITH FIRE. HELP KEEP THIS AREA CLEAN AND NATURAL
115TH AVE

Procedures--In each population, five plants were chosen for the crossing experiment. In the V. angustifolium population, these plants were chosen based on whether they produced enough flowers to perform the crosses. In the V. corymbosum population, the plants were chosen to represent a variety of selfing abilities as determined from crosses in previous years (Schott, 2000). A sixth plant was chosen in each population to serve as the outcross donor for the other five plants.

On each of the five experimental plants, pollen was collected from opened flowers, and 30-40 unopened flowers were emasculated. Approximately 2/3 of these emasculated flowers were self-pollinated and the remainder were pollinated with the out-cross pollen from the same species. The inflorescence with pollinated flowers were covered with a fine mesh nylon bag to prevent fruit loss and to exclude pollinators.

To get a temporal picture of the range in sizes of developing ovules, self- and out-cross fruits were sampled from each plant at one, two, three and 4 1/2 week intervals following pollination, and the remaining fruit were allowed to completely mature. In some cases, there were insufficient self pollinated fruit to sample at all four dates on an individual plant. When this occurred, the sampling was aborted when there was only one fruit remaining, and it was allowed to mature. The harvesting of mature fruits began approximately 60 days after pollination. Two open pollinated fruits were also collected at maturity from each plant.

To process the immature and mature fruits, an approximately 1-2 mm slice of the top of the fruit was removed with a razor blade, and the contents of the fruit were removed with a spatula. The number of maturing ovaries was quantified and all of the ovaries were spread out on a glass slide using a Wild M5 observation scope. The 'size' of each ovary was measured using the ocular micrometer of a Zeiss Axioskop. Blueberry ovules and seeds have a generally oblong shape with rounded ends (Figure 2), and the size was measured as the longest length of each ovule/seed.

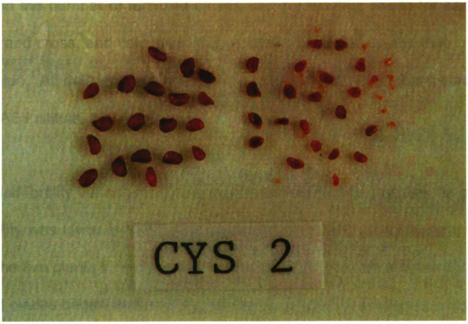


Figure 2. Picture of mature and aborted seeds from a Vaccinium corymbosum fruit.

The viability of aborted and mature seeds from ripe fruits was tested by transferring them to moistened filter paper in petri dishes. The seeds were kept moist with a solution of tap water and phosphoric acid (pH = 5.0). Low pH water was used to mimic field conditions. Seeds were than scored for their ability to germinate. Using this procedure, I was able to quantify the approximate size a seed must reach in order to germinate.

Data analysis--Data were compiled on a species, cross and individual plant basis. The mean size of seed that germinated was calculated and species and crosses were compared with a nested analysis of variance. Sources of variation were species and cross within species. We also quantified the number of fertilized ovules and germinating seeds for each species and cross, and calculated the percent of fertilized ovules that germinated. All data analyses were performed using JMP 3.1.6 software for the MAC (SAS Institute, 1994).

RESULTS

Self-fertility varied greatly among individuals of both species. In general, self fertility was lower in *Vaccinium angustifolium* than *V. corymbosum*, with four of the five plants failing to mature any self-pollinated fruit (Figure 3). The fertilized ovules began to develop in all the *V. angustifolium* plants, but ceased developing at variable times depending on genotype. Seed development ceased by day 23 in AAW 1 and AAW 5, and by day 33 in AAW 4. No ripe fruit were collected from AAW 2, and most of its seeds had aborted by day 23, but a few of its developing seeds had already gotten rather large by that time.

Figure 3. Ovule/seed size for self and outcross pollinations in five Vaccinium angustifolium plants. Rows represent the five plants. The first column is the mean ovule size for selfed and outcrossed fruits sampled at several dates before maturity, and at maturity (day 60). In addition, ovule size was quantified in unopened flowers, that were not pollinated, and in flowers that were naturally pollinated (OP). The second and third columns represents the distribution of ovule sizes for self and outcross pollinated fruits, respectively, at different sample dates.

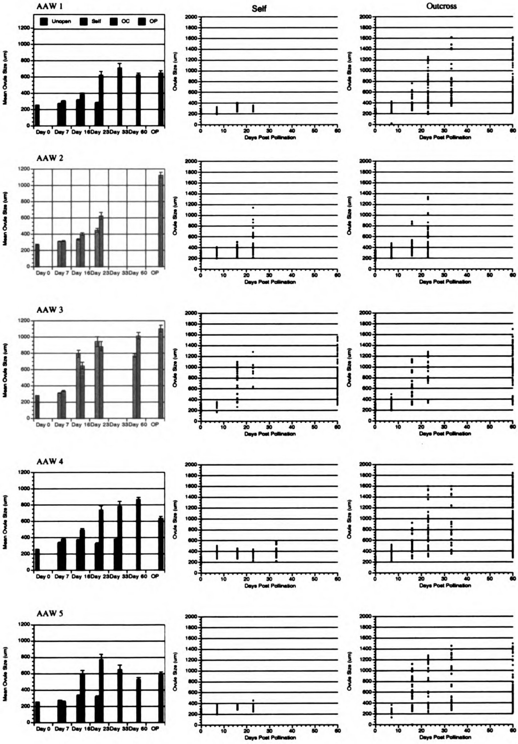
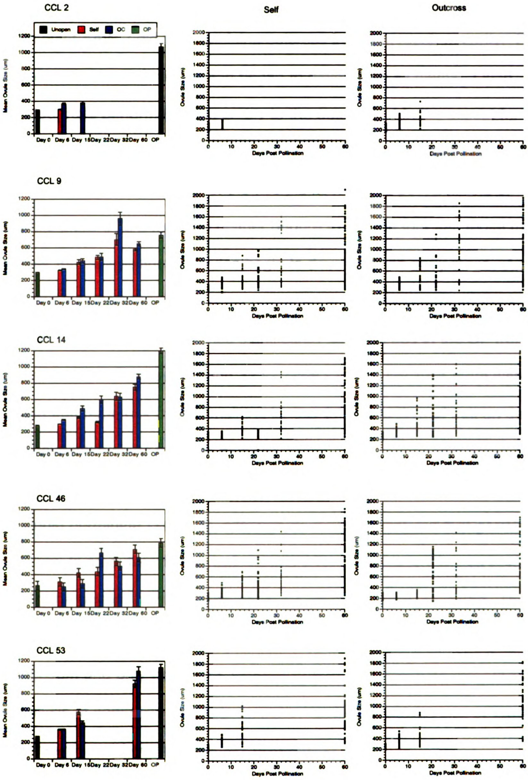

AAW 1
Self
Outcross
Unopen
Self
OC
OP
Mean Ovule Size (um)
Ovule Size (um)
Days Post Pollination
Day 0
Day 7
Day 16
Day 23
Day 33
Day 60
OP
0
200
400
600
800
1000
1200
1400
1600
1800
2000
10
20
30
40
50
60
AAW 2
AAW 3
AAW 4
AAW 5

Figure 4. Ovule/seed size for self and outcross pollinations in five Vaccinium corymbosum plants. Rows represent the five plants. The first column is the mean ovule size for selfed and outcrossed fruits sampled at several dates before maturity, and at maturity (day 60). In addition, ovule size was quantified in unopened flowers, that were not pollinated, and in flowers that were naturally pollinated (OP). The second and third columns represents the distribution of ovule sizes for self and outcross pollinated fruits, respectively, at different sample dates.


CCL 2
Self
Outcross
Unopen
Self
OC
OP
Mean Ovule Size (um)
Ovule Size (um)
Day 0
Day 6
Day 15
Day 22
Day 32
Day 60
OP
Days Post Pollination
CCL 9
CCL 14
CCL 46
CCL 53
0
200
400
600
800
1000
1200
1400
1600
1800
2000
10
20
30
40
50
60

AAW 3 was the only V. angustifolium that had large seeds and produced fully mature fruits. In four of the five plants, cross pollinated fruits reached maturity. Only in plant AAW 2 did the remaining outcross fruits not reach maturity. Outcrossed ovules were fertilized and began to develop on all plants. Open pollinated fruits performed comparably to the outcrossed fruits, except for plant AAW 4, where the open pollinated fruits had significantly smaller seeds.

In V. corymbosum, four of the five plants matured both self and outcross fruit while plant CCL 2 matured neither (Figure 4). In the four self-fertile plants, it is clear that self-pollinated ovules begin to develop at an early stage. Again, seed size was comparable in open-pollinated and cross-pollinated fruits, except in plant CCL 14, which had significantly larger seeds in the OP fruit. No data are available for days 22 and 32 for plant CCL 53 because there were too few self- and out-cross fruits remaining by day 22. Collection of fruit on day 22 would have left nothing to reach maturity.

In general, seeds of both species had to reach a size of 1100 um before they would germinate (Table 3), although an outlier was found that germinated at 820 um, is an outlier, falling well outside 95% confidence intervals (1433 <-> 1579) in the bottom 0.5% quantile of selfed seeds in V. corymbosum. The widest range in size of germinable seeds was found in self pollinated V. corymbosum and only one of the selfed seeds germinated in V. angustifolium. An analysis of variance revealed no differences between species or crosses in the mean size of germinating seeds (Table 4).

Table 3: Seed germination data for V. angustifolium (V. a.) and V. corymbosum (V. c.). The cross types are self pollen, outcross pollen (OC) or open pollinated (OP). Also included are the maximum (Max) and minimum (Min) size of germinating seeds, the number of seeds germinating (n), the mean size of germinating seeds, and the standard error of the mean (SEM). All seed sizes are in micrometers.

	Cross	Max	Min	n	Mean	SEM
	Self	1320	1320	1	1320	
V. a.	OC	2000	1140	96	1556	17.4
	OP	1860	1140	10	1594	61.9
	Self	2080	820	44	1506	36.2
V. c.	OC	1800	1280	19	1547	30.1
	OP	1980	1100	57	1579	28.4

Table 4. Effect of species and pollination treatment on the size of germinating seeds.

Source of variation	d.f.	MS	F
Species	1	1435	.03^{ns}
Cross(Species)	4	50899.6	1.32^{ns}
Error	221	38428.6	

Table 5. Data for fruits that reached maturity. Plant represents the 10 experimental plants. AAW represents the V. angustifolium population and CCL represents the V. corymbosum population. Cross represents self, outcross (OC) and open (OP) pollinated flowers/fruits. # fruits = the number of mature fruit sampled, # ovules is the total number of fertilized ovules in all of the mature fruits, # germ is the number of ovules that germinated, % germ is the percent of ovules that germinated, #>1100 is the number of seeds that were larger than 1100 um, #>820 is the number of seeds that were larger than 820 um, #>11/fruit is the number of seeds larger than 1100 um in each fruit, #>820/fruit is the number of seeds larger than 820 um in each fruit.

Plant	Cross	# fruits	# ovules	# germ	% germ	# ≥ 1100	# ≥ 820	#≥1100/fruit	#≥820/fruit
AAW 1	Self	0							
	OC	4	210	21	10.0	26	32	6.5	8.0
	OP	2	89	2	2.2	9	20	4.5	10.0
AAW 2	Self	0							
	OC	0							
	OP	2	114	5	4.4	67	94	33.5	47.0
AAW 3	Self	2	143	1	0.7	22	59	11.0	29.5
	OC	2	72	3	4.2	30	56	15.0	28.0
	OP	2	75	0	0.0	45	57	22.5	28.5
AAW 4	Self	0							
	OC	5	347	62	17.9	93	145	18.6	29.0
	OP	2	104	2	1.9	9	19	4.5	9.5
AAW 5	Self	0							
	OC	3	153	10	6.5	16	21	5.3	7.0
	OP	2	94	1	1.1	15	26	7.5	13.0
CCL 2	Self	0							
	OC	0							
	OP	2	124	4	3.2	63	95	31.5	47.5
CCL 9	Self	5	316	10	3.2	33	55	6.6	11.0
	OC	5	227	2	0.9	32	57	6.4	11.4
	OP	2	117	1	0.8	26	43	13.0	21.5
CCL 14	Self	2	145	5	3.4	32	46	16.0	23.0
	OC	3	190	2	1.0	43	84	14.3	28.0
	OP	2	135	13	9.6	86	105	43.0	52.5
CCL 46	Self	6	323	26	8.0	71	87	11.8	14.5
	OC	2	127	11	8.7	13	17	6.5	8.5
	OP	2	128	15	11.7	41	53	20.5	26.5
CCL 53	Self	1	67	3	4.5	15	43	15.0	43.0
	OC	1	50	4	8.0	25	38	25.0	38.0
	OP	2	123	24	19.5	68	96	34.0	48.0

Average germination rates for fertilized ovules were quite variable across plants and ranged from 0.0-19.5% (Table 5). For most fruits, a majority of seeds fall below the germination threshold of 1100 um. Thus, it is not surprising that such a small percentage of fertilized ovules germinate. The number of seeds exceeding the germination thresholds of 1100 um and 820 um is comparable between self and outcross pollinations in the majority of plants. The exceptions are selfed seeds in plant CCL 46, and outcross seeds in plant CCL 53, which had greater number of seeds above the germination threshold Figure 4.

DISCUSSION

The data presented in this study address the first two criteria for differentiating between inbreeding depression (ID) and ovular self-incompatibility (OSI), as outlined by Seavey and Bawa (1986). First, there is evidence for abortion occurring at various stages of development. Second, the response to inbreeding varied between plants. These data, in conjunction with the ID data of Hokanson and Hancock (2000) and Schott (2000) which show a range in self-fertility across plants (criteria #3 of Seavey and Bawa), clearly demonstrate that the blueberry species suffer from ID, not OSI. While some plants appear to be self-incompatible, or to have low self-fertility (AAW 1, AAW 4, AAW 5, CCL 2), this is by no means a universal trait in either V. angustifolium or V. corymbosum. In fact, plant CCL 2 has demonstrated high self and outcross fertility in previous years (Schott, 2000), relative to these data from 2000.

Seavey and Bawa (1986) suggest that uniform failure of a wide sample of ovules, at a comparable stage of development, suggests an OSI system. Naaborgh and Willemse (1992) demonstrated uniform rejection of ovules two days after fertilization in Gasteria verrucosa. They believe it is a 2+ loci incompatibility system that results in abortion due to the termination of maternal resources. While the majority of self-pollinated blueberry ovules are aborted early on, a number of them continue to develop and a small percentage do become mature seed.

Species suffering from ID will show individual variation in self-fertility (Seavey and Bawa, 1996). Species that are truly self-incompatible show little or no variation in self-fertility among plants. Plants with only a few sublethal recessives can have close to normal seed production when selfed. It is possible that a few self-fertile plants might arise through mutation in an otherwise self-infertile population, but the frequency of self-fertile plants found in our blueberry populations, and their variability in self-fertility from year to year (Schott, 2000), suggests that we are dealing with ID rather than rare self-fertile mutants in otherwise self-incompatible populations.

Where detrimental recessives lead to ID, mature selfed seeds are likely to express ID at later stages of development. Examples include: seed weight, days to germination, plant height and number of flowers in Collinsia heterophylla (Mayer et al., 1996); germination rate, seedling survival and seedling mass in Epilobium angustifolium (Husband and Schemske, 1997); and growth and survival of progeny in Spartina alterniflora (Daehler 1999). In blueberries, inbreeding depression was observed for germination rate in V. myrtilloides and V. corymbosum and growth rate in V. corymbosum (Schott, 2000).

The criteria outlined above eliminates SI as operating in blueberries; however, they are not completely self-fertile and may suffer from pseudo-self-fertility. According to Levin's definition of true self-fertility, similar seed sets follow self and outcross pollination (Table 1), which is not the case in blueberries. In addition, self pollen is less vigorous than outcross pollen in blueberry (Hokanson and Hancock, 2000), a further criteria for pseudo-self-fertility according to Levin (1996). Anecdotal observations also suggest that self-fertility varies from year to year, and is probably environmentally dependent (Schott, 2000).

Seavey and Bawa (1986) discuss species (Lilium sp.) for which self-incompatibility is not always complete. The mechanism for this leaky SI likely involves signaling, or lack there of, between embryonic and/or endosperm tissue and maternal tissue. The self-infertility modifier alleles conferring pseudo-self-fertility in Asclepias exaltata (Lipow and Wyatt, 2000) and other species (Levin, 1996), are possibly the basis for leaky SI. Smaller, more remote milkweed populations have higher frequencies of self-fertile individuals (Lipow and Wyatt, 2000), fitting with the notion

Why would long-lived, vegetatively reproducing species evolve the ability to self-fertilize? It is possible that the evolution from diploidy to polyploidy is a causal factor. A rare polyploid appearing in a diploid population will increase in numbers only if it is self-fertile (Thompson and Lumaret, 1992). Being long-lived and clonal will help it persist in the short-term, but only the tolerance of extreme inbreeding will allow it to develop into a distinct species that has evolutionary potential. Jain (1976) and Stebbins (1974) maintain that self-fertile lines that develop from self-infertile progenitors are almost always evolutionary dead ends. However, the disruption in mating system associated with polyploidy may allow a newly derived species to initially persist by selfing, before ultimately developing into a primarily outcrossed species. Such species would maintain the ability to self, and might maintain intraspecific variation in selfing ability.

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CONCLUSIONS

These data support previous work showing that there is significant inbreeding depression in wild blueberries (Hokanson and Hancock, 2000; Schott, 2000a). I am not aware of any published estimates of outcrossing rates in blueberries, but bee pollination is thought to promote many geitonogamous pollinations (Vander Kloet and Lyrene, 1987) and high cumulative inbreeding depression would suggest that successful progeny are, in general, outcrossed. The source of outcross pollen is irrelevant in the reproductive success of tetraploid V. corymbosum, although the performance of outcrossed progeny is superior to selfed ones when progeny are outcrossed. As a result, I suggest that selection will move these populations towards decreased self-fertility, which is counter to the common direction in which plant mating systems are thought to evolve (Stebbins, 1974). However, there are a few individuals that are relatively self-fertile, and even though cumulative inbreeding depression across life-history stage can be high (Schott, 2000a), self-pollinated progeny do emerge that are comparable or superior to outcrossed progeny (Schott, 2000a). Whether or not the existence of these few self-fertile plants is enough to maintain self fertility in the population as a whole is still an open question.

The genetic system of most blueberry plants and pollinator behavior seem to be at odds. Self-fertile plants would benefit from pollinator behavior that encouraged geitonogamous pollinations; however, relatively self-infertile plants would not benefit. Bee pollination in blueberries is thought to promote many geitonogamous pollinations, and this is the main reason suggested for why blueberries mature so few of their available ovules (Vander Kloet and Lyrene, 1987). The resulting situation is an evolutionary tug-of-war between two genetic systems in the plants, self-fertility versus self-infertility, and a pollinator foraging method that favors geitonogamous pollinations. Superimposed on this system are the survival rates of self and outcross progeny under natural conditions. It is suggested that variable pollinator service, due perhaps to environmental variation, will favor mixed mating (Kalisz et al., 1999). Whether or not such a force can mold the mating system of a long-lived perennial, like blueberries, is debatable. Blueberries can, and often do, tolerate years of complete reproductive failure (Vander Kloet and Cabilio, 1984). Likewise, they can persist for long periods of time whether or not they produce outcross progeny. It seems reasonable to surmise that the mating system of an annual must be more flexible than that of a perennial.

There are several questions about blueberry mating systems that remain to be answered: 1) What are outcrossing rates in natural populations of blueberries? It is presumed that their outcrossing rates are relatively high; however, evidence for outbreeding depression in V. myrtilloides and the relative indifference to source of outcross pollen in V. corymbosum (Schott, 2000b) suggest that a wide range of intermediate crossing distances are possible in these species. High inbreeding depression in V. angustifolium, relative to V. corymbosum (Hokanson and Hancock, 2000) suggest that the former species will have relatively high outcrossing rates. 2) What is the exact nature of self-infertility in blueberries? Interpreting previous studies, in light of my work and others, strongly suggests that blueberries are not self-incompatible, but suffer low self-fertility due possibly to pseudo-self-fertility. Only a detailed crossing experiment using individuals of various levels of self-fertility, as per Lipow and Wyatt (2000), and examining embryonic development, will fully address this question. 3) In what direction are blueberry mating systems evolving? I believe that they are evolving in the direction of decreased self-fertility. However, obtaining a definitive answer in such a long-lived perennial would be difficult. We can gain insight into this question through a series of field and greenhouse experiments. First, you would want to estimate outcrossing rates including the intrapopulational variation in outcrossing rate. You would then generate an array of progeny with of various levels of outcrossing, in the same proportion as found in the natural population. You would then establish a common garden of these progeny in the field and monitor their progress over several years taking note of survival, growth rates, and various aspects of fertility. Comparing the progress of self and outcross progeny would give us insights into the question of mating system evolution. If we find that selfed progeny are at a disadvantage, compared to outcross progeny, we might suspect that the self-fertility found in blueberries is left-over from the time polyploid species first arose, and is of little ecological and evolutionary importance.

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