# POLLEN-MEDIATED GENE FLOW AND GENETIC VARIATION WITHIN *MANFREDA VIRGINICA* POPULATIONS OCCURRING IN ADAMS COUNTY, OHIO

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# POLLEN-MEDIATED GENE FLOW AND GENETIC VARIATION WITHIN *MANFREDA VIRGINICA* POPULATIONS OCCURRING IN ADAMS COUNTY, OHIO

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POLLEN-MEDIATED GENE FLOW AND GENETIC VARIATION WITHIN *MANFREDA VIRGINICA* POPULATIONS OCCURRING IN ADAMS COUNTY, OHIO. (64 pp.)

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## Abstract

The purpose of this thesis was to investigate pollen-mediated gene flow and genetic variation within populations of *Manfreda virginica* that occur in prairie openings in Adams County, Ohio. *M. virginica* was chosen because of its historic co-dominance in the xeric prairies and its role as a dominant plant in globally rare limestone seep habitats. A pollen surrogate was used to estimate pollen dispersal and inferred pollen movement is frequent within populations, but not among populations. A single species of Halictid bee appears to be the primary and perhaps sole pollinator. Genetic analysis was performed using Inter-Simple Sequence Repeat (ISSR) markers. No significant relationship was found between geographic distance and genetic distance and substantial genetic variation is found between all populations regardless of size or geographic position. Heterozygosity and polymorphism was low within all populations. Analysis indicates substantial fixation of alleles within populations, perhaps driven by drift due to genetic isolation.


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Dedication

This thesis is dedicated to all of those who seek to understand, preserve and protect the diversity of life that graces our sacred planet Earth.

## Acknowledgments

I would like to thank foremost my advisor Harvey Ballard, whose support, advice and inspiration made my study at Ohio University possible, and without whose editing this thesis would be very hard to read. I would also like to thank the other members of my thesis committee, Matt White and James Cavender for their guidance and consideration. I am grateful for the assistance of my colleagues Ross McCauley and Cheryl McCreary for their advice and instruction. I would like to thank Darren Cohen of ILGARD for assistance with the maps and Kelly Johnston of Ohio University for helping me work out the bugs. I would like to thank The Nature Conservancy staff at the Edge of Appalachia, particularly Peter Whan, as well as Chris Beedle, his counterpart with the Cincinnati Museum of Natural History, for filling me in, sharing their maps, and letting me use their truck. Special thanks to Rick and Vicky Potts of Adams County, and finally, I would like to thank my field assistants Oren Frost of Carlton College and Dillon Ghawi. This research was funded by the Department of Environmental and Plant Biology, Ohio University, a John Houk Memorial Grant and a grant in aid of research from the Powaser and Jackson Trust.

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# Introduction

Within a managed ecosystem, maintaining the genetic viability and evolutionary potential of plant populations requires conservation strategies aimed at protecting the flow of genes across and within subpopulations of the plant's metapopulation. Physical and spatial barriers between colonies may limit the exchange of genetic material between populations. In many areas, barriers to gene flow can isolate subpopulations and thereby remove them from the metapopulation gene pool. An isolated population is subject to genetic drift and inbreeding depression, resulting in a loss of heterozygosity in the localized breeding population (Loveless and Hamrick 1994). Isolated plant populations have been shown to have divergence in genotypes in a number of studies (Fore et al 1999, Bai et al. 1997). In some cases, separated populations will exhibit high or low inter-population diversity, with the appearance of alleles unique to some population isolates (Travis et al. 1996). In other cases, isolated populations may experience a loss of heterozygosity relative to other populations or the species in its entirety (Fleishman et al. 2001). The loss of heterozygosity, which is generally considered to represent the ecological integrity and evolutionary potential of the population, may lead to localized extinctions of isolated populations, even if suitable habitat is available. In order to maintain gene flow between isolated populations within a metapopulation, there must be connectivity between the disparate populations. Gene flow in plants is carried out maternally through seed or genet dispersal, or paternally through pollen dispersal. Connections through pollen-mediated gene flow may have a substantial effect on the persistence of isolated colonies, and help homogenize the metapopulation (Richards 1999). Gene movement through pollen flow may

be scale dependent, with greater distances realized by larger population groups (St. Amand et al. 2000).

Recognizing the relationship between distance and barriers to pollen dispersal is problematic to each species, as each has differing reproductive strategies, and connectivity for each species patches is dependent on the filter effect of barriers and the degree of the species representation and dispersal ability. From a conservation standpoint, this requires an assessment of each plant in a community or group of communities in order to determine its likelihood of persistence in the managed ecosystem. However, from a practical standpoint, the resources and expertise required to assess each species individually are seldom, if ever, available. An alternative may be to assess and compare different types of plants occurring in the same habitat, or alternatively, to choose a plant that appears to be the most limited in terms of gene movement, or is most representative of a specific habitat. With these considerations in mind, research was conducted to investigate the relationship of pollen flow and genetic diversity within and between populations of *Manfreda virginica* occurring on calcareous prairie barrens in Adams County, Ohio.

*M. virginica* (Fig. 1) is a succulent perennial in the family Agavaceae, and is most closely related in its genus to *Agave lechuguilla* and *Prochnaythes mexicana* (Bogler and Simpson 1995). It is an obligate CAM plant (Martin et al. 1983), the only such native plant found in the prairie barrens communities of Adams County. The literature reveals a single study (Baskin and Baskin 1971) on the ecology of *M. virginica*. It is a dioecious monocot that has a long-day (short-night) flowering physiology and is protandrous. The seeds have an obligate cold requirement for germination and may remain dormant for some time. Seedlings emerge in April or May and have high mortality through summer.

The plant over-winters as a caudex with buds. A basal rosette forms in April. There may be a juvenility period of one or more years. Adult plants have an obligate cold requirement for flowering, although only a portion of the plants flower in any given year, independent of age class. Flowering plants begin to bolt and produce a scape in late May or early June. Flowering occurs in July and August, with sequential opening of flowers from the bottom up (Harris 1924). The light green flowers are very fragrant, with fragrance increasing toward nightfall.

The natural range of *M. virginica* is from southern Ohio to Missouri to North Carolina, Florida and Texas (Gleason and Cronquist 242). It is a southern and southeastern xerophyte whose northern range ceases in southern Ohio, where it is a local dominant (Braun, 1929). The absence of *M. virginica* and other southeastern xerophytes from other Ohio prairies led Braun to speculate that the communities in Adams County prairies were never continuous with prairies in glaciated Ohio, but are relicts of interglacial plant migration.

Other than a single visitation by a bumblebee reported by Baskin and Baskin (1971) the pollinators of *M. virginica* were unknown, or at least were not found in the initial literature review, although hawk moth pollination was implied (Robertson, 1928). Reference to the pollination system of *M. virginica* was later found in the literature (Groman and Pellmyr 1999). That detailed study examined diurnal-nocturnal pollinator partitioning utilizing populations of *M. virginica* that occur in the Cedar Glade region of central Tennessee. The identification of a guild of pollinators associated with *M. virginica* in that study is not supported in this research. The means of seed dispersal for *M. virginica* do not appear in the literature, other than speculation that the falling of the

previous year's scape the following spring disperses the seeds (Baskin and Baskin 1971). The seeds have no eliasome that might indicate ant dispersal, offer little food reward for other animals, have no means of attachment, and are not aerial.

Field research was conducted during July and August of 2001 at The Nature Conservancy's Edge of Appalachia Preserve System in Adams County, Ohio (Fig. 2). This is a complex of preserves amounting to approximately 13,000 acres owned or managed by the Conservancy, of which about 3000 belongs to the Cincinnati Museum of Natural History. The preserve system features prairie openings within a matrix of second- and third-growth mesophytic forests of rather southern floristic composition (Braun, 1928). There are two types of prairie communities in Adams County, xeric and mesophytic. Braun considered these openings to be relic colonies remaining from formerly widespread vegetation with western affinities. The xeric openings occur on Silurian limestone substrates.

The Nature Conservancy has categorized over 100 openings as having xerophytic prairie communities, ranging from a few hundred square feet to several acres in size. The prairie communities occur on dolomitic limestone substrate on upper and mid slopes amid the mesophytic forests, and along limestone and dolomite cliffs of the plateau escarpment (Jones, 1944).

Four sites were identified which feature populations of *M. virginica* separated by differing distances and cover. These sites, used for pollen flow studies with fluorescent dye powder, included populations at Desert Prairie, the Teakettle Knob area of the Wilderness Preserve, the Lynx Preserve, and Big Slump. *M. virginica* population groups are found in several places in each of the areas. At least two of these populations, at

Teakettle Knob East and Desert Prairie, occur as dominant plants in limestone seeps, a habitat type classified by the Nature Conservancy as G1, or globally imperiled. The four sites utilized for study of pollen dispersal were included in the ten sites within the preserve system from which samples were taken for genetic analysis. Additionally, samples from two sites located outside the preserve were included in the genetic analysis.

A study of gene flow through transfer of pollen requires a separation of seed mediated and pollen-mediated gene flow (Ouberg et al. 1999). Pollen transfer can be measured experimentally by counting the number of pollen grains transferred (Schulke and Wasser 2001), by genetic analysis utilizing molecular markers (with various assumptions made) and by progeny phenotype analysis (Staniland et al. 2000). Another method used to estimate gene flow through pollen transfer is the dusting of dehiscing anthers with fluorescent dye powder. Pollinators transfer the pollen analogue dye along with the pollen with properties very similar to actual pollen transfer (Wasser 1988). Fluorescent dye has been used to investigate the distance pollen is carried by bumblebees and hummingbirds (Schulke and Wasser 2000); the preference of pollinators for plants with more flowers (Rademaker and De Jong 1998); the pollination success of flowers in hermaphroditic plants (Campbell 1989); and the negative effect of pollinator competition on pollen dispersal (Campbell 1985). Fluorescent dye as a substitute for pollen was found to be effective in measuring pollen carry-over as well as direct pollination events (Waser and Price 1982), but may underestimate actual pollen dispersal distances in some cases (Waser 1988).

Inter-Simple Series Repeats (ISSR) were chosen as the marker system for the genetic analysis portion of the study. ISSR primers utilize hypervariable microsatellites in

the nuclear genome. Microsatellites, also known as simple sequence repeats (SSRs), are short (1-5 bp) tandemly repeated DNA sequences of di-, tri-, tetra or penta-nucleotide repeats (Prevost and Wilkinson 1999). SSRs are common in plant nuclear genome, but largely absent from chloroplast and mitochondrial genome. The anchored primers anneal directly to SSRs and access variation of the tandem-repeat motifs within populations, and therefore prior knowledge of the genome is not required. Polymerase chain reaction (PCR) amplifies the intervening region between pairs of primer annealing sites, which generates a band of a particular molecular weight. The banding patterns are discriminated by gel electrophoresis, and the bands are scored as dominant loci for the purposes of analysis. Typically, several to many microsatellite regions exist in the nuclear genome of a particular sample, so each primer generates a band pattern specific to that sample. The ISSR method generates fewer genotypic profiles than Amplified Length Polymorphisms (AFLP), and Random Amplified Polymorphic DNA (RAPD) methods (McGregor et al. 2000). However, in comparison with Restriction Fragment Length Polymorphisms (RFLPs) and RAPDs, ISSRs are more sensitive to inter-population variation, have greater repeatability, and are less expensive and time consuming (Li and Ge 2001) (Nagaoka and Ogihara 1997). The ISSR marker system is useful in assessing genetic variability within and among plant populations and is particularly useful when little or nothing is previously known about the genome being studied.

ISSR markers are neutral markers: they do not directly access known coding regions, and do not directly estimate quantitative genetic differentiation between individuals. However, there is no evidence that microsatellites are less informative than allozymes (Morgan et al. 2001). Neutral markers have been shown to be conservative

estimators of population structure for quantitative traits, are valid indicators of population subdivision for quantitative characters (Pfrender et al. 2000), and for small populations may underestimate evolutionary potential (Petit et al. 2001). Neutral markers such as ISSR are particularly useful for small populations (Cruzan 2001) that occur in restricted areas such as those of *M. virginica* used in this study.

## Field Methods

*Pollen movement*. Four sites in the preserve system were used to investigate pollen flow: Desert Prairie (N 38 45.869 W83 26.892), Teakettle East Prairie (N 38 46.596 W 83026.108), Lynx Prairie (N 38 45.668 W 83 24.673), and Big Slump Prairie (N 38 44.695 N 38 44.695). The dehiscing anthers of one plant in each group were treated with a different color fluorescent dye powder – orange, green, pink or red - several hours prior to sunset on July 14, 2001. The dye powder was applied with a small modeling paintbrush, with a different brush used for each color. Each of the 10 sites within the preserve was visited between 10:00 p.m. and 5:00 a.m. July 14-13 and on two subsequent nights. During these visits each flowering plant was examined with a ultra-violet flashlight for presence of fluorescent dye powder. Plants having dye powder were marked with tape corresponding to the color of the dye powder. Rain occurring between July 18 and July 23 smeared or removed dye powder on the treated and receiving plants. On July 23 a second application of dye powder was made to plants that had received dye powder on the first application and represented the greatest distance of dye transfer from first treatment. The population at Lynx Prairie was excluded from this, as no powder from the treated plant appeared on any other *M. virginica* plants. The populations within the preserve were again examined with the portable flashlight between 10:00 p.m. and

5:00 a.m. July 23-24, and on two subsequent evenings. Receiving plants were again marked with color-corresponding tape. Distance measurements for movement of the fluorescent dye powder were recorded during daylight hours using a 100-meter measuring tape. After all the plants at each of the four sites used for pollen flow measurement had finished flowering fruit set and total flower number were recorded.

*Pollinator identification*. The pollination strategy of *M. virginica* is largely unknown, so visual observations were made by walking transects among the various populations at all times of day and night. A 16.8-volt rechargeable worklight (Craftsman # 982140-001) was used intermittently during nighttime observation. Insect visitation to the flowering *M. virginica* plants was noted for time and location. Approximately 40 hours of visual observations were made. To assist in validating pollinator interactions individual plants were videotaped using an infra-red capable digital video camera (Sony TRV 330) for one-hour intervals at all times of day and night. One-hour intervals were chosen as it matched the running length of the videotape, was most convenient in terms of set-up, and allowed visual observation to be made in other areas during recording. The camera was set on a tripod and recorded daylight conditions under normal mode, and was switched to IR during darkness. A total of 25 hours of videotape observations were made. Ten hours of recordings made during mid-day and mid-night hours were devoid of insect visitors and were re-used. Pollinator types were confirmed through field observations, viewing the video record and lab identification of collected specimens.

*Tissue collection*. Leaf tissue of *M. virginica* of thirty-two plants from each of ten populations within the preserve and two more distant populations outside the preserve was collected on September 16, 2001 for ISSR analysis (Davis, N 38 56.163,W 83

21.548; Shivener, N 38 46.954,W 83 24.830; Teakettle East, N 38 46.596,W 83 26 108; Teakettle West, N 38 46.596,W 83 26.08; Lynx, N 38 45.668, W 83 24.673; Shooting Star, N 38 45.721,W 83 26.593; Unity N 38 54.484,W 83 30.950; Post, N 38 45.869,W 83 26.803; Homestead, N 38 45.894,W 83 26.813; Desert, N 38 45.921,W 83 26.892; Crossroads, N 38 44.840,W 83 26.504; Big Slump, N 38 44.695,N 38 44.695). Leaf tissue was randomly sampled across populations from plants of various size and maturity. A portion of leaf from each plant, amounting to approximately 4 $cm^2$, was stuffed into 2.5 ml micro-centrifuge tubes, which were then filled with silica gel desiccant (Fisher Scientific). The desiccant in each of the tubes was changed periodically until the tissue samples were completely dry. Coordinate positions (latitude-longitude) of each of the populations from which leaf tissue was collected were taken with a Garmin E-Trex Vista hand-held global positioning device.

## Laboratory Methods

*DNA extraction and ISSR amplification*. Genomic DNA was extracted for analysis. Approximately 2$cm^2$ of desiccated leaf tissue from each sample was placed into labeled sterile 1.5 ml micro centrifuge tubes. Liquid nitrogen was added and the frozen tissue was ground in the tube with a pestle. 400µl of SDS buffer was added to each tube and the tissue was ground again. 200µl more SDS buffer was added to each tube following removal of the pestle and the tube was vortexed. The tubes were floated in a 37$^o$ C hot water bath for 15-30 minutes and then removed to the fume hood. 400µl 24:1 chloroform-isoamyl alcohol mixture was pipetted into each tube. The tubes were inverted, the lids opened to release pressure, and sealed again. The tubes were then vigorously shaken until the contents formed a milky emulsion, and were then unsealed

slowly to release pressure. Following this, the tubes were centrifuged for five minutes at 13,000 RPM (Marathon 24-well microcentrifuge). A second set of labeled 1.5 ml tubes was prepared, and 400µl of clear upper aqueous phase was transferred from the chloroform-isoamyl tubes to the second set. 400µl ice-cold isopropanol from freezer was pipetted into each new tube, the lids were closed, and each tube was inverted 10-20 times. The tubes were then placed in a –20° C freezer for up to several days to precipitate DNA. Following the precipitation period, samples were removed from the freezer and centrifuged for 5 minutes at 13,000 rpm. The lids were then opened and the supernatant was poured off, leaving a pellet of DNA in the bottom of the tube. 100µl TE buffer was pipetted into each tube. The tubes were soaked briefly in the 37° C hot water bath and then inverted gently to break up the DNA pellet. Dissolution of the pellet was hastened using a clean stainless steel spatula for each sample. 50µl 7.5M ammonium acetate, 10µl 3M sodium acetate, and 300µl 95% ethanol were pipetted into each tube. The tubes were briefly vortexed and then placed in a 20° C freezer. After several days the samples were removed from the freezer centrifuged at 13,000 rpm for 5min and the supernatant was poured off. 100µl 70% ethanol was pipetted into each tube, then the tube was left undisturbed for 5-10 minutes before the ethanol was poured off. Inverting and tapping the tubes on a piece of brown paper towel removed excess alcohol; and the samples were dried in a vacuum for approximately 20 minutes at 25 psi. Once dried, 100µl TE buffer was pipetted into each tube to complete the extraction, and the tubes were placed in the 20° C freezer for storage until analysis with PCR amplification.

*ISSR analysis.* 15 samples of purified DNA were randomly selected from each population set and were tested for comparative bands using ISSR primers in a PCR

reaction. Ten primers, based on or derived from sequences given by Wolfe et al. (1998) or Harvey Ballard and produced by Operon Technologies, were screened for high variation, distinct bands and unambiguous interpretation using eight random samples from the twelve populations. The primer sequences selected for further analysis were one tri-nucleotide repeat motif ($(GTG)_3GC$)[HB 15] and two di-nucleotide repeat motifs ($(GA)_6CC$ [HB10], $(CA)_6GT$[Wolfe 14799B]). One primer was used for each reaction, and each DNA sample was treated with the same three primers in individual PCR reactions.

ISSR amplification was done with replicated single-primer 25 µl reactions. Constituents for the PCR reactions were 16µl distilled and autoclaved water, 3µl buffer, 5µl $MgCl_2$, 4µl dNTP mix, 2.5µl BSA, 0.5µl primer and 0.25µl Taq (Promega) for each sample. PCR was carried out in a Stratagene Robocycler thermocycler programmed for an initial denaturing stage of 2 minutes at $94^o$ C followed by 40 cycled with a 30 second denaturing stage at $94^o$ C, a 45 second annealing stage at $44^o$ C, and a one minute extension stage at $72^o$ C. After the 40 cycles there was a final extension stage of 20 minutes at $72^o$ C.

## Analytical Methods

*Visualization of ISSR banding patterns.* Following PCR amplification, ISSR banding patterns were visualized by electrophoresis in a 0.5 x TBE buffer in a 1.3% agarose midi-gel. Gel wells were loaded with 10 µl of each PCR reaction for each of the 15 samples of each population. Each of the 15-well series were flanked by 10 µl of 250bp ladder standard (Promega) mixed with tracking dyes. The gels were run at 60 volts (EC105 Fisher Biotech) for approximately 2.5 hours on an EC 350 Midigel rig (E-C

Technologies). After electrophoresis the gels were soaked with a 1:500 mixture of ethidium bromide for 5-7 minutes and imaged with the FluorS Max gel documentation system from. Gel analysis was performed with Quantity One software (Version 4.3.1) from BioRad Laboratories, which is matched to the imaging system. Data was scored by presence/absence across all samples for each primer. Questionable bands were eliminated. Bands below 400 base pairs were eliminated as possibly representing incomplete PCR fragments. Bands larger than1750 base pairs were eliminated because they exceeded reliable scoring in comparison to the DNA ladder standard. The three primers resolved 95 informative bands (Table 1). Band reports were generated and band data was exported from Quantity One as binary data matrices to Microsoft Excel files.

*Genetic diversity within and among populations*. Estimated heterozygosity and percent polymorphic loci within each population were calculated using Tools for Population Genetic Analysis (TFPGA) 1.3 (Miller 1997), with the assumption of Hardy-Weinburg equilibrium within all populations. Because ISSRs are dominant markers they are unable to express recessive alleles in heterozygotes; heterozygosity was estimated following the procedure of Weir (1990), in which the frequency of the recessive allele is calculated as the square root of the frequency of blanks for a locus across all populations.

*Calculation of genetic differentiation.* Genetic differentiation was assessed using an Analysis of Molecular Variance (AMOVA) performed with GenAlEx (version 5.04 for PC, Peakal and Smouse 2002) and Winamova 155 (Excoffier 1992, 1993). As ISSR data is dominant, and Winamova is engineered for co-dominant data, modified files were prepared using a Euclidian distance metric (Excoffier et al. 1992) in AMOVA-PREP 1.01 (Miller 1998). The AMOVA made pairwise comparisons between each of the twelve

populations. The AMOVA generated variance values, partitioned the values within and between populations, tested the significance of the variation and calculated a statistic for dominant markers, $\Phi_{st}$, equivalent to Wrights (1921) fixation index $F_{st}$, which is the statistic used for co-dominant genetic data. $F_{st}$ is the most inclusive measure of population substructure. AMOVA also produced a value for Bartlett's test for homogeneity of variances. This test generates a B value that is corrected to produce a $X^2$ value as an indication of heteroscedasticity, or variation from homogeneity in the distribution of genetic variation among the samples. The influence of population size on genetic differentiation was tested using a three-way AMOVA that divided the populations into three groups based on relative hierarchical size. The three-way AMOVA partitioned values among regions, among populations and regions, and individuals within populations, tested the significance and generated $\Phi_{st}$ as well as calculating the Bartlett statistic. Frequency distributions of permuted $\Phi_{st}$ versus observed $\Phi_{st}$ were produced to test the significance of the differentiation in each AMOVA.

*Comparing genetic distance and geographic position.* A Mantel test was performed using the GenAlEx macro program (Peakal and Smouse 2002) to examine genetic vs. geographic relationships. The Mantel test used a pairwise genetic distance matrix from the 2-way AMOVA procedure and a geographic distance matrix based on the latitude-longitude coordinate position of each of the twelve populations. The Mantel test computed a correlation between the two matrices as an alternative to the Mantel *z*-value (Sokal and Rohlf 813).

*Inferring phylogeographic relationships.* In addition to a second 3-level AMOVA focusing on central and marginal population groups, two multivariate tests allowed

inference into the spatial genetic and geographic relationships among the 12 populations of *M. virginica.* UPGMA cluster analysis and Principal Coordinates Analysis (PCoA) were performed in NtSys-pc 2.02J (Rohfl 1999). Cluster analysis was based on a Jaccard similarity matrix of band frequency in each population as a means of examining relationships among populations only. PCoA analyses based on the DICE coefficent examined relationships among populations as well as between individuals across populations.

## Results

*Pollen movement.* Pollen movement was estimated using fluorescent dye powder as a pollen surrogate. Pollen movement was tracked successfully within three of the four study groups. Pollen movement was similar within populations of Desert, Teakettle East and Big Slump. No pollen movement was noted at Lynx prairie, where only two flowering plants were found in the opening. These plants were only three meters apart, and one of them was dusted with the dye powder. Several other plants were nearby Lynx in small openings about 30 meters distant through cover of cedar, small hardwoods and shrubs. These satellite openings are within pollen carry distance as indicated in the other populations, but no dye was detected on any of the *M. virginica* occurring there following the initial dusting of the source plant. Neither was there seed set at Lynx or in the adjacent openings. No pollen movement was noted between any of the populations regardless of distance or cover types between the populations.

Within the populations at Desert, Big Slump, and Teakettle East, dye powder was demonstrated to have moved between plants and patches of plants within populations, including movement between light cover of shrubs and small trees. Pollen traveled most

frequently between closest plants, with frequency decreasing with distance. The greatest distance realized was 38.93 meters (Fig. 3). Due to heavy rain, the powder was washed from the anthers of treated plants several days after the application. At Desert and Big Slump Prairies, the dye powder was then applied to the plant in each population that had the greatest transfer distance from the initial application. The dye was transported from the plants receiving the second dusting, demonstrating potential pollen carryover. The greatest inferred potential pollen travel distance (the initial application distance plus the carryover distance from the second application) was 49.3 meters.

*Pollinator Identification.* Plant-pollinator interactions were almost entirely limited to a single species of bee from the family Halictidae. Specimens collected in the field were identified to genus with the assistance of Dr. Kelly Johnson, Associate Professor in the Department of Biological Sciences at Ohio University. The halictids were identified by a medial arch in the vein structure of the wing that is not present in the other 500 or so known members of the family. The halictids were present in all populations of the plant at predictable times in the mornings and late afternoons. They were observed foraging on dehiscent anthers, and frequently landed on other floral parts of the plant, including receptive stigmas. The first bees arrived at the plants minutes before solar sunrise. It should be noted that sunrise in the populations was effectively 30-45 minutes later than solar sunrise, due to the populations occurring in openings in forest as well as being deeply shaded morning and evening by surrounding hills. The halictids were largely absent from two hours before solar noon to several hours after. The bees abruptly departed within minutes of solar sunset, at which time the populations were already in deep twilight. No other insect visitor was recorded during the 25 hours of videotaping.

During visual observations, a single large bee visited three flowers of a single *M. virginica* on July 30 at approximately 9:30 a.m. This was the only other possible pollinator interaction observed during the entire study period, even though Hymenoptera, Diptera and Lepidoptera species were present in the prairie barrens at various times. Ants were seen foraging on one plant. Grasshoppers were noted resting on the stems of *M. virginica* on two occasions.

*Seed set*. Flower number and seed set were recorded in 2001 for the populations in Desert, Big Slump, Lynx and Teakettle East prairies. In 2002 seed set was recorded for the populations at Crossroads, Shooting Star, Post West, Teakettle West, Shivener and Davis prairies (Fig. 5). Across all populations the plants had an average of 18 flowers per plant that could have potentially set seed. Seed set as a percentage of flowers ranged from a high of 0.21 to a low of 0.03. Large and medium size populations had an average seed set of 0.16. This is in concordance with the seed set findings of Baskin and Baskin (1971) for *M. virginica* occurring in large (>100 flowering individuals) populations in the Cedar Glades region of Tennessee.

*Genetic diversity*. Estimated heterozygosity was relatively low in all 12 populations, and was not dependent on class size or geographic location (Table 2). Across all individuals in all populations heterozygosity was 0.18, and % polymorphic loci (P) is 98.95. Both heterozygosity and % polymorphic loci are higher across all populations than within populations. The populations with the highest heterozygosity are a small population at Lynx Prairie (0.14) and a large population at Post West Prairie (0.15), which also have the highest polymorphism (41% and 43%, respectively). Desert Prairie's large population has low heterozygosity and polymorphism (0.09 and 26.31).

The small population at Homestead prairie, which is within inferred pollen carry distance to, but separated by heavy cover from, Desert and Post West, has much lower heterozygosity and polymorphism than Post West but in both categories ranks higher than Desert. The largest populations were average in both heterozygosity and polymorphism. The populations at Teakettle East and West both had average heterozygosity and higher than average polymorphic loci relative to the others. The small, isolated population at Unity had lower than average heterozygosity and polymorphism, as did the isolated medium sized population at Davis. The adjacent medium-sized populations at Big Slump and Crossroads have below average heterozygosity and the lowest polymorphism of the study group (0.10, 26% and 0.09, 25% respectively). Low heterozygosity and polymorphism within populations and high estimated heterozygosity and high polymorphism among populations indicates substantial drift and fixation of alleles within populations resulting from isolation by distance.

*Population structure*. Three analyses of molecular variance (AMOVA) were made based on the data obtained from 15 samples from each of the 12 populations (Table 3). The first analysis is a two level AMOVA that explains genetic variation as being distributed 44% among populations and 56% between populations (Table 3 A.). The second AMOVA is a three-level analysis of the data divided into three size classes of small (<10 flowering individuals), medium (10-20 flowering individuals) or large (>20 flowering individuals). This analysis shows that 44% of genetic variation is explained within populations and 56% among populations (Table 3 B.) The third AMOVA (Table 3 C.) examined two *a priori* regions representing populations central to the local range (populations 8, 9 and 10) as well as population 7. Population 7 the population clustered

closely with populations 8, 9 and 10 in the PCoA, although it is most distant from them. The remaining eight populations were included in the marginal population region. AMOVA was also performed excluding population 7 from the central population region, but the results were identical. The *a priori* central and marginal regions explained 0% of the variation, with 50% of the variation being distributed within populations, and 50% distributed among populations. Random permutations of the data for each AMOVA (Fig. 6, 7 and 8) demonstrate partitioning is non-random. The results of the AMOVAs indicate that across the study area genetic differentiation was significant and is about equally distributed both within and among populations, but no differentiation is correlated with population size, position within the local range, or geographic remoteness among populations. The value of $\Phi_{st}$ (0.54) indicates significant differentiation and low gene flow across all populations (Hartl and Clark 1997).

Comparisons of $\Phi$ values between pairs of populations (Table 3) show levels of differentiation between all populations. The lowest value is for population 5 and 6 (0.37), which is interesting given their relative distance and isolation, suggesting more recent common ancestry or dispersal between these populations (Fig. 2). A relatively low value of 0.43 is found between populations 7 and 8, which are very distant from each other, suggesting relatively recent dispersal. Other relatively low values are found between adjacent populations: 3 and 4 (0.45), adjacent 11 and 12 (0.41).

Bartlett's statistic for the two-way AMOVA (3.94743, $\div^2_{0.05,\ 11}$ = 19.675), and for 3-way AMOVAs for size class (3.94743, $\div^2_{0.05,\ 11}$ = 19.675) and geographic position (3.37685, $\div^2_{0.05,\ 11}$ = 19.675) (Table 3) indicate that there is homogeneity of variance, or

low heteroscedasticity between the 12 populations, suggesting evenness in the degree of differentiation between populations.

*Population relationships*. UPGMA Cluster Analysis was performed in NTSys using the Dice coefficient. Shared bands in each population often manifested in clustering of adjacent populations (Fig. 9). Populations 9 and 10 form a group, and are geographically adjacent. Populations 11 and 12, which are close to each other but are well isolated from the other populations, form a group. Populations 3 and 4 are closely related, and are in fact adjacent to each other by a matter of meters but are separated by heavy cover. Populations 3 and 4 are geographically the closest to population 7 and they share certain genotypes with it, but population 7 is geographically quite distant from these. Populations 5 and 6 form a close group, although they are relatively distant from each other. Among all samples, the cluster analysis organizes individuals from the respective populations into groups with no overlap in relationships between the populations, indicating substantial differentiation among all populations.

*Principal coordinates analysis.* Ordination was performed with principal coordinates analysis (PCoA) based on ISSR band frequency in individuals (Fig. 10). It reveals substantial clustering and significant genetic distance between groups of individuals from populations. Axis 1 vs. axis 2 positions populations 5,6, 8, 9 and 10 together, these being geographically central to the local range. Population 7 clusters with the central group in first vs. second axis as well, but it is west-northwest of the central population group about 25 kilometers and this geographical separation is illustrated by the position of 7 in first vs. third axis. Populations 3 and 4 cluster together, and are separated from each other by 15 meters of heavy cover about 1.5 kilometers north of the

central group. Populations 1 and 2 are north and northeast of the central group, and are separated from them by a distance of about 11 kilometers and 13 kilometers respectively; and are positioned as genotypic extremes in the ordination. Populations 5 and 6, separated from each other by 2.5 kilometers, are positioned with the central group in first vs. second axis and are geographically related to them, however, differentiation from that group and relatedness to each other is shown in axis 1 vs. 3. Populations 11 and 12 are positioned together as another set of genotypic extremes. They are south of the central group (8, 9, and 10) by about 3.5 kilometers and are separated from each other by 100 meters of heavy cover by direct distance, but are connected without cover by a more circuitous route.

*Mantel Test*. A mantel test was performed (Fig. 10) based on a genetic distance matrix using the Nei (1978) coefficient between populations/individuals and a matrix of the geographic distances between pairs of populations/individuals. The test indicates no significant relationship ($R^2 = 0.1562$) between genetic and geographic distance for populations of *M. virginica* included in the study.

## Discussion

Field observations of plant-pollinator interactions and tracking of pollen movement using a dye surrogate, coupled with analysis of genetic variation within and among the populations using ISSR, indicate that gene flow by pollen across the range of *Manfreda virginica* in Adams County, Ohio is intra-populational and inter-population differentiation is substantial. The largest of the populations are small and generally limited to less than a hectare and 20-30 flowering individuals in any given year. The smallest populations in the study had only a few flowering individuals growing in patches

of only several meters square. Gene flow by way of pollen movement was found to be limited to *M. virginica* growing in discreet populations. No pollen movement was detected between any populations, even if they are only tens of meters apart but separated by heavy cover of as little as 15 meters across. Gene flow by way of seed dispersal was not investigated, but is presumed to be lower than that of pollen in order to explain the large differentiation among populations indicated by ISSR variation. Genetic differentiation among the populations is considerable and low levels of estimated heterozygosity and polymorphic loci were calculated within populations. The pattern of genetic variation and relationships among the study populations suggest fragmentation and differentiation of a once larger population into three genetically coherent groups, with groups and populations within groups showing genetically and geographically congruent relationships. Low gene flow among the *M. virginica* in the region and the resulting genetic and geographic isolation of all the populations should be of concern to land managers.

*Pollinator limitation on gene flow and seed set.* Careful observation of plant-pollinator interactions and tracking of potential pollen movement demonstrated pollen limitations on gene flow between populations. A single insect - a small halictid bee - was identified as a frequent visitor to *M. virginica* in the study populations. The bees do not appear to travel across areas shaded by heavy cover of trees. The bees evidently transport pollen readily across individual populations, with the greatest effect being on those plants closest to the source of the pollen. In visual observations, the pollen-predator halictids were seen to move from one plant to another apparently searching for dehiscing anthers. The bees visited most of the flowers on a plant as they searched for pollen, most

frequently landing on and moving around on unopened or emerging stamens. Contact by the bees with receptive stigmas was noted by visual and video observation but was infrequent compared to contact with the protandrous male floral structures. The bees departed from plants when either pollen was unavailable, they had full pollen loads, the dehiscing anthers of the plant were either largely cleared of pollen by other halictids or the concentration of bees on a plant were such that the bee was "pushed off". These observations would initially suggest potential for substantial within-population gene flow, but analytical results show the contrary: within population genetic differentiation is roughly equivalent to between population differentiation. Prohibitions on sib-mating, highly sedentary seed dispersal and limited pollinator contact with receptive stigmas are possible contributing factors.

It is of interest that this insect was never before identified as a pollinator of *M. virginica*, in spite of frequent observations by Baskin and Baskin (1971) and a detailed study made of the plant in Tennessee (Groman and Pellmyr 1999). The Baskins acknowledged seeing only a single bumblebee visit the plant in all of the time they spent in the field, and this may be attributable to their observations being made during the afternoon hours. In the case of Groman and Pellmyr, their study purported to identify a guild of pollinators associated with *M. virginica*. In July and August 2002 I made visual and video observations among the same Cedar Glade populations used by Groman and Pellmyr (1999). No members of the suggested guild were observed visiting the *M. virginica*. Although many members of the proposed guild were present in the vicinity of the *M. virginica*, they were visiting other flowering plant species. In the Cedar Glade

region, the sole observed insect visitor landing on *M. virginica* was a halictid bee, apparently the same species as in the Adams County, Ohio populations.

Further supporting pollinator limitations on gene flow are the behaviors of the halictids. In addition to their apparent aversion to moving through cover, the bees behave as efficient pollen predators. The bees identify those flowers that have dehiscing anthers and descend on the plants in groups and rapidly strip the anthers of pollen, leaving little for other insects to carry off, even if other insects were more interested in the pollen than the nectar reward offered by the protandrous stigma on flowers lower on the inflorescence. The number of halictids seems limited as well. No more than six were seen visiting any one plant at one time, and few more than this were evident in a population at any one time. Given the limited number of plants flowering in any population in a season (7-24) and the average number of flowers per plant (17), a single patch of *M. virginica* does not seem to represent a resource capable of supporting very many of the halictids if they are using the plant as their sole food source. However, the evidence suggests a specialized relationship between *M. virginica* and the pollen-predator halictid.

It may be to the advantage of *M. virginica* to have a close relationship with the small pollen predator halictid, even though the insect appears to be a limiting factor in seed set for the plant. *M. virginica* is a plant that grows in extreme habitats, essentially deserts or similar sites on thin, dry soils. The plant has been found to grow slowly, and typically flowers only after several years when it has built sufficient energy reserves. As a perennial, the plant has to allocate a portion of its annual photosynthate production to storage for maintenance and growth the following year (Baskin and Baskin, 1971). If every flower on the plant is seeded, it is possible that the plant will have to use too great a

portion of its reserves and have insufficient storage to persist another growing season (Baskin and Baskin, 1971). In this way it may be advantageous for *M. virginica* to produce copious amounts of pollen for insects whose behavior limits seed set in the plant, yet distribute some of its pollen broadly within the populations.

The behavior of the halictid bees is surely a contributing factor to the genetic structure of *M. virginica* populations in the study area. While *M. virginica* is an obligate outcrosser, the foraging bees have a leptokurtic pattern of visitation as evidenced by dye powder transfers. This pattern of pollen movement promotes subdivision and inbreeding, contributes to isolation by distance and may create patchiness that persists over several generations (Loveless and Hamrick 1984).

Two of the small populations had very low seed set of 0.04% and 0.03%. Both of these populations had very few flowering plants, but the plants were not separated by cover and were well within determined pollen travel distance of each other. It may be that the flowering plants in those populations are too closely related to mate. *M. virginica* is an obligate outcrosser, and may have mechanisms to prevent sib-mating. In two locations pairs of flowering plants were found of equal size and development that were growing almost on top of each other, and these were hypothesized as being siblings. Both pairs were somewhat isolated from other flowering plants, and of the four mature plants only one flower set seed between them, supporting the notion that *M. virginica* is an obligate outcrosser that has poor success mating with near relatives. It may be that in the populations with few individuals, the flowering plants were too closely related to further interbreed.

Occasional long-distance pollen dispersal by other insects should not be ruled out. A hawk moth was noted flying well above the population at Crossroads on one occasion. Hawk moth pollination was hypothesized by Robertson (1928) based on the pollinators of relatives in the Agaveaceae, and visitation by a hawk moth was reported by Groman and Pellmyr (1999). Pollination by moths could be possible: moths were frequently seen in the barrens communities during the study, although they were never observed visiting *M. virginica*. Large bees were seen infrequently in the prairie habitats of Adams County during the flowering period of the *M. virginica*, and one observation was made of a single large bee visiting the styles of one plant, but its behavior did not bring it into contact with dehiscent anthers higher on the scape. Large bees were quite active on other flowering plants during observations in the Cedar Glade region of Tennessee, but none were observed visiting *M. virginica*. Visitation by potential long-distance pollinators has been noted as infrequent by Baskin and Baskin (1971) and in this study. While it is possible that the number and frequency of potential long-distance pollinators noted by Groman and Pellmyr (1999) may occur in any given year, field observation and analysis of genetic differentiation suggests that long-distance pollen movement does not play a significant role in the genetic structure of *M. virginica* populations in Adams County.

*Genetic differentiation.* In theory genetic diversity should decline in recently derived populations, and in the literature there have been findings of decreased genetic variation in peripheral versus of central populations (Lande 1999). In this case, if small populations are hypothesized to represent colonies, they do not exhibit lower heterozygosity or lower polymorphism relative to larger populations, which would be expected for more recently established populations. As small populations have

maintained the same or greater levels of heterozygosity and polymorphism as larger populations, these may represent remnants of once larger populations. One of the most central populations, Post West (8), does have relatively high heterozygosity and polymorphic loci, however, two other populations most central to the range, Homestead (9) and Desert (10), are both below average in those categories. There is no clear pattern to heterozygosity and polymorphic loci within populations that is attributable to either population size or relative location. Error may be involved in the calculation of differentiation between populations in this analysis, as expected levels of heterozygosity using neutral genetic markers declines linearly with inbreeding (Morgan et al 2001). While *M. virginica* is an obligate outcrosser and the small effective populations of the study sites suggests that inbreeding is likely, the low levels of detected heterozygosity may be exaggerated by the limitations of the neutral marker system employed in the analysis.

Multivariate analysis of the genetic data demonstrates a fragmented pattern of genetic variation among the populations of *M. virginica.* The Mantel test did not identify a statistically significant correlation between geographic distance and genetic distance between populations, suggesting that these populations do not conform to an isolation by distance model to explain patterns of differentiation. Cluster analysis supported relationships among adjacent populations while clearly defining individuals as belonging to discrete populations. PCoA showed significant spatial genetic structure among adjacent populations and placed populations in three broad genetic groups. The two level AMOVAs found approximately equal partitioning of genetic variation among the populations. Bartlett's statistic for the two-level AMOVAs indicates a similar degree of

differentiation across populations. The three-level AMOVA did not find any significant effect by size class or in central versus peripheral populations, removing overall population size and geographic position within the local range as important factors accounting for the pattern of genetic differentiation. Barlett's statistic for the 3-level AMOVAs did not find heteroscedasticity to be a component of variation among the *a proiri* regions.

Together, these analyses and tests indicate that substantial genetic differentiation has occurred among these populations with fixation of alleles independent of spatial or demographic factors. Levels of differentiation appear to be even among all populations. Recent migration, colonization and founder events through seed dispersal are probably not a component of the pattern of genetic variation in the study area. Limitations on pollen mediated gene flow and high levels of differentiation among populations indicate that a metapopulation of the species may not operate in the region, and that *M. virginica* occurs in Adams County in genetically isolated populations.

*Conservation concerns*. Emma Lucy Braun first hypothesized that the plant communities of the Barrens in Adams County Ohio contained relict species, such as *M. virginica,* that remain following post-glacial plant migrations (Braun 1927). 4000-8000 years ago there was a hot, dry climatic period in this region known as the hypsithermal (Brown and Lomolino). During the hypsithermal prairie systems were extensive in the Ohio valley and *M. virginica*, a plant of southeastern origin, is thought to have extended its range into southern Ohio during this period. Climate change since that time brought warmer wetter conditions, allowing the growth of eastern deciduous forest communities (Braun 1927).

Prior to settlement, the region featured densely forested valleys and dry ridge-tops of some extent (Strittholt and Boerner 1995). Two hundred years ago a settler wrote an account of being chased by Native Americans for several miles through an "agave desert" in this region. Settlement of southern Ohio put pressure on the forest resources, and by the end of the American Civil War only a few patches of forest remained in the area (Strittholt and Boerner 1995). It is likely that larger cleared areas provided heightened opportunity for gene flow via pollen movement between populations of *M. virginica*, and that larger habitat patches with greater connectivity existed for the plant prior to the reforestation efforts of the late nineteenth century. Since then much of the eastern deciduous forest has returned, and 75% of the region has some form of natural cover (Strittholt 1994). Now, only fragments of the plant communities typified by the little American agave *Manfreda virginica* persist in isolated barrens along the most western edge of the Appalachian Plateau.

Recent history has shown that local populations of *M. virginica* have diminished due to natural succession and reforestation in areas like Agave Ridge, the site of Braun's famous transect. *M. virginica*, once a dominant plant on Agave Ridge, is no longer found along Braun's original transect there. Land managers are currently working to maintain the prairie openings with fire and tree and shrub removal, and in places are reclaiming overgrown openings by removal of trees and understory, and consideration should be given to increasing the connectivity of areas with adjacent populations of *M. virginica*, such as the satellite prairies at Lynx, the Teakettle prairies, and Homestead-Post West prairies.

This study found that the small populations at Teakettle West and Lynx Prairies had very low seed set, and this may be due to inbreeding prohibitions in the mating system of *M. virginica.* While identification of possible metapopulation dynamics among the populations of *M. virginica* in Adams County requires further research, creating connectivity for pollen movement from nearby populations may enhance seed set in these areas.

*M. virginica* is an obligate outbreeder and quite likely has near-sib mating prohibitions as well. These mechanisms may present a limitation on seed set in small populations. Small effective populations and limited gene flow through pollen movement as well as seed dispersal should be of concern to land managers in regards to *M. virginica* in the region. Management planners may wish to consider the implications of genetic isolation for *M. virginica* populations in their preserves, and existing populations should be monitored for maintenance of effective population size, fecundity, and persistence in the prairie barren communities.

## Conclusions

High levels of genetic variation among populations of *M. virginica* in this study are consistent with low gene flow between populations (Preecha and Baimai 1999) and may be explained by limited gene flow through pollen and/or seed dispersal. From the evidence it appears that the fragmented populations of *M. virginica* have very limited gene flow and differentiation has been promoted through genetic isolation. Clear differentiation between all populations in the study independent of population size characteristics and relative location suggests substantial drift as a contributing factor in the genetic substructure within populations.

In populations in equilibrium between drift and migration, genetic differentiation among populations is expected to increase with geographic distance (Slatkin 1994). No such simple linear relationship was found in the Mantel test among these populations of *M. virginica*, although geographically proximal populations are grouped together in the PCoA and UPGMA analyses. Additionally, the *M. virginica* populations in this study exhibit similarly high levels of differentiation as well as low heterozygosity and polymorphism regardless of spatial position or size class. Such a complex pattern of genetic fragmentation could conceivably be the end product of range expansion and contraction.

Plant metapopulations are connected by gene flow through dispersal of seed and pollen, and a relationship between spatial geographic and spatial genetic structure is expected in some form (Gonzalez-Astorga and Nunez-Farfan 2001). The spatial genetic structure of *M. virginica* in Adams County does not suggest connectivity among populations. Plant populations in fragmented habitats are expected to become genetically divergent due to reduced gene flow, genetic drift and inbreeding (Templeton et al. 1990). The effects of habitat fragmentation are implicated in the highly differentiated genetic structure among populations of *M. virginica* in Adams County, Ohio. Pollinator limitations and behavior, probable sedentary seed dispersal and historical factors of range expansion and habitat fragmentation through climate change and human activity have likely played roles in the distribution of genetic diversity among *M.* virginica in this region.

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Table 1. ISSR primer sequences used for analysis of genetic variation in 12 populations of *M. virginica* in Adams County, Ohio. Values are for bands generated in this study.

| Primer Sequence | Number of Informative Bands | Range of Fragment Size (bp) |
|---|---|---|
| $(GA)_6CC$ | 32 | 450-1735 |
| $(GTG)_3GC$ | 32 | 370-1615 |
| $(CA)_6GG$ | 31 | 400-1535 |

Table 2. Estimated heterozygosity and % polymorphic loci for 12 populations of *Manfreda virginica* in Adams County, Ohio. Size class indicates the relative number of flowering individuals (S=<10; M=10-20; L=>20) in the populations in 2001.

| Population Sample Number/Name | | Population Size Class | Estimated Heterozygosity | % Polymorphic Loci |
|---|---|---|---|---|
| | All Pops | | 0.17 | 98.95 |
| 1. | Davis | M | 0.08 | 27.37 |
| 2. | Shivener | L | 0.10 | 33.68 |
| 3. | Teakettle E. | M | 0.13 | 41.05 |
| 4. | Teakettle W. | S | 0.12 | 34.77 |
| 5. | Lynx | S | 0.14 | 41.05 |
| 6. | Shooting Star | L | 0.15 | 36.84 |
| 7. | Unity | S | 0.09 | 28.42 |
| 8. | Post W. | L | 0.15 | 42.11 |
| 9. | Homestead | S | 0.09 | 29.47 |
| 10. | Desert | L | 0.09 | 26.32 |
| 11. | Big Slump | M | 0.10 | 26.32 |
| 12. | Crossroads | M | 0.09 | 25.26 |

Table 3. Analysis of Molecular Variance (AMOVA) for 12 populations of *Manfreda virginica* in Adams County, Ohio. A. Two-way analysis of variation within and among populations. B. Three-way analysis comparing three size classes of populations. C. Three-way analysis comparing central and marginal populations.

| A. Source of Variation | df | Variance Component | Bartlett's Statistic | Φ Statistic | Value | P-value |
|---|---|---|---|---|---|---|
| Among Pops. | 11 | 7.323 | 3.947 | | | |
| Within Pops. | 168 | 6.327 | | $Phi_{PT}$ | 0.536 | 0.010 |

| B. Source of Variation | df | Variance Component | Bartlett's Statistic | Φ Statistic | Value | P-value |
|---|---|---|---|---|---|---|
| Among Regions | 2 | -0.302 | 3.947 | $Phi_{RT}$ | 0.022 | 1.000 |
| Among Pops./Regions | 9 | 7.546 | 0.429 | $Phi_{PR}$ | 0.563 | 0.001 |
| Indiv./Within Pops. | 168 | 6.283 | | $Phi_{PT}$ | 0.464 | 0.001 |

| C. Source of Variation | df | Variance Component | Bartlett's Statistic | Φ Statistic | Value | P-value |
|---|---|---|---|---|---|---|
| Among Regions | 1 | 0.005 | 3.377 | $Phi_{RT}$ | 0.000 | 0.415 |
| Among Pops./Regions | 10 | 6.979 | 0.649 | $Phi_{PR}$ | 0.498 | 0.001 |
| Individuals Within Pops. | 168 | 7.026 | | $Phi_{PT}$ | 0.498 | 0.001 |

Table 4. Pair-wise Phi-values showing genetic differentiation between 12 populations of *Manfreda virginica* in Adams County, Ohio.

| Pairwise Population PhiPT Values | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Pop 1 | Pop 2 | Pop 3 | Pop 4 | Pop 5 | Pop 6 | Pop 7 | Pop 8 | Pop 9 | Pop 10 | Pop 11 | Pop 12 |
| Pop 1 | 0.0000 | | | | | | | | | | | |
| Pop 2 | 0.5332 | 0.0000 | | | | | | | | | | |
| Pop 3 | 0.5621 | 0.5156 | 0.0000 | | | | | | | | | |
| Pop 4 | 0.5911 | 0.5743 | 0.4497 | 0.0000 | | | | | | | | |
| Pop 5 | 0.5722 | 0.5734 | 0.4709 | 0.5430 | 0.0000 | | | | | | | |
| Pop 6 | 0.5564 | 0.6014 | 0.4634 | 0.5746 | 0.3695 | 0.0000 | | | | | | |
| Pop 7 | 0.5831 | 0.5487 | 0.5054 | 0.5646 | 0.4865 | 0.5360 | 0.0000 | | | | | |
| Pop 8 | 0.5237 | 0.3819 | 0.4204 | 0.4859 | 0.4763 | 0.5070 | 0.4258 | 0.0000 | | | | |
| Pop 9 | 0.5731 | 0.5176 | 0.5019 | 0.4931 | 0.5247 | 0.5375 | 0.5322 | 0.4439 | 0.0000 | | | |
| Pop 10 | 0.5524 | 0.5481 | 0.4948 | 0.6128 | 0.5368 | 0.5457 | 0.5882 | 0.4073 | 0.5096 | 0.0000 | | |
| Pop 11 | 0.6623 | 0.6032 | 0.5480 | 0.6120 | 0.5502 | 0.5676 | 0.5812 | 0.4484 | 0.5319 | 0.5901 | 0.0000 | |
| Pop 12 | 0.6300 | 0.6359 | 0.5664 | 0.6122 | 0.5727 | 0.5632 | 0.5909 | 0.5237 | 0.5760 | 0.5775 | 0.4076 | 0.0000 |

Figure 1. *Manfreda virginica*. Modified from Braun (1967).

Figure 2. Map of study area in Adams County, Ohio. Stars indicate locations of populations from which samples were taken for genetic analysis

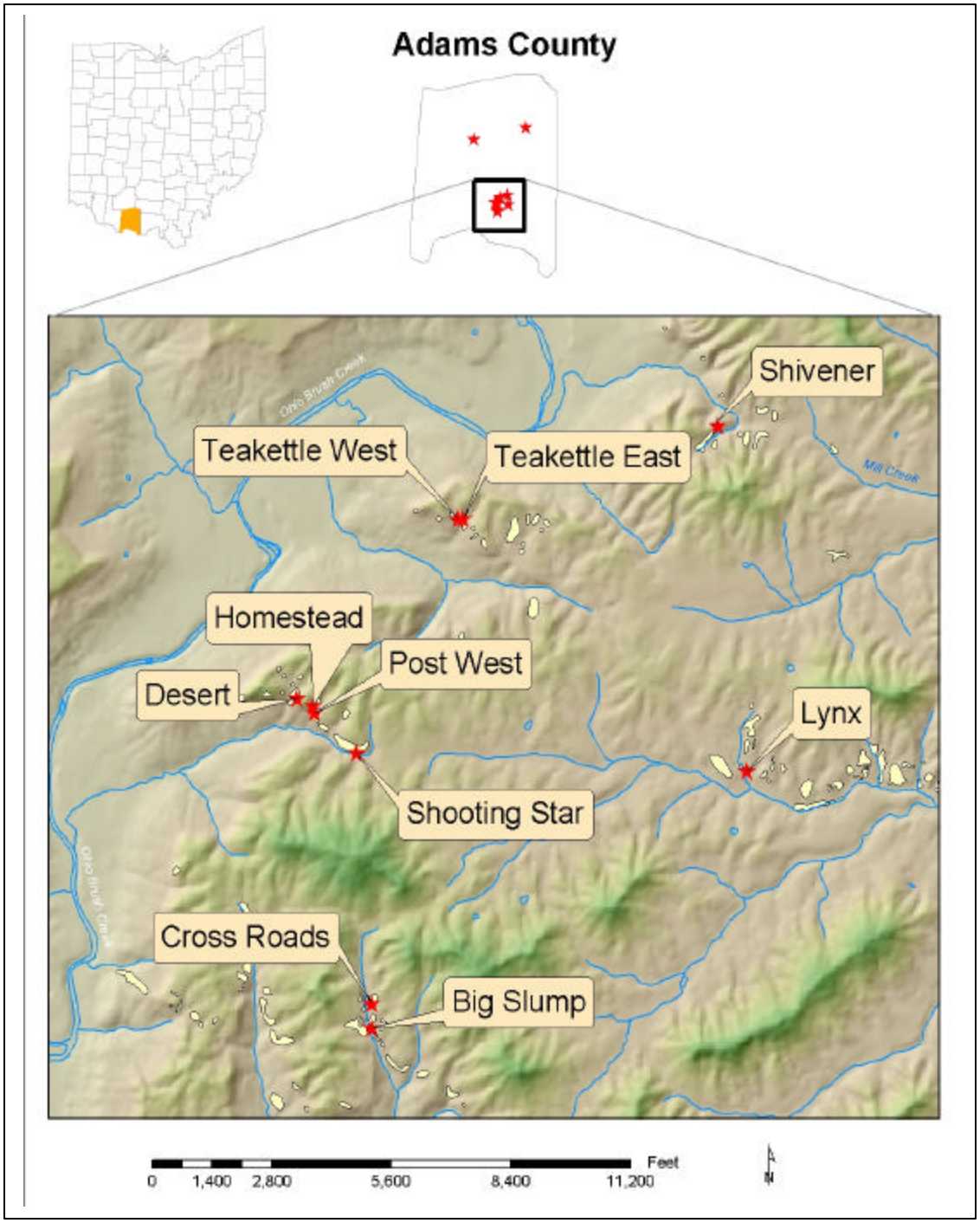


Figure 3. Inferred pollen travel distance in three populations of *Manfreda virginica* in Adams County, Ohio.

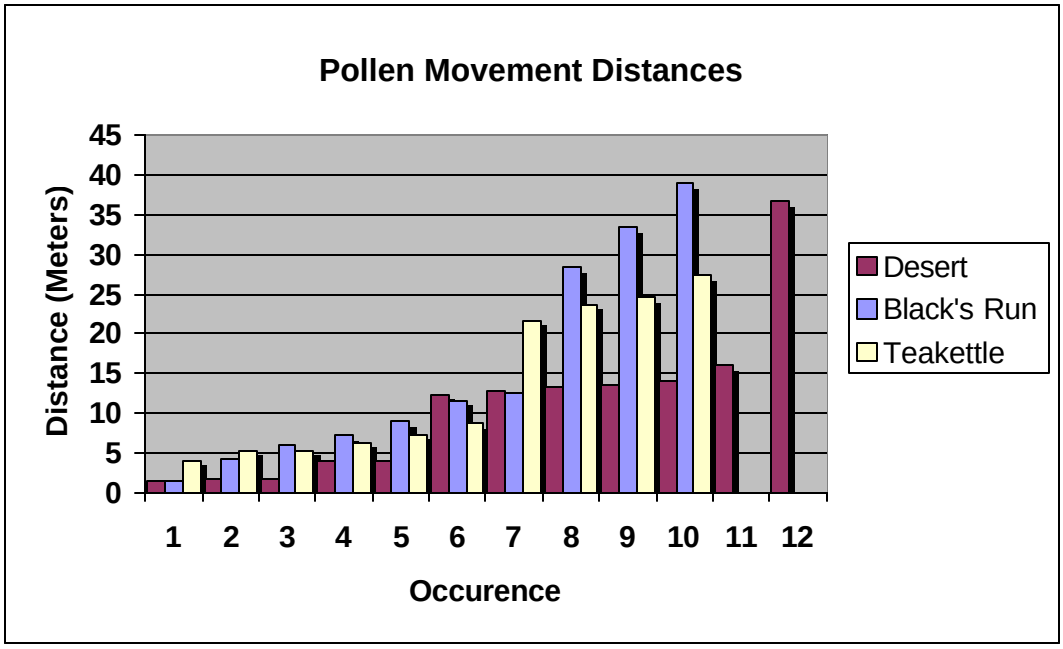


Figure 4. Pollinator visitation profile for 3 populations of *Manfreda virginica* in Adams County, Ohio. Numbers of visitors represents the number of halictid bees present on a plant at one time during a one-hour interval.

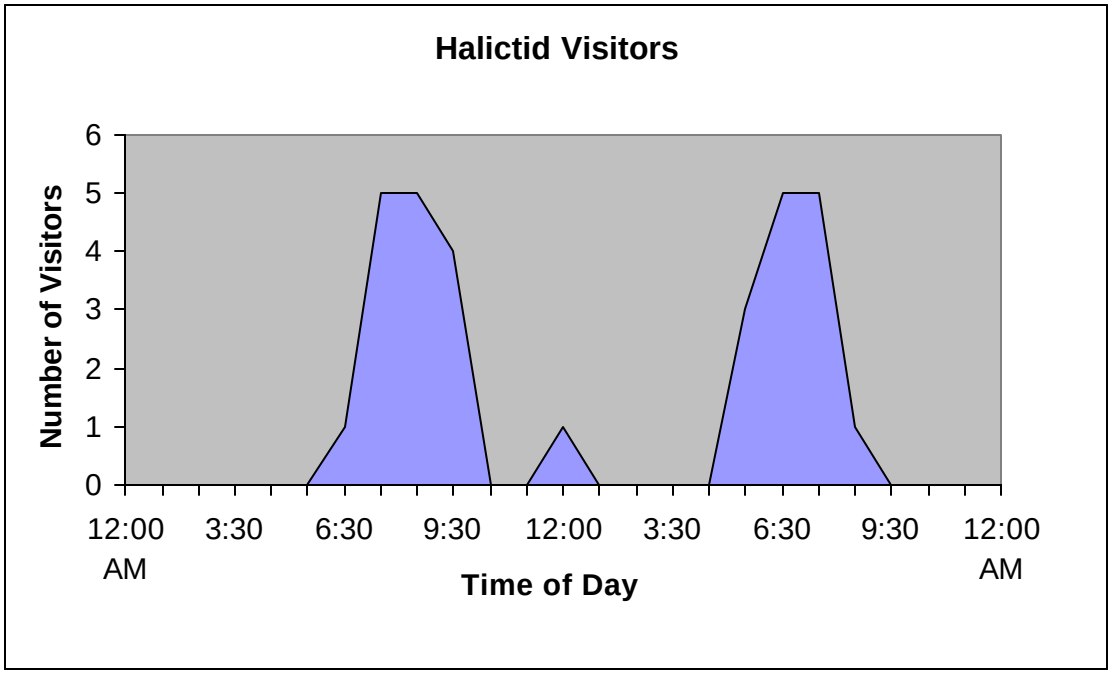


Figure 5. Seed set as a percentage of flowers in 10 populations of *Manfreda virginica* in Adams County, Ohio. An average of 18 flowers per plant were available for pollination.

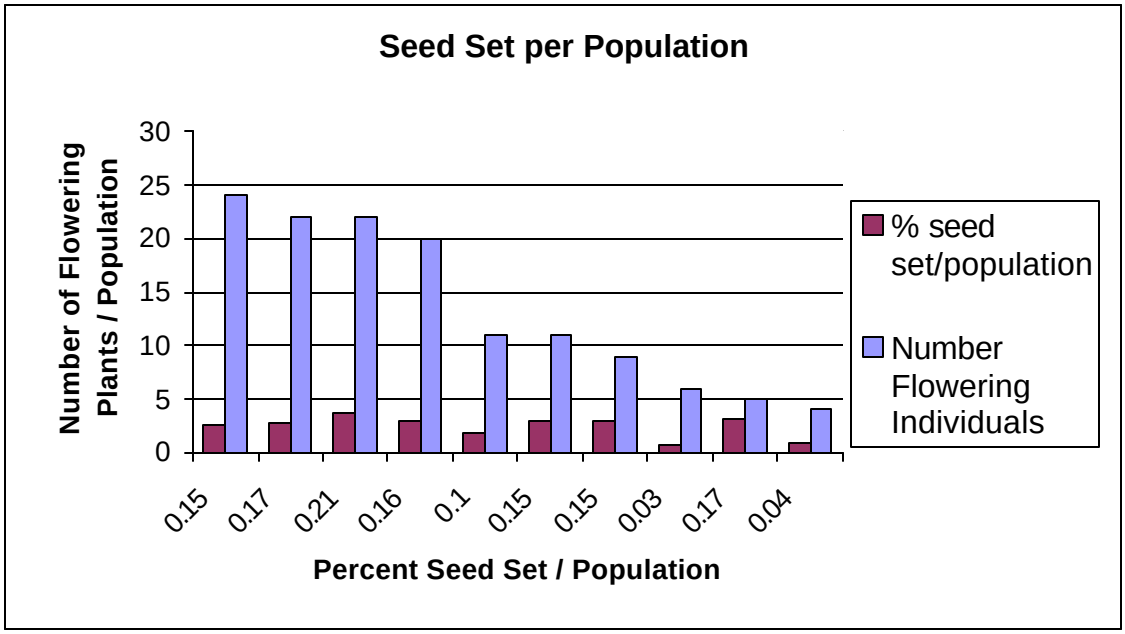


Figure 6. Frequency distribution for two level AMOVA of ISSR band variation for 12 populations of *Manfreda virginica* in Adams County, Ohio.

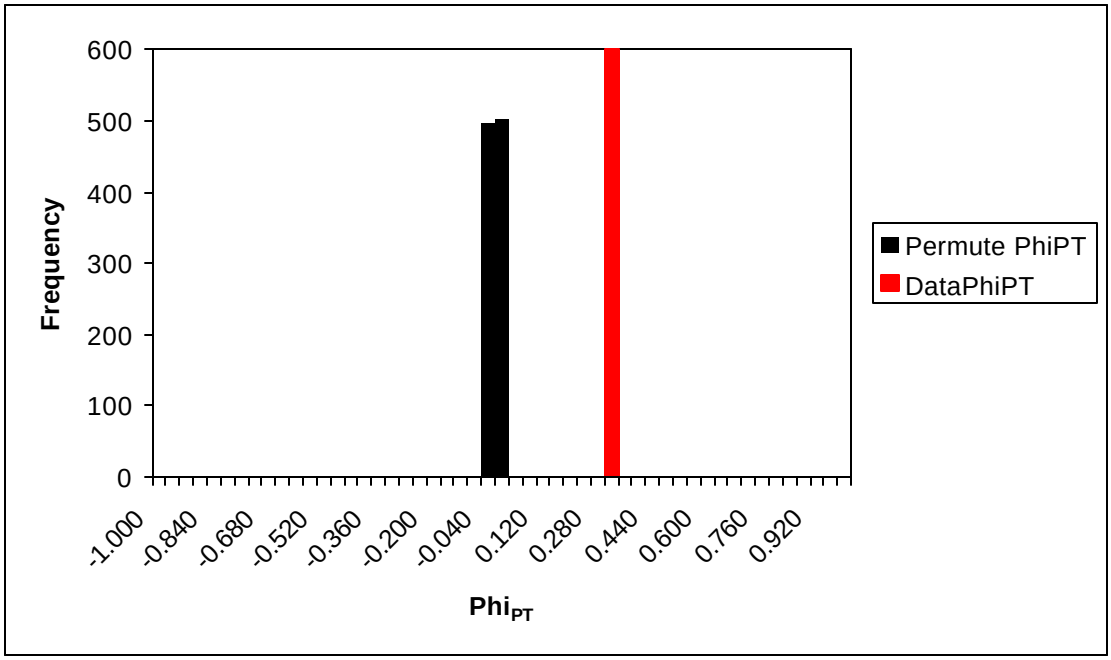


Figure 7. Frequency distribution for three level AMOVA of ISSR band variation with regions based on class size for 12 populations of *Manfreda virginica* in Adams County, Ohio.

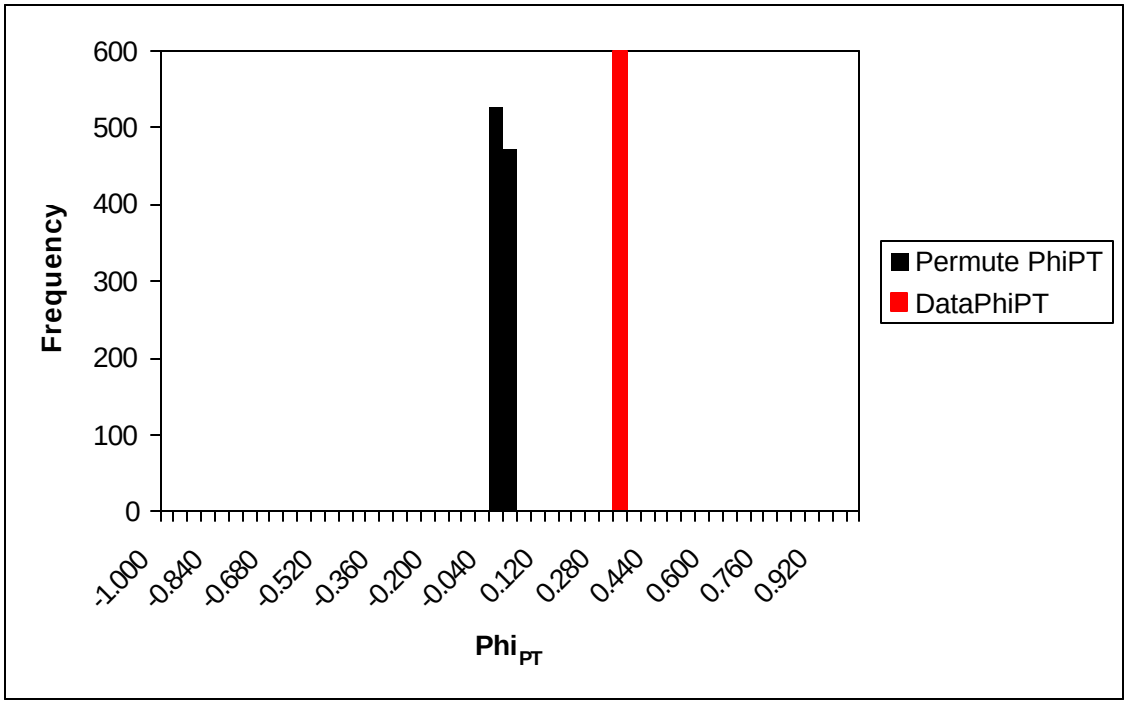


Figure 8. Frequency distribution for three level AMOVA of ISSR band variation with regions based on central and marginal population for 12 populations of *Manfreda virginica* in Adams County, Ohio.

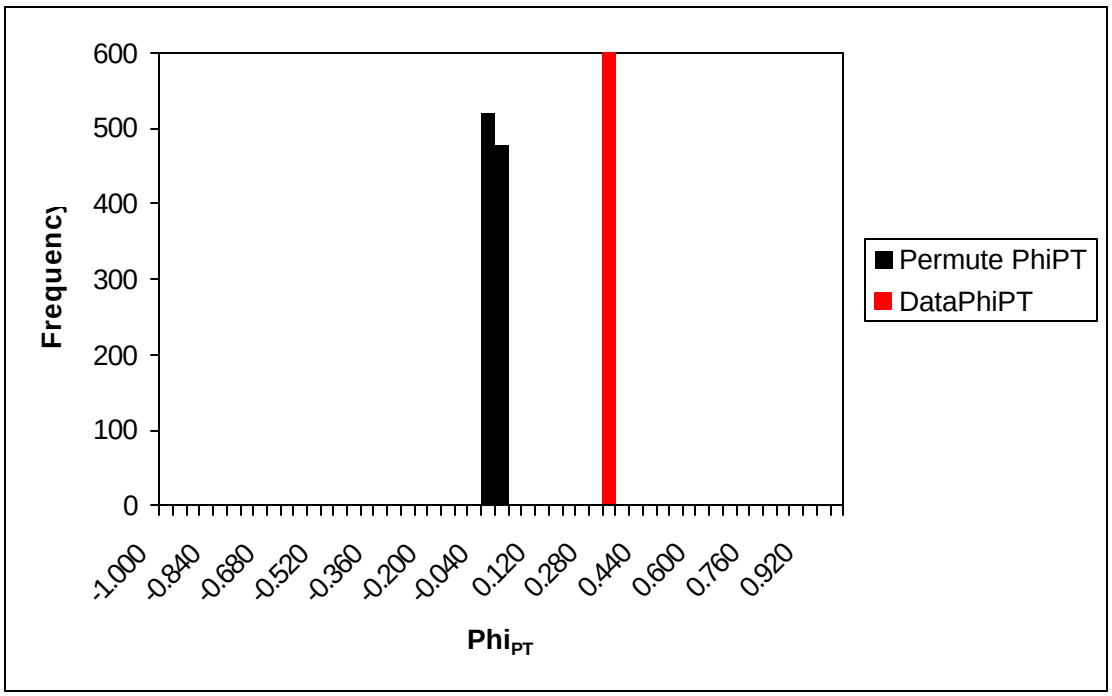


Figure 9. UPGMA cluster analysis for 15 individuals within 12 populations of *Manfreda virginica* in Adams County, Ohio.

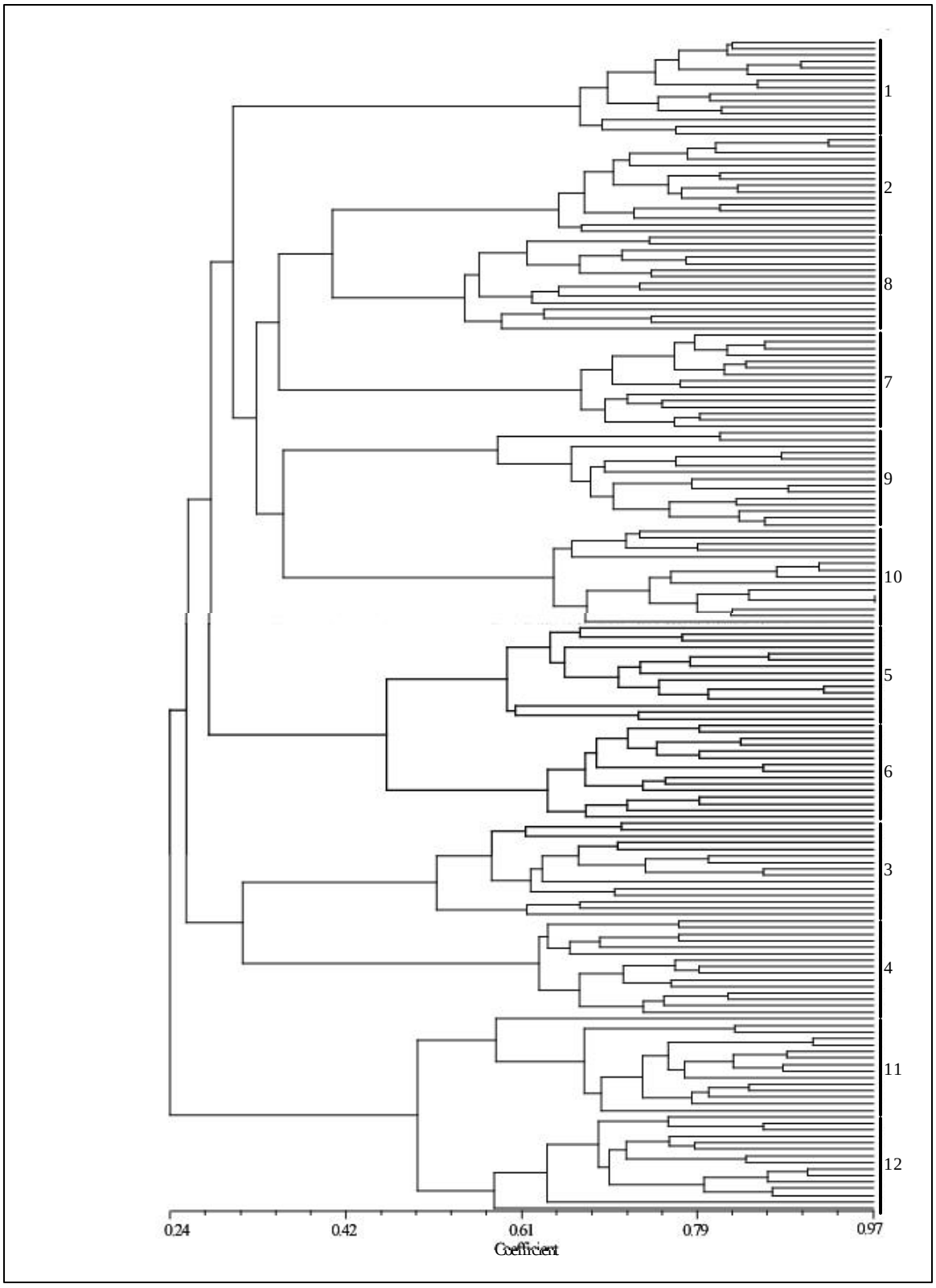


Figure 10. Principle coordinates analysis (PcoA) for 12 populations of *Manfreda virginica* in Adams County, Ohio. Numbers indicate 15 individuals from 12 populations.

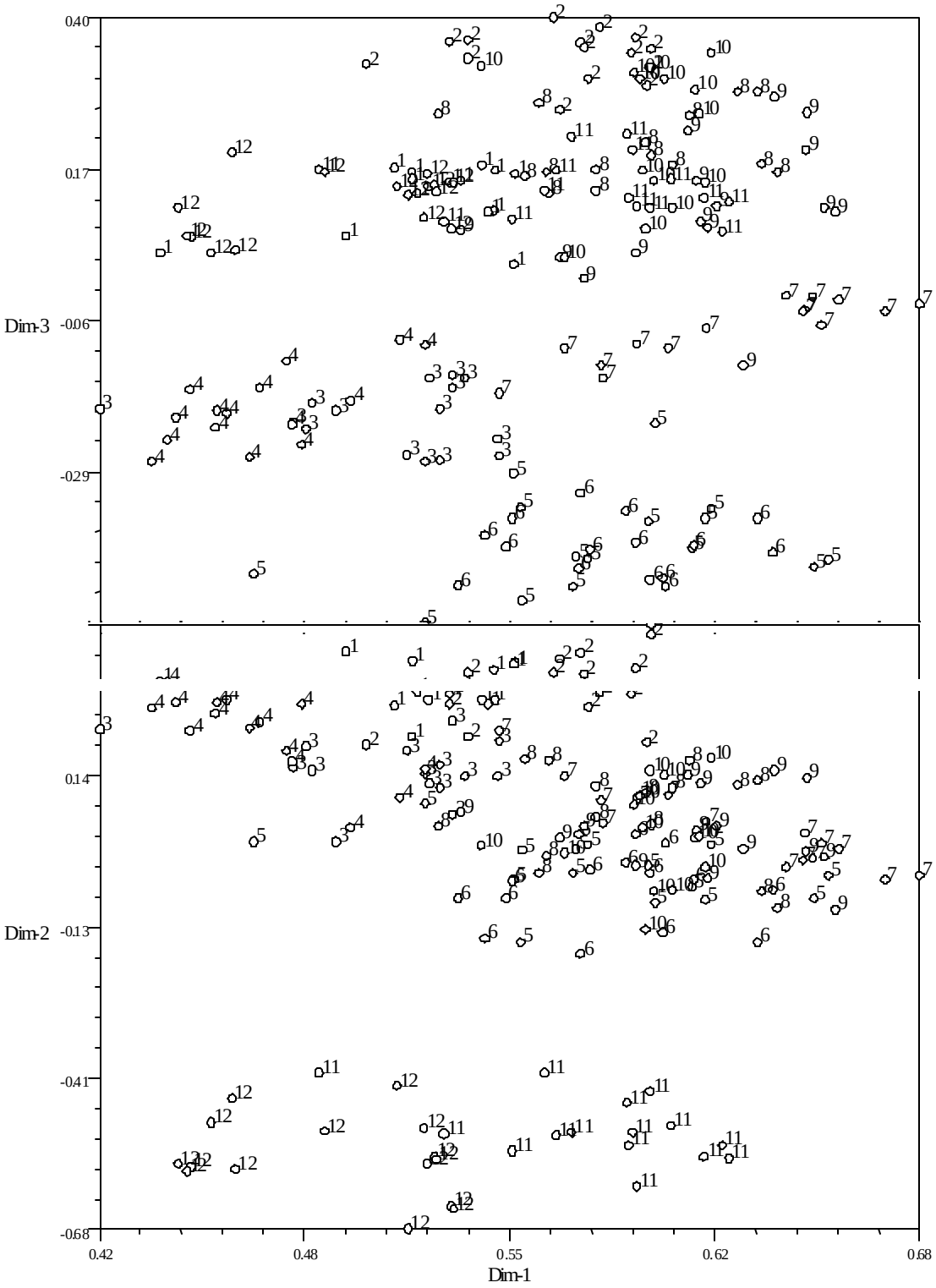
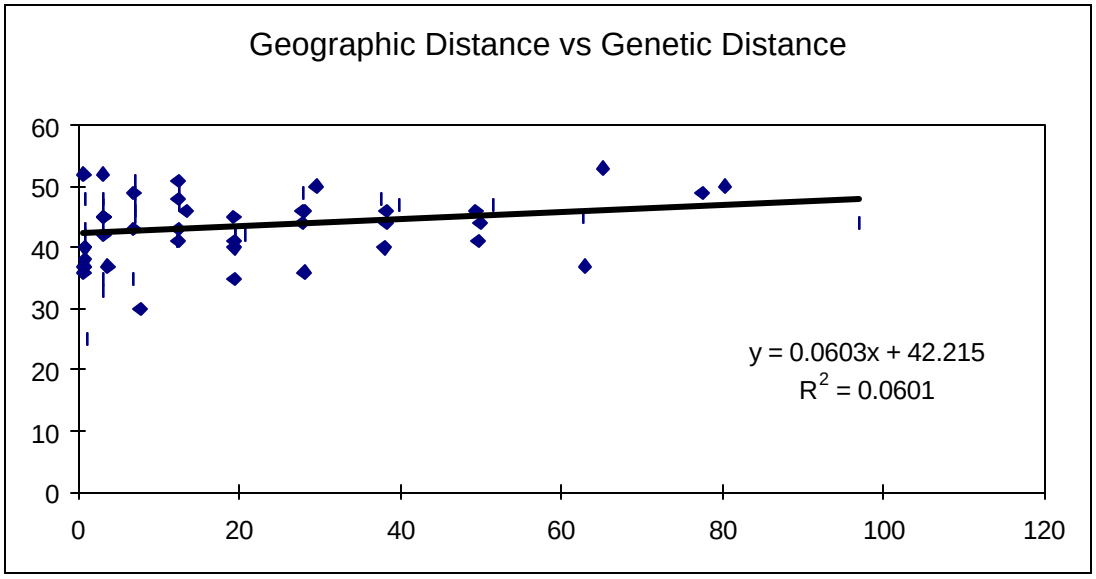


Figure 11. Mantel test of ISSR genetic distance matrix vs. geographic distance matrix for 12 populations of *M. virginica* in Adams County, Ohio.



Appendix. Raw data from ISSR analysis of 12 populations of *Manfreda virginica*.

| | Primer | Primer | Primer |
|---|---|---|---|
| | [---------14799B----------]----------HB15----------[----------HB10---------] | | |
| | 11111111111111111 111111111111111 111111111111111 | | |
| | 77665543332221100099988776665544655543322211100988777666555443354444322221100098888777666654443070525962950749629507391850638517304839506406304607407517407497394164752074730396317539640252050000050005055005500505000055505050505000500500550000500050050055505000500055000050500050000500 | | |
| Sample | | | |
| 1.1 | 00100000000001000100100001001000000000000000100010000100010000000000010000010100000000101001000 | | |
| 1.2 | 00000100001001000100100001001000000000100000001000010001000000001000000000100001000000001000 | | |
| 1.3 | 00000000001001000100000101001000000000000010000010000100010000000000010000010100000000101001000 | | |
| 1.4 | 00000101001001000000100101001010000010000010000010000100010000000010000000010100000000101001000 | | |
| 1.5 | 00100010001001000000100001001000000010000010100000000100010001000010000000101000010001010010000 | | |
| 1.6 | 00000010000001000100100001001000000010000010000010000100010000000000010000010101000000101001000 | | |
| 1.7 | 00010001000001000100100001001000000000000010000000000100010000000000010000010000000101001000 | | |
| 1.8 | 00010000001001000100100001001000000010000010001000000100010001000000000100010100001000101001000 | | |
| 1.9 | 00000100001001000000100001001010000010000010000010000100010000000000010000010100000000101001000 | | |
| 1.10 | 00100100001001000000100001001000000010000010000010000100010000000000010000000100001000101001000 | | |
| 1.11 | 00100000001001000000100101001010000010000000100000000100010001000000010000010100001000101001000 | | |
| 1.12 | 00010001000001000000100001001000000000001000000100100010001000000000000000010100000000101001000 | | |
| 1.13 | 00000010000001000100100001001000000000010000100001000100010000000000100000001010100000010100100 | | |
| 1.14 | 00000100000001000000100001001000000010000010001001000100010000000000000000010100000000101001000 | | |
| 1.15 | 00010010001001000000100001001000000000010000100010000100010000000000000000010100000000100001000 | | |
| 2.1 | 00010001000010010000100010010010000010000000100000000100010100000000000000100100100001001001010 | | |
| 2.2 | 00000001000010010000100010010000000001000001000010001000100000000010000001001000010100100100000 | | |
| 2.3 | 00001000000010010000100010010010000000010000100001000100010010000000000001001000001010010010000 | | |
| 2.4 | 10000010000000010000100010010000000000000001000010001000100000000010000001001000010010010010100 | | |
| 2.5 | 00010000000010010000100010010000010000010000010001000100010100000000010000100100100001001001010 | | |
| 2.6 | 00000011000000000010000010010010000010000000100001000100010000000000100001001001000010010010000 | | |
| 2.7 | 10000001000010010000010010010000000000010000100001000100010000000000000000100100100001001001010 | | |
| 2.8 | 00001001000010010000100010010000000010000000100000000100010010000000010000100100000101001001010 | | |
| 2.9 | 00010001000010010000100010010010000000000000100001000100010000000000000000100100100001001001010 | | |
| 2.10 | 00010001000010000010000010010010000000100000100001000100010000000000010000100100001001001001000 | | |
| 2.11 | 00001000000010010000100010110010000010000000100100000100010010000000010000100100001001001001000 | | |
| 2.12 | 10000000000010010010001000010010000010010000100001000100000010000100000000000100100001001001010 | | |
| 2.13 | 00001001000010000010001000010000000010000000100101000100010000000100000000100100100001001001000 | | |
| 2.14 | 10001000000010010000010000110010000010000000100101000100010000000100100000000100100001010010000 | | |
| 2.15 | 00010010000010010010000010010000010000000000100100000100010010000000000000000100100001001001010 | | |
| 3.1 | 00010000000010000000100010001010000000000001000001001000000000000000000010000100010001001000100 | | |
| 3.2 | 00000001000010000000100010001001100000001001000000001000100000000001000100001000000100010001000 | | |
| 3.3 | 00010000010000000000100010001010000010001001010000001000100001000001000000001000100100010001000 | | |
| 3.4 | 00000000000010000000010010001001000010000100010001001000100001000000000100001000100100010001000 | | |
| 3.5 | 01010000000010000100010010001010000000100000000001001000100100000000000100001001000100010001100 | | |
| 3.6 | 00000100000010000100100010001001000000100000100001001000100001000001000000001001000100010001100 | | |
| 3.7 | 00010001000010000000100010001000000000001000100001001000100000000000000001010010001000100010000 | | |
| 3.8 | 00000100010010000000100010001001100000000001000001000000100000000000000000010001000100100010000 | | |
| 3.9 | 01000100010010000000100010001010000100000100010001001000100000000001000000001001000000010001000 | | |
| 3.10 | 00000100000010000000100010001000000000000010001000100100010000000000100000000100100010001001000 | | |
| 3.11 | 00000001000010000000100010001001000100100000010001001000100101000001000000001001000100010001000 | | |
| 3.12 | 00000100010010000010001001000001010000000010001000100100010000000000000000000100100001000001100 | | |
| 3.13 | 01000001000010000100010010001001000010000100010000001000100100000001000000001010000100000001000 | | |
| 3.14 | 00000100010010000010010001000101000001000010010000100100010100000001000000001010100010000010000 | | |
| 3.15 | 00000100010010000010001001000100100000000010000000100100000101000001000000100010000100000010000 | | |
| 4.1 | 00010001000010001000100010001010000001000001000010100010001010000000001000010001000010000010000 | | |
| 4.2 | 00010001000010000000100010001000000001000001000010100010001000100000010000010000100001000010100 | | |
| 4.3 | 00000100000010001000100010001000100000000001000010000010001000100100001000000100100001000010100 | | |
| 4.4 | 00010000010010001000100010001010100000000100000010100010001000000000000000010000000010000000110 | | |
| 4.5 | 00010000000010001000000010001010100000000010000001010001000100000010000000000100000000010010100 | | |
| 4.6 | 00010000010010001000100010001010000001000100000000100010001010000000010000010010000001000010110 | | |
| 4.7 | 00000101000010001000100010001000001000010100000010000010001000000000001000010100001000010010100 | | |
| 4.8 | 00010001000010000000100010001010000000010001000010100010001000000000000000010000101000100010110 | | |
| 4.9 | 00010000010010000000100010001001100000000001000010100010001010000000001000001010001000010010110 | | |
| 4.10 | 00000100000010000000100010001010001000000000000010100010001000000000001000010100001000010010110 | | |
| 4.11 | 00000101000010001000100010001010001000000001000010100010001000000000001000001001000000101001011 0 | | |
| 4.12 | 00000100000010000000100010001001100000010000010010100010001000000000000000010100000000010010100 | | |
| 4.13 | 00000100000010001000100010001001000001000001010010000010001000100000010000010100000001010010100 | | |
| 4.14 | 00000100010010001000100010001010100000000010000101000100010000000001000001010010000001000010100 | | |
| 4.15 | 00000100010010001000100010001001100000000001000010000010001010100100000000010010000001010010100 | | |

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5.2 0000100010010000000010001000100100010010000010010000101100110010001000000001000000001001000100
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5.4 0001100010010000101000101000100101000100000010010100101100110010000000100000000000000101001000
5.5 0001000000010100010000101010100100010010000010010000101100110010000000000001100100000001001000
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5.13 0001000010010100010000101010100101000010000010010000100100100010100000001000000100000010100101 0
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6.3 0000010000000100001100000100100000000001000001010000100110110010001000000100100000100000100100 0
6.4 0000000010000000000001001000100000100000010001010000100111010001000001000000000100100010100100 0
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6.6 0000001000010000001000001000100000010000000001010000100110110010000010000001000100000010100101 0
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6.10 0000010000010100010001001100101000010000000001010000100111010001000000100001000010000010100100 0
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6.12 0000011000010000000100000100100000010001000001010000100111011001000100010000000010000001010010 0
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9.8 0001000000000010000000001000100100000000010000010000001000001000000010000000010000000100100100 1
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9.10 0001000000000010100000001000100000000010000100001001000100000101000001000000001000000010010010 01

9.11 0001000000000010000001000000100100000000001000010000001001001010000010000000010000000010010010000
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9.15 0001000000000010000001001000100100000000010000000010001000101000000000000100010000000010010010001
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10.3 0000000000000000001000010100100110000000000010010000100000010100000000001000100000000100100100000
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