Genetic Structure of the Common Milkweed, *Asclepias syriaca* L.

By

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Abstract: Chapter 2

- *Premise of the study:* Microsatellite primers were developed for the common milkweed, *Asclepias syriaca*, to assist in genet identification and the analysis of spatial genetic structure.
- *Methods and Results:* Using an enrichment cloning protocol, eight microsatellite loci were isolated and characterized in a North American population of *A. syriaca*. The primers amplified di- and tri-nucleotide repeats with 4-13 alleles per locus.
- *Conclusions:* The primers will be useful for studies of clonality and gene flow in natural populations.

Abstract: Chapter 3

Spatial genetic structure (SGS) is largely determined by the reproductive strategies of species. Many plant species, including *Asclepias syriaca*, the common milkweed, reproduce both sexually and asexually and there can be great variation in SGS among species that reproduce by both methods. SGS was assessed within an old field population of *A. syriaca* in northern Michigan. Strong SGS was detected to 38 m when multiple identical genotypes were included in the analysis; this signal was lost when identical genotypes were removed. This suggests that clonal reproduction in *A. syriaca* has a greater impact on spatial genetic structure than does sexual reproduction over the spatial scale of an old field.

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Chapter 1: Literature Review

Introduction

It remains a fundamental goal of evolutionary biology to understand the causes and consequences of genetic variation within and among populations of organisms. Genetic variation is present in natural populations of all organisms and, most often, the genotype interacts with the surrounding environment to produce a phenotype. For example, plants of identical genotype may produce different phenotypes when grown in different environments (Callaway, Pennings, and Richards, 2003; Aremu, Ariyo, and Adewale, 2007). Such variation is often described in terms of the natural or historic range of variability in environmental conditions such as weather, disturbance events, resource availability, population sizes of competitors, etc (White and Walker, 1997). Since differences among individuals are determined at least partly by genotype, population genetic theory predicts that in variable environments a broader range of genetic variation will persist.

The extent of gene flow among local populations determines their potential for genetic differentiation (Slatkin, 1985). How much gene flow is of evolutionary importance depends on the other mechanisms of genetic change (selection, drift, etc) at work (Slatkin, 1985). The ability of species to respond to selection is dependent upon the presence of heritable variation (Bever and Morton, 1999). If genetic variation is present within a species, any change in selective pressures due to environmental changes will allow some individuals to survive and reproduce at an advantage over others (Darwin, 1859). Additionally, genetic drift can change allele frequencies within small populations from one generation to the next because of the random sampling of gametes (Lande,

1976). As a consequence, human activities that change environmental conditions and/or reduce population sizes will likely influence the genetic architecture of populations (Huyghe, 1998; Robinson et al., 2009). Therefore, as the human population continues to grow, understanding patterns of genetic structure in natural populations is becoming increasingly important. The over-arching goal of my thesis work is to describe and understand the genetic architecture within a single population of a widespread native plant species, *Asclepias syriaca* L. (Apocynaceae). Because *A. syriaca* is widely distributed throughout the US, it may serve as a useful model for changes in distribution and genetic architecture under environmental change. However, before such studies can be undertaken, we need a basic understanding of its population genetics, and that has prompted the work described in this literature review.

There are many ecological factors that can affect the amount of genetic variation that is present within and among populations. Before describing the research that I conducted for my thesis, I discuss several of the more common factors and their implications. Although I could not address all of these factors explicitly in my own work, they provide background and context for the research that I conducted.

Common Factors Affecting Genetic Variation and Genetic Architecture

Pollinator limitation can reduce genetic variation in plant populations (Kirchner et al., 2005). Without suitable pollinators, transfer of genetic material to suitable mates is inhibited; this can also lead to higher instances of self pollination, which reduces genetic variation. Frequent self pollination can lead to inbreeding, which increases homozygosity within populations causing alleles to become fixed (Kirchner et al., 2005). Clonal

reproduction can also reduce genetic variation over time, due to a lack of outcrossing (Stenstrom et al., 2001). Clonal reproduction can also maintain genetic variation over time, since the variants are not lost; they are maintained within the population (Pomper et al., 2003; Sole et al., 2004; Chen et al., 2006). Further implications of clonality and its effects upon genetic diversity will be discussed later in this document.

Biological invasions may cause serious damage to native environments, threaten native biodiversity and change genetic diversity. For example, morphologically indistinguishable – but genetically distinct – cryptic species may be introduced without human recognition of their uniqueness, thereby becoming a ‘‘cryptic invasion’’ (Miura, 2007; Lee and Gelembiuk, 2008). The ecological and evolutionary impacts of cryptic invaders may be largely overlooked because they have the same phenotype as their congeners (Lee and Gelembiuk, 2008). Additionally, invasive species may fragment native populations (below) or disrupt pollination services (above), both of which influence genetic architecture of populations. Molecular comparisons can be used to identify endemic populations, invasive populations, and possible source populations, which can then be applied to improve management plans.

Global environmental change is altering selection regimes for all biota (Reusch and Wood, 2007). Biological consequences of rapid climate change can include range shifts, behavioral changes, altered phenology and local extinctions (Reusch and Wood, 2007). Local adaptation of populations to climate has been demonstrated many times in the past (Owuor et al., 1997; Li et al., 1999). Levels of climate-related genetic variation in natural populations may be high, but are not unlimited in the face of intense directional selection that will result from rapid climate change (Jump and Penuelas, 2005). It is

possible therefore that rapid warming will decrease genetic diversity within populations beyond those loci directly involved in determining the species response to climate (Jump and Penuelas, 2005). Maintenance of genetic diversity within populations is a key conservation aim, as it will enhance their ability to adapt to future environmental changes. Reduction of genetic diversity within populations may significantly reduce the ability of populations to resist and recover from perturbations such as pest and disease outbreaks (Altizer, Harvell, and Friedle, 2003) or extreme weather events (Reusch et al., 2005). Although the consequences of reduced genetic diversity will vary among species and populations, decreased climate-related diversity is likely to reduce the ability of populations to withstand and recover from future climatic perturbations (Jump and Penuelas, 2005). It is likely therefore that in many cases plant adaptation will fail to match the pace or magnitude of predicted changes in climate (Jump and Penuelas, 2005), leading to extinction and territory loss.

Finally, habitat fragmentation amplifies the effect of intense selection by reducing the introduction of genetic novelty by gene flow from neighboring populations. Small fragments may show (i) lower within-population genetic diversity (ii) lower allelic richness or (iii) loss of low-frequency alleles (Pautasso, 2009). These patterns can be expected from the positive relationship between genetic diversity and area (Zhou et al., 2008); as area increases so does the amount of genetic diversity contained within populations.

Choosing Molecular Markers for Studies of Population Genetic Structure

In order to establish the effects that ecological factors have upon genetic diversity, a molecular marker must be chosen. There are many kinds of genetic markers available, each with its own pros and cons. An ideal genetic marker for an ecological genetic study should meet certain criteria for analysis. Weising et al. (1995) propose a list of six characters that should be met for ecological genetic studies.

- There should be direct qualitative or quantitative variation. The marker in question should either be absent or present, or the level of its expression should show discrete variation.
- The genetic marker chosen should show no environmental or developmental influences. For example, if an individual is moved to three separate environments, then it should show the same genotype irrespective of environment. If the marker is found in the juvenile it should be found in the adult as well.
- It should show simple codominant inheritance. In a diploid organism, both alleles at a locus should be visible in the heterozygote condition. Under complete dominance, one allele is expressed and it is impossible to distinguish between the dominant homozygote and the heterozygote condition.
- The marker that you choose should also be able to detect silent nucleotide changes. It should be capable of detecting changes in the coding region of a genome that results in synonymous amino acid substitutions.

- It should be able to detect changes in coding and non-coding regions of the genome. The markers should be randomly distributed across the genome and not restricted to just one class of DNA.
- Finally, the marker should detect evolutionary homologous changes. The markers that are used for genetic analysis should be homologous or similar by descent from a common ancestor.

None of the markers that are currently available meet all of these criteria but each one is useful for certain problems. Based on the criteria above and my research goals (below), I chose to use microsatellite markers. In the past few years microsatellite markers have become some of the most popular molecular markers with applications in many fields, not just ecology. High polymorphism and the relative ease of scoring represent the two major features that make microsatellites appropriate for many genetic studies (Zane, Bergelloni, and Patarnello, 2002). The major drawback of microsatellites is that they need to be isolated *de novo* from species that are being examined for the first time (Zane, Bergelloni, and Patarnello, 2002). Microsatellites are short tandem repeats of mono- to tetra-nucleotide repeats which are assumed to be randomly distributed throughout the nDNA, cpDNA, and mtDNA (Zane, Bergelloni, and Patarnello, 2002). Microsatellites exhibit length variation that results from changes in the number of repeat units, to which stepwise mutation models can be applied (Schlotterer and Tautz, 1992). Microsatellites have proven to be an extremely valuable tool for genome mapping in many organisms. Additionally, they are applied in projects ranging from ancient and forensic DNA studies, to population genetics and conservation/management of biological resources (Jarne and Lagoda, 1996; Zane, Bergelloni, and Patarnello, 2002).

Analysis of Fine Scale Spatial Genetic Structure and Plant Reproduction

Here, the intended application of microsatellite markers is for use in the analysis of fine scale spatial genetic structure (SGS) in a plant that reproduces sexually and vegetatively. SGS is defined as the non random spatial distribution of individuals with respect to relatedness of allele frequencies (Frantz, Hamann, and Klein, 2008). The genetic structure of natural populations is largely mediated by their reproductive strategies. Measurements of SGS are used to study effects of various ecological factors on populations. Limited seed dispersal within populations is one example of an ecological factor that leads to spatial clustering of full- and half-sibs, a type of SGS (Shimono et al., 2006). Depending on the extent of seed shadow overlap within populations, a high density of reproducing adults is just one more ecological factor that influences the SGS of future generations, (Knowles, Perry, and Foster, 1992).

Isolation by distance models (Wright, 1943) can be used to interpret fine scale SGS within populations (Vekemans and Hardy, 2004). The observed level of SGS within populations depends on the degree of overlap between gene shadows (i.e. the spatial distribution of seed- and pollen-mediated gene dispersal events around each parent) of adjacent individuals (Hardy et al., 2006). The lower the overlap, the greater the probability that nearby offspring are sibs; SGS therefore builds in successive generations until an equilibrium SGS is reached (Hardy et al., 2006).

While effects of sexual reproduction on SGS are commonly studied in flowering plants, effects of vegetative reproduction (cloning) are often overlooked. Cloning most often refers simply to vegetative reproduction of individuals (Cook, 1979). Most plants

reproduce vegetatively to some extent, and some species reproduce almost exclusively by vegetative means, such as the aspen, *Populus tremuloides (Barnes, 1975a, b)*. Each individual within a clone is called a ramet, and when a ramet is genetically identical to the surrounding ramets, the system is classified as a genet (Cook, 1979). In non-clonal plants, individuals are likely to experience the same microenvironments over their lifetime because individuals don't move significantly after seed germination. In contrast, clonal plants possess dispersal ability by means of vegetative growth (Skalova et al., 1997). Under vegetative growth, individual genets can change their spatial position and therefore environment over time.

The plant species studied here, *Asclepias syriaca*, reproduces both asexually and sexually. It is self incompatible (Morse and Fritz, 1983) and, during sexual reproduction, pollen grains are packaged in discrete units called pollinia, which contain enough pollen to ensure full seed-set of a single flower (Ivey, Martinez, and Wyatt, 2003). The wind dispersed seeds are attached to long, white flossy hairs and encased in large follicles. Asexual reproduction occurs by the elaboration of underground rhizomes. Accordingly, sexual reproduction gives rise to new genets whereas asexual reproduction can produce multiple ramets per genet. The reproductive habits of *A. syriaca* will be discussed in further detail later in this document.

One might expect the SGS of clonal plants to be higher than that of non-clonal plants, because nearby individuals are more likely to have identical genotypes. It is widely accepted that populations dominated by clonal reproduction are genotypically almost as diverse as fully sexually reproducing populations (Araki, Shimatani, and Ohara, 2007). Clonal reproduction can retard the loss of genetic diversity within populations by

reducing the probability of genet death (Chung and Epperson, 1999). Clonal growth is advantageous in establishing offspring without pollination, whereas sexual reproduction with pollen and seed dispersal helps to maintain genetic diversity in plant populations (Araki, Shimatani, and Ohara, 2007). For long lived clonal plants, a small number of seedling recruits each year is sufficient to maintain clonal diversity in populations (Araki, Shimatani, and Ohara, 2007).

Clonal organisms can change their size and shape as they grow, and the details and nature of their breeding systems are not static, but change in association with the growth of the clone (Handel, 1985). White clover, *Trifolium repens* (Fabaceae), like the common milkweed is a stoloniferous, self incompatible plant that is common in pastures. As the size of a clone of white clover increases, it becomes more limited by restricted pollen flow, which leads to a higher level of SGS (Handel, 1985). In some clonal species, there is an additional relationship between clonal spread and pollination biology when the ratio of vegetative growth to sexual reproduction is affected by increasing density of shoots in the population. In some cases, a greater allocation to asexual, clonal growth occurs at lower densities and relatively fewer flowers are produced (Handel, 1985). By favoring vegetative growth as opposed to sexual reproduction, the species will have lower genetic variation than those species that do not exhibit clonal reproduction.

In the case of *Aralia hispida*, another self incompatible species, large clones are present but with synchronized cycles of protandry within the clone, a condition to promote outbreeeding in which the anthers release the pollen before the stigma is receptive (Barrett and Thomson, 1982). In this way, *A. hispida* shows developmental escape from the increased inbreeding associated with clonality. Barrett and Thompson

(1982) suggest that in species whose demography includes the occurrence of very large clones, there may be selection for dioecy. Clonality may in this way act as a selective force for a change in breeding system (Handel, 1985).

Clonal structure influences patterns of pollen dispersal and mating opportunities of individual plants and has an impact on reproductive success. In outcrossing insect pollinated plants, large clone size and multiplication of flowering shoots from clonal growth may enhance geitonogamy (pollination of a flower with the pollen from another flower on the same flowering plant) (Araki, Shimatani, and Ohara, 2007). In a large clone, frequency of pollen deposit within the same clone is higher than that for smaller clones (Araki, Shimatani, and Ohara, 2007). Thus, high flowering density should attract pollinators. However, reproductive success should be affected by fine scale spatial population structure, because the amount of compatible pollen depends on clonal structure as well as ramet distribution and density (Araki, Shimatani, and Ohara, 2007). This impact also depends on the magnitude of pollen dispersal, which is affected by the distance pollinators travel between successive flowers (Araki, Shimatani, and Ohara, 2007). Therefore, self incompatible species rather than self compatible species are exposed to the risks of a deficiency in compatible pollen (Araki, Shimatani, and Ohara, 2007).

New statistics are also being developed for analysis of SGS and the impacts of clonality as well. Genetic resolution, defined as the ability to resolve clonal ramets, is a function of the number of polymorphic loci and population level genetic diversity at each locus (Gonzales et al., 2006). Genet richness can be described as the number of genets divided by the number of samples in each plot and genet diversity is a complement to

Simpson's index (Simpson, 1949), but instead is corrected for finite sample size (Gonzales et al., 2006).

The majority of *Populus tremuloides* clones found in the intermountain west are thought to represent ancient genets that have potentially persisted since the last glacial maximum (Barnes, 1975a) and since that time have likely experienced limited sexual reproduction. In time, we might expect the following two patterns. First, genets that have achieved a large spatial extent at some point during their existence will have undergone fragmentation as a result of small-scale disturbance and encroachment of other clones and vegetation types. Second, genets will have accumulated many mutations, particularly at rapidly evolving neutral loci, such as microsatellite regions (Mock et al., 2008). These mutations should result in a strong spatial clustering of mutational variants arising from the same parent genet, a pronounced deviation from Hardy–Weinberg expectations in local populations, genotypic disequilibrium among loci, and pronounced differences in allele frequencies between regions that would otherwise be homogenized by gene flow via wind dispersed pollen and seeds (Mock et al., 2008).

Microsatellites are commonly used in studies of SGS. Since their description by Karp et al. (1998) as a useful tool for measurements of SGS, they now comprise the majority of markers used in these studies (Arnaud-Haond et al., 2007). These markers are useful in discriminating between individuals and populations, which makes them ideal for use with SGS. Microsatellites are also common for use in clonal species, as they can be used to identify which ramets belong to which genet (Suvanto and Latva-Karjanmaa, 2005). The highly variable microsatellites are often used in conjunction with amplified fragment length polymorphism (AFLP) to recognize clones more precisely. This

technique of combining microsatellite with multi-locus AFLP technique has been used to successfully detect clones in natural populations of black poplar (*Populus nigra* L.) (Arens et al., 1998) and eelgrass (*Zostera marina* L.) (Reusch et al., 1999). Microsatellites can also detect clonal structure that is more than 1000 years old (Reusch et al., 1999) which is useful for long lived tree species.

System of Study

The goal of the current study is to assess fine scale SGS of the common milkweed, *Asclepias syriaca* L. In most *Asclepias* species, stems produce many paired umbels, which contain numerous flowers that are typically pollinated by wasps and bees. Most flowers on an umbel open simultaneously, but different umbels on a plant may open over a period of four weeks or longer (Himes and Wyatt, 2005). The inflorescences typically bloom in sequence up the stem. The flowers consist of five showy, reflexed petals that cover the small green sepals. Two free and superior ovaries are joined by their styles to form a gynostegium with five lateral stigmatic surfaces. These surfaces are enclosed by the tightly adjoining wings of adjacent anthers to produce five stigmatic chambers. Coronal extensions from the base of the stamen (hoods), each of which may contain an arching structure of varying length (horns), retain nectar secreted by nectaries located inside the stigmatic chambers (Wyatt, 1978).

Asclepias plants are pollinated by a wide variety of pollinators. Flowers of some species secrete nectar continually, over much of the day and night (Morse and Fritz, 1983). As a result they attract both diurnal and nocturnal pollinators. The most abundant pollinators are bumble bees, honey bees, yellow jackets and various butterflies (Morse

and Fritz, 1983), which make up the majority of diurnal visitors. Moths, primarily noctuids and geometrids make up the majority of nocturnal visitors (Morse and Fritz, 1983). Moths transfer pollen to stigmas more effectively and transport it further than do diurnal visitors (Gimenez-Benavides et al., 2007). Only insects large enough to remove and carry pollinaria (below) are capable of effective pollination (Ivey, Martinez, and Wyatt, 2003). Nectar secretion is substantial in both the daytime and the nighttime, for some species production is higher during the day (Morse and Fritz, 1983) while in others, such as *Asclepias syriaca,* it is higher at night (Jennersten and Morse, 1991).

Milkweed pollen grains are dispersed within discrete, aggregated packages, called pollinia (Ivey, Martinez, and Wyatt, 2003). Only milkweed and orchids share this trait which makes them unique among angiosperms There are five adjacent pollinaria per flower, each consisting of pollinia (paired pollen sacs) from adjacent anthers joined by translator arms to a corpusculum located just above the stigmatic chamber (Wyatt, 1978). A pollinium is a tiny flat, wing-like body, narrow at its apex, close to the translator arms, and wide at the base. The pollinium is of a waxy consistency, protected on the outside by a thick cuticularized membrane (Galil and Zeroni, 1969). A single pollinium contains enough pollen grains to ensure full seed-set of a single flower (Ivey, Martinez, and Wyatt, 2003). The number of pollinia removed is higher than the number of pollinia inserted in all cases except for hybrid species (Stevens, 1945). Each milkweed flower has only five effective sets of pollinia.

Milkweed pollination can be considered in a two-stage process. The first step in fertilization is the removal of a pollinarium, which occurs when a groove in the corpusculum catches on a bristle or other appendage of an insect and is pulled from the

flower. The second step includes insertion, which is successful when a pollinium lodges in a stigmatic chamber (Jennersten and Morse, 1991; Wyatt and Broyles, 1994; Kephart and Theiss, 2004). Three adjacent stigmatic chambers transmit pollen tubes to one of the two separate ovaries, whereas the other two chambers transmit to the second ovary (Wyatt and Broyles, 1994). Each milkweed flower contains two ovaries, but in nature two follicles rarely develop from one flower (Himes and Wyatt, 2005). Pollination of two adjacent stigmatic chambers is necessary for pod formation (Moore, 1946).

Fruit set is typically very low in milkweeds, usually around 1% of all pollinations result in mature fruit for almost all species (Wyatt, 1976). Hand pollination typically increase the fruit set but generally only to 15-20%, which is well above field levels (Wyatt et al., 1998). It is generally thought of as a combination between mechanical and physiological factors that interact together to produce the low fruits set seen in *Asclepias* (Wyatt, 1976). The physiological factors are usually postulated to operate through some form of competitive interaction among fertilized ovules (Wyatt, 1976). It also appears likely that failure of the pollen tubes to penetrate the ovary and effect fertilization, and similar failures at various stages of development are responsible for the physiological contribution to low fruit set (Wyatt, 1976).

Milkweeds generally fall into two reproductive types; species that are largely self compatible and species that are largely self incompatible. Species that are entirely or largely self incompatible include: *A. exaltata* (Lipow and Wyatt, 2000), *A. perennis* (Wyatt et al., 1998), *A. subulata* (Wyatt, Ivey, and Lipow, 1996), *A. syriaca* (Morse and Fritz, 1983), *A. texana* (Wyatt et al., 1998), *A. tuberosa* (Wyatt, 1976), and *A. verticillata* (Wyatt and Broyles, 1994). In four of these species, ovarian rejection of self-pollen has

been demonstrated, including *A. syriaca* (Lipow and Wyatt, 2000). Several studies of self-pollinated flowers of *A. syriaca* and *A. exaltata* show that male gametes are released into the female gametophyte and that initial development of endosperm occurs (Lipow and Wyatt, 2000). After this occurs, the selfed ovules consistently fail; selfed zygotes do not undergo mitosis, and the endosperm stops growing (Lipow and Wyatt, 2000). Self incompatibility in *A. exaltata* is caused by a single gene, the S-gene and incompatibility occurs when two plants share one or more alleles of this gene (Lipow and Wyatt, 2000). It appears that self incompatibility is a highly variable trait as seen in *A. exaltata*. Although most individuals of *A. exaltata* express a functional genetic self-incompatibility system, remarkably high variation in self-fertility exists within and among populations (Lipow, Broyles, and Wyatt, 1999). Self incompatibility also appears to be a shared trait in closely related taxa including, *Apocynum cannabinum, Gonolobus suberosus,* and *Periploca aphylla* (Lipow and Wyatt, 1999).

Species that are entirely or largely self compatible include *A. curassavica* (Wyatt and Broyles, 1997), *A. fruticosa* (Wyatt and Broyles, 1997), and *A. incarnata* (Wyatt and Broyles, 1994). *A. curassavica* and *A. fruticosa* have been shown to be entirely self-compatible; fruit set percentages from self pollination are as high as with cross pollination (Wyatt and Broyles, 1997). Likewise, seed viability from self- and cross-pollinations are equally high and comparable (Wyatt and Broyles, 1997). Self-pollinated fruits generally contain fewer seeds than cross-pollinated fruits, but germination percentages are 100 percent for both of crosses (Wyatt and Broyles, 1997).

The genus *Asclepias* is distributed amongst three centers of dispersal: temperate to tropical North America, subtropical South America, and southern and eastern Africa

(Woodson Jr, 1954). Of these, the North American and the African are the largest, including over 100 species a piece. *Asclepias* L. probably arrived in the new world through the Bering Strait at 20 Ma; dispersing to South America before the emergence of the Isthmus of Panama (Rapini, van den Berg, and Liede-Schumann, 2007). The North American species of *Asclepias* are adapted to a wide range of environments. Since they are essentially subtropical plants, their altitudinal and latitudinal preferences are somewhat restricted; few extend to elevations over 2000 meters and only a few cross the southern borders into Canada (Woodson Jr, 1954). The individual species are narrow in their ecologic preferences, and only *A. syriaca, A. curassavica,* and *A. fasciculata* can be classified as weeds (Woodson Jr, 1954). Most species prefer open dry woods, glades, barrens and plains. Several species can be found in the deserts of the southwestern United States and adjacent into Mexico. A few species including, *A. perennis* and *A. incarnata* are subaquatics, and only *A. i. pulchra* is known for its tolerance of brackish water (Woodson Jr, 1954; Ivey, Lipow, and Wyatt, 1999).

The widespread distribution of *A. syriaca* makes it an ideal study plant. It can be found throughout the Great Plains from southern Canada south to NE Oklahoma, NW Georgia, and Texas, and east from North Carolina to Maine. Its range continues to move south and specimens have now been collected from Georgia and Louisiana (Wyatt et al., 1993; Wyatt, 1996). Its large distribution makes it ideal for comparisons across large spatial regions.

Plant species that have a wide geographical range tend to have higher levels of genetic variation than species with a narrow distribution (Stenstrom et al., 2001). Genetic differences between northern and southern population should also be noticeable through

genetic analysis. Northern populations may be less genetically diverse than southern populations due to repeated founder effects (Cwynar and Macdonald, 1987). One would expect higher levels of genetic diversity within *A. syriaca* than within other *Asclepias* species due to its extensive distribution but there would be declining genetic variation in samples moving northward.

Individual genets of *A. syriaca* can be hard to distinguish from one another in large field populations. Previous techniques have included digging up the root systems to determine which ramets belonged to a given genet. The use of microsatellite markers allows genotyping in a much less invasive way. By being able to distinguish individual genotypes in this manner it will be easier to establish future experiments based on inheritance and clonal habit. Ten polymorphic loci have already been isolated and characterized from *A. syriaca*. These loci have also been successfully cross-amplified on *A. exaltata* (O'Quinn and Fishbein, 2008). Isolation of additional microsatellites from *A. syriaca* may be useful in other milkweed species and assist in conservation and management plans of rare and endangered species.

Another trait common within the genus *Asclepias* is the production of cardenolides, bitter tasting steroids that occur in all tissues, including the latex. Cardenolides act by disrupting the sodium and potassium flux in cells and have toxic effects on most animals (Malcolm, 1991). Preliminary data has shown that cardenolide concentrations vary by genotype (Hunter *unpublished data*). The development of microsatellite markers would allow us to analyze genotypes and cardenolide concentrations in a more efficient way, and examine more critically the genetic contributions to chemical phenotype in field populations. This will only be possible if the

microsatellite loci are linked to the genes that are responsible for cardenolide production; otherwise the genotypes will change each generation and not necessarily be associated with cardenolides.

The herbivore community that feeds upon *Asclepias* consists of species that are primarily host specific. The herbivores fill almost every available feeding guild: aphids feed on phloem, beetles and caterpillars chew the leaves, flies mine the leaves, hemipterans eat the seeds, weevils bore through the stem and eat the pith and beetle larvae bore through the roots (Agrawal and Fishbein, 2006). *Asclepias syriaca* has 12 reported insect herbivores including members of all the above guilds. Many can sequester the cardenolides that are found in the tissues they consume. There are several other traits of milkweed that are considered as defensive attributes; these include leaf toughness, trichrome density, water content, nitrogen content and specific leaf area (Mattson, 1980; Coley, 1983; Haddad and Hicks, 2000; Lavoie and Oberhauser, 2004) and analysis of SGS in *A. syriaca* will aid in determining the role of genetic variation in their expression.

Using this study system, I address the following questions: 1. Can ramets be assigned to genets efficiently using microsatellite markers? 2. At what scale does genetic architecture occur within populations? In Chapter 2, I focus on the development of microsatellite markers for the common milkweed, *Asclepias syriaca*. In Chapter 3, I use microsatellites to analyze the spatial genetic structure of an old field in northern Michigan. In the final chapter, I propose further research and topics that will need to be addressed in the future.

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Chapter 2:
Isolation and characterization of microsatellite loci for Common Milkweed, *Asclepias syriaca* L. (Apocynaceae) [1]

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ABSTRACT

- *Premise of the study:* Microsatellite primers were developed for the common milkweed, *Asclepias syriaca*, to assist in genet identification and the analysis of spatial genetic structure.
- *Methods and Results:* Using an enrichment cloning protocol, eight microsatellite loci were isolated and characterized in a North American population of *A. syriaca*. The primers amplified di- and tri-nucleotide repeats with 4-13 alleles per locus.
- *Conclusions:* The primers will be useful for studies of clonality and gene flow in natural populations.

Key words: Apocynaceae; *Asclepias syriaca*; microsatellite; milkweed.

INTRODUCTION

Common Milkweed, *Asclepias syriaca* L. (Apocynaceae), is one of the most common and widely distributed of approximately 100 North American milkweed species. *Asclepias syriaca* can be found throughout the Great Plains from southern Canada south to NE Oklahoma, NW Georgia, and Texas, and east from North Carolina to Maine. Its range continues to move south; in the last two decades specimens have been collected from Georgia and Louisiana (Wyatt et al., 1993; Wyatt, 1996), and it has become naturalized in the Western U.S. and invasive in parts of Europe. Although *A. syriaca* produces milky latex with toxic steroid glycosides, it hosts several specialist insect herbivores, including the monarch butterfly (*Danaus plexippus*), the milkweed beetle (*Tetraopes tetraophtalmus*), large milkweed bug (*Oncopeltus fasciatus*), small milkweed bug (*Lygaeus kalmii*) and milkweed leaf beetle (*Labidomera clivicollis*). Its broad geographic range and specialized ecological interactions makes *A. syriaca* an ideal species with which to examine geographic patterns of coevolution.

Asclepias syriaca reproduces both asexually and sexually. It is self incompatible (Morse and Fritz, 1983), and, during sexual reproduction, pollen grains are packaged in discrete units called pollinia, which contain enough pollen to ensure full seed-set of a single flower (Ivey, Martinez, and Wyatt, 2003). The wind-dispersed seeds are attached to long, white flossy hairs and encased in large follicles. Asexual reproduction occurs by the elaboration of underground rhizomes. Accordingly, sexual reproduction gives rise to new genets whereas asexual reproduction can produce multiple ramets per genet. Assigning ramets to genets is challenging under field conditions, and microsatellite markers have been used to differentiate among genets in a variety of systems [e.g. quaking aspen (*Populus tremuloides*) (Namroud et al., 2005)]. Using molecular

markers to distinguish among genets of *A. syriaca* would facilitate studies of its ecology and evolutionary biology (Helms, Connelly, and Hunter, 2004; Van Zandt and Agrawal, 2004). Previous microsatellite markers have been isolated from *A. syriaca* (O'Quinn and Fishbein, 2008) and here we describe additional markers that will increase resolution of clonality and genetic structure in natural populations.

METHODS AND RESULTS

DNA was extracted (DNeasy Plant Kit, Qiagen Corporation, Valencia, CA USA) from one *A. syriaca* ramet collected at the University of Michigan Biological Station (UMBS), Pellston, Michigan (45.55 N, -84.67 E). DNA was enriched twice for simple sequence repeats using the Oligomix 2 mixture of repeat units [$(AG)_{12}$, $(TG)_{12}$, $(AAC)_6$, $(AAG)_8$, $(AAT)_{12}$, $(ACT)_{12}$, $(ATC)_8$] and protocol of Glenn and Schable (2005). Polymerase chain reaction (PCR) products were ligated to a plasmid vector using the TOPO TA Cloning Kit (Invitrogen Corporation, Carlsbad, CA USA). Plasmid inserts were amplified and sequenced (BI Model 3730 Sequencer). Thirty-four of the 105 sequenced clones (32%) contained microsatellites. Primers were designed using the software OligoCalc (Kibbe, 2007). Polymorphism was screened in thirty *A. syriaca* genets from the University of Michigan Biological Station for twelve loci.

PCR was carried out in a volume of 10 µL containing ~30 ng of template DNA, 2 µL 10X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 U *Taq* polymerase and 0.2 µm of each primer. Hot Start *Taq* Polymerase (Qiagen, Valencia, CA USA) was used for loci AS94 and ASF2, whereas GoTaq (Promega Corporation, Madison, WI USA) was used for all other loci. PCR for ASC5 and ASG5 included 25 µg⁄mL of BSA. The thermal cycle began with a 4-min denaturation step at 94°C, followed by 45 cycles of 30 s at 94°C, 30 s at 52°C, and 60 s at 72°C,

and a final extension at 72°C for 10 min. A 15-min denaturation step was used for loci AS94 and ASF2.

Forward or reverse primers were end-labeled with FAM, HEX or TAMRA. Eight of the 12 loci were found to be polymorphic and generated consistent and easily scored amplification products of the expected size (Table 1). Amplified products were genotyped on an ABI 3730 Sequencer and analyzed using GeneMarker v 1.8 (SoftGenetics LLC, State College, PA USA).

GenAlEx v 6.2 (Peakall and Smouse, 2006) was used to calculate the mean number of alleles observed (A), observed heterozygosity (H_o) (Table 2) and Probability of Identity (PI) for each locus. Linkage disequilibrium (LD) and deviations from Hardy-Weinberg (HW) were assessed in GenePop v 4 (Raymond and Rousset, 1995) using the Markov chain method with 1000 dememorizations, 100 batches and 1000 iterations per batch.

The loci contained 3 to 13 alleles in a sample of 30 individuals from the UMBS, with observed heterozygosity ranging from 0.33 to 0.83 (Table 2). Three loci (ASG6, ASB5, ASG5) showed significant excess of heterozygotes. Two loci (ASH8 and AS94) showed a significant excess of homozygotes. Significant LD was detected between loci ASF9 and ASH8. Probability of identity (PI) using all eight markers was 1.5E-7. An assessment of clonality was run incorporating GPS coordinates (Trimble GeoExplorer 3 QuickStart, Trimble Navigation Limited, Sunnyvale, CA) obtained at the time of collection using GenAlEx v 6.2 (Peakall and Smouse, 2006) which showed no multilocus clones within this data set.

CONCLUSION

The excesses in heterozygosity in three loci may reflect biological properties particular to the UMBS population, such as the level of clonality, while the excess homozygosity in two loci may be caused by null alleles. Even though *A. syriaca* can reproduce asexually there is no indication that the samples used in this study came from repeated genets. However due to the relative isolation from other populations of *A. syriaca,* it is likely that the population is inbred which could cause the homozygote excess, although inbreeding should cause homozygote excess in all the loci studied. The high probability of identity indicates that these markers will provide clear resolution of genet and ramet structure in the UMBS and other natural populations of *A. syriaca*. These markers will also be useful in assessing population genetic structure and gene flow at local and regional spatial scales.

Table 1. Characteristics of 8 microsatellite primers developed in *Asclepias syriaca*. Shown for each primer pair are the forward and reverse sequences, repeat type in the cloned fragment, size range (bp) in a sample of 30 individuals, annealing temperature (T_a) and the GenBank accession number.

Locus	Sequence	Repeat Motif	Size Range (bp)	T_a	GenBank Acc No.
ASC5	F: TTGGAAGCTCAATTCTATACT R: CAAAGATGTAGAGGGTAAGTC	$(GAT)_{20}$	102-121	52	HM004507
ASF2	F: TGAACAAGATCCTGCGAATG R: TCATTAGCAACAAAGGTATCC	$(AGA)_{10}$	87-118	52	HM004509
ASF9	F: CACAGAAACAAGGTGAAATG R: TACTTTGCTTAATCAGCTCC	$(AAG)_9$	107-125	52	HM004508
ASH8	F: AAATCGCATACAGTGGAAAG R: GACTACTTTCGCTAAATCAG	$(AAG)_{11}$	157-171	52	HM004502
ASG6	F: CTATGCAAACTCCTCATGAT R: GAAGGCTGTTTCAGATCTTG	$(TGG)_9$	171-205	52	HM004506
AS94	F: TTCTTCGAGTAGGTAGGAATG R: CACCCCTACAAACAATCCT	$(AAG)_{19}$	139-171	52	HM004505
ASB5	F: CCATGAAATTAGCTCAAGATC R: CAAAGTCCGATTCGGGTAA	$(GAA)_{11}$	187-193	52	HM004504
ASG5	F: CTGACAGAATCACTGCTC R: CTTTATATCGCTGACATTACT	$(TTC)_{11}$	157-176	52	HM004503

Table 2. Results of initial primer screening in a single population of *Asclepias syriaca*. Shown for each locus are the number of alleles (A), observed heterozygosity (H_o) and expected heterozygosity (He) in a population of 30 putative genets. Deviations from Hardy-Weinberg * P<0.05, ** P<0.01, ***P<0.001

Locus	A	H_o	H_e
ASC5	4	0.70	0.55
ASF2	9	0.77	0.74
ASF9	8	0.77	0.62
ASH8	7	0.33*	0.49
ASG6	12	0.83***	0.82
AS94	13	0.53***	0.75
ASB5	3	0.80***	0.50
ASG5	11	0.83*	0.78

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Chapter 3:
Spatial genetic structure of the common milkweed, *Asclepias syriaca* L., in an old field in northern Michigan.

Abstract

Spatial genetic structure (SGS) is largely determined by the reproductive strategies of species. Many plant species, including *Asclepias syriaca*, the common milkweed, reproduce both sexually and asexually and there can be great variation in SGS among species that reproduce by both methods. SGS was assessed within an old field population of *A. syriaca* in northern Michigan. Strong SGS was detected to 38 m when multiple identical genotypes were included in the analysis; this signal was lost when identical genotypes were removed. This suggests that clonal reproduction in *A. syriaca* has a greater impact on spatial genetic structure than does sexual reproduction over the spatial scale of an old field.

Introduction

It remains a fundamental goal of evolutionary biology to understand the causes and consequences of genetic variation within and among populations of organisms. Genetic variation is present in natural populations of all organisms and, most often, the genotype interacts with the surrounding environment to produce a phenotype. For example, plants of identical genotype may produce different phenotypes when grown in different environments (Callaway, Pennings, and Richards, 2003; Aremu, Ariyo, and Adewale, 2007). Since differences among individuals are determined at least partly by genotype, population genetic theory predicts that in variable environments a broader range of genetic variation will persist.

Spatial genetic structure (SGS) is defined as the non random spatial distribution of genotypes as estimated by similarities in allele frequencies (Frantz, Hamann, and Klein, 2008). The genetic structure of natural populations is mediated by their reproductive strategies, such as the mating system, pollen and seed dispersal distances, and natural selection. While effects of sexual reproduction on SGS are commonly studied in flowering plants, effects of vegetative reproduction (cloning) are often overlooked. Cloning refers simply to vegetative reproduction of

individuals (Cook, 1979). Most plants reproduce vegetatively to some extent, and some "populations" reproduce almost exclusively by vegetative means, such as in the aspen, *Populus tremuloides* (Barnes, 1975). Each individual within a clone is called a ramet, and when a ramet is genetically identical to the surrounding ramets, the system is classified as a genet (Cook, 1979). In non-clonal plants, individuals are likely to experience the same microenvironments over their lifetime because individuals do not move significantly after seed germination. In contrast, clonal plants possess dispersal ability by means of vegetative growth (Skalova et al., 1997). Under vegetative growth, individual genets can change their spatial position and therefore environment over time.

The plant species studied here, the common milkweed, *Asclepias syriaca* L. (Aponcynaceae), reproduces both asexually and sexually. It is self incompatible (Morse and Fritz, 1983) and, during sexual reproduction, pollen grains are packaged in discrete units called pollinia, which contain enough pollen to ensure full seed-set of a single flower (Ivey, Martinez, and Wyatt, 2003). The wind dispersed seeds are attached to long, white flossy hairs and encased in large follicles. Asexual reproduction occurs by the elaboration of underground rhizomes. Accordingly, sexual reproduction gives rise to new genets whereas asexual reproduction can produce multiple ramets per genet.

Asclepias syriaca is one of the most common and widely distributed of approximately 75 North American milkweed species. *Asclepias syriaca* can be found throughout the Great Plains from southern Canada south to NE Oklahoma, NW Georgia, and Texas, and east from North Carolina to Maine. Its range continues to move south; in the last two decades specimens have been collected from Georgia and Louisiana (Wyatt et al., 1993; Broyles, Schnabel, and Wyatt, 1994; Wyatt, 1996; Wyatt, Ivey, and Lipow, 1996), and it has become naturalized in the Western

U.S. and invasive in parts of Europe. Although *A. syriaca* produces milky latex and toxic steroid glycosides, it hosts several specialist insect herbivores, including the monarch butterfly (*Danaus plexippus*), the milkweed beetle (*Tetraopes tetraophtalmus*), large milkweed bug (*Oncopeltus fasciatus*), small milkweed bug (*Lygaeus kalmii*) and milkweed leaf beetle (*Labidomera clivicollis*). Its broad geographic range and specialized ecological interactions makes *A. syriaca* an ideal species with which to examine geographic patterns of coevolution. In this study we attempt to assess the spatial genetic structure of *A. syriaca* in an old field in Northern Michigan.

Materials and Methods

Study site and sampling for genotyping- Collections of *A. syriaca* were conducted on June 27 2007 in an old field at the University of Michigan Biological Station (UMBS), Pellston, MI (45.55 N, 84.67 E). Two perpendicular transects were established in order to maximize collections for spatial genetic analysis. Transect 1 ran along a North/South gradient for 80 m and transect 2 ran along a West/East gradient for 135 m (Figure 1). One leaf per ramet of up to ten ramets per sampling site were collected every five meters along each transect. Samples were collected at random at each distance location. All collections were made within one meter of the appropriate distance class. Leaf material was immediately placed on ice and then stored in a -10°C freezer until DNA could be extracted.

Microsatellite genotyping- After DNA was extracted (DNeasy Plant Kit, Qaigen Corporation, Valencia, CA USA), microsatellite loci ASH8, ASF9, ASF2, and ASG6 were amplified using the protocol of Kabat et al (in press). Microsatellite loci C109 and B121 were amplified using the protocol of O'Quinn and Fishbein (2008). Forward or reverse primers were

end-labeled with FAM, HEX or TAMRA. Amplified products were genotyped on an ABI 3730 Sequencer and analyzed using GeneMarker v 1.8 (SoftGenetics LLC, State College, PA USA).

Data analysis- Not every 5m collection site on our transects hosted a ramet of *A. syriaca.* Transect 1 yielded 8 occupied sites and a total of 55 samples and transect 2 yielded 21 occupied sites and a total of 138 samples for subsequent analyses. Genotypic richness was estimated according to the equation $R=(G-1)/(N-1)$, where G is the number of distinct genotypes and N is the sample size. The number of distinct genotypes, which provide a genetic measure of the degree of clonality, was estimated in GenAlEx v 6.2 (Peakall and Smouse, 2006). Expected and observed heterozygosity (H_e and H_o) were calculated using GenAlEx v 6.2 and assessed with and without repeated genotypes.

Spatial autocorrelation- SGS was assessed using the kinship coefficient r with GenAlEx v 6.2 (Peakall and Smouse, 2006). Genet level analysis was first performed using all the ramets for a given genet with their corresponding coordinates. Additionally, genet level analysis was performed with a single ramet per genet (and its corresponding coordinates). The ramet was chosen so that each site that was included in the first analysis still had a ramet in the second analysis. Average kinship for each distance class was plotted against mean spatial distance for each class.

Spatial autocorrelation was also analyzed using the kinship coefficient F_{ij} of Loiselle et al. (1995) using the software SPAGEDI (Hardy and Vekemans, 2002). Average kinship coefficients were estimated using even distances classes of 5 m, with 28 total distance classes for the analyses with and without repeated genotypes. To further quantify the level of SGS, I calculated the Sp statistic as $b/(1-F_{(1)})$, where b is the regression slope of F_{ij} on the natural

logarithm of the spatial distance and $F_{(1)}$ is the mean F_{ij} between individuals belonging to the first distance interval (Hardy and Vekemans, 2002).

Results

Clonal diversity and structure- On our transects, we detected a total of 23 clones ranging in size from 2 ramets to 50 ramets. Median clone size was 1 ramet per clone with clone size ranging from >1 m^2 to 30 m^2. 48 ramets were not genetically matched with any other ramets along the transects for a grand total of 71 clones. The frequency distribution of clone sizes is illustrated in Figure 2. Figures 3 and 4 represent clonal identity and distribution of ramets from transects 1 and 2. The distribution of ramets belonging to each genet was skewed towards small genets with 68% having only one ramet. Genet richness, calculated as $R= (G-1)/ (N-1)$ was 0.361.

Genetic diversity- Between six and fifteen alleles were detected per microsatellite locus. (Table 1). Heterozygosity values (observed and expected) are shown in Tables 1 and 2; all loci were found to be out of Hardy-Weinberg Equilibrium at $p<0.001$. Expected heterozygosity for all ramets ranged from 0.326 to 0.743 with an average of 0.546. Expected heterozygosity when repeated genotypes were removed ranged from 0.387 to 0.805 with an average value of 0.545. The average probability of identity (PI) for all ramets was 1.7E-2 and the PI when repeated genotypes were removed was 4.8E-2.

Spatial genetic structure- Significant SGS was detected until the 35-40 m distance class when multiple ramets per genet were included in the analysis (Figure 5). The results obtained through GenAlEx were corroborated with SPAGEDI. When the largest clone of 50 ramets was removed from the analysis, significant SGS was detected up to a distance class of 15-20 m

(Figure 6). No significant SGS was detected when identical genotypes were removed from the analysis (Figure 7). Again, the same result was obtained in analyses using either GenAlEx or SPAGEDI. When multiple identical genotypes were included in the analysis, the Sp statistic value was 0.092 (based on b= -0.74 and $F_{(1)}$= 0.1877). When only the largest clone was removed (containing 50 identical genotypes) the Sp value was 0.103 (based on b= -0.078 and $F_{(1)}$ = 0.2401). When identical genotypes were removed from the analysis, the Sp value was 0.0025 (based on b = -0.0024 and $F_{(1)}$ = 0.0381.

Discussion

Spatial autocorrelation analyses reveal that *A. syriaca* in this old field in Northern Michigan shows significant SGS at the ramet level up to the distance class of 35-40 meters (Figure 5). This pattern of SGS may arise in part because of one large clone with 50 ramets that occurs near the intersection of our two transects. When the largest clone is removed from the analysis, significant SGS at the ramet level is detectable up to 15-20 meters (Figure 6). That the pattern of SGS on our transects can be determined in large part by a single clone attests to the power of asexual reproduction in generating SGS in *A. syriaca*. However, reproductive strategy may interact with disturbance regime to influence SGS. The old field in which we sampled was originally forest that was cleared in 1980 and has been disturbed repeatedly since, including the top layer of soil being removed by backhoe (Bob Vande Kopple, personal communication). *A. syriaca* is capable of sprouting from its rhizome as long as a node is present on the rhizome (Cook, 1979; Wyatt and Broyles, 1994). Repeated disturbance may have fragmented *A. syriaca* rhizomes, and led to the current distribution of genets within the field (Figure 3 and 4).

With the removal of repeated genotypes from the analysis, the SGS signal is lost; this is corroborated by the Sp statistics which quantifies the strength of the SGS and allows comparisons among species. The Sp value is related to the mating system of plants (higher in selfing species) and to their life form (higher in herbs than trees), as well as to the population density (higher under low density) (Vekemans and Hardy, 2004). For trees with wind based pollen and seed dispersal, the observed Sp for *Populus alba* was observed to be 0.00414, which is an extremely low value (van Loo et al, 2008). Other wind pollinated species range from 0.00196 to 0.00460 (Vekemans and Hardy 2004). *A. syriaca* has a high level of SGS primarily because of its life form and reproductive strategies. Although SGS can result from a variety of factors, gene flow through pollen and seed dispersal is a key determinant in its establishment (Wright, 1943; Schnabel and Hamrick, 1995; Vekemans and Hardy 2004; van Loo et al, 2008).

The high level of clonal richness on our transects is an indicator of high genetic variability in this population of *A. syriaca*. Estimates of genetic diversity based on microsatellites between sample sites is generally high in *A. syriaca*, and similar to levels found in other studies applying the same techniques (Byars, Parsons, and Hoffman, 2009; Ohsako, 2010). It is widely accepted that populations dominated by clonal reproduction are genotypically almost as diverse as fully sexually reproducing populations (Araki, Shimatani, and Ohara, 2007). Clonal reproduction can retard the loss of genetic diversity within populations by reducing the probability of genet death (Chung and Epperson, 1999). Clonal growth is advantageous in establishing offspring without pollination, whereas sexual reproduction with pollen and seed dispersal helps to maintain genetic diversity in plant populations (Araki, Shimatani, and Ohara, 2007). For long lived clonal plants, a small number of seedling recruits each year is sufficient to maintain clonal diversity in populations (Araki, Shimatani, and Ohara, 2007).

Several studies have investigated clonal reproduction in natural populations of perennial herbaceous species (Cheplick and Gutierrez, 2000; Brzosko et al, 2002), but little is known about the processes that determine intraspecific variation in asexual reproduction, either within or among sites (Gonzales et al, 2008). In some species vegetative spread is associated with stressful environmental conditions and is interpreted as an important adaptation for survival under unfavorable conditions (Tybjerg and Vestergaard 1992; Gonzales et al, 2008). Plant species that have a wide geographical range tend to have higher levels of genetic variation than species with a narrow distribution (Stenstrom et al., 2001). *A. syriaca* can be found throughout the Great Plains from southern Canada south to NE Oklahoma, NW Georgia, and Texas, and east from North Carolina to Maine. Its range continues to move south and specimens have now been collected from Georgia and Louisiana (Wyatt et al., 1993; Wyatt, 1996). Its wide distribution makes it ideal for comparisons across large spatial regions. Genetic differentiation between northern and southern population should also be noticeable through genetic analysis and will be the subject of future work. Northern populations may be less genetically diverse than southern populations due to repeated founder effects (Cwynar and Macdonald, 1987). One would expect higher levels of genetic diversity within *A. syriaca* than within other *Asclepias* species due to its extensive distribution but there may be declining genetic variation in samples moving northward. Our overall findings seem to corroborate theoretical predictions and empirical studies that asexual reproduction can lead to increases in spatial structure (Epperson 1992; Chung and Epperson 1999; Thompson et al, 2008).

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Locus	A(n)	H_o	H_e
H8	7(71)	0.352	0.495***
F9	6(71)	0.408	0.464***
F2	7(71)	0.268	0.387***
G6	15(71)	0.803	0.805***
C109	12(71)	0.507	0.712***
B121	8(71)	0.958	0.704***

Table 1. Observed and expected heterozygosity values calculated without repeated genotypes. All values were found to be significantly out of Hardy-Weinberg equilibrium at $P<0.001$. A(n) represents the number of alleles per locus and number of individuals tested.

Locus	A(n)	H_o	H_e
H8	7(194)	0.325	0.407***
F9	6(194)	0.356	0.393***
F2	7(194)	0.320	0.326***
G6	15(194)	0.887	0.735***
C109	12(194)	0.737	0.743***
B121	8(194)	0.959	0.671***

Table 2. Observed and expected heterozygosity values calculated with repeated genotypes. All values were found to be significantly out of Hardy-Weinberg equilibrium at $P<0.001$. A(n) represents the number of alleles per locus and number of individuals tested.

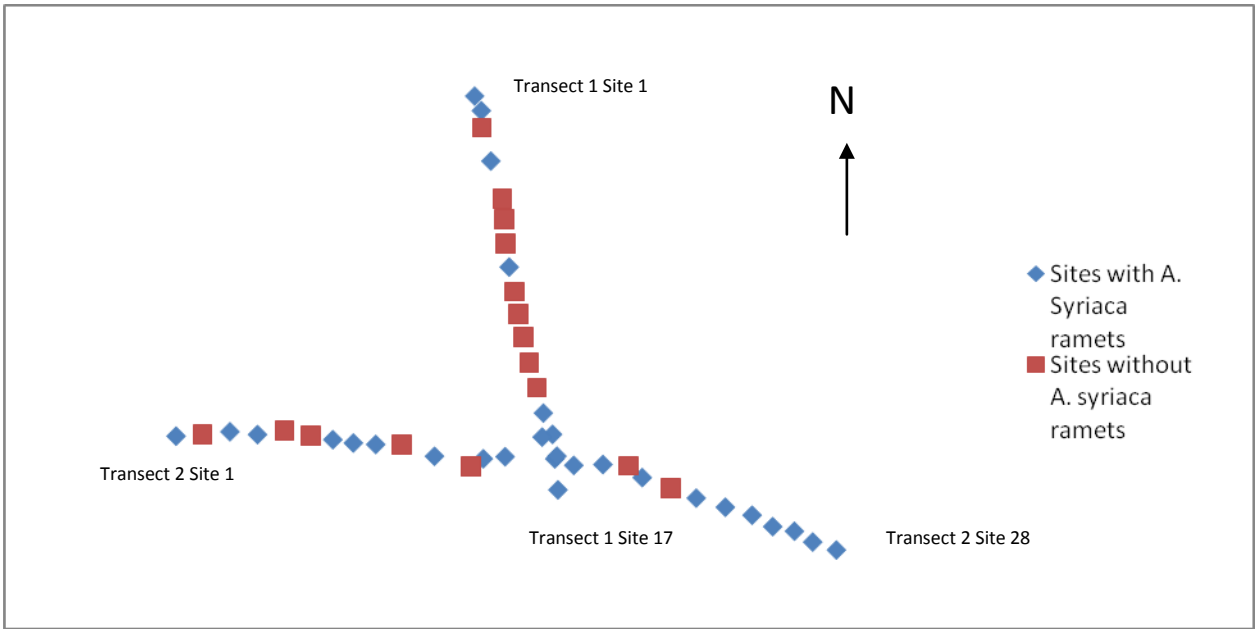


Figure 1 Collection sites within an old field for analysis of the spatial genetic structure of *Asclepias syriaca* at the University of Michigan Biological Station. Transect 1, represented by sites 1 to 17 runs north to south. Transect 2, represented by sites 1 to 28 runs west to east. Sites that were included in the analysis contained at least one ramet per location. Collections occurred at 5 meter intervals.

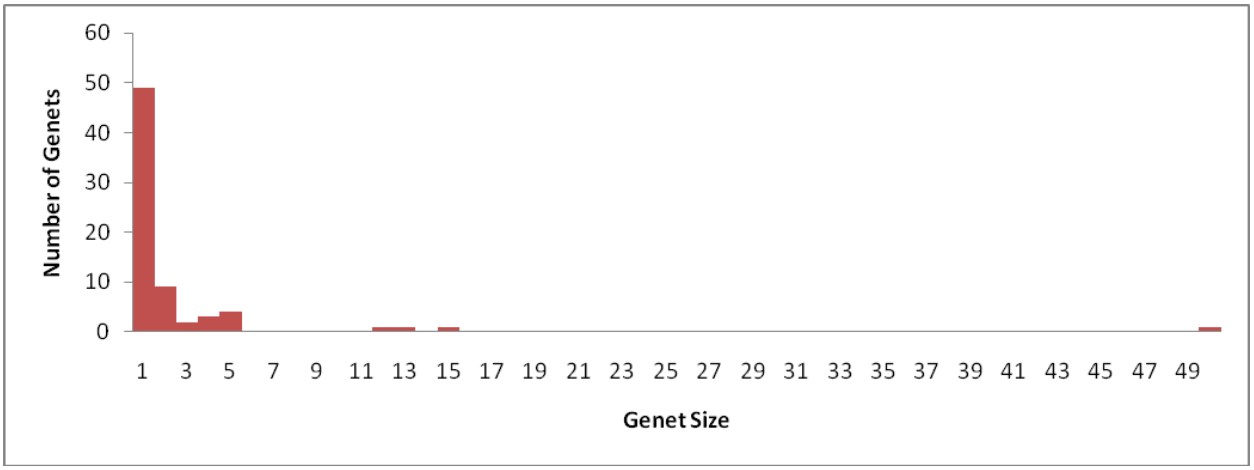


Figure 2. A frequency distribution of the number of ramets belonging to genets of *Asclepias syriaca* in an old field in northern Michigan.

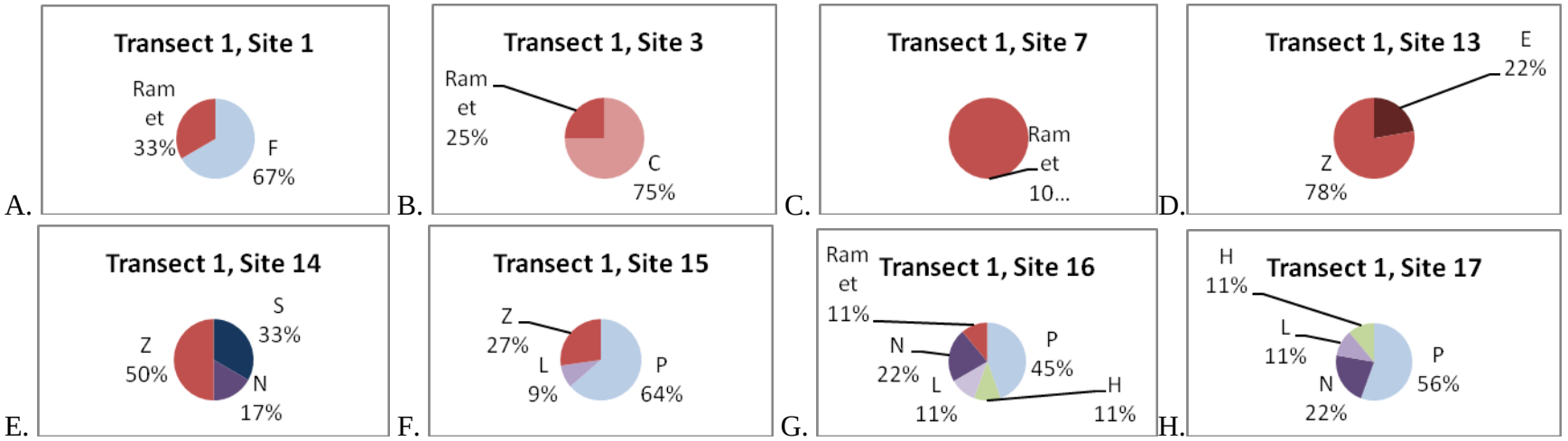
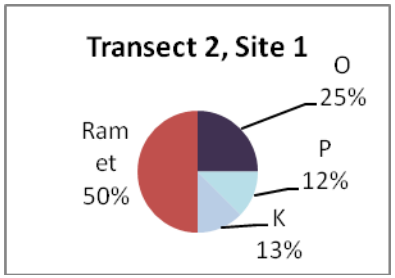
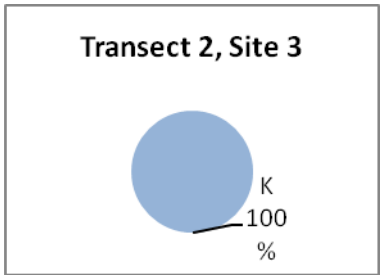


Figure 3 (A-H). Clonal identity of sites form transect 1 including repeated genotypes, results of clonality assessment using GenAlEx v 6.2 (Peakall and Smouse, 2006).

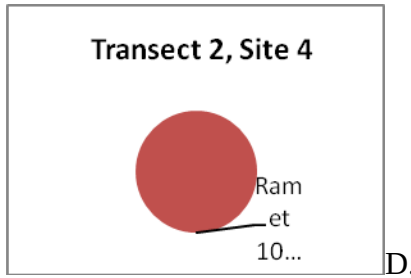
A.



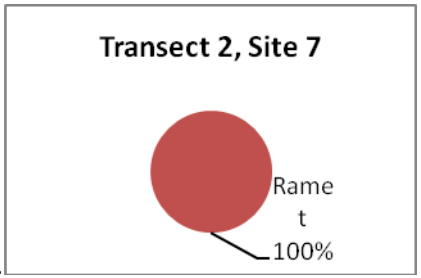
B.



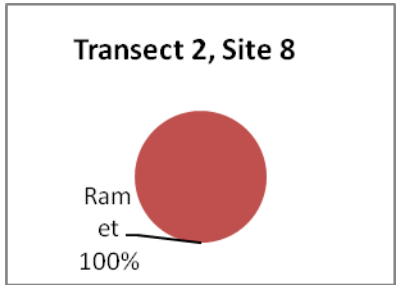
C.



D.



E.



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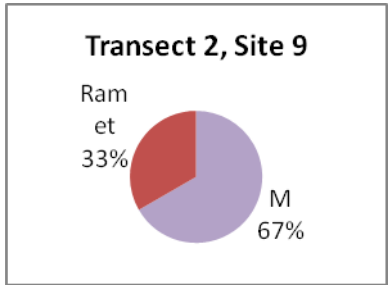
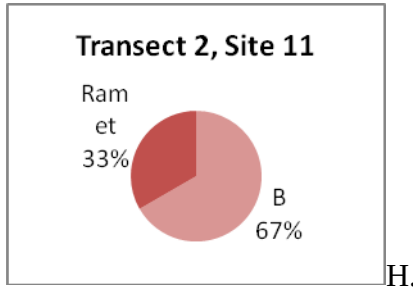
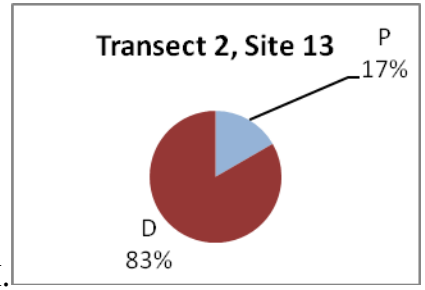


G.



H.



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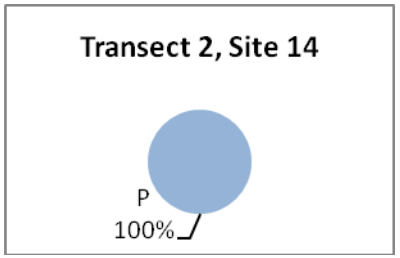
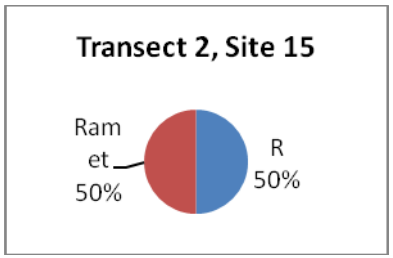
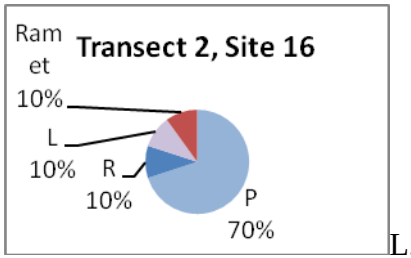


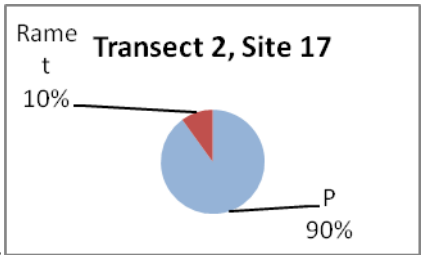
J.



K.



L.



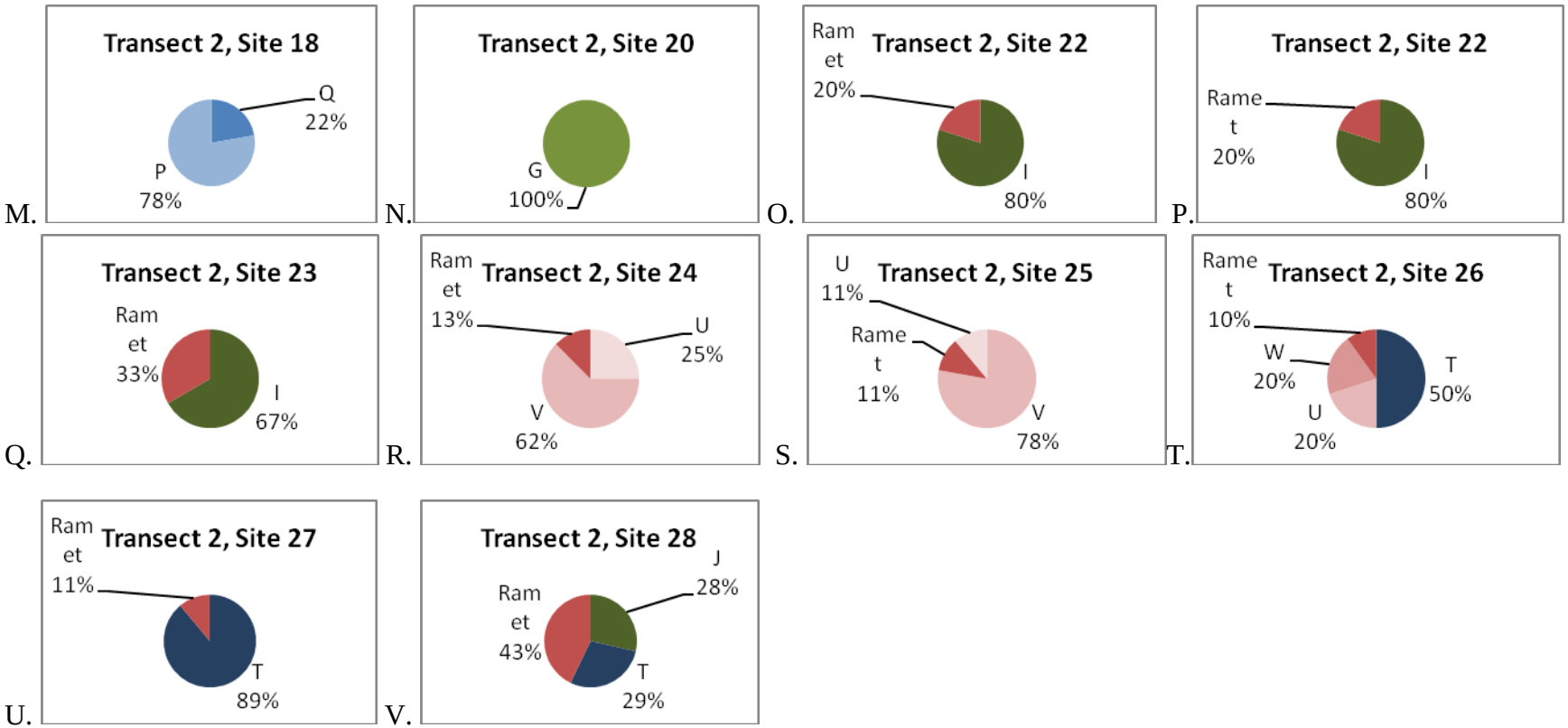


Figure 4 (A-U). Clonal identity of sites form transect 2 including repeated genotypes, results of clonality assessment using GenAlEx v 6.2 (Peakall and Smouse, 2006).

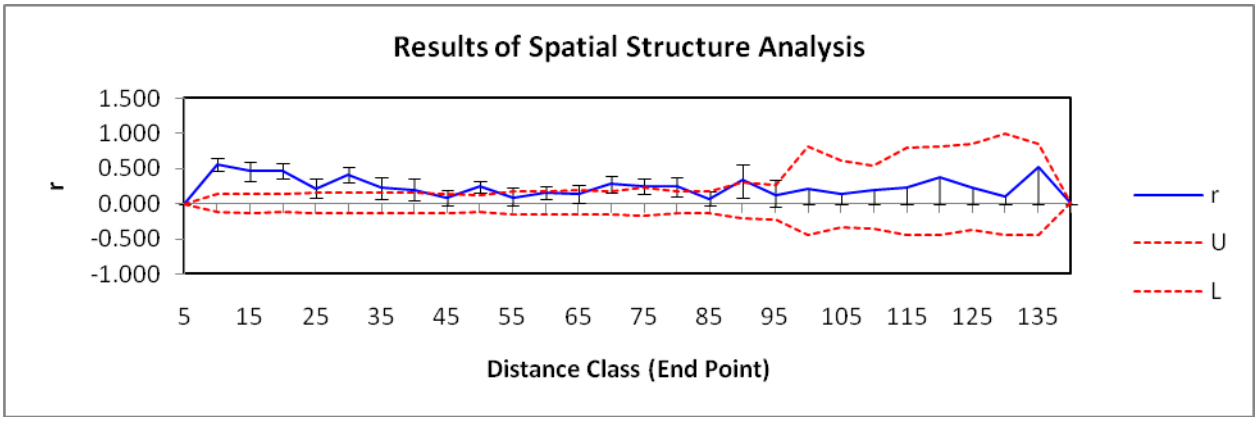


Figure 5. Results of spatial structure analysis run with GenAlEx v 6.2 (Peakall and Smouse, 2006) which includes multiple ramets per genet. U and L represent the 95th confidence intervals through with significant SGS was detected.

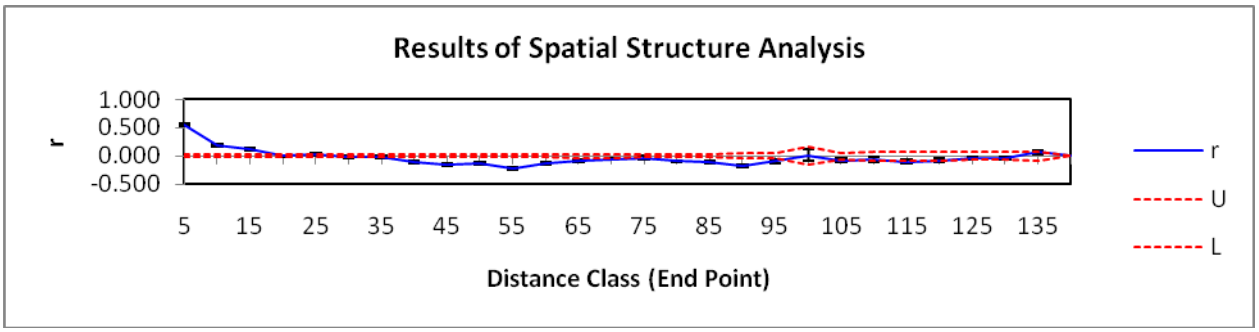


Figure 6. Results of spatial structure analysis run with GenAlEx v 6.2 (Peakall and Smouse, 2006) which includes the removal of the largest clone (50 individuals). U and L represent the 95th confidence intervals through with significant SGS was detected.

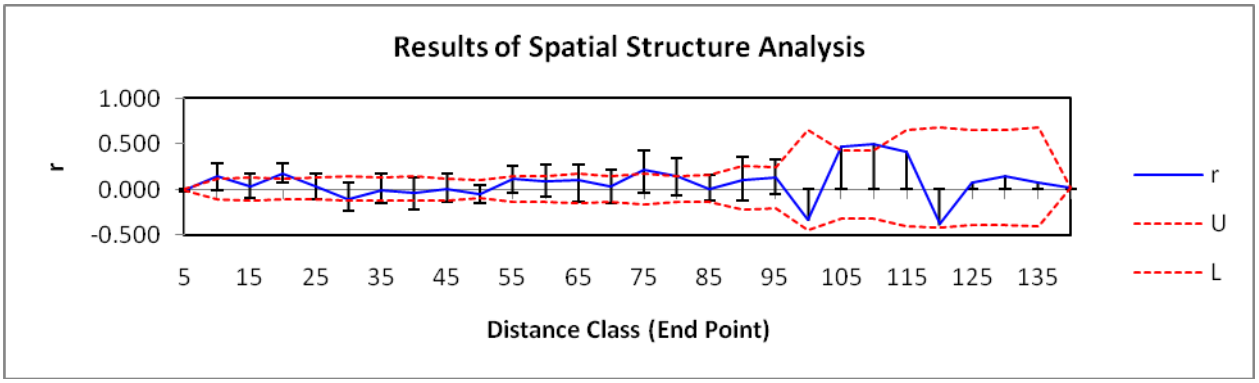


Figure 7. Results of spatial structure analysis run with GenAlEx v 6.2 (Peakall and Smouse, 2006) which does not include multiple ramets per genet. U and L represent the 95th confidence intervals through with significant SGS was detected.

Chapter 4: Future Directions

If there was an unlimited amount of time and of course an unlimited funding source, these are the directions that I would like to take this research in.

At the time of collection at UMBS on June 27 2007, I collected enough tissue so that the appropriate chemical analyses could be preformed. With the data obtained in chapter 3, it would make for a nice comparison to the chemical composition of the same ramets and determine which is factor is more important in determining cardenolide concentrations; the environment or the genes.

I would also like to assess spatial genetic structure at a larger spatial scale. During my time at Michigan I was fortunate enough to collect data along a transect that runs the entire state of Michigan along Interstate 75. Conducting SGS analyses on these samples would help explain if the results obtained in chapter three are a result of scale or some other yet unknown factor. Cardenolide analysis has already been performed on these samples and as mentioned above, it would hopefully corroborate the evidence obtained from these samples and the one mentioned above.