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Kira I. Sussman
University of Montana, kira.sussman@umontana.edu

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EXAMINING DRIVERS OF PHENOTYPIC VARIATION IN THE PERENNIAL HERB SHOWY MILKWEED (*ASCLEPIAS SPECIOSA*).

By

KIRA IRENE SUSSMAN

Undergraduate Thesis
presented in partial fulfillment of the requirements
for the University Scholar distinction

Davidson Honors College
University of Montana
Missoula, MT

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Approved by:

Dr. Lila Fishman, Faculty Mentor
Division of Biological Sciences

ABSTRACT

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Faculty Mentor: Dr. Lila Fishman

Many plant species show variation in phenotypic traits, such as traits related to growth and defense against herbivores, across environmental gradients. Differences in phenotypic traits can be due to selection, leading to local adaptation, or due to the random process of genetic drift. To examine the driver of phenotypic variation, I conducted a Qst (a measure of phenotypic variation among populations) vs. Fst (a measure of genetic variation among populations) analysis for 13 populations of *A. speciosa* grown in a common garden, using five growth and defense traits and seven microsatellite markers. I found relatively low differentiation at the neutral markers (mean Fst = 0.005), and population differentiation of plant height, leaf shape, and latex production traits (but not trichome density or specific leaf area). These results suggest that the three highly differentiated growth and defense traits are responding to population-specific selection pressures, indicating local adaptation of *A. speciosa* distributed across an environmental gradient.

Examining Drivers Of Phenotypic Variation In The Perennial Herb Showy Milkweed (*Asclepias Speciosa*).

Introduction

Plant species are often distributed as separate populations that occur in a variety of different environments, with each population experiencing different abiotic and biotic conditions (Gould et al., 2014; Kooyers et al., 2015). One common feature of spatially separated populations is that they often evolve and exhibit different growth and defense traits, called phenotypic traits (Anderson et al., 2015). If selection by the biotic or abiotic environment is strong, then the population should reflect traits that match the environmental conditions. For instance, if selection by a biotic factor, such as high herbivore pressure, is strong in a certain population then the population should evolve strong defense traits (Abdala-Roberts et al., 2016). If selection by an abiotic factor is strong, such as rainfall, then the population should evolve strong growth or stress tolerance traits. Such selection for traits that match the environmental conditions leads to locally adapted populations (Kawecki and Ebert, 2004; Blanquart et al., 2013; Richardson et al., 2014).). Local adaptation is the process through which one population develops higher fitness in its environment compared to another population of the same species introduced to that environment (Kawecki and Ebert, 2004; Blanquart et al., 2013; Richardson et al., 2014).

Differences in phenotypic traits between populations can also be due to other evolutionary forces, but this has received considerably less attention as a mechanism for describing differentiation of traits among populations than local adaptation. Genetic drift, which occurs due to random genetic changes by sampling error during reproduction, can also create differences in traits among spatially separated populated (Hendry et al., 2001; McKay and Latta, 2002; Kawecki and Ebert, 2004). Some separated populations may show little or no trait variation between populations, which can occur due to the stabilizing effects of gene flow or similar environmental conditions (Kawecki and Ebert, 2004). In order for local adaptation to occur, traits must be affected to a greater degree by selection than by drift or gene flow (Hendry et al., 2001; McKay and Latta, 2002; Kawecki and Ebert, 2004). Both biotic and abiotic factors can act as selective forces; biotic factors tend to act on defense traits, while abiotic factors tend to act of growth traits (Abdala-Roberts et al., 2016). While studies have addressed the individual effects of either biotic or abiotic factors as selective forces, there has been little work has compared how these factors might select for different traits.

Showy Milkweed (*Asclepias speciosa*) is a self-incompatible perennial plant that occurs across much of the western part of North America, and can reproduce through underground clonal roots as well as seed production (Wyatt and Broyles, 1994). Pollination occurs mainly by bees and flies which visit the flower for its abundant nectar (Wyatt and Broyles, 1994). Pollen is dispersed in pollinia, units of hundreds of pollen grains, and dispersal distance largely depends on pollinator behavior, but can be upwards of several kilometers (Wyatt and Broyles, 1994). Fruits typically contain 50-100 plumed seeds (Bookman, 1983), all of which are full siblings (Morse and Schmitt, 1985). Seeds are wind dispersed, and the dispersal distance depends on seed mass, wind speed, and height of release, and can result in long-distance dispersal events (Morse and Schmitt, 1985). *Asclepias speciosa* is attacked by a suite of specialist herbivores, including the Monarch Butterfly, which uses *A. speciosa* for ovipositation, cardenolide sequestration, and food (Ackery and Vane-Wright, 1984; Malcolm, 1994). *A. speciosa* is an ideal species to study local adaptation, as it occurs across a variety of environmental conditions and exhibits diverse growth and defense traits between populations. Previous work in other milkweed species has shown a genetic component to defense trait variation, but the mechanism behind the variation has not been determined. This study builds on previous work that address the mechanisms of phenotypic variation between populations of *Asclepias* found on different continents (Agrawal et al., 2016).

In this study, I analyzed variation phenotypic traits between 13 different populations across the Northern United States grown in a common garden. I tested whether growth and physiological traits (specific leaf area, leaf length:width ratio, and height) and defense traits (latex production and trichomes) were differentiated between the populations, and calculated Qst. In order to test for the effects of selection on differentiation of these traits, relative to other evolutionary processes, I analyzed the population genetic structure using microsatellite DNA markers. Microsatellites are 1-6 nucleotide repeats that are found in nuclear DNA (Selkoe and Toonen, 2006). Because they are generally found in noncoding regions and have high mutation rates (Selkoe and Toonen, 2006), microsatellites are putatively neutral and often highly variable, which makes them suitable for population genetic analysis. As putatively neutral markers, microsatellites reflect demographic processes (drift and migration or gene flow, mating system) that affect all parts of the genome equally (Selkoe and Toonen, 2006). I then conducted a Qst vs. Fst analysis to determine which evolutionary mechanism is more important in the resultant trait

differentiation. If Qst (measure of phenotypic trait differentiation) is greater than Fst (measure of neutral genetic variation), populations are more distinct that expected from drift alone, indicating local adaptation as the cause of the trait differentiation (Hendry et al., 2001; Whitlock and Guillaume, 2009). If Qst is equal to Fst, the result indicates that the amount of phenotypic variation between populations is equal to the amount of neutral genetic variation, so drift cannot be ruled out as the cause of differentiation. If Qst is less than Fst, the result indicates that there is less phenotypic variation than neutral genetic variation, which could indicate stabilizing or balancing selection. Overall, this work integrates selection by biotic and abiotic factors with population genetic structure to analyze the mechanisms and forces behind the phenotypic differentiation between different populations of *A. speciosa*.

Methods

Common Garden Set-up

The plants from the common garden were collected from 13 different populations with origins between Eastern Washington and North Dakota (Figure 1). Each population contained 1-9 of individuals from 2-7 families, with 6-24 total individuals per population. Seeds were collected from the field populations in 2015 and planted directly into the common garden in May 2016.

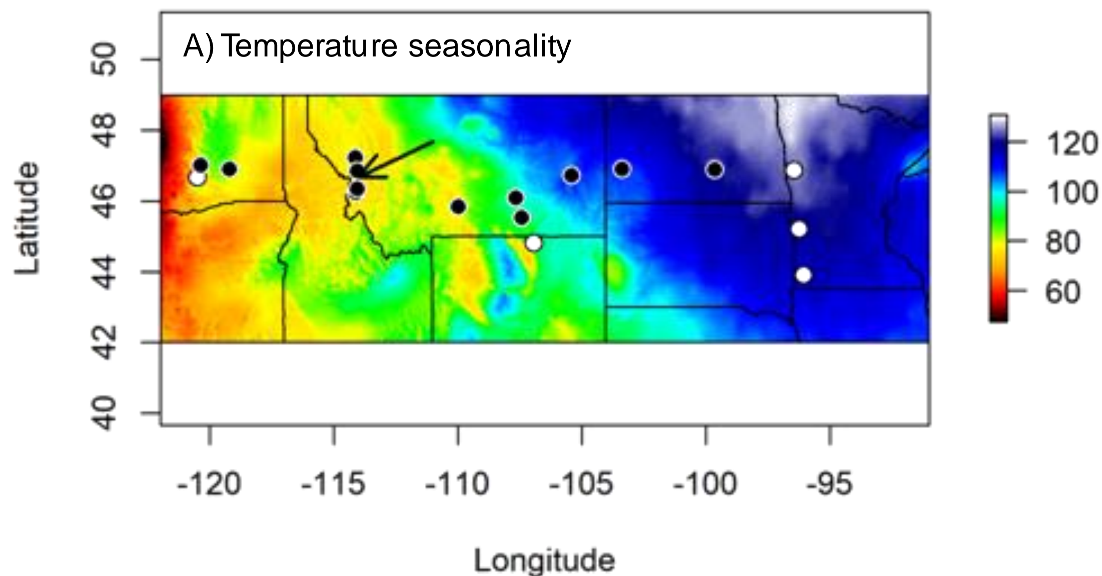


Figure 1. Map of study populations used in the common garden (black dots). Arrow points to the location of the common garden, near Missoula, MT. Colors show 'temperature seasonality,'

which is a measure of variation in monthly average temperatures, where higher values indicate more seasonal climates.

DNA extraction and PCR genotyping

On July 18th, 2017 I collected a single piece of leaf tissue (~1 cm^2) from 192 individual plants in the common garden, representing all 13 populations. The tissue was dried on silica beads prior to extraction. DNA was extracted from the dried tissue using a standard CTAB protocol (Doyle and Doyle, 1997) and , quantified using a fluorometer, and diluted to a concentration of 2-10 ng/microL . The extracted DNA was then amplified in a polymerase chain reaction (PCR) for 7 microsatellite markers that had previously been developed and tested in *Asclepias syriaca*: ASH8, ASF2, and ASF9 from Kabat et al. (2010) and A106, B5, C102, and B121 from O'Quinn and Fishbein (2009). PCR solution contained 2µl of diluted genomic DNA with 8µl of standard PCR solution. The samples were amplified with a touchdown PCR program. Once completed, 2µl of PCR product were diluted with 50µl of H_2O for fragment analysis using a 3130 Genetic Analyzer at the University of Montana Genomics Core Center. Allele sizes were determined using Genemapper Software (Applied Biosystems), and all allele calls were hand-checked.

Measurement of phenotypic traits

I measured phenotypic traits on plants in the common garden on July 18th. Plant height was measured from the ground to the apical meristem on the tallest stem, and the number of stems were also counted. To measure latex, I clipped ~1 cm from the tip of one of the youngest fully expanded leaves. I collected the latex that exuded from the cut leaf on a disc of pre-weighed filter paper that was contained in pre-weighed tubes. I collected the opposite leaf for determining trichome density and specific leaf area (SLA). The number of trichomes in 1/4th of a $33cm^2$ circle were counted under dissecting microscope, and the estimated percentage of leaf area covered by trichomes was estimated visually. The leaves were then scanned and uploaded to the software program ImageJ, where area, length and width were determined. The full leaves were dried in a drying oven at 55°C for 7 (seven) days and weighed. The dried leaves were weighed for dry mass to calculate SLA. SLA was calculated as the area (cm^2) divided by the dry mass (mg^2).

Neutral and quantitative trait comparison

To test marker viability, I screened the markers in GenAlEx 6.5 to test for expected heterozygosity (H_e), observed heterozygosity (H_o), and probability of deviation from Hardy-Weinberg equilibrium (Peakall and Smouse 2006, 2012). Next, I estimated population structure using the program Structure (Prichard et al., 2000), with 10,000 burn-in and 100,000 MCMC iterations. This procedure detects the underlying genetic population individuals by first clustering genotypes, and then assigning individuals proportioally to clusters based on their genotype. I tested numbers of populations (K) from K=13 to K=2 to see if the individuals sorted into genetically distinct groups. I then estimated isolation by geographic distance and genetic distance using the R package ADGENT, and performed a Mantel test for correlations between distance matrices. This analysis tests for correlations between matrices of pairwise genetic distance and pairwise geographic distance.

To compare neutral genetic differentiation to quantitative genetic variation I used the R package "QstFstComp" (Gilbert and Whitlock 2015). Briefly, Qst is the proportion of variation in phenotypic traits attributed to among-population differences, and Fst is the proportion of genetic variation in neutral genetic markers attributed to among-population differences. QstFst Comp uses the null hypothesis Qst = Fst and conducts parametric resampling of Qst and bootstrap sampling of Fst to generate a null distribution for each variable. The observed values are then compared to the null distributions. I used an unbalanced full-sib design with a shared dam and relatedness between siblings of 0.5. I randomly sampled one individual from each family for calculation of Fst.

Table 1. Characteristics of 7 microsatellite markers and results from testing in 192 samples of *Asclepias speciosa*. Shown for each locus is the number of alleles, size range, expected heterozygosity (He), observed heterozygosity (Ho), and source of the marker. Deviations from Hardy-Weinberg * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Marker	Number of Alleles	Size Range (bp)	He	Ho	Source
ASH8	7	157-171	0.52	0.51***	Kabat
A106	14	234-270	0.83	0.74***	OQuinn
B5	9	253-268	0.42	0.42	OQuinn

ASF2	5	87-118	0.58	0.79***	Kabat
C102	4	236-242	0.29	0.34	OQuinn
ASF9	4	107-125	0.50	0.43	Kabat
B121	2	226-255	0.49	0.76***	OQuinn

Results

Analyzing the population genetic data through STRUCTURE showed no differentiation between populations, indicating that there is little variation in the microsatellite markers is described by population (Figure 2). Similarly, the Mantel test showed no correlation between genetic geographic distance (R = -0.08, P = 0.634, Figure 3).

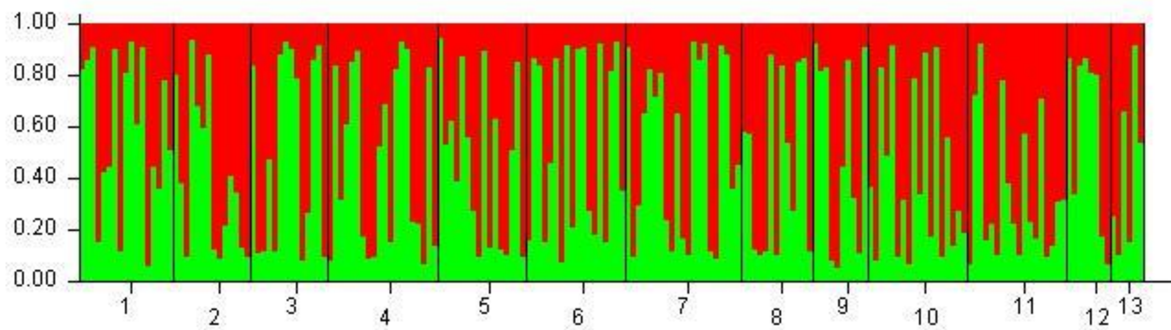


Figure 2. STRUCTURE results with K = 2. Red and green bars represent two genetically distinct populations. X-axis numbers are actual populations and y-axis numbers are the proportion of each individual's genotype that could be assigned to the green population. This analysis indicates that there is are no significant differences in genetic structure among the populations.

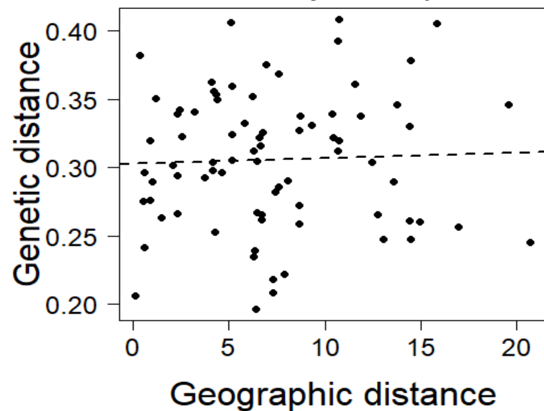
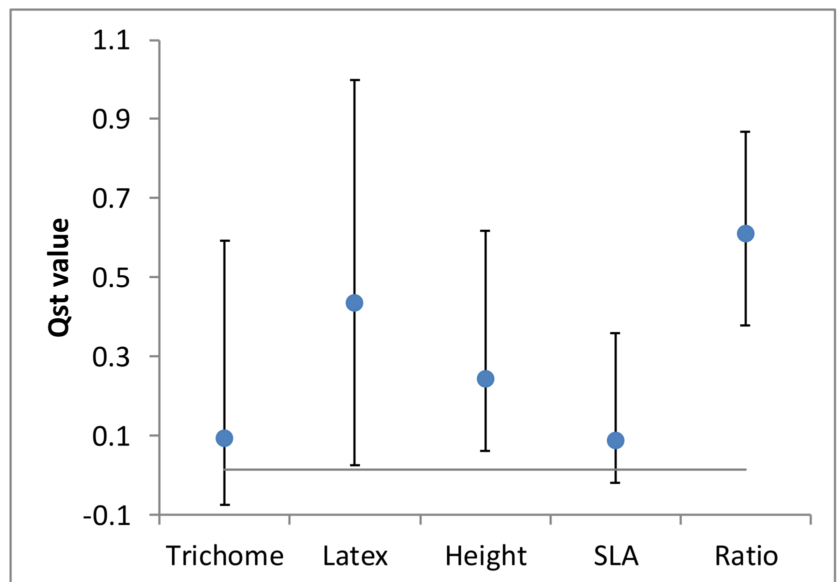


Figure 3. Isolation by distance plot measuring correlation between geographic distance and genetic distance.

Qst-Fst comparison

Analysis of genetic differentiation showed no evidence for population structure (mean Fst = 0.005). This finding is consistent with the lack of identifiable populations determined by STRUCTURE (Figure 2), indicating that the populations are not differentiated.



Qst values were significantly higher than Fst values for three of the traits: latex (Qst = 0.436, P = 0.0006), plant height (Qst = 0.2444, P = 0.0003) and leaf ratio (Qst = 0.6119, P =0.00001) (Figure 4). Qst values were not significantly higher than Fst values for trichomes (Qst = 0.0942, P = 0.07) and SLA (Qst = 0.0895, P = 0.1) (Figure 4).

Figure 4. Qst values for each of the five traits plotted with the overall Fst value. Error bars at 95% confidence intervals.

<u>Discussion</u>

I present evidence for local adaptation of several leaf and defense traits in A. *speciosa* across the Northern United States. The low Fst value of 0.005 indicates that there is no neutral genetic differentiation between populations, which could be due to extensive gene flow across the entire

geographical range I studied. Gene flow is generally thought to work against local adaptation, as one genotype can invade all of the populations and swamp out phenotypic diversity (Lenormand, 2002; Yeaman and Otto, 2011; Blanquart et al., 2013). However, local adaptation can be maintained in the face of gene flow if selection is sufficiently strong to overcome swamping effects (Isik and Nelson, 1997; Kawecki and Ebert, 2004; Sambatti and Rice, 2006; Saenz-Romero et al., 2006; Gonzalo-Turpin and Hazard, 2009; Richardson et al., 2014). Thus, my result of the comparatively high Qst values show strong evidence for divergent selection of both growth and defense traits, indicating that *A. speciosa* is under selective pressure likely by both the abiotic and biotic environment, leading to the visible phenotypic differences between the populations despite gene flow.

The low Fst value and lack of population structure was surprising given that the populations are dispersed across a gradient of 1500km. However, studies conducted in similar species of *Asclepias* found that populations of *A. perennis* and *A. texana* had Gst values (a related measure of genetic diversity partitioned by population) of 0.082 and 0.068 respectively across a gradient of 1,700 km (Edwards et al., 1994). One likely cause of the lack of neutral genetic variation could be extensive gene flow between the populations. Gene flow in *A. speciosa* can occur from distribution of pollinia between populations by pollinators, or wind-dispersal of the feathery seeds (Morse and Schmitt, 1985). Pollen in *A. speciosa* is dispersed by pollinia, containing hundreds of grains of pollen, which can persist on a pollinator for 24 hours (Morse, 1982; Broyles 1994). The possibility that pollinia can be transported and deposited 24 hours after it is picked up with a bee, coupled with the long-distance wind dispersal of the seeds, points to the strong possibility that genetic material could be dispersed across a gradient as large at 1500km over generations.

Latex differentiation among populations could be due to differing levels of herbivory in the plants' source environment. Latex is a sticky, toxic, mixture that has no role in a plant's primary metabolism (consisting of resource acquisition and allocation), but has strong evidence as a defense against herbivorous insects (Dussourd & Eisner, 1987; Zalucki & Malcolm, 1999; Zalucki et al., 2001; Agrawal et al., 2008). Latex production has been found to correlate with greater resistance to monarch larvae (Zalucki et al., 2001; Woods et al. 2012). For trichome density, the low Qst value suggests that populations are not significantly differentiated in their production of trichomes. Trichomes can function as a physical barrier to defend against chewing

insects, although there is no negative impact on sucking insects (Malcolm, 1994; Fordyce and Agrawal, 2001; Agrawal, 2004), as well as a mechanism to reduce UV absorbance and heat by shading leaves (Ehleringer et al., 1976). Weaker evidence for selection on trichomes could be due to similar levels of UV radiation in the different environments, or due to the prevalence of insects that suck on the plants rather than chew. My results point to strong selection on latex, possibly by varied herbivore pressure in different environments. While selection may still be acting on trichome density, the differentiation is not consistent with local adaptation.

I found that plant height was significantly differentiated by population (Qst = 0.2444, P = 0.0003), suggesting that height is undergoing selection leading to local adaptation. Plant height is a growth trait that responds to biotic conditions such as competition from other plants (Weiher et al., 1999; Nicotra et al., 2010), as well as abiotic conditions such as light acquisition (Weiher et al., 1999) and soil nutrient content, which impacts overall plant productivity and growth (Grime, 1977). Given that the plants originate from populations that are distributed across an environment gradient (Figure 1), the among-population variation in height could be due to varying levels of competition in the different environments which could lead to selective pressure for taller plants in order to reduce light competition. The variation could also be due to differences in nutrients across the environmental cline, which could lead to selection for faster- or slower-growing plants.

Leaf ratio was the most highly differentiated trait, suggesting strong selection in each population. Leaf ratio is a method of quantifying leaf shape, which has exhibited clinal variation in various species of *Asclepias* (Woodson 1962; Wyatt and Antonovics 1981). Selection on leaf shape can be due to herbivore pressure, such as in a study conducted by Rausher (1978), which showed that *Battus philenor* butterflies search for specific leaf shapes to ovaposit on, potentially leading to modification of leaf shapes. Monarch butterflies show preference for particular milkweed plants in part due to the size and strength of their leaves, but no studies have shown a preference for particular leaf shape in Monarchs (Ladner and Altizer, 2005). Leaf shape could also be selected on by average temperature of the plant's home environment. Leaf shape impacts the size of the boundary layer, which is the immobile air next to a leaf's surface, which increases in thickness with distance from the leaf edge (Schuepp, 1993). The thinner boundary layer created by narrower leaves is advantageous in warmer environments, as it allows the leaves to

cool via convective exchange without increased transpiration (Schuepp, 1993; Ferris et al., 2015).

Conclusion

Overall, my results show strong evidence for local adaptation of a suite of traits in *A. speciosa*, despite strong gene flow. Growth traits (height, leaf shape) and defense traits (latex) both show evidence for local adaptation, indicating that *A. speciosa* experiences selection by both biotic and abiotic factors. However, microsatellite markers have a limited ability to pick out subtle population structure, which could further be explored using more sophisticated approaches such as SNPs or rad-seq. Whole genome studies of different populations of *A. speciosa* could provide insight into the actual rates of gene flow between populations and could identify the genes under selection. Nevertheless, my work provides an important step towards understanding the drivers of local adaptation in *A. speciosa.*

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