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Population Genetic Structure of *Asclepias Tuberosa* In Northwest Iowa: A Comparison Within and Between Remnant Prairies and Commercially Available Seed

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Isolated in scattered remnants, less than 0.1% of Iowa's original tallgrass prairie remains. The small populations remaining are at risk for reduced genetic diversity, inbreeding depression, and outbreeding depression. In light of these concerns, we used microsatellite analysis to assess the genetic structure of butterfly milkweed (*Asclepias tuberosa*) populations on prairie remnants in northwest Iowa. We compared remnant populations with a restoration population at Dordt College in Sioux Center, Iowa, and with an Oklahoma seed source. Microsatellites identified for use in common milkweed (*Asclepias syriaca)* had sufficient polymorphism information content (PIC) across the butterfly milkweed (*A. tuberosa*) populations sampled (mean PIC = 0.624). The F_{IS} values indicated a lack of inbreeding (mean F_{IS} = –0.1455) even in the commercially expanded seed. The pairwise F_{ST} values showed a low degree of differentiation among the remnants (mean $F_{ST} = 0.0453$) but a moderate degree (mean $F_{ST} = 0.105$) of differentiation when comparing the remnants to the Dordt restoration or to seed from Oklahoma. Despite massive loss and fragmentation of the tallgrass prairie, our microsatellite analysis revealed no evidence of inbreeding in *A. tuberosa*. However, evidence of genetic differentiation suggests that effort should be made to preserve the diversity still present. Seed expansion efforts appear to have had minimal impact on overall genetic diversity, although the diversity in particular selectable traits may be reduced. The differences between the genetics of the propagated seed at the Dordt restoration and the Oklahoma seed when compared to native remnants support the usefulness of source-identified seed.

INDEX DESCRIPTORS: tallgrass prairie, butterfly milkweed, microsatellite, inbreeding, outbreeding

INTRODUCTION

The prairie that once covered Iowa has been reduced by more than 99% due to the establishment of agricultural fields, towns, and roads (Fletcher and Koford 2002). Before European settlement, 79% of Iowa's landscape was tallgrass prairie; now, less than 0.1% of native prairie remains (Fletcher and Koford 2002), making the tallgrass prairie one of the most endangered ecosystems in North America (Johnson et al. 2003). The changes in land use practices over the last 150 years have left Iowa with a scattered patchwork of remnant prairies. Consequently, plants native to the tallgrass prairie exist in small populations and risk extinction from habitat loss and fragmentation. Reduced population sizes can lead to a loss of gene flow and genetic diversity within populations, which may reduce the potential for adaptation under changing environmental conditions. Similarly, fragmented populations with reduced diversity can genetically differentiate from other populations and become more susceptible to inbreeding depression. Loss of allelic diversity in a population can increase the likelihood of homozygosity. Researchers disagree as to whether increased homozygosity is intrinsically harmful (overdominance hypothesis) or if it is harmful because of the increased expression of deleterious recessives (Roff 2002). In either case, many researchers broadly and demonstrably associate an increase in homozygosity with a reduction in fitness (Edmands 2007).

Though the risk of inbreeding depression is of particular concern, attempts to increase genetic diversity in remnant prairies and restoration efforts by utilizing diverse source populations can put local native populations at risk for outbreeding depression. When non-local genotypes are introduced to native populations, the potential dilution and resorting of local genotypes may result in a loss of alleles and disruption of co-adapted gene complexes that reflect the native population's adaptation to a specific environment (Edmands 2007).

In restoration efforts, seed sources shape the genetic structure of the prairie. Seed expansion efforts can decrease genetic diversity by producing large populations and many seeds that originate from only a few plants. Additionally, fixed seed collection and harvest practices can further reduce the genetic diversity of a seed supply. Seed originating from ecologically distinct locations may diminish the future resilience and stability of the restored prairie. While novel genotypes may initially thrive in a new environment, introduced plants may exhibit reduced fitness over time because they are not equipped to survive the dynamic conditions of the new environment (Aldrich et al. 1998). Genetic interactions that occur in successive generations where parental co-adapted gene complexes are recombined can also reduce fitness (Whitlock et al. 1995).

The potential for outbreeding depression is intensely debated. Both the understanding that inbreeding is more detrimental than the potential for outbreeding and the ambiguous description of "local" genotypes fuel the debate. The extent of local adaptation and ecotypic variation is poorly understood for most prairie species and varies considerably for species that have been studied (Cortese et al. 2010). Consequently, restrictive seed transfer zones, which have been suggested, would not be practical for prairie conservation (Hufford et al. 2012). The extent of local adaptation is also difficult to assess in recently fragmented habitats, such as the tallgrass prairie, where observed genetic differentiation among isolated populations may be a glimpse of historic patterns rather than an indication of reduced gene flow.

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Table 1. Sampling locations in northwest Iowa.

Prairie	Area (ha)*	No. Plants Sampled	Latitude	Longitude
Brewer	4	38	42.70850	−95.57345
Broken Kettle	1214	17	42.69694	−96.57611
Dordt College	8	47	43.08086	−96.16543
Freda Haffner	45	45	43.34500	−95.22500
Steele	81	40	42.87659	−95.58233

*Approximate

Although the current understanding of local adaptation and outbreeding in prairie conservation is limited, many conservationists encourage the use of local seed to reduce the potential for outbreeding depression (Crémieux et al. 2010; Montalvo and Ellstrand 2001). This applies to both the addition of seed to remnant populations and to reconstruction efforts near remnants. Similarly, research described by McKay et al. (2005) suggests that the small, isolated populations of plants present in restoration sites are especially susceptible to outbreeding depression and the addition of maladaptive genotypes. In response to the potential for outbreeding and the need for local seed, the Iowa Ecotype Project (Natural Selections) was developed in 1990 to increase the availability of source-identified seed (Houseal 2003). This project aims to limit the introduction of potentially maladaptive genetic material by specifying the physical area in which local seeds should be planted. The Iowa Ecotype Project divides the state of Iowa into three latitudinal zones for seed transfer in an attempt to conserve the existing local ecotypes (Houseal 2003).

Hufford and Mazer (2003) argue that the physical distance separating populations cannot solely account for the genetic differentiation between them; rather, genetic differences between populations more likely result from environmental differences such as elevation, soil characteristics, climate, and predators. The idea of local ecotypes in relation to seed transfer zones is relatively new, and limited research concerning ecotypic variation between populations of native wildflowers has been published.

In light of concerns regarding the likelihood of inbreeding and outbreeding depression in the tallgrass prairie, we investigated the population genetics of butterfly milkweed (*Asclepias tuberosa* L.*)* from 4 remnant prairies, an out-of-state seed supplier, and a prairie restoration project on the campus of Dordt College. This limited study provides important information regarding the viability of the molecular markers used, as well as the genetic structure of restored and remnant tallgrass prairies.

Butterfly milkweed was chosen as the study species because it is a common wildflower native to the tallgrass prairie; it is easily identifiable, often a component of seed collections used in the conservation reserve program; and molecular markers were available (O'Quinn and Fishbein 2008).

Milkweeds have unique reproductive characteristics. Pollen is carried in pollinia, which must be inserted into a closed stigmatic chamber in order for pollination to occur. The rate of successful fruit-set is low across the *Asclepias* genus, and questions still remain concerning the extent of self-compatibility in butterfly milkweed (Wyatt and Broyles 1994; Ivey et al. 1999). Seeds are dispersed by the wind, and large-winged butterflies are major pollinators of butterfly milkweed. (Luna and Dumroese 2013). Though evidence exists for long-distance pollen dispersal (Broyles et al. 1994), direct measurements taken by Pleasants (1991) using radioactively labelled pollen demonstrate a much smaller range for pollen dispersal. Seed dispersion and pollination have important implications concerning gene flow and the genetic structure of populations.

In this study, the genetic structure of butterfly milkweed populations was examined using microsatellite analysis. Ten microsatellite loci in common milkweed (*A. syriaca* L.) had earlier been identified (O'Quinn and Fishbein 2008), and we were able to establish the viability of these markers for work with butterfly milkweed (*A. tuberosa* L.). These heritable short sequence repeats (SSRs) are ideal for investigating genetic diversity because they detect high levels of polymorphism, are neutral and locus specific, and relatively easy to use (Wei et al. 2013).

We looked for evidence of inbreeding by comparing the allelic and genotypic frequencies within the populations. Additionally, we examined the overall allelic variability within and across remnant populations to evaluate the degree of genetic distinctiveness of remnant prairies. We also compared the allelic composition of populations from remnant prairies and those containing seed originating outside the state of Iowa as an initial indication of the potential for outbreeding depression from introduced seed. Greater insight into population structure, the prevalence of inbreeding, and the potential for outbreeding depression is critical to enable land managers to make appropriate management decisions in the face of continued habitat loss and fragmentation. This work can serve as a platform for field studies of fitness, self-compatibility, and heterosis.

METHODS

Prairie Selection

Four remnant prairies in northwest Iowa were selected for sample collection (Table 1). The Steele prairie state preserve (81 ha) and Brewer prairie (4 ha) in Cherokee County and Freda Haffner State Preserve (45 ha) in Dickinson County were selected as representative remnant prairies. Broken Kettle Grasslands (1,214 ha) in Plymouth County is also a remnant prairie but is part of the Loess Hills region (Fig. 1). This location was chosen because the soil composition differs significantly from the other prairie locations and may constitute a distinct selection mechanism. In addition, the population is small and thus more subject to loss of allelic diversity. We also analyzed material from the Dordt College restored prairie (8 ha, seed provided by The Prairie Flower, Spencer, Iowa) and from Lorenz's OK Seeds, LLC, of Okeene, Oklahoma. This source provided an opportunity to look for different allelic frequencies and composition characteristic of a distant region. In this case we extracted DNA from germinated seeds rather than growing plants.

DNA Extraction

Approximately 1–cm^2 sections of leaf tissue were collected from Steele (40 plants), Brewer (38 plants), Broken Kettle (17 plants), Freda Haffner (45 plants) and Dordt College (47 plants) prairies (Table 2). Seed was ordered from seed suppliers in Oklahoma (Lorenz's OK Seeds, LLC) and was germinated. DNA was extracted using a modified phenol-chloroform extraction protocol developed by Nalini et al. (2004).

PCR and Visualization

A LI-COR® 4300 DNA Analysis system was used to perform microsatellite analyses. Ten microsatellites have been identified for common milkweed (O'Quinn and Fishbein 2008). This system requires the use of polymerase chain reaction (PCR) and infrared labels to detect the products. This reaction has been optimized in

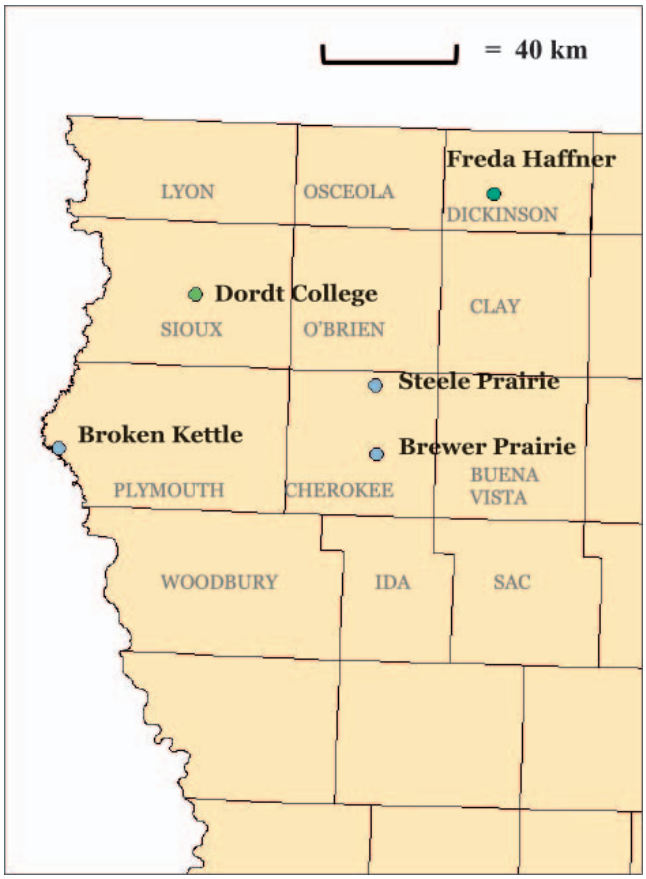

Fig. 1. Prairie locations in northwest Iowa.

our lab for 6 of the 10 available microsatellite sequences to be used with butterfly milkweed (Table 2). Saga®, the software accompanying the LI-COR® DNA analysis system, was employed to store microsatellite information and generate data sets.

To amplify specific microsatellite loci, a two-step PCR reaction process was used. Step 1 involved PCR amplification of microsatellite sequences in a reaction volume of 12µl containing: 1µl of each primer (F/R) diluted to 1µM, 5µl of GoTaq® Green Master Mix (Promega), 3µl of water, and 2µl of template DNA extracted from the samples. PCR for Step 1 was performed on a Bio-Rad Gene Cycler™ with the following cycling conditions: 94°C for 2 min (one cycle); 94°C for 30 s, 51°C for 30 s, and 72°C for 30 s (8 cycles); 72°C for 2 min (one cycle). The specific microsatellite sequences were further amplified during the second step of the PCR reaction with the addition of M13 tails. The microsatellites were amplified in a reaction volume of 10µl containing1µl of product from Step 1, 0.05µl of M13 (F/R), labeled with IRDye® 800 primers, 5µl of GoTaq® Green Master Mix, and 4µl of water. Step 2 PCR had the following cycling schedule: 94°C for 2 min (one cycle); 94°C for 30 s, 51°C for 30 s, and 72°C for 30 s (30 cycles); 72°C for 2 min (one cycle).

Statistical Analysis

Arlequin 3.5.1.3© and Genepop 4.1.3© were used to carry out a variety of analyses on the data collected (Excoffier and Lischer 2010; Rousset 2008). The number of individuals scored per locus and the allelic variability at each locus were investigated to confirm the appropriateness of the molecular markers for analytical use. Per-locus gene diversities were analysed using Genepop 4.1.3© through comparison of observed and expected heterozygosities under Hardy-Weinberg equilibrium. Polymorphism Information Content (PIC) values were calculated for each locus as well.

Allele frequencies and gene diversity based on allele identity were quantified for each population using Genepop. F_{IS} statistics were calculated as in Weir and Cockerham (1984). F_{ST} values based on allele identity were used for pairwise comparison of the genetic differentiation of populations. An AMOVA test was run using Arlequin to examine the overall structure of variability among all samples (Weir and Cockerham 1984). The frequency of private alleles, present in only one population, is also reported.

RESULTS

Application of microsatellite markers identified in *A. syriaca* to *A. tuberosa*

To test the performance of cross-species molecular markers, we amplified 6 microsatellite loci originally identified for common milkweed in butterfly milkweed. A total of 66 alleles were detected across all 6 loci in the 216 plants genotyped (Table 3). All markers were highly informative (mean PIC = 0.624, SD = 0.157), which

Table 2. Primer sequences and characteristics for six microsatellite loci developed in *Asclepias syriaca* used in *Asclepias tuberosa* (O'Quinn and Fishbein 2008).

Locus	Primer sequence (5′-3′)	Repeat motif	Size range (bp)	Melting temperature (Tm)
Asyr-B2	F: GCGTGGAATCTTGTCAAATTAAC *	(AAC)8	231-297	52
FJ478395	R: CCAAAGAATTGTGTACGATACC			51
Asyr-B5	F: CTCTTCAACCCCTACTCCTC *	(AAC)10	253-268	54
FJ478396	R: CCATCAATAACCATCCGTCTC			52
Asyr-B121	F: GTCAATCCAGAATTACTCGACTC *	(AAC)9	226-255	54
FJ478398	R: GAGTTCATCGTCACCTGATACT			53
Asyr-C102	F: CTTTCCGTACACTTCAAATTATGG **	(ATG)8	236-242	52
FJ478400	R: TACAAGATAAAATGACGGCTAAAG			51
Asyr-C109	F: TCAAACGCTGTGGAAGATAAACC **	(ATG)8	115-130	53
FJ478402	R: ATCATCATCCCCCAACTCTC			52
Asyr-C124	F: AGTCCAAACTAATCCCAGAGC **	(ATG)8	238-257	52
FJ478403	R: ATGAAAACAAGAACAGCAAGAAAG			51

*M13F tail 5′-CACGACGTTGTAAAACGAC-3′
**M13R tail 5′-GGATAACAATTTCACACAGG-3′

Table 3. Genetic variability per locus in *Asclepias tuberosa*.

Locus	k	N	H_e	H_o	PIC
B2	13	162	0.6467	0.7768	0.695
B5	8	177	0.3033	0.2522	0.329
B121	10	193	0.5770	0.6282	0.618
C102	13	194	0.6121	0.7253	0.641
C109	11	201	0.6337	0.7110	0.670
C124	11	185	0.7444	0.8249	0.793

k number of alleles, N total number of individuals scored for a given locus, H_e expected heterozygosity, H_o observed heterozygosity, PIC polymorphism information content

confirms their usefulness for analysing genetic diversity in this species. The number of alleles per locus was satisfactory (mean = 11.0, SD = 1.90). Expected heterozygosities at each locus were also within a desirable range (mean = 0.5862, SD = 0.1495). Locus B5 exhibited lower polymorphism but was still informative alongside the other markers. In addition to establishing the viability of these markers in butterfly milkweed, we also determined that the markers are not suitable for analyzing whorled milkweed (*A. verticillata*) or swamp milkweed (*A. incarnata*). Allelic diversity was quite low for many of the loci in the populations of whorled and swamp milkweed we sampled (data not shown).

Diversity within populations

We investigated the genetic diversity in populations of butterfly milkweed in 4 remnant prairies in Iowa, 1 population from a prairie restoration at Dordt College, and 1 seed source population from Oklahoma. Overall, we sampled 6 populations with an average of 36 individuals per location (Table 4). The average number of alleles per population ranged from 4.00 to 7.67. Interestingly, the average number of alleles in the Dordt College prairie restoration and Oklahoma seed source were comparable to the remnant prairies.

Comparisons of expected heterozygosity to observed heterozygosity gave no evidence of inbreeding. Across populations, the mean expected heterozygosity was 0.5814 (SD = 0.0406), while

Table 4. Within population diversity indices calculated from microsatellite data.

Population (n)	Number of Alleles	H_e	H_o	F_{IS}
Steele (40)	5.00	0.5660	0.6763	−0.1948[b]
Brewer (38)	5.83	0.6228	0.6585	−0.0574[b]
Br. Kettle (17)[a]	4.17	0.5500	0.7442	−0.3530[b]
Oklahoma (29)	4.00	0.5567	0.6417	−0.1527[b]
Dordt (47)	6.67	0.5501	0.5516	−0.0027[b]
Freda Haffner (45)	7.67	0.6425	0.7149	−0.1126[b]

H_e expected heterozygosity, H_o observed heterozygosity, F_{IS} inbreeding coefficient (Weir and Cockerham 1984)
[a]Sampled entire population at this location
[b]No significant departure from Hardy-Weinberg equilibrium using H1=heterozygote deficit with $p<0.001$

the mean observed heterozygosity was 0.6645 (SD = 0.0668). Heterozygosity deficit, as measured by Wright's inbreeding coefficient F_{IS}, was negative in all populations when averaged across loci. The average value of F_{IS} across loci and populations was −0.1455 (SD = 0.1223, all p values < 0.001). Based on these data, we found no genotypic evidence of inbreeding within the populations. In fact, there appears to be an excess of heterozygotes relative to expectations based on Hardy-Weinberg equilibrium.

Between population diversity

We examined the population structure of the remnant prairie fragments by comparing allelic and genotypic frequencies across different locations, including the restoration at Dordt College and a seed supplier from Oklahoma. A comparison of the allelic diversity across locations (Fig. 2) revealed relative consistency in significant alleles across populations (mean At 5% = 3.75, SD = 0.53). The average number of rare alleles per locus (alleles representing less than 5% of the alleles at a particular locus) varied substantially by location (mean = 1.80, SD = 1.15). There was no relationship between the size of the remnant and the allelic diversity or frequency of rare alleles. A comprehensive census of plants was not conducted for each location.

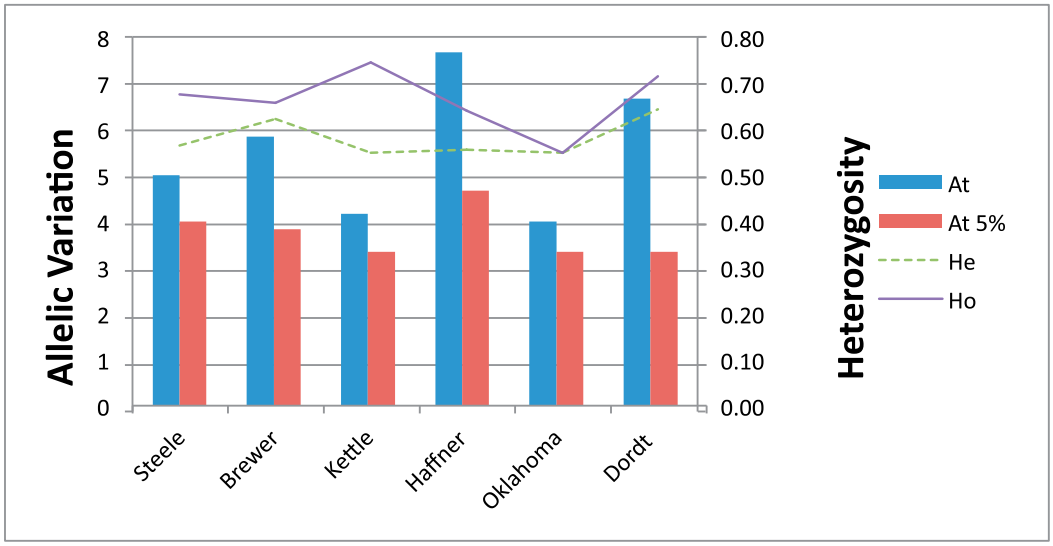

Fig. 2. Allele patterns for 6 microsatellite loci. At is average number of alleles, At 5% is the number of alleles with frequencies above 5%, H_e is the unbiased expected heterozygosity, H_o is the observed heterozygosity.

	Steele	Brewer	Br. Kettle	Fr. Haffner	Oklahoma	Dordt
Steele						
Brewer	0.03163					
Br. Kettle	0.05656	0.0339				
Fr. Haffner	0.04278	0.03308	0.07396			
Oklahoma	0.1068	0.09812	0.1041	0.1379		
Dordt	0.1122	0.07841	0.1006	0.1107	0.04387	

Fig. 3. Genetic differentiation between all pairs of populations obtained from microsatellite data. The blue and green colors indicate little (approximately 0.05 or less) and moderate (approximately 0.05 to 0.2) genetic differentiation, respectively, based on pairwise F_{ST} estimates (Rousset 2008).

Therefore, the relative abundance or distribution of butterfly milkweed at each location is unknown with the exception of the Broken Kettle sample, which represented the entire population.

Pairwise F_{ST} values were generated for each pair of locations (Fig. 3), and significant differentiation was detected for all of the pairwise comparisons ($p < 0.001$). Among remnants, the differentiation was generally low ($F_{ST} < 0.055$) with the exception of Freda Haffner-Broken Kettle ($F_{ST} = 0.074$), which are the 2 most distant from one another geographically. Pairwise F_{ST} values comparing the Dordt College restoration to the remnants were moderate (mean $F_{ST} = 0.100$). Pairwise F_{ST} values comparing the Oklahoma seed source to the remnants also showed a moderate degree of differentiation (mean $F_{ST} = 0.111$). Somewhat surprisingly, the Dordt College restoration and the seed from Oklahoma showed a low degree of differentiation ($F_{ST} = 0.04387$). The frequency of private alleles overall is relatively low among the populations sampled, given the inclusion of the Oklahoma and Dordt College samples in the analysis ($p(1) = 0.0358548$). There was a significant positive regression ($R^2 = 0.6411$, $p = 0.055$) between the level of differentiation as measured by F_{ST} and the geographical distance between remnants (Fig. 4).

DISCUSSION

Given the severe fragmentation and reduction of native prairies in Iowa, the possibility of both inbreeding and outbreeding depression constitutes a significant threat for many species (Edmands 2007). Among the populations tested there was no evidence of inbreeding, which would be shown by an excess of homozygous individuals. F_{IS} values were near 0 and/or negative (range = –0.353 to 0) indicating heterozygosity is at or above levels expected under Hardy-Weinberg equilibrium. The populations also show a low degree of differentiation, which may be an indication of adequate gene flow. More likely, there has been little opportunity for genetic drift to cause differentiation because of the relatively recent fragmentation of the prairie in Iowa (Bossart and Prowell 1998). A comparison study of butterfly milkweed across continuous prairies similar to that carried out by Williams et al. (2003) could clarify this issue.

Differentiation among prairies should inform the choice of seed sources for restoration efforts, particularly when population structure reflects historical patterns rather than genetic drift due to isolation. The relatively high frequency of rare alleles (those representing less than 5% of total alleles) coupled with the recent fragmentation of the prairie may indicate that there has not been a significant reduction in the genetic diversity of *A. tuberosa* across Midwestern prairies (Luikart and Cornuet 1998). The relatively low pairwise F_{ST} values among native remnants in the area compared to the moderate F_{ST} values between remnants and our restoration or the commercial seed source indicate that care should be taken to maintain local genetics during restoration efforts. Though plants in our restoration appear to be thriving, the prairie is a very dynamic environment, and non-adaptive genotypes have not been exposed to the full spectrum of environmental conditions typical of the area. Because the history of

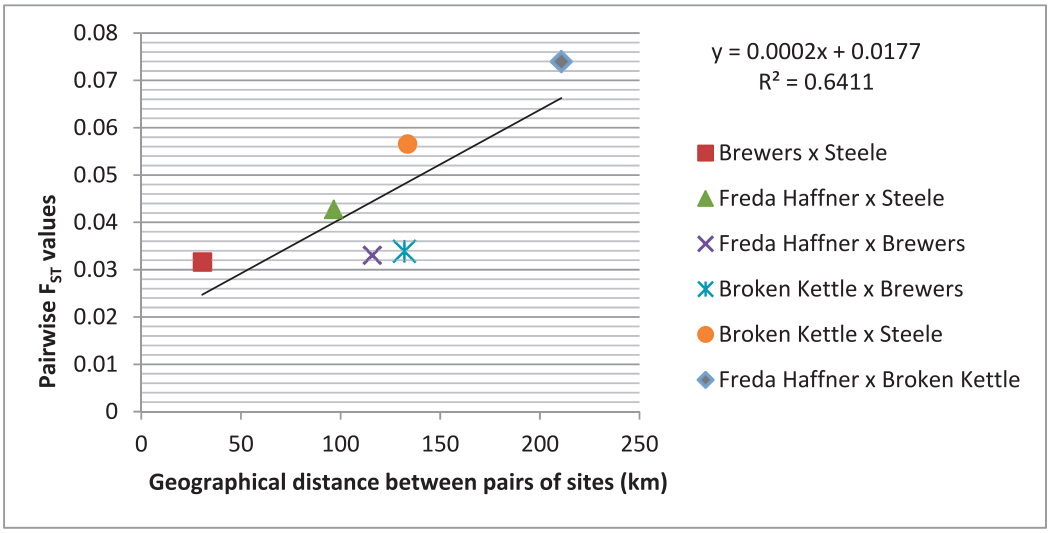

Fig. 4. Relationship between pairwise F_{ST} estimates and their geographical distance among butterfly milkweed populations from 4 remnant prairies.

seed production and restoration is not completely documented, it is possible that deleterious remixing of potentially co-adapted gene complexes will happen but is as yet undetected. Given the suggestion that most *Asclepias* species tend to out-cross (Ivey et al. 1999; Kephart et al. 1988), this is less likely to be a problem but is still worth considering, particularly if these results influence decisions about prairie restoration in general.

Genetic diversity might be lost when seed is collected and expanded commercially to provide the volume needed for restoration efforts. Our results indicate that in the case of both the Dordt College restoration and the seed from Oklahoma, allelic diversity is comparable to the populations found in native remnants. However, selection may have occurred on traits like timing of flowering and fruit set, seed size, seed weight, and dormancy factors through the seed collection and propagation process, which microsatellite analysis cannot address. A single selection event may have influenced a particular trait without overtly affecting the diversity of alleles across multiple neutral loci.

Despite the lack of evidence for inbreeding depression and reduced genetic diversity, efforts should be made to preserve the remaining diversity. It is possible that processes undetectable by microsatellite analysis are affecting fecundity. Direct evidence of breeding success from controlled crosses made in the field, within and between remnants, would be of great value. Furthermore, an examination of the relationship between specific environmental characteristics and genotypic variation could also be very helpful in the selection of seed sources for conservation and restoration efforts.

Seed expansion efforts appear to have had minimal impact on overall genetic diversity, indicating that source seed collection in the populations tested was sufficiently diverse. This should not be taken to mean that selection on particular traits has not occurred during the initial seed collection or post-expansion harvest processes. Depending on how widely such propagated seed is used, it still constitutes a potential danger in terms of outbreeding depression. The differences between the genetics of the propagated seed in the Dordt College restoration and the Oklahoma seed when compared to native remnants suggests that genotype-environment interactions and hybrid fitness are worth investigating.

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