Reproductive Biology and Ecological Genetics of Three Prairie Forbs

BY

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THESIS

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Eun Sun Kim

I collected *Asclepias lanuginosa* and *A. viridiflora* samples and extracted DNA for microsatellite genotyping. I optimized primers and conducted polymerase chain reaction. I scored microsatellite genotypes and conducted genetic data analyses. I wrote the manuscript.

David N. Zaya

DNZ helped with sample collection, DNA extraction, primer optimization, and scoring microsatellite genotypes. He conducted population viability analyses.

Jeremie B. Fant

JBF helped with sample collection and proofreading the manuscript.

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I contributed to conceptualization, data curation, data analysis, investigation, methodology, writing of original draft, and proofreading the manuscript.

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LIST OF ABBREVIATIONS

ABC	Approximate Bayesian Computation
AIC	Akaike information criterion
AICc	Akaike information criterion corrected for small sample size
AMOVA	Analysis of molecular variance
BN	Bagged, no hand pollination
DAPC	Discriminant analysis of principal components
DD	Different color, different populations
ddRADseq	double digest Restriction-Site Associated DNA sequencing
DNA	Deoxyribonucleic acid
DP	Dropseed Prairie
DS	Different color, same population
GLMM	Generalized mixed-effects model
GM	Gensburg-Markham Prairie
HP	Hoosier Prairie
IB1	Illinois Beach State Park 1
IB2	Illinois Beach State Park 2

LIST OF ABBREVIATIONS (continued)

LMM	Linear mixed-effects model
MC	Meissner Prairie-Corron Forest Preserve
MLG	Multilocus genotype
MW	Miller Woods, Indiana Dunes National Lakeshore
NR	Newark Road Prairie
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PS	Pine Station Nature Preserve
PVA	Population viability analysis
SD	Same color, different populations
SNP	Single nucleotide polymorphism
SP	Self-pollination
SP	Shaw Prairie, Skokie River Nature Preserve
SR	Sand Ridge Savana Nature Preserve
SS	Same color, same population
UPGMA	Unweighted pair group method with arithmetic mean

SUMMARY

Less than 1% of native tall grass prairie remains in the United States, and many native prairie plants have suffered drastic declines. Such extreme habitat loss poses several threats to native plant species, including population isolation, reduced gene flow, loss of genetic diversity, disruption of mutualisms or other biotic interactions, and limited mate availability. My work aimed at investigating the aspects of reproductive biology and population genetics of three iconic prairie forbs. One of the *Asclepias* species was endangered and I focused on genetic variability and clonal structure. For my work on scarlet Indian paintbrush, the focus was understanding the biology underlying bract color polymorphism.

In chapter 1, I investigated the role of genetic, reproductive and demographic factors in the decline of two co-occurring milkweeds, *Asclepias lanuginosa* and *A. viridiflora*, in fragmented populations in Illinois and Wisconsin. *Asclepias lanuginosa* flowers but does not set seed while seed set is regularly observed in *A. viridiflora*. I used microsatellite genotypes to determine the extent of clonal growth, genetic diversity, and genetic structure in nine populations of *A. lanuginosa* and five populations of *A. viridiflora*. Microsatellite genotyping revealed extremely high clonality in *A. lanuginosa*. For *A. lanuginosa*, only 32 multilocus genotypes occurred among more than 300 ramets, compared to 118 multilocus genotypes among 124 ramets for *A. viridiflora*. Four *A. lanuginosa* populations were monoclonal.

While there was no evidence for inbreeding, *A. lanuginosa* had significantly lower expected heterozygosity and a lower mean number of effective alleles than *A. viridiflora*.

Population Viability Analysis (PVA) conducted at one site indicated a high probability of persistence, although the population was comprised of only two clones. Because PVA does not distinguish between ramets and genets, results should be interpreted with caution when conducted on highly clonal species. A nearly complete shift in the mode of reproduction, from sexual to asexual, appears to be the most immediate threat to survival of *A. lanuginosa* in these fragmented prairie remnants. Conservation management strategies should include actions to increase clonal diversity in remnant populations.

In chapter 2, I investigated a possible mechanism for the maintenance of bract color polymorphism in scarlet Indian paintbrush (*Castilleja coccinea*). Populations of *C. coccinea* in the Midwestern United States exhibit a bract color polymorphism, with each population having predominantly yellow or scarlet bracts. I conducted hand-pollination experiments in two nearby populations, one predominantly yellow and one predominantly scarlet. The hand-pollination treatments were either self-pollination or cross pollination using pollen from within and between populations. Both color morphs were used as pollen donors for the within and between crosses.

I found that both color morphs of *C. coccinea* were self-compatible. When the scarlet morph was the maternal plant it had higher seed set. When pollinators were excluded, the yellow morph outperformed the scarlet morph in fruit set and seed set. The apparent trade-offs between a higher reproductive output in the scarlet morph and a reproductive assurance advantage in the yellow morph may explain the maintenance of the polymorphism in *C. coccinea*. While many

previous studies have provided evidence for pollinator preference playing a role in floral color polymorphism, the results of the current study indicate that reproductive assurance, which would be important for fluctuations in pollinator abundance or colonizing new areas, may act as a selective agent to maintain such polymorphisms.

Castilleja coccinea displays showy scarlet colored bracts throughout its range in eastern North America, but yellow colored bracts are predominant colors of some populations in the Midwestern United States. In chapter 3, I conducted population genomics of the Midwestern *C. coccinea* using 958 single nucleotide polymorphisms (SNPs) that were variable among 10 populations and 5,532 SNPs that were variable between the color morphs, which were discovered by double digest Restriction-Site Associated DNA sequencing (ddRADseq). I evaluated possible evolutionary history scenarios in this region using approximate Bayesian computations. Results suggest the distribution of bract colors is not correlated to the patterns of population structure and genetic diversity. Further, the two color morphs were not genetically differentiated. Although geographic distance or evolutionary history scenarios failed to explain the apparent population structure and genetic differentiation, I found some indirect evidence that neutral processes may be responsible.

In chapter 4, a hand-pollination experiment was used to determine inheritance patterns of bract colors in *C. coccinea*. I conducted a hand-pollination experiment among yellow, orange, and scarlet bract colored individuals. Bract colors of pollen donors (paternal plants) and

recipients (maternal plants) were varied to determine transmission patterns of parental bract colors to offspring. Seeds resulting from hand-pollinations were collected, sown, and grown until offspring plants flowered. Assessment of the resulting offspring bract colors suggests that bract color of *C. coccinea* is likely primarily controlled by multiple alleles at a single locus and follows an autosomal dominant mode of inheritance with yellow dominant over scarlet and orange. Anthocyanins and carotenoids are most likely responsible for bract colors in *C. coccinea*.

My original plans for studying bract color polymorphism in *Castilleja coccinea* included: 1) a larger scale study of population structure across the species range, including populations in the unglaciated regions; 2) a paternity study using 22 maternal plants and offspring plants from an experimental garden to assess mating patterns; and 3) comparison of outcrossing rates, selfing rates, and effective number of pollen donors between the color morphs using samples from natural populations. However, microsatellite markers did not consistently amplify between samples and showed reduced genetic variation. These technical difficulties with microsatellites impeded my original plans and research objectives. This unforeseen hindrance eventually led me to explore a different genetic marker (SNPs) and broaden my research scope to include hand-pollination experiments.

1. GENETIC FACTORS ACCELERATE DEMOGRAPHIC DECLINE IN RARE *ASCLEPIAS* SPECIES

The chapter is a reprint (with minimal reformatting) of an original article published in the journal *Conservation Genetics*. The citation is as follows:

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1.1 Introduction

Controversy exists over the relative importance of genetic factors in driving extinctions (Lande 1988; Avise 1989; Caro and Laurenson 1994; Spielman et al. 2004; Frankham 2005). Depending on taxa, genetic factors may be more important than demographic factors, or they may interact to exacerbate the decline of a species (Spielman et al. 2004; Ouborg et al. 2006). In the case of plant species impacted by anthropogenic habitat fragmentation, it can be particularly difficult to disentangle genetic and demographic factors, and comprehensive research may be required to best inform conservation management strategies.

Habitat fragmentation leads to creation of small remnants that are separated by unsuitable habitat, and has been shown to cause reproductive (Cunningham 2000; Wilcock and Neiland 2002; Newman et al. 2013) and demographic (Bruna 2002; Bruna and Kress 2002; Hobbs and Yates 2003) decline in a number of plant species. However, the specific impact or impacts of fragmentation will depend on the plant's breeding system, mating pattern, pollen and seed

dispersal, and life history traits (Aguilar et al. 2008). As a consequence of fragmentation, a population may experience a decline in gene flow and reduction in effective population size. Genetic drift and chance fixation of alleles in these smaller populations may erode genetic diversity. Fragmented plant populations can experience changes in mating patterns, with the possibility of increased selfing, breeding among relatives, or a shift towards clonal reproduction rather than seed production (Honnay and Bossuyt 2005). These processes can lead to further genetic decline and potentially inbreeding depression, with a reduction in individual and population fitness.

For self-incompatible plant species, reduction in genotypic diversity can lead to mate limitation (Young et al. 2012). For example, DeMauro (1993) demonstrated the role of limited compatible mates in lack of seed set for *Hymenoxys acaulis*, a sporophytic self-incompatible species. A remnant population that had not set seeds for over 15 years consisted of a single mating type. In remnant populations of the self-incompatible prairie perennial *Echinacea angustifolia*, mean mate compatibility was correlated with population size, and was as low as 25% in the smallest remnants (Wagenius et al. 2007).

Pronounced shifts to clonal reproduction may pose another threat to outcrossing clonal plant species in fragmented landscapes (Honnay and Bossuyt 2005), a threat that has been largely unappreciated and understudied. Shifts to clonal reproduction have been observed in self-incompatible species in small, fragmented populations (Aguilar et al. 2006; Gitzendanner et al. 2012) as well as in self-compatible species in peripheral populations (Dorken and Eckert 2001). Management practices can also alter the ratio between sexual and clonal reproduction, as shown

for *Asclepias meadii*, where mowing inhibited sexual reproduction, leading to exclusively clonal spread and attrition of genotypes compared to burning (Tecic et al. 1998). Although clonal growth can allow a species to persist under stressful conditions, uneven selection and clonal competition can greatly reduce the genotypic diversity of a population even when the number of ramets remains relatively constant. Conservation risk assessment approaches such as population viability analysis (PVA) typically do not incorporate shifts to asexual reproduction in estimating the likelihood of extinction. Often only genetic tests can determine the level of clonality versus sexual reproduction (Wilk et al. 2009) and distinguish between ramet and genet counts.

Habitat fragmentation may also cause population declines irrespective of genetic issues. It may cause edge effects that reduce fitness by introducing invasive species or by altering soil moisture, light, or herbivore communities, thus reducing the suitability of habitat (Fahrig 2003). Random fluctuations in population size are expected to be greater in smaller populations, which increase the likelihood of small populations falling to unsustainably low numbers (Lande 1988). Small populations are more susceptible to being wiped out by environmental stochasticity and chance events associated with weather or other external factors (Morris and Doak 2002). Pollen limitation may also occur because of reduced pollinator activity (Moody-Weis and Heywood 2001; Gonzalez-Varo et al. 2009; Potts et al. 2010).

The milkweed genus *Asclepias* contains over 140 species of perennial, herbaceous plants best known for their production of a milky juice containing latex and alkaloid compounds that are often poisonous. *Asclepias* species have a complex pollination mechanism that requires insect pollinators to remove and insert pollinia (pollen sacs), effected primarily by

hymenopterans and lepidopterans. The genus contains both self-compatible and self-incompatible species. Milkweed seeds are relatively large with a tuft of comose hairs to aid in dispersal by wind. A number of *Asclepias* species are federally and state-listed rare species. Some populations of these threatened species rarely produce seed and are currently being maintained only through asexual reproduction, including *A. lanuginosa*, *A. meadii*, and *A. welshii* (Betz 1989; Palmer and Armstrong 2000).

In this study, we investigate the impact of habitat loss and habitat fragmentation on two species of *Ascleipias*, *A. lanuginosa* (woolly milkweed) and *A. viridiflora* (green milkweed), that are rare in the Midwestern United States and restricted to high quality remnant prairies (Swink and Wilhelm 1994). At our study sites, *A. lanuginosa* seldom produces seed pods (Betz and Lamp 1990), but *A. viridiflora* regularly sets seed. This provides a useful comparison because abiotic and biotic conditions, including pollinators, will be similar where the two species co-occur. We used nuclear microsatellite DNA markers to address four main objectives. The first was to determine the extent of clonal growth in *A. lanuginosa* and *A. viridiflora*. If a shift towards asexual reproduction is more pronounced in *A. lanuginosa* relative to *A. viridiflora*, this may be associated with poor seed set in *A. lanuginosa*. Second, we compared genetic variability of *A. lanuginosa* to *A. viridiflora*. Lower levels of diversity in *A. lanuginosa* could suggest genetic drift, biparental inbreeding and/or selfing as potential factors contributing to lack of seed set. Third, we compared the genetic variability of *A. lanuginosa* populations that have recently set seed to those that have not, to look for correlations between genetic diversity and reproductive failure. Finally, the results of the genetic analyses from one site were compared to population viability analysis conducted by using 20 years of monitoring data to compare the

outcomes of the two approaches. The results of this study will be used to provide recommendations for an *A. lanuginosa* recovery plan and address the importance of genetic diversity in declining, fragmented species.

1.2 Materials and methods

1.2.1 Study species

Asclepias lanuginosa (woolly milkweed) and *A. viridiflora* (green milkweed) are rare prairie forbs with declining populations as a result of the loss of native prairie habitat. In Illinois they are both restricted to high quality dry remnant prairie (Swink and Wilhelm 1994). Both species are short perennial milkweeds, ranging from 15 to 30 cm tall for *A. lanuginosa* and 30 to 60 cm tall for *A. viridiflora*. Of the two, *A. lanuginosa* has seen a more severe decline throughout most of its range, which extends from Wisconsin to North Dakota and south to Kansas. It is a state-listed endangered species in Illinois and threatened in Iowa and Wisconsin. In Illinois, the remaining populations are fragmented and isolated, and while profuse flowering is observed there is almost no seed set. For example, in 1990 researchers found only two out of 437 flowering stems set seeds in Illinois and Indiana populations (Betz and Lamp 1990). *Asclepias viridiflora* is rare in Illinois (Plants of Concern 2013), listed as special concern in Connecticut, endangered in Florida, and threatened in New York (USDA, NRCS 2013). Betz and Lamp (1990) reported seed set was as high in this species as in the widespread common milkweed (*Asclepias syriaca*). The breeding system of these two species has not been investigated, but self-incompatibility has been reported for several congeners, including *A. exaltata, A. perennis, A.*

subulata, A. texana, A. tuberosa, and A. meadii (Kephart 1981; Wyatt and Broyles 1994; Lipow and Wyatt 2000). Self-compatibility has also been observed in a few *Asclepias* species, including *A. incarnata* (Willson and Price 1977), *A. curassavica,* and *A. fruticosa* (Wyatt and Broyles 1997). Both focal species can spread clonally.

1.2.2 Study sites, sample collection, and pollinator observation

In May and June, 2011, we surveyed thirteen sites in Illinois and Wisconsin where *A. lanuginosa* had been reported within the past 20 years. Only eight of these sites had *A. lanuginosa*, four in Illinois and four in Wisconsin (Table I, Figure 1). A ninth site was discovered and sampled in June, 2012 (IL5, Table I, Figure 1). At most sites the plants had a clustered distribution. We collected multiple leaf samples from each cluster and recorded the distance between the closest ramets. Leaves from a total of 324 ramets of *A. lanuginosa* were collected and dried in silica gel for later DNA analysis (Table I). We sampled *A. viridiflora* at five sites identified from the Plants of Concern database in June 2011 and June 2012 (Plants of Concern 2013). Leaf samples from a total of 124 *A. viridiflora* ramets were collected for DNA analysis (Table I). At four of the sites (IL2, IL3, IL4, and IL5) *A. viridiflora* co-occurred with *A. lanuginosa*. Information on seed set was obtained from the Plants of Concern database and/or resource managers. The actual site names and locations are kept confidential to protect both species; the coded site names are listed in Table I. Pollinator observations on *A. lanuginosa* were conducted on June 8, 2012 at IL4, one of the sites where the species co-occurred. The observer recorded each visitor, frequency of visit, and whether pollinia was removed and attached to the floral visitor.

Table I. Indices of clonal diversity and genetic diversity of *Asclepias lanuginosa* and *A. viridiflora*.

Populations	Reproductive	No. of ramets genotyped	No. of MLGs	Range of ramets/ genet	D	$\frac{G-1}{N-1}$	A_e	H_o	H_e	F_{IS} (W&C)
A. lanuginosa										
(a) Illinois										
IL1	Y (+++)	63	6	4-14	0.82	0.08	2.31	0.63	0.55	-
IL2	Y (+)	41	6	1-94	0.59	0.03	2.36	0.56	0.50	-
IL3	N	8	1	8	0	0	1.98	0.63	0.31	n/a
IL4	N	13	2	7-19	0.41	0.04	1.63	0.63	0.44	-
IL5	N	5	1	5	0	0	1.38	0.38	0.19	n/a
IL Mean (SE)							1.93 (0.19)	0.57 (0.048)	0.40 (0.066)	
(b) Wisconsin										
WI1	N	39	1	39	0	0	1.50	0.50	0.25	n/a
WI2	Y (++)	78	3	9-50	0.52	0.03	1.79	0.42	0.40	-
WI3	N	4	1	4	0	0	1.50	0.50	0.25	n/a
WI4	Y	73	11	1-14	0.88	0.14	2.65	0.52	0.52	C124* (0.48)
WI Mean (SE)							1.86 (0.27)	0.49 (0.022)	0.36 (0.065)	
Mean (SE)							1.90 (0.095)*	0.53 (0.046)	0.38 (0.029)*	
A. viridiflora										
IL2	Y	19	16	1-3	0.98	0.83	2.38	0.59	0.54	B102* (-0.46) C4* (0.15)
IL3	Y	24	23	1-2	1.00	0.96	2.28	0.58	0.54	C102*(0.015)
IL4	Y	43	43	1	1	1	2.39	0.58	0.55	C124* (0.18) C102** (-0.35)
IL5	Y	25	23	1-3	0.99	0.92	2.72	0.67	0.56	B102* (-0.45)
IL6	Y	13	13	1	1	1	2.49	0.55	0.54	-
Mean (SE)							2.45 (0.16)*	0.59 (0.026)	0.55 (0.022)*	

Notes: All *A. viridiflora* sites are in Illinois. Each population was considered reproductive if seed pods have been reported in last 10 years (+ 1 pod was seen, recorded, and disappeared; ++ at least 1 pod was seen and recorded; +++ multiple pods were observed in one patch). MLG is multilocus genotype, *D* is the complement of Simpson's index corrected for finite size. In $\frac{G-1}{N-1}$, *G* is number of genets and N is number of ramets. A_e is number of effective alleles, H_o is observed heterozygosity, H_e is expected heterozygosity, *SE* is standard error, and F_{IS} is inbreeding coefficient (Weir and Cockerham 1984). For A_e, H_o, and H_e, means were calculated separately for Illinois (IL mean) and Wisconsin populations (WI mean). Asterisks indicate significant differences between species. In column F_{IS}, only loci that had significant F_{IS} values are listed with F_{IS} values in the parentheses. F_{IS} values are not applicable (n/a) in monoclonal populations (* $p < 0.05$ and ** $p < 0.02$).

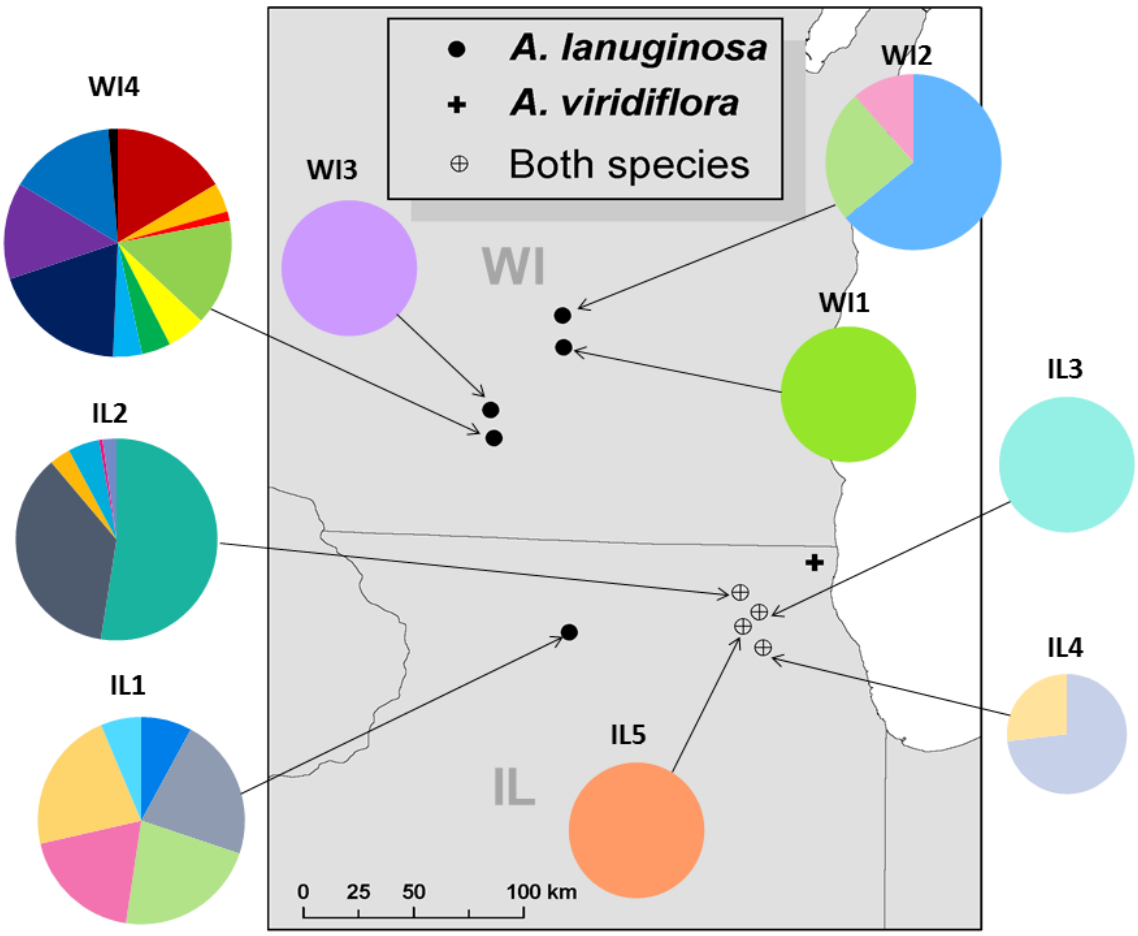

Figure 1.1. Map showing ten sites in Illinois (IL) and Wisconsin (WI) where samples of *Asclepias lanuginosa* and *A. viridiflora* were collected. Pie charts show the number of genets found at each site for *A. lanuginosa* and the ramets per genet ratio. Areas of pie charts are proportional to the total number of ramets at each site.

1.2.3 DNA extraction and microsatellite analysis

Genomic DNA was extracted from leaf samples using the DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA). The concentration of DNA in each extraction was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). We used eight microsatellite primer pairs previously developed for other *Asclepias* species. Seven primers, B5, B102, C4, C102, C103, C109, and C124, were developed in *A. syriaca* and cross-amplified in *A. exaltata* (O'Quinn and Fishbein 2009). One primer, ASF9, was developed for *A. syriaca* (Kabat et al. 2010). All eight primer pairs amplified polymorphic microsatellite loci in *A. lanuginosa*, and all except ASF9 consistently amplified *A. viridiflora* loci following polymerase chain reaction (PCR) optimization. PCR was conducted following the protocol from Schuelke (2000) and Abraham et al. (2011). We analyzed fragment sizes of PCR product (using 1.0 – 1.5 μL) with the ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA), using a LIZ500 ladder. We scored all microsatellite genotypes using GeneMapper software, version 3.7 (Applied Biosystems).

1.2.4 Genetic data analyses

The ALLELEMATCH package for R was used to identify matching multilocus genotypes (MLGs) and to determine P_{sib} (Galpern et al. 2012; R Core Team 2012). We used alleleMismatch parameter, $\hat{m}$, of 1 for both species which allowed two genotypes to differ at one allele and still be considered identical. P_{sib} is the probability of two individuals having identical MLGs due to sibling relationship rather than because they are clones. For subsequent analyses, we collapsed

ramets into genets, which resulted in 32 unique MLGs for *A. lanuginosa* and 118 unique MLGs for *A. viridiflora* ($\alpha < 0.05$). The range of number of ramets per genet was determined for each site. Clonal diversity was measured using the complement of the Simpson's index corrected for finite size, *D*, (Pielou 1969; Ellstrand and Roose 1987; Diggle et al. 1998) and also using $\frac{G-1}{N-1}$ where *G* is the number of genets and *N* is the number of ramets (Brzosko et al. 2002; Arnaud-Haond et al. 2005; Fant et al. 2008). To cluster populations based on genetic distances (θ) between populations, GDA 1.1 was used to conduct the unweighted pair group method with arithmetic mean (UPGMA) (Lewis and Zaykin 2001). Principal coordinate analysis (PCoA) was conducted to cluster MLGs based on genetic distance using GenAlEx V6.5 (Peakall and Smouse 2006, 2012). A Mantel test was conducted to test for association between genetic distances (using Jost's D_{est} values) and geographic distances using adegenet, mmod, and ade4 packages in R (Dray and Dufour 2007; Jombart 2008; Jombart and Ahmed 2011; Winter 2012). For the five sites of *A. lanuginosa* with two or more genets and all five sites of *A. viridiflora*, analysis of molecular variance (AMOVA) was conducted using GenAlEx V6.5 (Peakall and Smouse 2006, 2012) to identify hierarchical population genetic structure by determining genetic variation within and among populations. For populations with two or more MLGs, Nei's bias corrected G_{ST}, and Jost's D_{est} values, along with p-values based on bootstrapping of 1000 iterations, were estimated for pairwise comparisons of populations using DEMEtics in R (Nei and Chesser 1983; Jost 2008; Gerlach et al. 2010). When using highly variable markers such as microsatellites, Jost's D_{est} may be more informative than G_{ST} as a population differentiation measure because their maximum possible value G_{ST} is dependent on within population diversity (Jost 2008). We tested for heterozygote deficiency by determining Weir and Cockerham's (1984) estimate of F_{IS} for each site using GenePop on the Web Version 4.0.10 (Raymond and Rousset 1995; Rousset

2008). We tested for evidence of inbreeding by running binomial tests to determine the probability of finding a certain number of positive F_{IS} values that are significant with $\alpha < 0.05$. Permutation tests were conducted to compare the number of effective alleles, observed heterozygosity, expected heterozygosity, number of multilocus genotypes, Simpson's index for finite samples, and $\frac{G-1}{N-1}$ between the two species, between *A. lanuginosa* populations that had and had not set seed recently, and between Illinois and Wisconsin *A. lanuginosa* populations. All permutation tests were executed in R using the lmPerm package (Wheeler 2010).

1.2.5 Population viability analyses

A detailed record of *A. lanuginosa* at the IL2 site from 1991 to 2012 (except for 2007) was available from Barbara Wilson, a resource manager (data not shown). We used stem count data from one subpopulation at IL2 to conduct a count-based population viability analysis (PVA). We compared the fit of four models by calculating maximum log likelihoods and comparing AICc values (Burnham and Anderson 1998), as outlined in Morris and Doak (2002). The four models were a density-independent model and three density-dependent models: Ricker model, theta logistic model, and an Allee model that includes both negative density dependence and positive density dependence. Refer to Morris and Doak (2002; Chapter 4) for the details on each model.

The density-independent model was parameterized with a linear regression where the intercept was forced through zero. The density-dependent models were all parameterized with non-linear regression. AICc values for all models were used to calculate Akaike weights and to determine the best-fitting model.

We tested for temporal autocorrelation in λ (the finite rate of population growth) using two tests, the Durbin-Watson test and a linear regression estimating the first-order autocorrelation of residuals. We tested for outliers in the linear regression with visual inspection and t-test of residuals. For non-linear least squares regressions we used jackknife resampling with the nlstools package (Baty and Delignette-Muller 2013) in R (R Core Team 2012). We tested for normality of residuals in the best model with a Shapiro-Wilk test.

The cumulative probability of extinction was estimated in two ways. First, we simulated 99,999 *A. lanuginosa* populations with an initial population size of 163, the number of stems counted in 2011 when the samples for the genetic study were collected. We used randomly generated normal values to simulate inter-annual growth rate using the mean growth rate and standard deviations of rates (Morris and Doak 2002; Chapter 4). Second, we calculated the probability of extinction using the diffusion approximation of the stochastic exponential growth model (Dennis et al. 1991; Morris and Doak 2002), and parametric bootstrapping was used to determine 95% confidence limits through time (99,999 resamples). Again, we used an initial population size of 163. Populations were modeled 50 years into the future, with separate models or simulations for quasi-extinction levels of 3, 5, 10, and 20 individuals.

1.3 Results

1.3.1 Population surveys and pollinator observation

In 2011, *Asclepias lanuginosa* was found at only eight of 13 sites where the species was previously reported, indicating recent extirpations. Where *A. lanuginosa* occurred, the total number of ramets observed varied greatly among sites, from 4 at WI3 to 179 at IL2. Flowering *A. lanuginosa* ramets were observed in all sites except IL3 and IL5. *Asclepias viridiflora* co-occurred on the same hillside at IL3 and IL4, and on neighboring hillsides at IL2 and IL5. At IL6 there were no *A. lanuginosa* populations. The number of *A. viridiflora* stems censused ranged from 24-219, similar in size to populations of *A. lanuginosa*. Seed set was reported for *A. viridiflora* at all sites where it co-occurred with *A. lanuginosa*.

Pollinator observations at IL4 population found one species of prairie beetle, two species of bee, and seven species of ant found in inflorescences (Table II). Only one individual bee (*Bombus griseocollis)* carried pollinia.

Table II. Floral visitors seen on *Asclepias lanuginosa* at IL4 on June 8, 2012.

Family	Species name	Note
Apidae	*Bombus griseocollis**	Pollinia on face, mouth parts (labrum), and all six legs
Chrysomelidae	*Acanthoscelides seminulum*	Common on the inflorescences
Formicidae	*Dorymyrmex grandulus*	Common visitor to the inflorescences
Formicidae	*Crematogaster* sp	Common visitor to the inflorescences
Formicidae	*Formica incerta*	Common visitor to the inflorescences
Formicidae	*Formica vinculans*	Occasional visitor to the inflorescences
Formicidae	*Lasius neoniger*	Occasional visitor to the inflorescences
Formicidae	*Myrmica evanida*	Common visitor to the inflorescences
Formicidae	*Monomorium minimum*	Common visitor to the inflorescences
Formicidae	*Nylanderia parvula*	Occasional visitor to the inflorescences
Megachilidae	*Megachile brevis* Say	No pollinia on body

Note: Asterisk indicates pollinia found on body parts.

1.3.2 Clonal structure

We identified 32 unique MLGs for *A. lanuginosa* and 118 for *A. viridiflora*. The P_{sib} values were less than 0.05 for all MLG matches for both *A. lanuginosa* and *A. viridiflora*. No populations shared MLGs. The number of MLG per site ranged from 1 to 11 for *A. lanuginosa* and from 13 to 43 for *A. viridiflora* (Table I). The largest sampled genet for *A. lanuginosa* had 94 ramets in contrast to three ramets for the largest *A. viridiflora* genet. The Simpson's index for finite samples, *D*, ranged from 0 to 0.88 for *A. lanuginosa* and from 0.98 to 1 for *A. viridiflora*. The $\frac{G-1}{N-1}$ values ranged from 0 to 0.14 for *A. lanuginosa* and from 0.83 to 1 in *A. viridiflora* (Table I). Several measures of clonal diversity were significantly lower in *A. lanuginosa* populations ($n = 9$) than in *A. viridiflora* populations ($n = 5$). *Asclepias lanuginosa* had 84% fewer MLGs per population ($P < 0.001$), and the Simpson's indices were also significantly lower for *A. lanuginosa* (0.63 absolute difference, $P = 0.003$), as well as $\frac{G-1}{N-1}$ (0.91 absolute difference, $P < 0.001$).

We also found significant differences between reproductive and non-reproductive *A. lanuginosa* populations, with non-reproductive populations having 82% fewer MLGs than reproductive populations ($P = 0.009$), lower Simpson's indices (0.62 absolute difference, $P = 0.001$), and lower $\frac{G-1}{N-1}$ (0.062 absolute difference, $P = 0.040$). No significant differences were found between Illinois and Wisconsin *A. lanuginosa* populations in terms of the number of MLGs, Simpson's indices, and $\frac{G-1}{N-1}$ ($P > 0.7$).

1.3.3 Genetic variability

The number of effective alleles (A_e), observed heterozygosity (H_o), expected heterozygosity (H_e), and inbreeding coefficient (F_{IS}) for each site were calculated after collapsing ramets into genets (Table I). Permutation tests found significant differences between *A. lanuginosa* and *A. viridiflora* populations (n = 9 and 5, respectively), with *A. lanuginosa* having 22% fewer effective alleles (P = 0.023) and a 0.17 absolute difference in expected heterozygosity (P = 0.017). There was no significant difference in observed heterozygosity (0.06 absolute difference, P = 0.18).

Significant differences in genetic diversity were also found between reproductive and non-reproductive *A. lanuginosa* populations (n = 4 and 5, respectively), with a 30% decrease in non-reproductive populations for the number of effective alleles (P = 0.010) and 0.20 absolute difference in expected heterozygosity (P = 0.008). There was no significant difference in observed heterozygosity (0.005 absolute difference, P = 0.95). No significant differences were found between Illinois and Wisconsin *A. lanuginosa* populations in the number of effective alleles, expected heterozygosity, and observed heterozygosity (P > 0.2). Significantly positive F_{IS} values, an indication of inbreeding, were tested for each locus in each individual population. For *A. lanuginosa*, no loci had significantly positive F_{IS} values. For *A. viridiflora*, we found three significantly positive F_{IS} values: in IL2 at C4 (F_{IS} = 0.15, P = 0.030); in IL3 at C102 (F_{IS} = 0.015, P = 0.045); and in IL4 at C124 (F_{IS} = 0.18, P = 0.012). However, binomial tests indicate that

there is a relatively high probability of finding three positive F_{IS} values out of 35 F_{IS} values in the null case, $\alpha = 0.05$ ($P = 0.3$). Thus, neither species appears to be experiencing inbreeding at our study sites.

The level of population differentiation was low in both species, albeit significant in most of the pairwise comparisons for *A. viridiflora* (Table III). Negative G_{ST} and D_{est} values, likely resulting from small sample size and using bias-corrected estimates of H_T and H_S, were converted to zero (Table III). Pairwise G_{ST} ranged from 0 to 0.044 for *A. lanuginosa* and from 0.00079 to 0.060 in *A. viridiflora* (Table III). Pairwise Jost's D_{est} values were relatively low, ranging from 0 to 0.18 for *A. lanuginosa* and from 0.022 to 0.13 for *A. viridiflora*. Statistical tests could not be conducted for G_{ST} values from *A. lanuginosa* pairwise comparisons due to small sample sizes. Pairwise D_{est} values for *A. lanuginosa* were not significant (Bonferroni corrected $P > 0.05$). In contrast, most of the G_{ST} and D_{est} values obtained from *A. viridiflora* pairwise comparisons were significant (Bonferroni corrected $P < 0.05$). The Mantel test did not find evidence of isolation by distance in either species ($P = 0.7$ for *A. lanuginosa* and $P = 0.4$ for *A. viridiflora*, 999 replicates for both species) and the UPGMA phenogram did not cluster populations that are closer geographically (results not shown). The PCoA did not cluster MLGs that are from same populations (results not shown). The results of the AMOVA indicated that most of the genetic variation was found within populations (95% for *A. lanuginosa* and 94% for *A. viridiflora*) as opposed to among populations for both species.

Table III. Pairwise G_{ST} and Jost's D_{est} averaged across loci for populations with two or more MLGs.

A. lanuginose					
	IL1	IL2	IL4	WI2	WI4
IL1	-	*0.04474*	*0*	*0.02332*	*0.02419*
IL2	0.1811	-	*0*	*0.02614*	*0.01747*
IL4	0	0.08391	-	*0*	*0.009169*
WI2	0.1002	0.1258	0	-	*0.03225*
WI4	0.07297	0.07379	0	0.1292	-
A. viridiflora					
	IL2	IL3	IL4	IL5	IL6
IL2	-	0.04561*	0.01258	0.03465*	0.006777
IL3	0.1047*	-	0.03753*	0.06429*	0.04213*
IL4	0.04357*	0.1027*	-	0.03445*	0.003986
IL5	0.1052*	0.1429*	0.1092*	-	0.03862*
IL6	0.03857	0.1089*	0.007837	0.1099*	-

Notes: G_{ST} is shown above diagonal and D_{est} below diagonal. For populations with two or more MLGs, Nei's bias corrected G_{ST}, and Jost's D_{est} values, along with p-values based on bootstrapping of 1000 iterations, were estimated for pairwise comparisons of populations using DEMEtics in R. Asterisks indicate significant values (Bonferroni corrected $p < 0.05$). Negative G_{ST} and D_{est} values, likely resulting from small sample size and using bias-corrected estimates of H_T and H_S, were converted to zero. Bonferroni corrected p-values could not be determined for *A. lanuginosa* pairwise comparisons (italicized values) due to small sample sizes.

1.3.4 Population viability analysis

For the count-based PVA of one subpopulation at the IL2 site, the density-independent model had the best fit, with an Akaike weight of 0.729, compared to 0.188 for the next best-fitting model. There were no significant outliers, as determined after visual inspection of residuals, testing for normality using the Shapiro-Wilk normality test of standardized residuals

(W = 0.96, P = 0.57) and studentized residuals (W = 0.964, P = 0.65), and a t-test of the most extreme residual (t = 2.0, df = 17, unadjusted P = 0.06, Bonferroni corrected $P > 0.9$). There was no evidence for temporal autocorrelation of annual growth rates using either the Durbin-Watson test (DW = 1.8, P = 0.66) or a test of the first-order residuals (t = 0.034, df = 16, P = 0.86). Also, the distribution of log-annual growth rates was not significantly different than normal (Shapiro-Wilk normality test, W = 0.96, P = 0.57). The estimate of the overall finite rate of log population growth was 0.037. Estimates of the cumulative probability of quasi-extinction through time were similar for both the growth rate simulations and the diffusion approximation model. For the diffusion approximation model, the best estimate of the probability of extinction for a quasi-extinction threshold level of 10 after 10 years was 0.002, 0.023 after 25 years, and 0.059 after 50 years, although the confidence limits indicate uncertainty. The likelihood of extinction was sensitive to the quasi-extinction threshold, but the best estimate remained low regardless of the threshold used.

1.4 Discussion

Here we investigated potential causes for the lack of seed set in fragmented populations of *Asclepias langinosa*. Hypothesized causes include self-incompatibility, extensive clonal growth, inbreeding depression, and a reduced number of effective pollinators (Betz 1989). Our project was designed to distinguish among these potential causes and to develop effective management strategies. Our survey in 2011 indicated that *A. lanuginosa* was apparently extirpated at four of eight sites where the species had been recently reported in Illinois, indicating that the species is continuing to decline in this part of its range. However, at most of

the sites where the species still occurred, we observed a large number of flowering ramets. This suggests that resources are not limiting flowering and growth at these sites, but rather other factors are preventing seed set. Both *A. langinosa* and *A. viridiflora* have a complex pollination mechanism they share with other species of *Asclepias*. Fertilization requires insects that are able to both remove and insert pollinia, and only a few large insects can act as effective pollinators (Wyatt 1976; Kephart 1983; Wyatt and Broyles 1994). At four of our study sites, *A viridiflora* was growing nearby, flowering, and setting seed (Table I), indicating the presence of effective pollinators. Pollinator observations at the IL4 site also suggest that potential pollinators were present (Table II), although perhaps at reduced numbers. One bee (*Bombus griseocollis*) was observed carrying pollinia; it is a common native bee in Illinois (Grixti et al. 2009). Together these findings suggest that lack of pollinators cannot fully explain the absence of seed set for *A. lanuginosa*.

The most dramatic finding from our study was the difference in the clonal diversity of the two species. Compared to *A. viridiflora*, *A. lanuginosa* had far fewer MLGs per site and a much higher number of ramets per genet at each site. The extensive clonal growth found in *A. lanuginosa* populations suggest this species is persisting at many sites solely through clonal growth, with only 32 MLGs found among more than 300 ramets. In contrast, 118 MLGs were found among the 124 *A. viridiflora* samples that were genotyped. This difference is unlikely due to sampling differences because we used similar sampling methods for the two species. While *A. viridiflora* is capable of clonal growth, our results suggest that the current populations of this species were mostly established by sexual reproduction, whereas reproduction in *A. lanuginosa*

appears to be almost entirely asexual. This is consistent with monitoring efforts which have shown that seed pods are consistently formed in *A. viridiflora* but not in *A. lanuginosa*.

The high level of clonality of *A. lanuginosa* demonstrated through genotyping could not be detected in the field. Clones were spread across physically separated patches of ramets. For example, at IL2 we found four patches consisting of 159 ramets. Genotyping these ramets revealed that there were only two genets with 94 and 65 ramets each. Neighboring clusters separated from each other by over two meters were comprised of the same clone. One clonal patch found at WI2 consisted of 50 ramets of the same MLG, with some ramets separated by as much as six meters. These results show that genets of highly clonal species cannot be ascertained based on distances between patches, and attempts to do so may greatly overestimate clonal diversity (Wilk et al. 2009).

For *A. lanuginosa*, the greatest number of MLGs, 11, was found at WI4, the only site where seed set was annually observed as recently as 2010 (personal communication with R. A. Henderson). Although evidence from a single site must be interpreted with caution, this finding may be important when we consider the breeding system of *Asclepias* species. If *A. lanuginosa* is self-incompatible like many of its congeners, the limited number of compatible mates may explain poor seed set because most sites had less than three genets and four were monoclonal. Other studies of self-incompatible plants have demonstrated that small, isolated populations show reduced seed set or even complete failure of sexual reproduction (DeMauro 1993; Vekemans et al. 1998; Glemin et al. 2008; Scobie and Wilcock 2009). Our results suggest local "sexual extinction" for *A. lanuginosa*, a term coined by Honnay and Boussuyt (2005) for "the permanent inability of a population to reproduce sexually." The sexual reproductive failure

observed in *A. lanuginosa* is likely due to low genotypic diversity at each site, which may then accelerate the demographic decline of the species initially caused by prairie habitat fragmentation. Local sexual extinction and clonal competition would cause further loss of genotypic diversity. We did not find evidence of such a shift in reproductive mode in *A. viridiflora*, a co-occurring and ecologically similar congener. In this species, clonal patches were small, consisting of two or three ramets. This observation along with the much higher number of MLGs suggests recruitment occurring primarily through sexual reproduction.

Despite the low genotypic diversity at most sites, we found no evidence for inbreeding in *A. lanuginosa,* so inbreeding depression is unlikely to be the cause of sexual reproductive failure in *A. lanuginosa*. Likewise, we did not find evidence for inbreeding in populations of *A. viridiflora* we sampled.

We found significantly lower genetic variability in *A. lanuginosa* compared to *A. viridiflora* for two parameters, mean number of effective alleles and mean expected heterozygosity. Due to the extremely high degree of clonal reproduction in *A. lanuginosa,* the number of genotypes was small, which in turn resulted in low allelic diversity. Because the microsatellite loci used here were developed for other species of *Asclepias*, the possibility of ascertainment bias cannot be ruled out (Ellegren et al. 1995), and comparisons of genetic diversity between species must be interpreted cautiously.

The results of the count-based population viability analyses highlight the importance of understanding clonal structure when determining the population dynamics of highly clonal

species like *A. lanuginosa*. In all four models, the PVA, which did not incorporate clonality in the models, gave overly optimistic predictions. In general, PVA based on field observations of ramet numbers rather than on genetic identification of genets will provide overestimates of effective population size for species that propagate asexually. Our genetic analysis demonstrated that the monitored subpopulation used for the PVA consisted of only two genets. The negative consequences of local sexual extinction could not be detected based on the results of the count-based PVA, which suggested a stable or slowly growing population.

Efforts to promote effective pollinators are needed to ensure successful seed set in any milkweed population, due to their complex pollination mechanism. However, the results of this study suggest that the recovery plan for *A. lanuginosa* must focus on increasing the genotypic diversity at each site and thereby the number of compatible mates. This could be done by transplanting individuals between populations and introducing seeds or individuals from out-of-state populations as was done in conservation efforts for *A. meadii* (Tecic et al. 1998; Hayworth et al. 2001) and *Hymenoxys acaulis* (DeMauro 1993). Since no significant population differentiation was observed for *A. lanuginosa*, outbreeding depression may not be a major concern for moving individuals among sites.

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2. REPRODUCTIVE TRADE-OFFS MAINTAIN BRACT COLOR POLYMORPHISM IN SCARLET INDIAN PAINTBRUSH (*CASTILLEJA COCCINEA*)

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2.1 Introduction

Polymorphisms for floral traits occur in many angiosperm species, and the underlying evolutionary forces maintaining these polymorphisms have long been the subject of interest and debate among evolutionary biologists. Floral traits reported to vary intraspecifically include corolla length and corolla flare (Galen, 1999), calyx length (Cariveau et al., 2004), flower size and style length (Elmqvist et al., 1993), and floral color (Levin and Brack, 1995; Strauss et al., 2004; Schemske and Bierzychudek, 2007). Among these traits, floral color polymorphisms are the most visually striking and thus have drawn many researchers to investigate the cause and maintenance of intraspecific variation (Epling and Dobzhansky, 1942; Wright, 1943; Epling et al., 1960; Kay, 1978). Floral color polymorphisms vary both within (Levin and Brack, 1995; Gigord et al., 2001; Irwin and Strauss, 2005; Eckhart et al., 2006; Takahashi et al., 2015) and between populations (Miller, 1981; Streisfeld and Kohn, 2005; Schemske and Bierzychudek,

2007; Arista et al., 2013) and a variety of selective agents have been implicated in their maintenance.

Numerous studies have demonstrated that pollinators are often the primary selective agent maintaining floral color polymorphisms both within and between populations (Waser and Price, 1981, 1983; Schemske and Bradshaw, 1999; Gigord et al., 2001; Smithson, 2001; Fenster et al., 2004; Sobral et al., 2015). Pollinator preference and constancy may result in assortative mating, limiting gene flow between the morphs within a population (Stanton, 1987; Niovi Jones and Reithel, 2001). For *Ipomoea purpurea*, pollinator constancy by bumble bees resulted in assortative mating within a population (Brown and Clegg, 1984), while in *Clarkia xantiana*, floral color polymorphism is maintained via a combination of positive frequency-dependent pollinator preference by one bee species and negative frequency-dependent pollinator preferences by two other bee species (Eckhart et al., 2006). In *Mimulus aurantiacus*, where red and yellow ecotypes inhabit different habitats, hummingbirds and hawkmoths show strong preference for red and yellow morphs, respectively, hence both pollinator preferences and ecogeographic isolation has led to assortative mating, thereby maintaining the flower color polymorphism between populations (Streisfeld and Kohn, 2007).

Selection by non-pollinator agents can also lead to floral color polymorphism (Armbruster, 2002; Strauss and Whittall, 2006). Differences in seed set, seed weight, and seed predation under different environmental conditions have been documented between color morphs (Schemske and Bierzychudek, 2001, 2007, Carlson and Holsinger, 2010, 2015). Anthocyanins, a primary floral pigment (Grotewold, 2006), are related to tolerance against abiotic stresses such as

UV-B radiation (Steyn et al., 2002), heat (Coberly and Rausher, 2003), and drought (Warren and Mackenzie, 2001), as well as non-pollinator biotic pressures such as herbivore defense (Irwin et al., 2003; Strauss et al., 2004). Such pleiotropic effects will interact with the pollinator community to either maintain or enforce floral color polymorphism (Johnson, 2010).

In theory, floral color polymorphisms associated with differences in breeding system could also be maintained by selection. For example, autogamous selfing (within the same flower) provides reproductive assurance when vector-mediated cross pollination is insufficient, but the advantage is offset by pollen and/or seed discounting (Lloyd, 1992; Schoen and Lloyd, 1992). Color morphs associated with higher rates of selfing may therefore have a selective advantage when pollinators are limited but not when they are abundant. Numerous studies have demonstrated that intraspecific variation in other floral traits, such as herkogamy and protandry (Moeller, 2006), flower size (Elle and Carney, 2003; Dart et al., 2012) and even scent (Gervasi and Schiestl, 2017) can influence reproductive assurance within and between populations. We are aware of only one report of differences in selfing rates associated with variation in flower color. In *Ipomoea purpurea*, when the relative frequency of the white morph is low compared to the darkly and lightly pigmented morphs, the white morph had higher selfing rates (Brown and Clegg, 1984; Fry and Rausher, 1997). When morphs were at more similar frequencies, all three morphs had similar selfing rates, so the white morph seems to be maintained by negative frequency dependent selection on reproductive assurance (Epperson and Clegg, 1987; Rausher et al., 1993; Fry and Rausher, 1997).

Scarlet Indian paintbrush, *Castilleja coccinea* L. Sprengel (Orobanchaceae), is a hemiparasitic forb native to the Eastern United States. Showy bracts surround small, greenish flowers. Flowers are perfect with the style slightly exserted (Torrey, 1843). Individuals are annual or biennial and may produce multiple stems and inflorescences. A successful fertilization results in a capsule that contains an average of 150 seed. The bracts surrounding flowers display yellow or scarlet (orange-red) colors. Despite the common name, the yellow morph dominates some populations in the Midwestern United States. Populations in this region are predominantly one color or the other, with over 90% of the individuals typically having either yellow or scarlet bract colors (Braum, 2014). Although the basis of bract color has not been studied, seeds collected from natural populations and sown in a common garden grew into plants exhibiting maternal bract colors, suggesting that bract color is a heritable trait (Braum, 2014). Further, we have hand pollination data that indicates bract color shows simple Mendelian inheritance, with yellow dominant over scarlet (in prep). *Castilleja coccinea* has been reported to attract ruby-throated hummingbirds, *Archilochus colubris* (Robertson, 1891; Bertin, 1982; Williamson, 2001; Spira, 2011; Eastman, 2014) and insect pollinators such as bees and butterflies. It is tempting to hypothesize that pollinator preference might cause positive assortative mating and thus maintains the bract color polymorphism in *C. coccinea,* but there are no published studies demonstrating different rates of pollinator visitations and effectiveness to color morphs in this species.

We investigated the possible role of the breeding system in the maintenance of flower color polymorphism in *C. coccinea*. We used hand-pollination experiments at a site in northeastern Illinois (Illinois Beach State Park) where a yellow population and a scarlet population are found approximately 500 m apart. The color morphs grow on the same sandy

dune-swale complex under similar abiotic conditions, and likely share a pollinator community. Our overall goal was to characterize the breeding system of the species, and to identify differences between color morphs, if they occurred. We used pollinator exclusion and hand-pollination experiments to compare the color morphs with regard to 1) self-compatibility, 2) response to pollinator exclusion, 3) cross-compatibility between the color morphs, and 4) relative female fertility and male fitness.

2.2 Materials and methods

2.2.1 Study populations

A hand-pollination experiment was conducted at Illinois Beach State Park from May 29th to July 6th in 2013. Two populations in Illinois Beach State Park, separated by an oak savannah and approximately 500 m apart, differ in bract color. Population 1 (hereafter, the yellow population) is predominantly yellow (87% yellow) whereas population 2 (hereafter, the scarlet population) is predominantly scarlet (99.6% scarlet) (Braum, 2014). A limited pollinator observation study was conducted in both populations to determine the presence or absence of floral visitors to *C. coccinea*. A total of 24 observation sessions, each lasting 15 minutes, were conducted in the yellow population, and 17 observation sessions were conducted in the scarlet population from morning to late afternoon.

2.2.2 Hand-pollinations

We conducted hand-pollinations to study the breeding system of *C. coccinea* and to compare female fertility under different treatments. To exclude animal pollinators, we used nylon mesh bags (17.8 cm by 11.4 cm) to cover entire inflorescences for all six treatments. The pollen donors varied by bract color and population. There were six pollination treatments: 1) bagged, no hand pollination (BN); 2) self-pollination (SP); 3) same color, same population (SS); 4) same color, different populations (SD); 5) different color, same population (DS); and 6) different color, different populations (DD) (Figure 2.1). For self-pollination, the pollen was transferred to the stigma of the same flower (autogamous selfing). For all "same population" treatments, pollen donors were chosen at least 5 m apart from the pollen recipients to decrease chances of biparental inbreeding. Eighteen randomly selected individuals from each population were chosen as pollen recipients, providing three replicates for all six treatments. All 18 individuals were defined by the predominant color of the population, hence the "yellow morph" refers to a pollen recipient with yellow bract color from the yellow population and the "scarlet morph" refers to a pollen recipient with scarlet bract color from the scarlet population. For the treatment assigned "same color, between populations", the pollen donor was the same color as the predominate color of the recipient population but from the other population. Toothpicks and small plastic containers were used to remove and transfer pollen grains between individuals and populations. New pollen grains were collected for each day's hand-pollination. Leftover pollen grains were discarded. In addition to the experimental treatments, three open control plants were followed in each population.

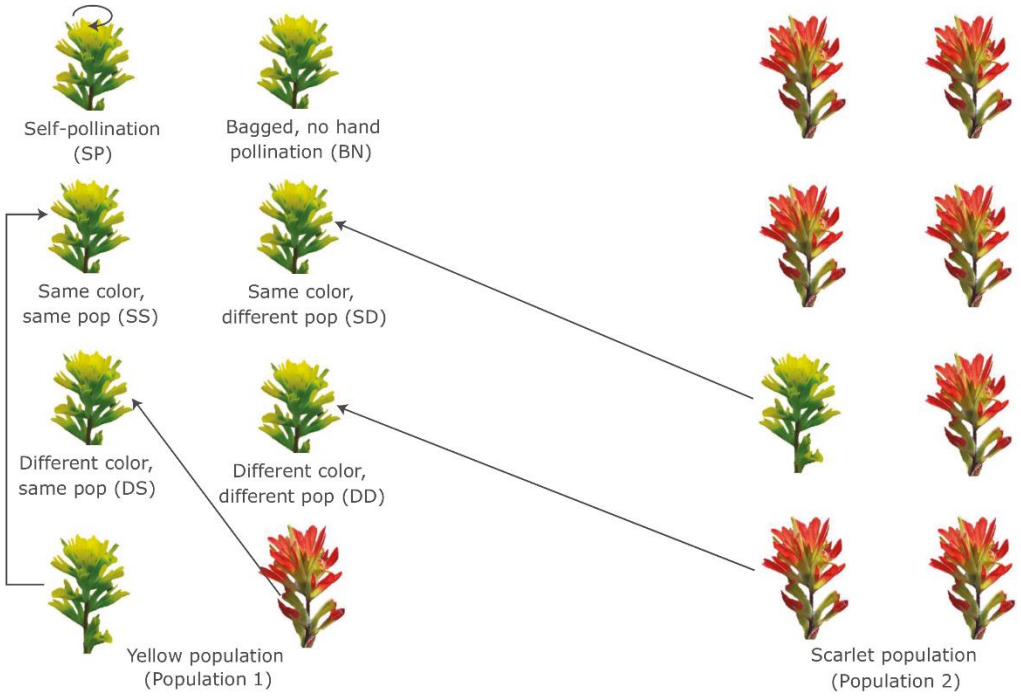

Figure 2.1. Illustration of six hand-pollination treatments for the yellow morph. All pollen recipients were bagged. The arrows move away from pollen donors and point to pollen recipients. The scarlet morph also received the corresponding six treatments but are not illustrated in this figure. There were three replicates for each treatment.

Flowers that were open prior to bagging were counted and marked by threads. Hand-pollinated flowers were marked with a black permanent marker. On each visit, the number of fertilized flowers was recorded for each individual. For the open control, the number of fertilized

flowers were divided by the total number of flowers to estimate the fruit set. When individuals receiving treatments were either removed or had a broken stem, they were replaced with other individuals to keep sample size consistent. A total of 35 individuals were used for the fruit set analyses. One yellow morph that received the SS treatment had a broken stem, but it was too late in the blooming season to replace it. After collecting the fruit set data, two additional individuals were found with broken stems, one yellow morph that received the DD treatment and one yellow morph that received the SD treatment. Excluding these two, a total of 33 individuals were used for the seed set analyses. To calculate seed set we collected mature capsules and counted the number of seeds for each capsule using a Contador seed counter (Pfeuffer GmbH, Kitzingen, Germany). After the seed count, the seeds were returned to the site.

2.2.3 Data analysis

Two variables were used as metrics of reproductive success, fruit set (the proportion of flowers that developed into fruits) and seed set (the number of seeds per fruit). Seed set excluded flowers that did not develop into fruits. We used linear mixed-effects models (LMM) for analyses involving seed set and generalized linear mixed-effects models (GLMM) for analyses involving fruit set. For GLMM relating to fruit set, fertilization of individual flowers was modeled as a binary (Bernoulli) response variable. Mixed-effects modeling was implemented with the *lme4* package for R version 3.5.0 (Bates et al., 2015; R Core Team, 2018). In all mixed-effects models, we used the individual plant as the random effect (random intercept), and maternal color as one of the fixed effects.

To address our questions regarding self-compatibility, we compared the reproductive success of plants that were self-pollinated to those that were cross-pollinated with plants of the same color and the same population (i.e., the SP and SS treatments, respectively). Self-compatibility indices were calculated for each population using the average seed set where the average seed set from SP was divided by the average seed set from SS (Lloyd and Schoen, 1992). To investigate the effects of pollinator exclusion, we compared the reproductive success of plants that were bagged and not hand-pollinated to those that were self-pollinated (i.e., the BN and SP treatments). For each model we included the interaction of treatment and bract color. Thus, the maximal model had two fixed effects (maternal color, pollination treatment) with two levels each, an interaction of the fixed effects, and a random effect of the individual.

Additionally, we compared the open control treatment (unmanipulated, unbagged plants) in each population. This analysis only included maternal color as a fixed effect (same as the source population).

We investigated cross-compatibility between color morphs with regard to the relative fitness of the sexes using a larger model, that added 1) whether pollen came from the same or different population, (i.e., the "population" fixed effect), and 2) whether pollen came from individuals of the same bract color, (i.e., the "color" fixed effect), in addition to maternal color. Both of these had two levels (same or different). These two fixed effects combined to describe four of the pollination treatments described earlier (SS, SD, DS, and DD). The interactions of maternal color with each of the other two fixed effects were also included in model testing.

For each test, we compared candidate models with every combination of fixed effects using the Akaike information criterion corrected for small sample size (AICc). Akaike weights were calculated and the best model, assuming one correct model exists in the candidate set, was determined by the maximum Akaike weight value. Estimates of mean values and 95% confidence intervals for a given factor in our mixed-effects models were presented as estimated marginal means calculated using the *lsmeans* package (Lenth, 2016).

We determined the relative influence of fixed effects and random effects by comparing the marginal and conditional R^2, following Nakagawa and Schielzeth (Nakagawa and Schielzeth, 2013). Calculation of the coefficients of determination was completed with the *MuMIn* package (Barton, 2018).

2.3 Results

Our limited pollinator observations confirmed the presence of insect floral visitors. In the yellow population, we observed black swallowtails (*Papilio polyxeneson*) and bumble bees (*Bombus* sp.) on the yellow morph. In the scarlet population, we observed only sweat bees on the scarlet morph. Bumble bees and butterflies were present in the scarlet population, but they did not visit scarlet individuals during our observation sessions. We did not observe ruby-throated hummingbirds at our study sites.

2.3.1 Self-compatibility

Both color morphs were self-compatible with no evidence of self-sterility. The average seed set for SP was 110.43 and 144.43, for the yellow morph and the scarlet morph, respectively. The average seed set for SS was 121.89 and 162.40, for the yellow morph and the scarlet morph, respectively. The self-compatibility indices were 0.91 for the yellow and 0.89 for the scarlet. The self-pollination (SP) treatment had similar fruit set to the same color, same population (SS) treatment (Figure 2.2 and Table IV, Appendix A). The average fruit set was 60% in all treatment-color combinations and 70% for the self-pollination treatment (Figure 2.2). In our statistical models for fruit set (Table V), the null model had the greatest support (Akaike weight=0.447; Table V). There was little support for a difference between treatments (ΔAICc=1.31, Akaike weight=0.232), or color morphs (ΔAICc=2.11, Akaike weight=0.155).

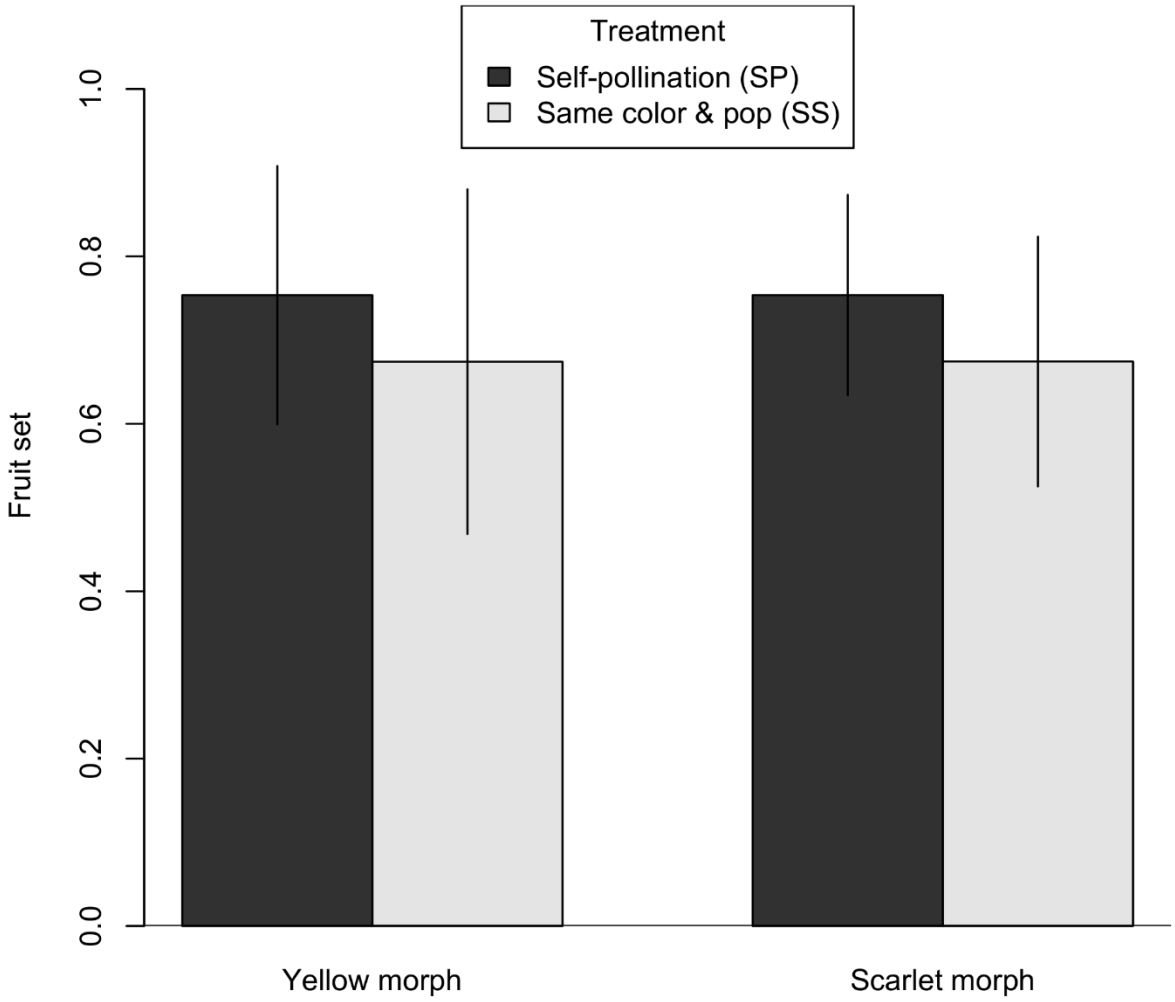

Figure 2.2. Fruit set comparison between self-pollination and outcrossing. For each maternal color, we compared fruit set in two treatments, SP and SS. Bar heights represent the estimated marginal means from the GLMM and error bars represent the 95% confidence interval.

Table V. Summary of model selection results for the relationship between fruit set and self-compatibility.

Model	df	AICc	ΔAICc	Weight
Null	2	131.7	0	0.447
Treatment	3	133	1.31	0.232
Maternal color	3	133.8	2.11	0.155
Maternal color + Treatment + Maternal color:Treatment	5	135	3.27	0.087
Maternal color + Treatment	4	135.2	3.47	0.079

Fruit set was predicted by two fixed effects and their interaction. This set of models includes the SP and SS treatments.

Seed set was about 18% lower in the self-pollination treatment (SP) compared to the same color, same population (SS) treatment (Figure 2.3 and Table VI, Appendix B), but there was little statistical support for including treatment in the best model (Table VII; for the best model with treatment, ΔAICc=1.51, Akaike weight=0.198). There was a more pronounced difference between color morphs, with scarlet individuals having 33% to 42% greater seed set for the cross-pollination (SS) and self-pollination (SP) treatments, respectively. The best model did include maternal color as a fixed effect (Akaike weight = 0.423), while the null model excluding maternal color and treatment was the next best model (ΔAICc=1.37, Akaike weight=0.213).

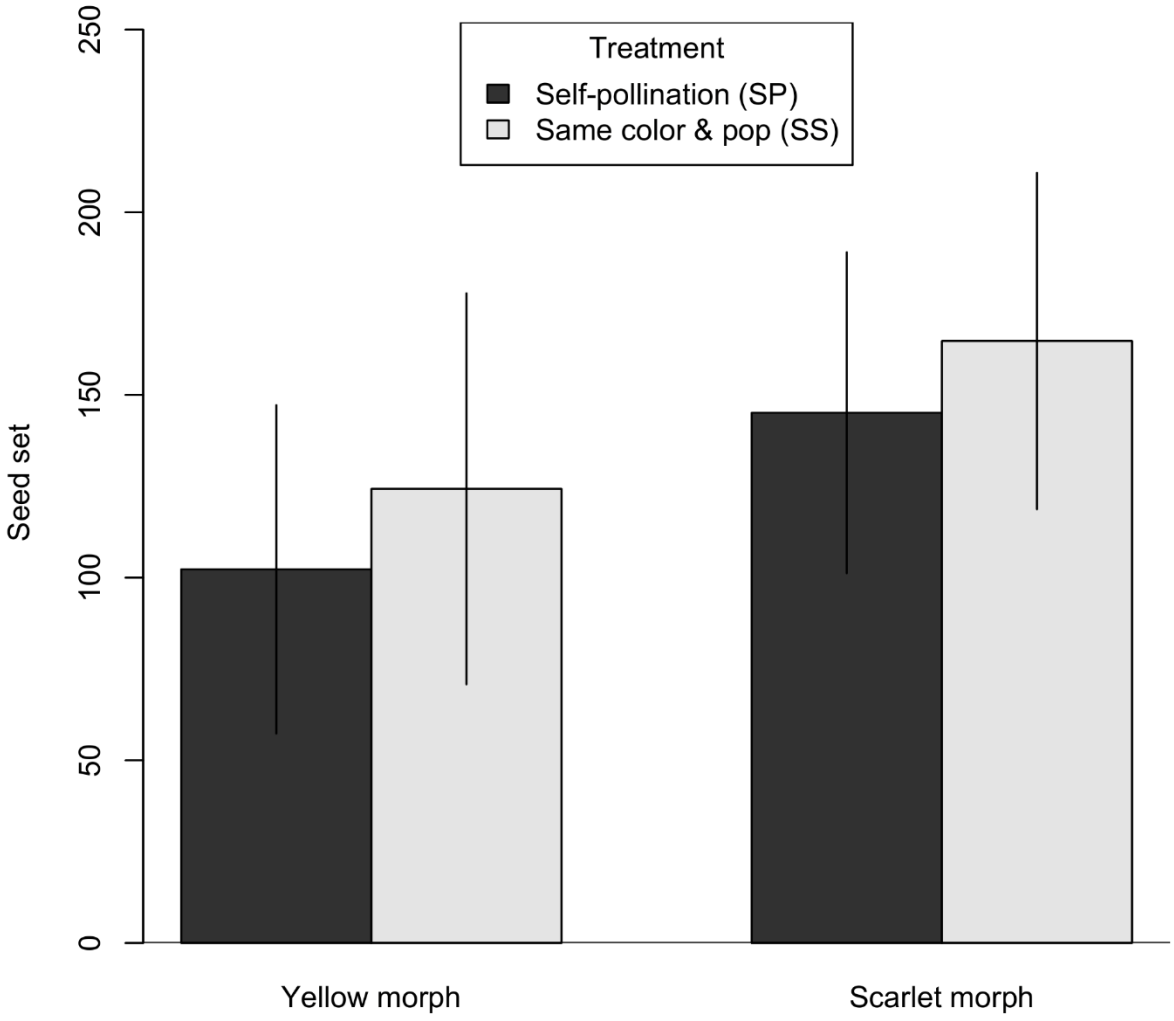

Figure 2.3. Seed set comparison between self-pollination and outcrossing. For each maternal color, we compared seed set in two treatments, SP and SS. Bar heights represent the estimated marginal means from the LMM and error bars represent the 95% confidence interval.

Table VII. Summary of model selection results for the relationship between seed set and self-compatibility.

Model	df	AICc	ΔAICc	Weight
Maternal color	4	540.8	0	0.423
Null	3	542.2	1.37	0.213
Maternal color + Treatment	5	542.4	1.51	0.198
Treatment	4	543.5	2.67	0.111
Maternal color + Treatment + Maternal color:Treatment	6	545	4.11	0.054

Seed set was predicted by two fixed effects and their interaction. This set of models includes the SP and SS treatments.

Among open control plants, one yellow plant had a broken stem and one scarlet plant could not be located after collecting the fruit set data (Table VIII, Appendix C and Table IX, Appendix D). For the remaining plants, fruit set in the open control was not different between the two populations, as the null model was preferred (ΔAICc=2.08, weight=0.739). Fruit set for open control plants was high in both populations: 80.6% in the yellow population (95% confidence interval, 66.7%-94.6%) and 82.8% in the scarlet population (73.0%-92.5%). When testing seed set, model selection showed a slight preference for the model that included population over the null model (ΔAICc=0.45, weight=0.556). Seed set in the scarlet population was estimated as 188.8 seeds per fruit (95% C.I., 117.3-260.4), much greater than the 96.3 seeds per fruit (27.40086-165.2658) estimated for the yellow population.

2.3.2 Pollinator exclusion

Both morphs were capable of self-fertilization; both the bagged, no hand pollination treatments (BN) and the self-pollination (SP) treatments yielded fruits and seeds. Fruit and seed production in the BN treatment indicates either autonomous self-pollination, a bag effect (accidental pollination when bags were placed or removed), or pollination by "squatters" (Lloyd and Schoen, 1992) (small, long-staying insects such as aphids and thrips already present when bags were placed). Interestingly, the color morphs differed markedly in comparisons of the BN and SP treatments. For the scarlet morph, the BN plants had a 43% reduction in fruit set (Figure 2.4, Table X, Appendix E) and a 66% decline in seed set compared to the SP plants (Figure 2.5, Table XI, Appendix F). For the yellow morph, the BN plants had no reduction in fruit set (Figure 2.4), and only a 12% reduction in seed set relative to the SP plants (Figure 2.5). Thus, pollinator exclusion had a greater effect on the scarlet morph with respect to both fruit set and seed set (Figures 2.4 and 2.5). Statistical modeling provides the most support for difference between morphs with respect to reliance on a pollen vector. For both fruit set (Table XII) and seed set (Table XIII), the best candidate model included the interaction between treatment and maternal color (fruit set, Akaike weight = 0.598; seed set, Akaike weight = 0.483).

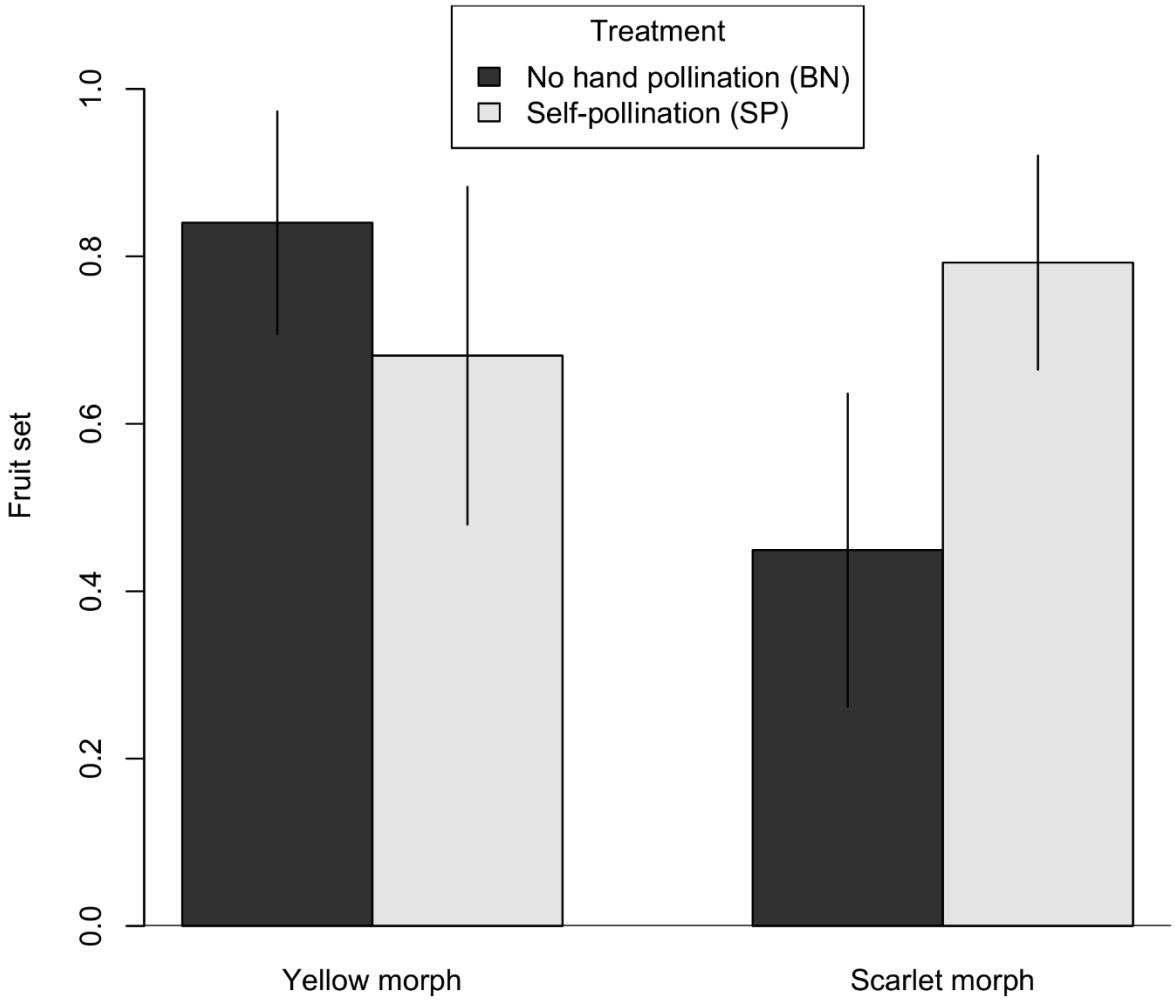

Figure 2.4. Fruit set comparison between bagged, no hand pollination and self-pollination. For each maternal color, we compared fruit set in two treatments, BN and SP. Bar heights represent the estimated marginal means from the GLMM and error bars represent the 95% confidence interval.

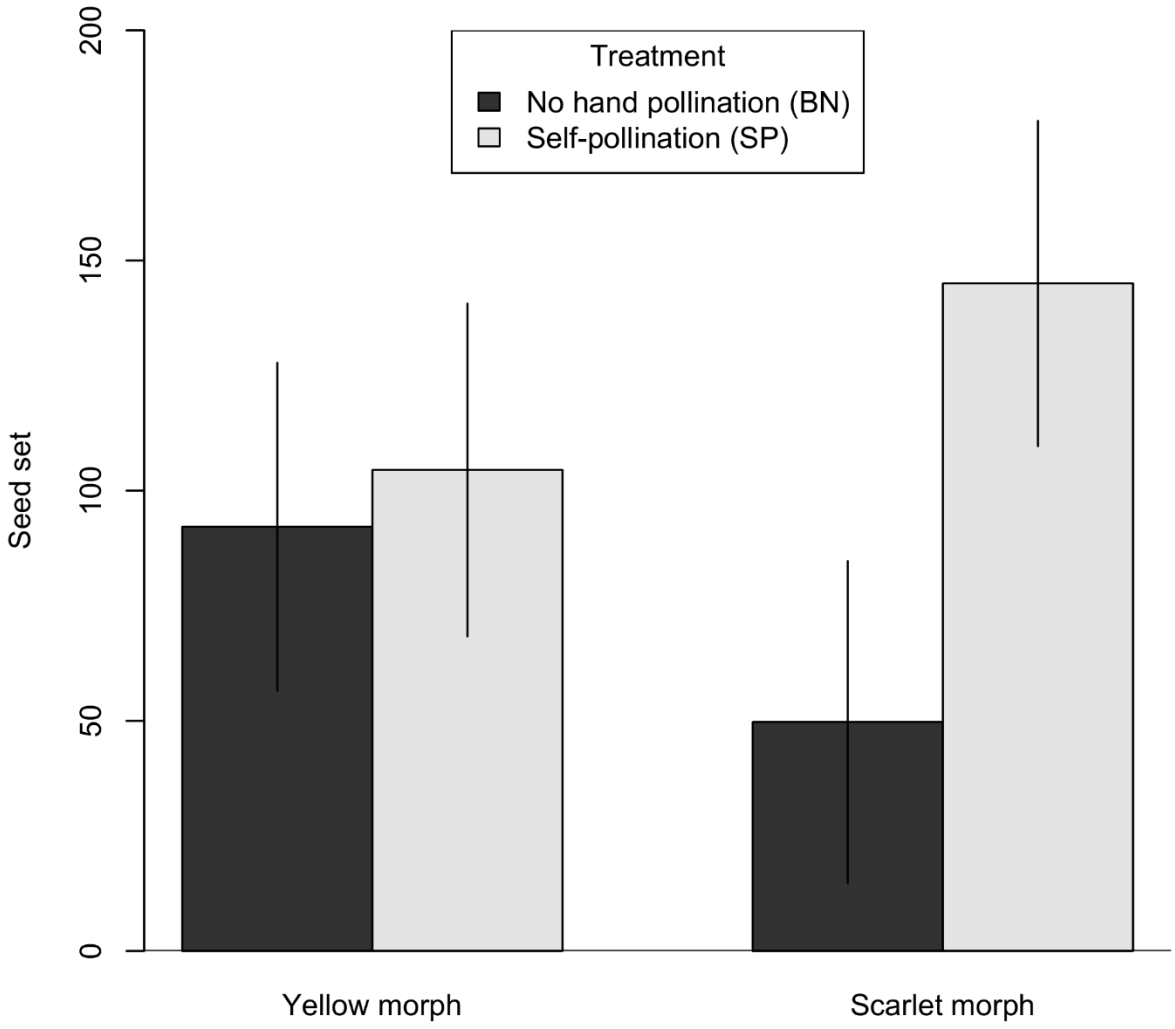

Figure 2.5. Seed set comparison between bagged, no hand pollination and self-pollination. For each maternal color, we compared seed set in two treatments, BN and SP. Bar heights represent the estimated marginal means from the LMM and error bars represent the 95% confidence interval.

Table XII. Summary of model selection results for the relationship between fruit set and pollinator exclusion.

Model	df	AICc	ΔAICc	Weight
Maternal color + Treatment + Maternal color:Treatment	5	155	0	0.598
Null	2	157.7	2.7	0.155
Maternal color	3	158.4	3.41	0.109
Treatment	3	159	4.08	0.078
Maternal color + treatment	4	159.5	4.58	0.061

Fruit set was predicted by two fixed effects and their interaction. This set of models includes the BN and SP treatments.

Table XIII. Summary of model selection results for the relationship between seed set and pollinator exclusion.

Model	df	AICc	ΔAICc	Weight
Maternal color + Treatment + Maternal color: Treatment	6	619.4	0	0.483
Treatment	4	620.2	0.85	0.316
Maternal color + Treatment	5	622.6	3.25	0.095
Null	3	622.9	3.56	0.081
Maternal color	4	625.2	5.87	0.026

Seed set was predicted by two fixed effects and their interaction. This set of models includes the BN and SP treatments.

2.3.3 Cross-compatibility and relative fitness

There was some evidence that fruit set was greatest for pollination between the same colors from same population (Figure 2.6, Table XIV, Appendix G). The manipulated movement of pollen between plants readily yielded fruits with a large number of seeds (Figure 2.7, Table XV, Appendix H). In all combinations, pollination with same source population but different color saw a 6-15% reduction in fruit set. However, no reduction in seed set for pollinations using different population of different color was observed for maternal plants with yellow bracts, and the reduction was less than 3% for maternal plants with scarlet bracts (Figure 2.7). The "color" fixed effect did appear in the best candidate model for describing fruit set (Akaike weight = 0.417; Table XVI), and the lack of interaction with maternal color suggests a consistent effect in both color morphs. The "color" fixed effect was not present in any of the top candidate models for seed set (best model that included "color", ΔAICc = 2.16, Akaike weight= 0.113; Table XVII).

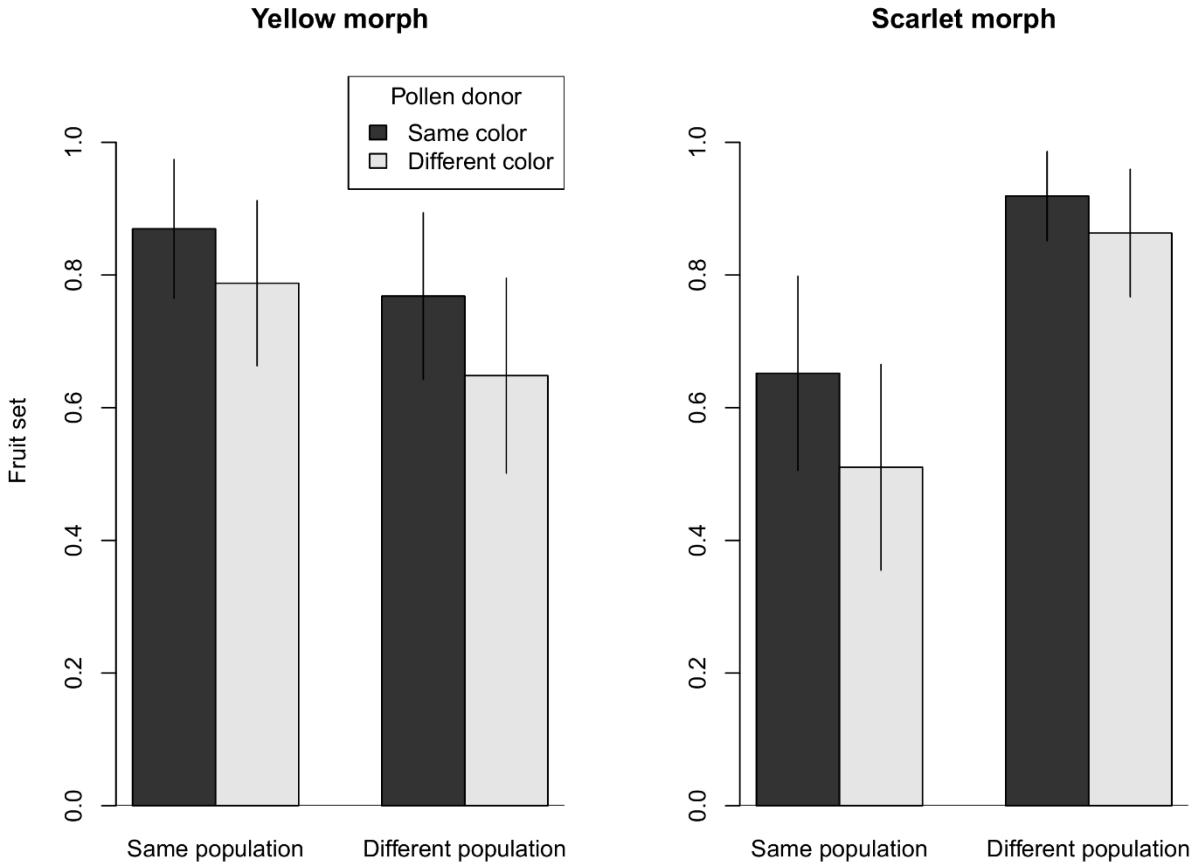

Figure 2.6. Fruit set comparison in inter-population and inter-morph crosses. For each maternal color, we compared fruit set in four treatments (SS, SD, DS, and DD) that combined two color morphs and two source populations for the pollen donors. Bar heights represent the estimated marginal means from the GLMM and error bars represent the 95% confidence interval.

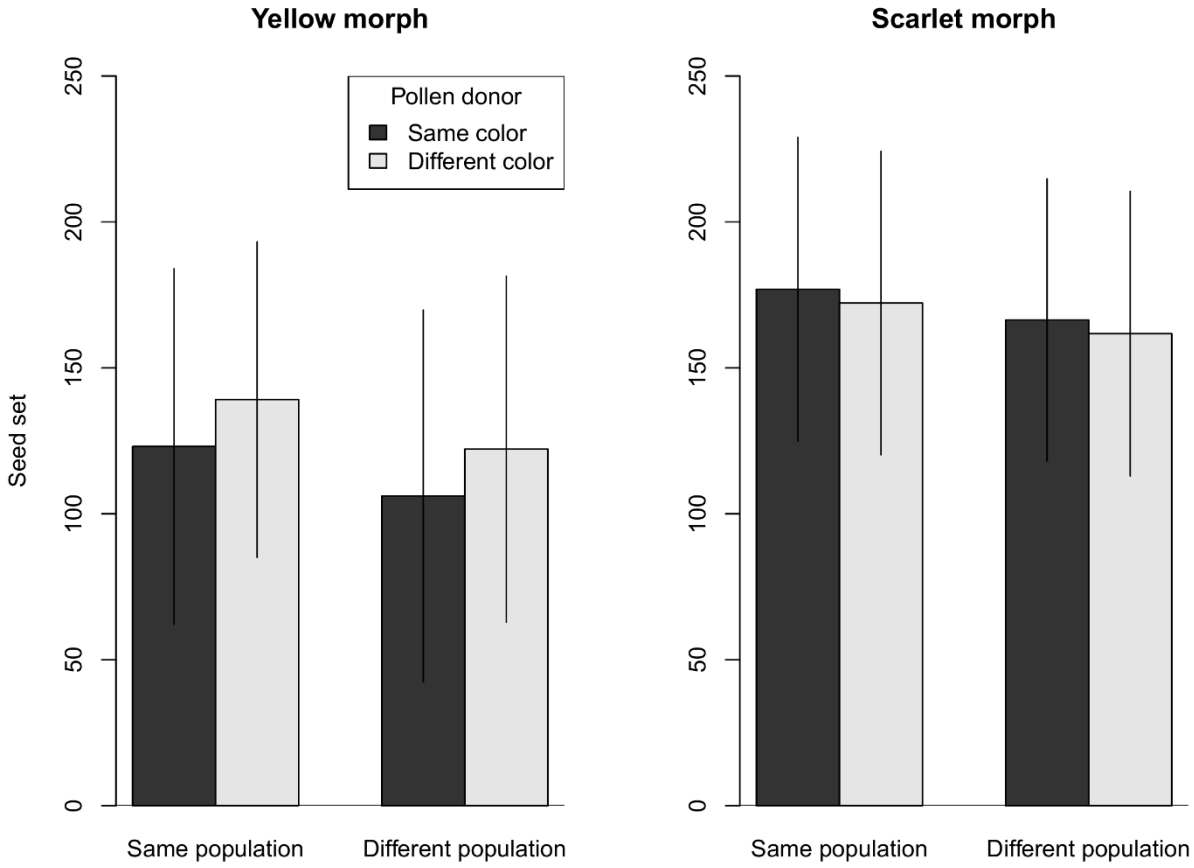

Figure 2.7. Seed set comparison in inter-population and inter-morph crosses. For each maternal color, we compared seed set in four treatments (SS, SD, DS, and DD) that combined two color morphs and two source populations for the pollen donors. Bar heights represent the estimated marginal means from the LMM and error bars represent the 95% confidence interval.

Table XVI. Summary of model selection results for fruit set and cross-compatibility between color morphs and populations.

Model	df	AICc	ΔAICc	Weight
Maternal color + Color + Pop + Maternal color: Pop	6	276.9	0	0.417
Maternal color + Pop + Maternal color: Pop	5	277.6	0.73	0.29
Maternal color + Color + Pop + Maternal color: Color + Maternal color: Pop	7	278.4	1.49	0.198
Pop	3	282.8	5.88	0.022
Null	2	283	6.12	0.02
Color + Pop	4	283.8	6.91	0.013
Color	3	284	7.07	0.012
Maternal color + Pop	4	284.8	7.93	0.008
Maternal color	3	285.1	8.17	0.007
Maternal color + Color + Pop	5	285.9	8.99	0.005
Maternal color + Color	4	286	9.12	0.004
Maternal color + Color + Maternal color: Color	5	287.4	10.47	0.002
Maternal color + Color + Pop + Maternal color: Color	6	287.5	10.62	0.002

Fruit set was predicted by three fixed effects and two of their interactions. The data for this set of models resulted from the SS, SD, DS, and DD treatments.

Table XVII. Summary of model selection results for seed set and cross-compatibility between color morphs and populations.

Model	df	AICc	ΔAICc	Weight
Maternal color	4	1267	0	0.332
Null	3	1268	1.73	0.14
Maternal color + Same pop	5	1269	1.81	0.134
Maternal color + Same color	5	1269	2.16	0.113
Same pop	4	1271	3.74	0.051
Same color	4	1271	3.88	0.048
Maternal color + Same color + Same Pop	6	1271	4.02	0.045
Maternal color + Same pop + Maternal color: Same pop	6	1271	4.02	0.044
Maternal color + Same color + Maternal color: Same color	6	1271	4.17	0.041
Same color + Same pop	5	1273	5.93	0.017
Maternal color + Same color + Same Pop + Maternal color: Same color	7	1273	6.06	0.016
Maternal color + Same color + Same pop + Maternal color: Same pop	7	1273	6.27	0.014
Maternal color + Same color + Same pop + Maternal color: Same color + Maternal color: Same pop	8	1275	8.36	0.005

Seed set was predicted by three fixed effects and two of their interactions. The data for this set of models resulted from the SS, SD, DS, and DD treatments.

Fruit set was lower when pollen donors came from the scarlet population, whether the pollen donors were scarlet or yellow. The decline was 12-18% for yellow maternal plants

("different population" pollen donors) and 29-41% for scarlet maternal plants ("same population" pollen donors). The differential success of pollen from the two populations was reflected in the strong support for models that included the interaction between "population" and maternal color (top three combined Akaike weights = 0.905; Table XVI).

Seed set was consistently 24-57% greater in maternal plants of the scarlet morph compared to the yellow morph (Figure 2.7), with seed set being slightly higher (by 6-16%) when the maternal plant and pollen donor came from the same population (Figure 2.7). Mixed-effects models provided little support for a role of pollen source in predicting seed set. Also, there is almost no support for an interaction between "population" and maternal color, as was found with fruit set. The variable with the strongest explanatory power for seed set was maternal color; the best performing candidate model had only maternal color as an explanatory variable (Akaike weight = 0.332, Table XVII).

2.3.4 Relative influence of fixed and random effects

For each statistical model, we estimated the amount of variance explained by the fixed effects (marginal R^2) and the combination of fixed and random effects (conditional R^2). The same random effect, a random intercept for individual plant, was present in all the models. When

there is a large discrepancy between the marginal R^2 and conditional R^2, we expect a large influence of individual plant on the response variable. We found that the two R^2 values were similar for all three of the fruit set models (Table XVIII), suggesting that the variance observed in fruit set was not explained by differences between individual plants. In the model testing the effect of pollinator exclusion on seed set (Table XIII, Figure 2.5) the marginal R^2 was more than half of the conditional R^2 (Table XVIII); the fixed effects alone accounted for more than half of the variance observed in seed set. For the seed set models that tested self-compatibility (Table VII, Figure 2.3) and cross-compatibility (Table XVII, Figure 2.7), the conditional R^2 was much greater than the marginal R^2 (Table XVIII), indicating a large influence of individual plants in the performance of the model.

Table XVIII. Comparison of the marginal (R^2m) and conditional (R^2c) coefficient of determination values

	Response variable	**Figure**	**R^2m**	**R^2c**
Self-compatibility	Fruit set	Fig 2.2	0.0444	0.0444
	Seed set	Fig 2.3	0.151	0.441
Pollinator exclusion	Fruit set	Fig 2.4	0.136	0.145
	Seed set	Fig 2.5	0.286	0.464
Cross-compatibility and relative fitness	Fruit set	Fig 2.6	0.142	0.159
	Seed set	Fig 2.7	0.0758	0.456

R^2m and R^2c are marginal R^2 and conditional R^2, respectively.

2.4 Discussion

Castilleja coccinea populations in the Midwestern region show intraspecific bract color polymorphism. Braum (2014) reported that the color morphs were also associated with morphological differences, with the scarlet morph consistently larger in several bract and flower measurements including stamen and style length. Differences in both floral color and morphology could impact the breeding system of *C. coccinea* in ways that might involve reproductive trade-offs under pollen or pollinator limitation. Hence, we chose to investigate whether factors related to the breeding system might play a role in maintaining the floral color polymorphism. We found that both color morphs were self-compatible, and fruit set and seed set did not differ between selfed (SP) and outcrossed (SS) pollinations (Figures 2.2 and 2.3). Both color morphs are also inter-morph cross-compatible, although there may be evidence of a small reduction in fruit set in inter-morph crosses (Figure 2.6). Two notable differences were found between the color morphs. First, they differed in their response to pollinator exclusion. In the control treatments (bagged, no hand pollination), the scarlet morph showed reduced fruit and seed set, whereas the yellow morph did not (Figures 2.4 and 2.5). Second, the scarlet morph set more seed than the yellow morph (Figure 2.7).

The genus *Castilleja* includes both self-incompatible (*C. levisecta, C. linariaefolia, C. miniata, C. rhexiifolia, and C. sulphurea*) (Carpenter, 1983; Kaye and Lawrence, 2003; Hersch-Green, 2012) and self-compatible (*C. attenuata*) (Chuang and Heckard, 1992) species, but breeding system had not been previously assessed in *C. coccinea*. Results of this study show that *C. coccinea* is highly self-compatible because fruit set and seed set were not reduced in individuals that received self-pollen compared to individuals that received outcross pollen

(Figures 2.2 and 2.3). This pattern is true in both color morphs as shown by the self-compatibility indices which are above the threshold of 0.75 to be described as self-compatible, following Lloyd and Schoen (Lloyd and Schoen, 1992). While self-incompatibility assures the genetic and evolutionary benefits of outcrossing (Igic et al., 2008), self-compatible species have the advantage of reproductive assurance when pollen is limited (Kalisz et al., 2004), especially when inbreeding depression is low.

Comparison of the self-pollination treatment (SP) and the negative control (BN) showed that, surprisingly, the pollinator exclusion treatment had little effect on the yellow morph, which showed only slight or no reduction in either fruit set and seed set (Figures 2.4 and 2.5). The scarlet morph showed reductions in both measures of female fertility under the pollinator exclusion treatment. Thus, the yellow morph would likely experience an advantage of reproductive assurance in cases of pollinator limitation, and perhaps even pollinator absence. Fruit set from the negative control might have been the result of true autonomous self-pollination. In the greenhouse, *C. coccinea* did not set fruits or seeds (J. Fant, personal observation). This suggests that the autogamous selfing we observed might be due to accidental transfer of self-pollen to stigma ("bag-effect"). Alternatively, predatory insects such as aphids and thrips (squatters) that dwell in flowers may have caused "quasi-autonomous" pollination (Lloyd and Schoen, 1992). Whatever the mechanism of autogamy, the yellow morph outperformed the scarlet morph in the negative control treatment, indicating the yellow morph's ability to tolerate limited pollen delivery. While there are many cases where different color flower morphs attract different pollinators (Kay, 1978; Streisfeld and Kohn, 2007; Hersch-Green, 2012), to our knowledge this is the first report of color morphs differing in their dependence on

pollinators for self-fertilization. The abundance of pollinators appears to act as a selective agent in *C. coccinea*, with the yellow morphs being favored when pollinator abundance is low.

There are different modes of self-pollination that offer different levels of reproductive assurance. Geitonogamy, where pollen is transferred between flowers, does not offer reproductive assurance because it relies upon the same pollinator activity as cross-pollination. Bagging experiments like ours investigate autogamous selfing (within flowers), but we did not investigate the precise timing and mechanism, factors that are important for determining the level of benefits provided by reproductive assurance (Lloyd and Schoen, 1992).

Greater seed set was observed for the scarlet morph compared to the yellow morph, suggesting that the two morphs also differ in potential reproductive output. Regardless of the bract color of the pollen donor or which population the pollen came from, the scarlet individuals consistently produced more seeds per capsule (Figure 2.7). This difference was also observed for individuals that were self-pollinated (Figure 2.3) (except for the negative control as already noted).

Our study was not ideally designed to distinguish the effects of genotypic differences among individual plants from the effects of treatment, since we could not apply every treatment to every individual. The small number of individuals in each treatment compounds this limitation. However, we made efforts to statistically assess the relative influence of fixed effects (experimental treatments, maternal plant color) and random effects (individual plant) in the models using marginal and conditional R^2 (Nakagawa and Schielzeth, 2013). Where the

difference between these values is large, there is the potential that genotypic differences between individual plants may be confounding our experimental findings. We found that differences in individual plants explained almost none of the variance observed in the fruit set analyses, as the marginal R^2 and conditional R^2 were nearly equal (Table XVIII). Additionally, for our analysis of pollinator exclusion and seed set, the marginal R^2 was more than half the value of the conditional R^2. These findings reinforce our conclusions regarding the differences in fruit set between bract colors and among experimental treatments. Also, the influence of pollinator exclusion on seed set (which differs between color morphs) is largely confirmed. However, the conditional R^2 was much greater for the other two analyses of seed set. Conclusions regarding seed set and the self-compatibility experiment, or the cross-compatibility experiment, must be made with caution. We cannot rule out the possibility that genotypic differences between plants randomly assigned to treatment were the primary drivers of the patterns we observed for the latter two tests.

Our study was conducted over a single flowering season, so we cannot say whether the higher seed set for the red morph would be maintained over multiple years or varying conditions. Differences in seed set were observed between color morphs when five floral color polymorphic species (*Cirsium palustris, Digitalis purpurea, Holcus lanatus, Polygonum persicaria, and Vicia sepium*) were under drought and well-watered treatments (Warren and Mackenzie, 2001). Under a drought treatment, pink/purple morphs had greater seed set, but under a well-watered treatment, white morphs had greater seed set. The year of our study (2013) was extremely wet from April through June but had near average temperature and precipitation from June through August (National Temperature and Precipitations Maps obtained from NOAA).

We also observed differences in male fertility between populations, though not between color morphs. Plants from the scarlet population were poor pollen donors; hand-pollinations using pollen from the scarlet population individuals consistently resulted in lower fruit set (Figure 2.6). The lower fruit set was not related to bract color but rather related to the source population of the pollen donor because both color morphs were tested as pollen donors. The poor quality or low quantity pollen of the scarlet population likely reduces the overall male fertility of the scarlet population. We did not directly test pollen viability or pollen competition in this study, and we do not know the cause of the reduced pollen performance. Different pollen viability among color morphs has been observed in *Claytonia virginica* (Frey, 2007). The reduced male fertility in the scarlet population might be due to higher inbreeding depression in that population (Krebs and Hancock, 1990), although we have no other evidence that suggests inbreeding levels or levels of genetic variability differ between the two populations.

While the color morphs were cross-compatible, there was slight evidence of reduced fruit set for inter-morph crosses (Figure 2.6). Reduced intermorph compatibility may lead to reproductive isolation and genetic divergence of the color morphs. For seed set, there was no evidence that inter-morph crosses produced fewer seeds per fruit (Figure 2.7). Further studies of pollinator behavior, mating system, and population genetics could reveal more about the reproductive interaction between the two morphs. We are currently conducting a genetic study, using a double digest Restriction-Site Associated DNA sequencing (ddRADseq) approach (Peterson et al., 2012), to address gene flow between color morphs, and compare genetic structure and inbreeding across morphs and populations.

This study was limited to two populations that differed strikingly in bract color frequency. The populations were very close to each other, but undetected site-specific effects might exist. Further investigations at additional sites would be needed to confirm that the reproductive differences we observed between scarlet and yellow bract colored *C. coccinea* extend across the species range. Based on findings from these two sites, we posit that these reproductive trade-offs maintain the bract color polymorphism in *C. coccinea*, where pollinators are selective agents. The scarlet morph has greater potential reproductive output, but the yellow morph has greater reproductive assurance when pollinators are limited. In the absence of pollen limitation, both bract colors develop fruit equally well, but the scarlet morph would yield greater seed set. This appeared to be the situation for our open control, where scarlet plants produced many more seeds per fruit (though fruit set was similar between morphs). When pollinators are limited, the yellow morph may self-pollinate at a higher rate. The differences we observed between the color morphs might also have conservation implications, with the scarlet morph more susceptible to the negative consequences of pollinator declines due to pollution, habitat loss, or habitat degradation (Steffan-Dewenter and Tscharntke, 1999; Potts et al., 2010).

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3. ddRADseq DATA REVEALS NO ASSOCIATION BETWEEN THE DISTRIBUTION OF BRACT COLORS AND THE POPULATION STRUCTURE OF THE MIDWESTERN *CASTILLEJA COCCINEA* (SCARLET INDIAN PAINTBRUSH)

3.1 Introduction

Given the importance of floral display for reproductive success in angiosperms, intraspecific polymorphism in floral traits has long been of interest to plant evolutionary biologists (Wright, 1943; Levin and Brack, 1995; Galen, 1999; Cariveau et al., 2004; Strauss et al., 2004; Schemske and Bierzychudek, 2007). In particular, selective agents driving floral color polymorphism have been extensively studied, and pollinators as well as non-pollinator agents have been shown to be important (Fenster et al., 2004; Strauss and Whittall, 2006). Pollinators can maintain floral color polymorphism through flower constancy leading to assortative mating (Niovi Jones and Reithel, 2001), frequency dependent selection (Epperson and Clegg, 1987; Gigord et al., 2001), and opposing preferences for different flower colors (Stanton, 1987; Streisfeld and Kohn, 2007). Selection by non-pollinator agents can also lead to floral color polymorphism (Armbruster, 2002; Strauss and Whittall, 2006). In some species, different color morphs exhibit different fitness measures (seed set, seed weight, and seed predation rates) under different environmental conditions (Schemske and Bierzychudek, 2001, 2007, Carlson and Holsinger, 2010, 2015). Anthocyanins are a common floral pigment (Grotewold, 2006), which are related to plant response against abiotic stresses such as UV-B radiation (Steyn et al., 2002), heat (Coberly and Rausher, 2003), and drought (Warren and Mackenzie, 2001). Anthocyanins also play a role in herbivore defense (Irwin et al., 2003; Strauss et al., 2004).

Whether pollinators or other agents are responsible for floral color polymorphism, gene flow between morphs may be reduced due to prezygotic and postzygotic barriers (Lowry et al., 2008). Preferences of different pollinators, if consistent, can result in assortative mating within color morphs and pollinator-mediated reproductive isolation (Levin and Kerster, 1967; Hoballah et al., 2007; Sobel and Streisfeld, 2015). If gene flow occurs almost exclusively within the same flower color, over time, the color morphs would become genetically differentiated from each other. Ecotypic specialization of different flower morphs could also lead to reduced gene flow between morphs, even without pollinator preferences, if specialized habitats were spatially separated and pollinator movements between habitats were limited (Sobel and Streisfeld, 2015). Postzygotic barriers such as reduced viability in seeds from between color morph crosses compared to within color morph crosses may hinder gene flow between color morphs (Losada et al., 2015). Over time, the color morphs may become genetically differentiated, eventually leading to speciation (Bradshaw and Schemske, 2003; Hopkins and Rausher, 2011).

Despite extensive research on flower color polymorphism and the potential for reduced gene flow between morphs, few studies have examined neutral genetic differentiation between morphs. In the few studies that have examined genetic variability within and between color morphs, there is little evidence that gene flow has been reduced sufficiently to result in genome-wide differentiation. For example, Schemske and Bierzychudek (2007) reported a steep cline in flower color for *Linanthus parryae* sampled along a transect, but allozyme frequencies were nearly uniform. In RADseq studies of *Mimulus aurantiacus*, most SNP markers show little differentiation between scarlet and yellow floral morphs despite strong and opposing pollinator preferences (Solbel and Streisfeld 2014, Stankowski et al. 2015). Yet incipient speciation

(reproductive isolation) based on floral morphology is paradigm in floral evolution (Schemske and Bradshaw, 1999; Sobel and Streisfeld, 2015).

Attempts to demonstrate that floral color or morphology indicates genetic differentiation can be confounded by ecological and evolutionary forces unrelated to floral characters. For example, if populations are relatively isolated from each other as a result of habitat fragmentation, genetic drift may drive differentiation regardless of floral display (Edh et al., 2007; Wang et al., 2016). Landscape features that are barriers to gene flow may cause patterns of genetic differentiation that complicate analyses, especially if they are cryptic or are coincident to other environmental shifts such as temperature or rainfall. Historical patterns of colonization or range expansion may also be important. A microsatellite study of *Hepatica nobilis* var *japonica*, which exhibits flower color polymorphism in Japan, indicated that the distribution of the color morphs is best explained by refugial isolation and subsequent post-glacial range expansion (Kameoka et al., 2017).

This study looked at the genetic structure and distribution of bract color morphs in scarlet Indian paintbrush, *Castilleja coccinea* (L.) Sprengel (Orobanchaceae), in the Midwestern United States. In *C. coccinea*, bract color polymorphism is observed between populations and within populations. Populations in the Midwest region are predominantly yellow or scarlet (Figure 3.1), with approximately 90% of the individuals typically being one color or the other (Braum, 2014). It is tempting to apply the "pollinator syndrome" paradigm to *C. coccinea* because the species has been reported to attract hummingbirds (Robertson, 1891), but detailed pollinator observation studies have not been published. Insect visitors, but not hummingbirds, were seen at both morphs

during a recent hand-pollination study (Kim et al., 2019). While the genes responsible for bract color have not been identified, a common garden experiment showed that bract color is a genetically inherited trait (Braum, 2014). Bract colors of plants grown in a common garden, using seeds collected from natural populations, resembled the maternal bract colors. Further, results of a hand-pollination experiment suggest the bract color follows simple Mendelian inheritance with yellow dominant over scarlet (Chapter 4).

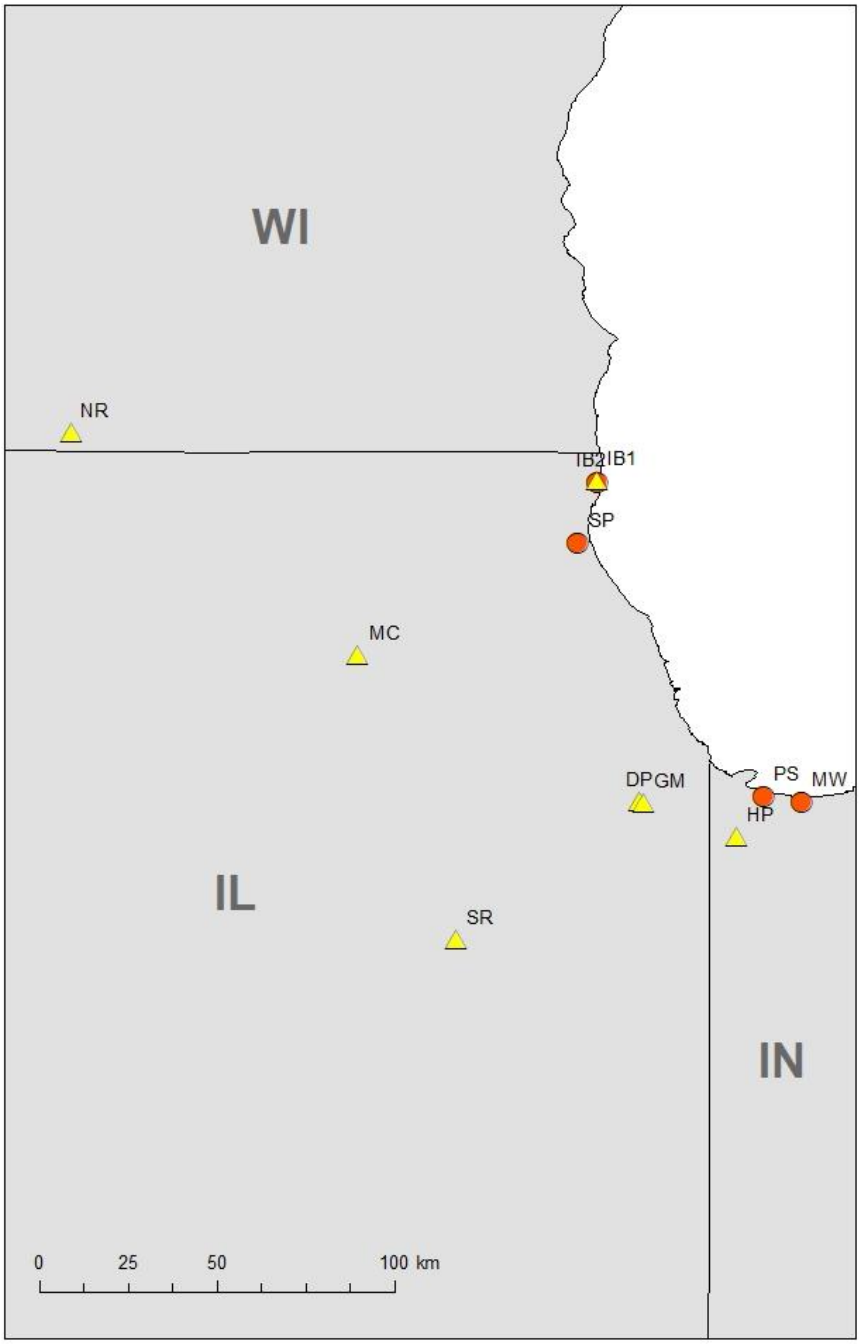

Figure 3.1. Map of 11 *Castilleja coccinea* collection sites.

The aim of this study was to understand factors underlying the distribution of scarlet and yellow populations of *C. coccinea* in the Midwest. These populations represent a unique system to attempt to tease apart ecological and evolutionary factors because there is no obvious explanation underlying their current spatial distributions. In this region two populations can be geographically close to each other and show striking differences in color frequencies (Figure 3.1). There is not a clear latitudinal gradient, and while scarlet populations tend to be closer to Lake Michigan, there are also yellow populations near the lake. The region I examined was covered by a glacier until 14,000 yBP (Gleason, 1922; Colman et al., 1994) so current populations must have arrived relatively recently. Populations close to Lake Michigan may have been established less than 4,500 years ago since lake levels were higher until that time (Larsen, 1985; Thompson and Baedke, 1997). A previous study found few differences in plant communities, edaphic factors, or herbivory between yellow and scarlet populations (Braum 2014).

I used single nucleotide polymorphism (SNP) markers discovered by restriction site-associated DNA sequencing (RADseq) to investigate the population genomics of *Castilleja coccinea*. RADseq is cost effective because it can discover hundreds of SNPs per individual, which then allows a smaller number of samples per population to conduct population genomics studies (Willing et al., 2012). RADseq has sources of errors like allelic dropout due to heterozygous restriction sites, resulting in overestimating genetic variation (Gautier et al., 2013), but such bias can be resolved by conservatively filtering SNP loci to discard loci with missing data (Davey et al., 2013). I used double digest Restriction Site-Associated DNA sequencing

(ddRADseq), a variant method of RADseq which uses two restriction enzymes, one common and one rare, to cut genomic DNA into smaller fragments (Peterson et al., 2012; Puritz et al., 2014).

My goal was to assess the relationship between bract color and the genetic structure of *C. coccinea* populations in the Midwestern United States. Using SNP markers, I addressed several questions regarding the distribution of scarlet and yellow populations: 1) Does floral color explain genetic structure in this region, with yellow populations forming one genetic cluster and scarlet populations another? 2) Is population genetic structure best explained by isolation by distance, regardless of color? 3) Does post-glacial range expansion explain patterns of genetic structure and genetic diversity? 4) Is there evidence for population isolation and genetic drift? I evaluated these questions by assessing patterns of genetic variation, genetic structure, isolation by distance, and bract color distribution. I also used Approximate Bayesian Computation (ABC) to evaluate support for different evolutionary histories.

3.2 Methods

3.2.1 Sampling sites

In 2012, leaf samples were collected from 11 sites, seven predominantly yellow and four predominantly scarlet bract-colored, in the Midwest region (Figure 3.1, Table XIX). Leaf samples were collected from plants with the prevalent bract color morph of each site. Four samples from nine sites (DP, GM, HP, MC, MW, NR, PS, SP, and SR) and six samples from two

sites (IB1 and IB2) (Figure 3.1 and Table XIX) were selected for ddRAD seq (48 samples total). Leaf samples were dried in silica gel.

Table XIX. Location and code of sampling sites, the predominant bract color of a given site, and number of leaf samples collected at each site.

Site	Code	Location	Predominant bract color	N
Dropseed Prairie	DP	Markham, IL	Yellow	4
Gensburg-Markham Prairie	GM	Markham, IL	Yellow	4
Hoosier Prairie	HP	Markham, IL	Yellow	4
Illinois Beach State Park 1	IB1	Zion, IL	Yellow	6
Illinois Beach State Park 2	IB2	Zion, IL	Scarlet	6
Meissner Prairie-Corron Forest Preserve	MC	St. Charles, IL	Yellow	4
Miller Woods, Indiana Dunes National Lakeshore	MW	Gary, IN	Scarlet	4
Newark Road Prairie	NR	Beloit, WI	Yellow	4
Pine Station Nature Preserve	PS	Gary, IN	Scarlet	4
Shaw Prairie, Skokie River Nature Preserve	SP	Lake Forest, IL	Scarlet	4
Sand Ridge Savanna Nature Preserve	SR	Braidwood, IL	Yellow	4

3.2.2. DNA extraction and ddRADseq library preparation

Dried leaf material (approximately 20 mg) were ground in liquid nitrogen and then homogenized using a Qiagen TissueRuptor. Genomic DNA was extracted using a Promega

Maxwell 16 (Madison, WI, USA) at University of Illinois at Chicago, Research Resources Center, DNA Services Facility. The extracted DNA quality was assessed using Agilent TapeStation 2200 (Santa Clara, CA, USA) and quantity was measured using Life Technologies Qubit. Global Biologics (Columbia, MO, USA) completed library preparation and conducted paired-end, double digest Restriction Site-Associated DNA sequencing (ddRADseq) of 100 base pairs length on an Illumina HiSeq 2500 platform. All 48 *C. coccinea* samples were analyzed on a single lane, along with 48 samples of a different plant species. The library preparation protocol followed Poland et al. (2012). The ddRADseq protocol followed Peterson et al. (2012). The restriction enzymes *Eco*RI and *Msp*I were used to generate double-digested fragments for sequencing.

3.2.3 SNP genotyping

I used *Stacks* v2.0b (Catchen et al., 2011, 2013) and followed the Rochette and Catchen (Rochette and Catchen, 2017) protocol to assess paired-end reads and SNP genotype individuals. Samples were demultiplexed using *process_radtags*. After demultiplexing, ten samples with less than one million retained reads were removed from downstream analyses. This resulted in only a single sample remaining from the GM population, so this population was excluded from the downstream analyses, reducing the total number of samples from 48 to 37, and sampled populations from 11 to 10. Since there was no reference genome available, I used the *de novo* genotyping pipeline of *Stacks* v2.0b (Catchen et al., 2011, 2013). I followed the protocol by Rochette and Catchen (2017) for parameter testing on choosing values of *M* (number of mismatches allowed between stacks of a single individual) and *n* (number of mismatches

allowed between stacks for building the catalog). Minimum stack depth (*m*) was fixed at the default value of 3. One sample each from DP, IB2, MC, MW, and NR was randomly chosen. I tested for optimal parameters by conducting test runs using *denovo_map.pl* with a range of parameter values ($M = n$, 1 to 6) and fixing $m = 3$. The *populations* unit of *Stacks* was used to filter the raw results to keep loci that were present in at least 80% of the five test samples. Based on the number of polymorphic loci shared by 80% of samples (Figure 3.2) and the distribution of the number of SNPs per locus (Figure 3.3), $M = n = 3$ were chosen as optimal parameter values.

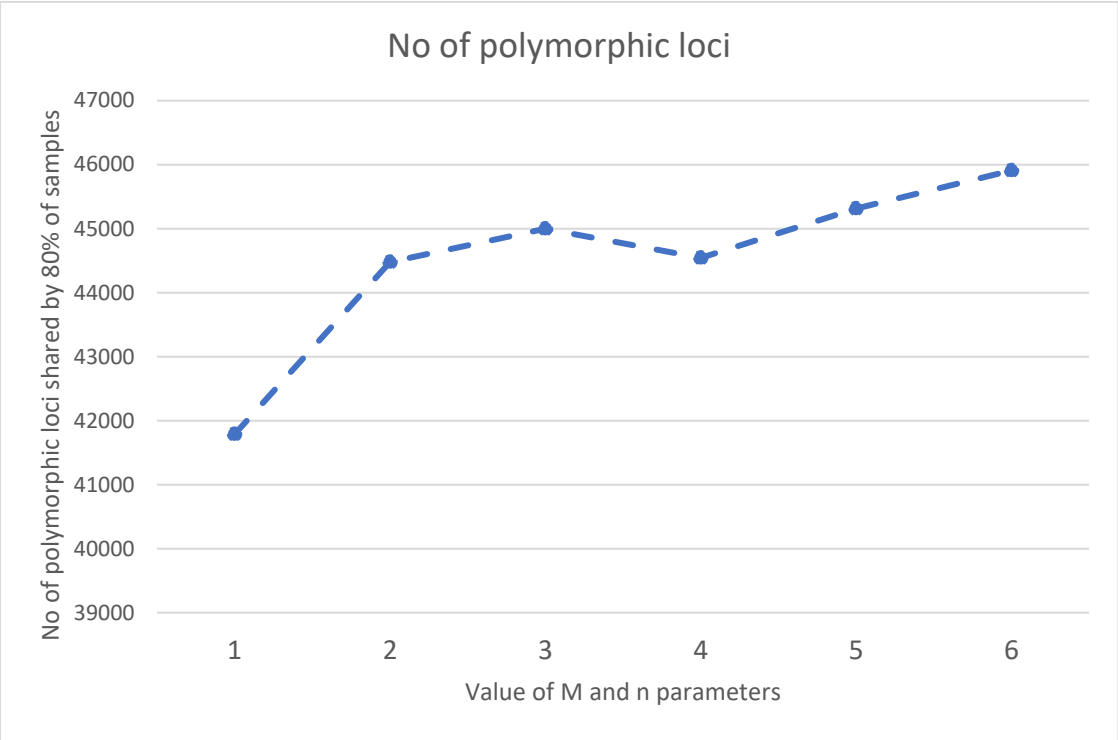

Figure 3.2. Number of polymorphic loci shared by 80% of samples as a function of values of *M* (number of mismatches allowed between stacks of a single individual) and *n* (number of mismatches allowed between stacks for building the catalog) parameters in Stacks. I kept *M* = *n* and *m* (minimum stack depth) = 3.

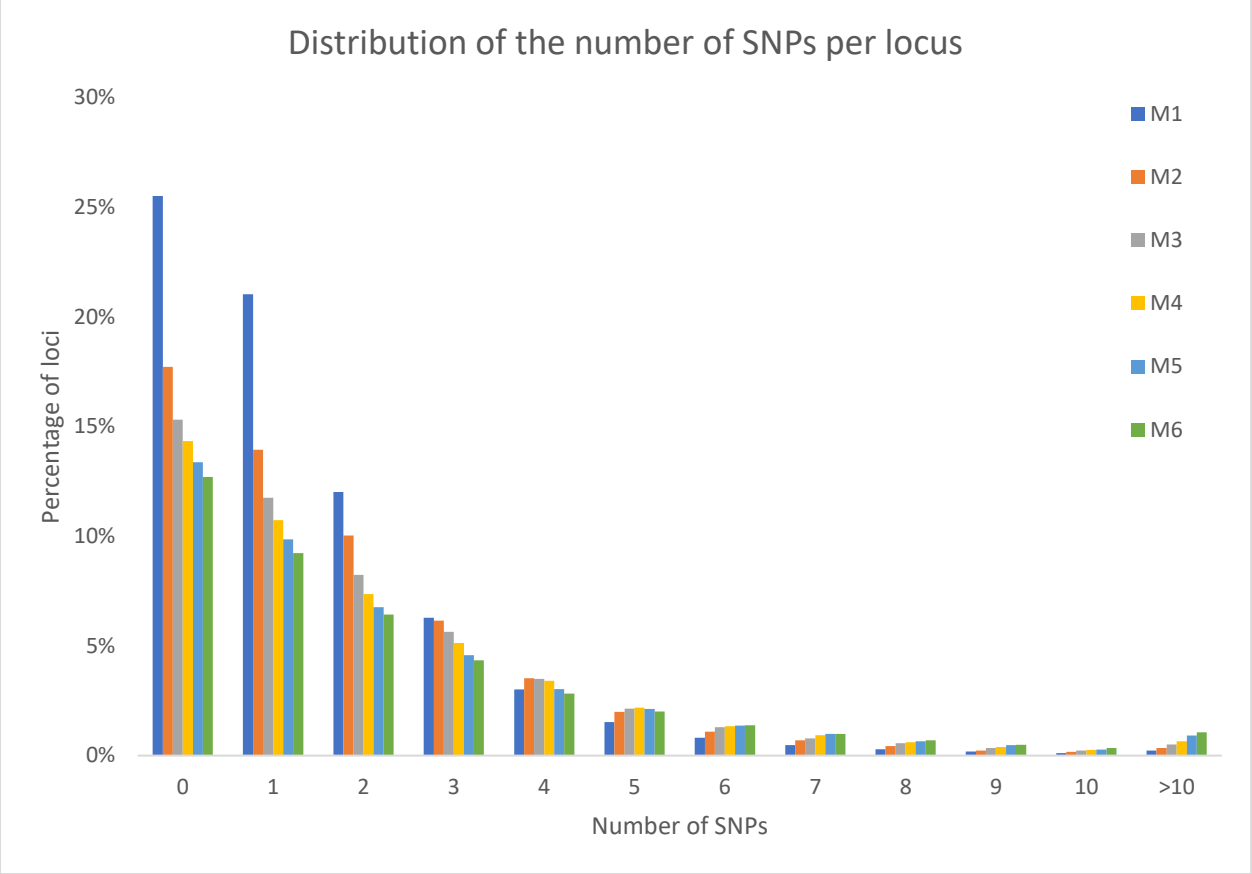

Figure 3.3. Distribution of the number of SNPs per locus and the percentage of loci. I kept *M* = *n*, *m* = 3, and compared different values of *M*. *M* = number of mismatches allowed between stacks of a single individual, *n* = number of mismatches allowed between stacks for building the catalog, and *m* = minimum stack depth)

After determining the optimal parameter values, I ran *ustacks* on 37 samples to find putative loci in each sample. Repetitive stacks with high coverage (greater than mean coverage + 2 x standard deviation) were removed in this step. I ran *cstacks* with 10 samples that had high coverages to generate the catalog. Then *sstacks*, *tsv2bam*, and *gstacks* were used to match the

putative loci in each sample to the catalog, to store the data by locus, to add paired-end reads, and to genotype each sample.

SNP loci were further filtered using the *populations* unit of *Stacks*. Two different data sets were generated by using different schemes to assign samples into groups. For the first data set, I grouped samples by the collection sites (10 collection sites) and retained loci that were present in all populations ($p = 10$) and 75% of the samples ($r = 0.75$), hereafter population data set. For the second data set, I grouped samples by bract color, either yellow or scarlet, and retained loci that were present in both groups ($p = 2$) and 75% of the samples ($r = 0.75$), hereafter color-morph data set. For both data sets, the minimum minor allele frequency (MAF) filter was 0.01 because MAF < 0.01 could be due to sequencing errors. Further, I removed SNPs with maximum observed heterozygosity greater than 0.5 as such excess of heterozygosity could be an indication of paralogous regions in the genome that differ by one nucleotide and are not true SNPs (Hohenlohe et al., 2011). I kept one random SNP per a single RAD locus. The SNP genotype data were exported in *VCF* and *Structure* format.

I searched for SNP loci under selection by identifying F_{ST} outliers between the color morphs using *BayeScan* (Foll and Gaggiotti, 2008). For the F_{ST} outlier analysis, I used a data set generated with less conservative Stacks parameter settings where SNP loci were retained if it was present in both color morphs ($p = 2$) and at least 50 % of the individuals in the group ($r = 0.5$). The SNP genotype file in *Structure* format generated by *Stacks* was converted to *BayeScan* format using *PGDSpider* (Lischer and Excoffier, 2012). I ran *BayeScan* at a default setting for

5000 iterations with a thinning interval of 10. The number of pilot runs was 20 with length of 5000 and burn-in length was 50,000. The prior odds for the neutral model were 100.

3.2.4 Population genetic analyses

Genetic analyses were conducted on both population and color-morph data sets. I compared within population genetic diversity measures presented in the Stacks output; the number of individuals per locus in each population or color morph, total number of nucleotide sites which included both fixed and variant, the number of variant sites, the number of private alleles, the number and proportion of polymorphic loci out of total number of nucleotide sites, observed heterozygosity, and expected heterozygosity. I tested whether the proportion of polymorphic loci was the same among 10 populations and between the two bract color morphs by using two-sample test for equality of proportions (using *prop.test* function) in R version 3.5.1 (R Core Team, 2018). Pairwise F_{st} and p-values were calculated using the StAMPP package (Pembleton et al., 2013) in R. The p-values were obtained using 1000 bootstraps. I generated neighbor joining tree using Ape package version 5.1 (Paradis and Schliep, 2018) in R to graphically represent Nei's distance between populations calculated by StAMPP package.

I used *STRUCTURE* version 2.3.4 (Pritchard et al., 2000) to identify genetic clusters by testing K from 1 to 12 with a burn-in of 100,000 and 500,000 MCMC, not using a LOCPRIOR. I generated 20 independent runs for each *K* using the population data set. I generated 10 independent runs for each *K* using the color-morph data set due to computational limitations.

Results of independent runs for each *K* were summarized using the main pipeline of *CLUMPAK* (Kopelman et al., 2015). Both the Δ*K* method (Evanno et al., 2005) and the method of plotting ln(Pr(X|K)) values (Pritchard et al., 2010), as implemented in the best *K* pipeline of *CLUMPAK* (Kopelman et al., 2015), were used to estimate the optimal *K*. Structure bar plots were graphed using *STRUCTURE PLOT* (Ramasamy et al., 2014).

I conducted a discriminant analysis of principal components (DAPC) using the *adegenet* package in R (Jombart and Ahmed, 2011) to visually assess population structure (Jombart et al., 2010). Samples were assigned to groups by populations because using *k*-means (*find.cluster* function) the lowest BIC value was observed at $K = 1$, which was not informative in determining the optimal number of clusters. I retained 8 PCs after performing cross-validation (*xvalDapc* function) to optimize the number of PCs.

I used *GenAlEx* (Peakall and Smouse, 2006, 2012) to generate the codominant genotypic distance matrix, which was then used to run a Principal Coordinates Analysis (PCoA) to visualize genetic relationship among samples without *a priori* information on population. Four samples that were missing 90 or more SNPs were excluded from PCoA because when included they appeared as clear outliers. The partitioning of hierarchical genetic variation was investigated by conducting analysis of molecular variance (AMOVA) implemented in *GenAlEx*. I also conducted a Mantel test with 9,999 permutations in *GenAlEx* to investigate the relationship between geographic distance and genetic distance in F_{ST} between populations (isolation by distance).

Approximate Bayesian Computation (ABC) was conducted using *DIYABC* 2.1.0 (Cornuet et al., 2014) to investigate the evolutionary history of the populations. I compared four scenarios of population divergence without admixture (Figure 3.4). Scenario 1 was a null hypothesis where all ten populations diverged simultaneously from an ancestral population. In scenario 2, a yellow lineage and a scarlet lineage evolved independently. In scenario 3, I assumed post-glacial expansion in northward direction with no role for bract color. In scenario 4, I assumed post-glacial migration in northeast direction with no role for bract color (Gleason, 1922). In the first comparison, I compared scenarios 1, 2, and 3. In the second comparison, I compared scenarios 1 and 4. I obtained one-sample summary statistics (proportion of monomorphic loci, mean and variance of gene diversity across polymorphic loci, and mean gene diversity across all loci) and two-sample summary statistics measured in F_{ST} distances with 3,000,000 simulated data sets for the first comparison and with 2,000,000 simulated data sets for the second comparison. The logistic regression was used to estimate and compare the posterior probabilities of each scenario.

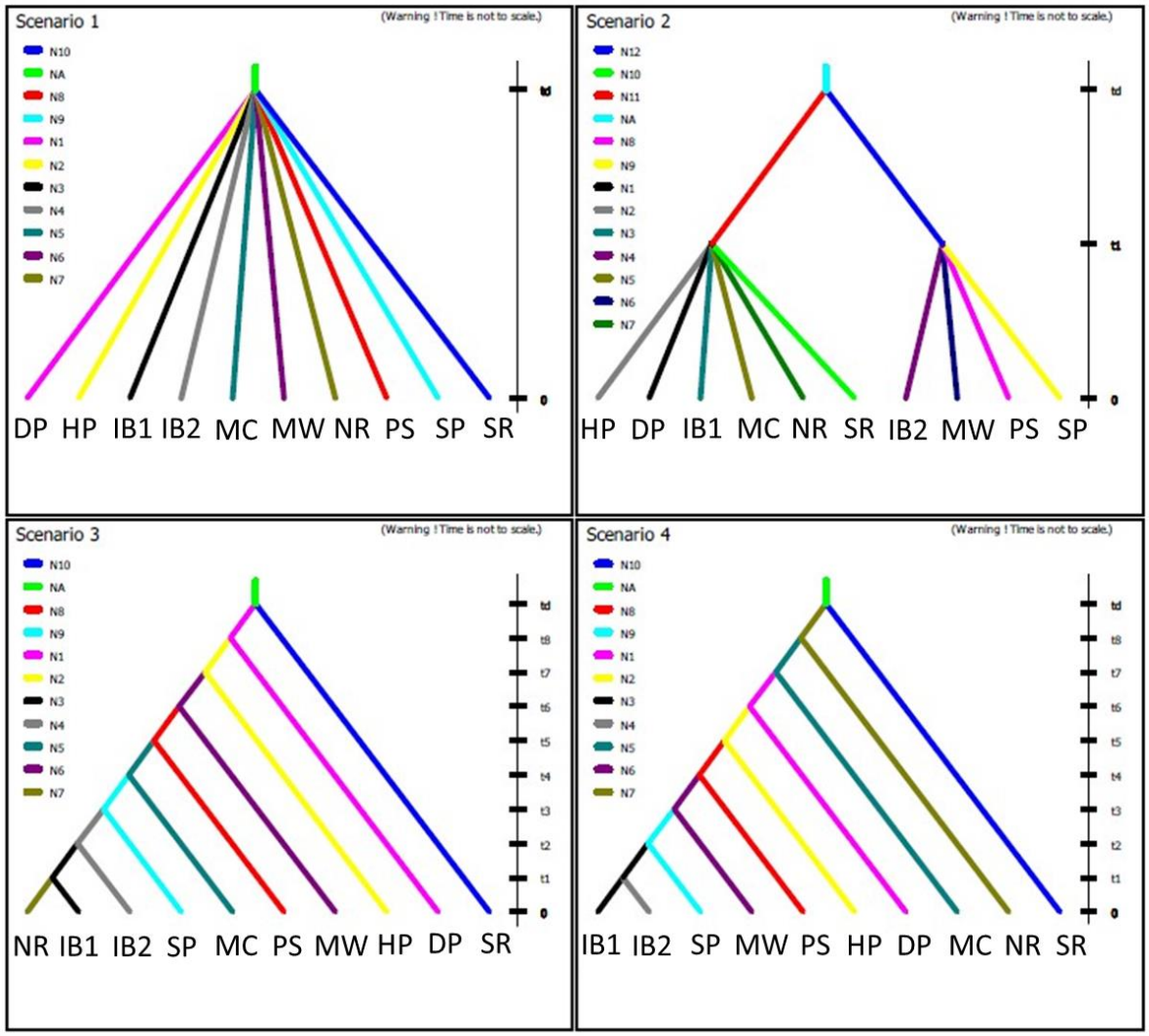

Figure 3.4. Graphical representation of four evolutionary history scenarios compared using DIYABC

3.3 Results

After the 10 individuals with less than one million reads, along with the only remaining GM individual, were removed, the final demultiplexed and quality filtered dataset consisted of 307,343,589 reads. The number of retained reads per individual ranged from 1,416,258 to 23,067,807 (median =7,812,210). For 127,591 loci that were called after merging paired-end contigs with the single-end locus using *gstacks*, the effective per sample coverage ranged from 12.2 to 49.3 (mean = 26.7, stdev = 10.7).

After further filtering using the *populations* program of *Stacks*, I found a total of 268,735 nucleotide sites, of which 958 were variant among populations in the population data set. Observed heterozygosity, which ranged from 0.0889 to 0.140 across loci, was greater than expected heterozygosity in all populations except in NR, suggesting the populations were predominantly outcrossing. The NR population also showed the lowest within population genetic diversity in terms of the proportion of polymorphic loci and the observed heterozygosity. The result of 10-sample test for equality of proportions indicated significant difference in the proportion of polymorphic loci among populations ($p < 0.0001$). For each population, the number of private alleles (variant sites unique to each population) ranged from 24 to 56 whereas the proportion of polymorphic loci ranged from 0.0915 to 0.149 (Table XX).

Table XX. Comparison of within population genetic diversity among 10 populations.

Pop	N	Private	No. polymorphic	%Polymorphic	H_o	H_e
DP	3	24	270	0.100	0.106	0.106
HP	3	29	246	0.0915	0.103	0.0966
IB1	4	33	348	0.130	0.139	0.125
IB2	6	53	400	0.149	0.138	0.127
MC	4	46	348	0.130	0.140	0.124
MW	3	46	305	0.113	0.124	0.113
NR	4	34	240	0.0893	0.0889	0.0949
PS	4	33	311	0.116	0.138	0.111
SP	3	34	300	0.112	0.125	0.113
SR	3	56	293	0.109	0.135	0.110

Pop= population (sampling site), N= number of individuals sampled, Total= total number of nucleotide sites including fixed and variant, Variant= number of variant sites, Private = variant sites unique to each population, No. polymorphic = the number of polymorphic loci out of 268,735 loci, % Polymorphic= the proportion of polymorphic SNP loci, H_o= observed heterozygosity, and H_e= expected heterozygosity. H_o and H_e are based on 958 variant sites only.

For the color-morph data set, I found a total of 1,390,221 nucleotide sites, of which 5532 were variant between the color morphs. The yellow color morph had a higher number of private alleles (variant sites only found in one color morph) and proportion of polymorphic loci compared to the scarlet color morph (0.325 for the yellow color morph and 0.279 for the scarlet morph, Table XXI). The result of 2-sample test for equality of proportions indicated the proportion of polymorphic loci was significantly higher for the yellow color morph ($p < 0.0001$). *BayeScan* did not detect any F_{ST} outliers from 12,134 SNP loci.

Table XXI. Comparison of within population genetic diversity between the bract color morphs.

Bract color	N	Private	No. polymorphic	%Polymorphic	H_e
Yellow	21	1649	4514	0.325	0.171
Scarlet	16	1018	3883	0.279	0.164

N= number of individuals sampled, Total= total number of nucleotide sites including fixed and variant, Variant= number of variant sites, Private = variant sites only found in one color morph, No. polymorphic = the number of polymorphic loci out of 1,390,221 loci, % Polymorphic= the proportion of polymorphic SNP loci, and H_e= expected heterozygosity (genic diversity). H_e is based on 5532 variant sites only.

I found significant levels of genetic differentiation between populations and between the color morphs. Pairwise F_{ST} values ranged from 0.0491 to 0.269 (Table XXII), and all values were statistically significant (the confidence intervals of all pairwise F_{ST} values did not include zero). The lowest pairwise F_{ST} was observed between IB1 and MW, and the highest pairwise F_{ST} was observed between NR and PS. The F_{ST} value between the yellow individuals and the scarlet individuals was lower than any of the pairwise population comparisons (0.0383) but was significant as indicated by non-inclusion of zero in the confidence interval.

Table XXII. Pairwise F_{ST} values below the diagonal and Nei's distance values above the diagonal between pairwise comparison of 10 populations. The green color scales indicate the lowest value in the light green to the highest value in the dark green.

	DP	HP	IB1	MC	NR	SR	IB2	MW	PS	SP
DP	-	0.0743	0.0605	0.0527	0.0739	0.0698	0.0593	0.0635	0.0753	0.0599
HP	0.242	-	0.0518	0.0557	0.0672	0.0697	0.0491	0.0484	0.0619	0.0586
IB1	0.169	0.147	-	0.0457	0.0582	0.0584	0.0299	0.0356	0.0474	0.0364
MC	0.141	0.168	0.113	-	0.0538	0.0547	0.0414	0.0508	0.0562	0.0496
NR	0.261	0.25	0.194	0.179	-	0.0636	0.0503	0.0565	0.0703	0.0522
SR	0.218	0.236	0.17	0.16	0.229	-	0.0509	0.0492	0.0643	0.0535
IB2	0.191	0.158	0.0594	0.121	0.18	0.162	-	0.0356	0.0433	0.0357
MW	0.176	0.125	0.0491	0.131	0.185	0.127	0.0811	-	0.0461	0.0427
PS	0.256	0.22	0.138	0.184	0.269	0.224	0.142	0.131	-	0.054
SP	0.161	0.174	0.0539	0.124	0.165	0.147	0.0815	0.0782	0.168	-

Nei's distance values ranged from 0.0299 to 0.0753 (Table XXII). The lowest Nei's distance value was between IB1 and IB2, and the highest value was between DP and PS. The neighbor joining tree generated from Nei's distance values did not group populations by color (Figure 3.5). The shortest branch lengths were observed between IB1 and IB2. Although four yellow populations (DP, MC, NR, and SR) are connected on the neighbor joining tree, long branch lengths indicate these populations are less genetically similar than the IB1 and IB2 pair with different bract colors.

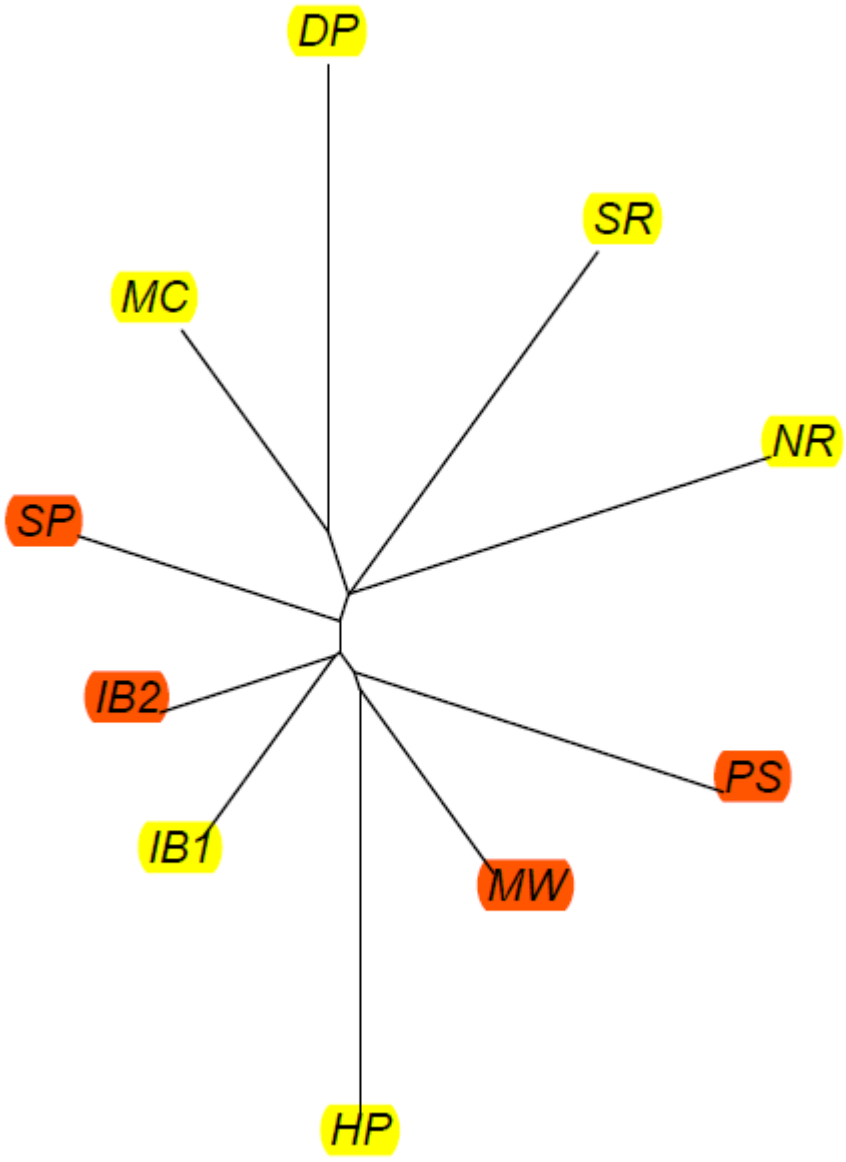

Figure 3.5. Neighbor joining tree generated from Nei's distance between populations

The results of Structure analyses showed a weak pattern of genetic clustering (Figures 3.6 and 3.7). Based on the population data set, the best *K* chosen by the Evanno method and the probability of *K* method were 4 and 8, respectively (Figure 3.8, Appendix I and Figure 3.9, Appendix J). Many individuals were admixed and again there was no evidence of genetic clustering by bract color. Using the color-morph data set, the best *K* chosen by the Evanno method and the probability of *K* method were 2 and 8, respectively (Figure 3.10, Appendix K and Figure 3.11, Appendix L). The Structure bar plots graphed using $K = 2$ (Figure 3.12) and $K = 8$ (Figure 3.13) indicated that samples did not cluster by bract colors.

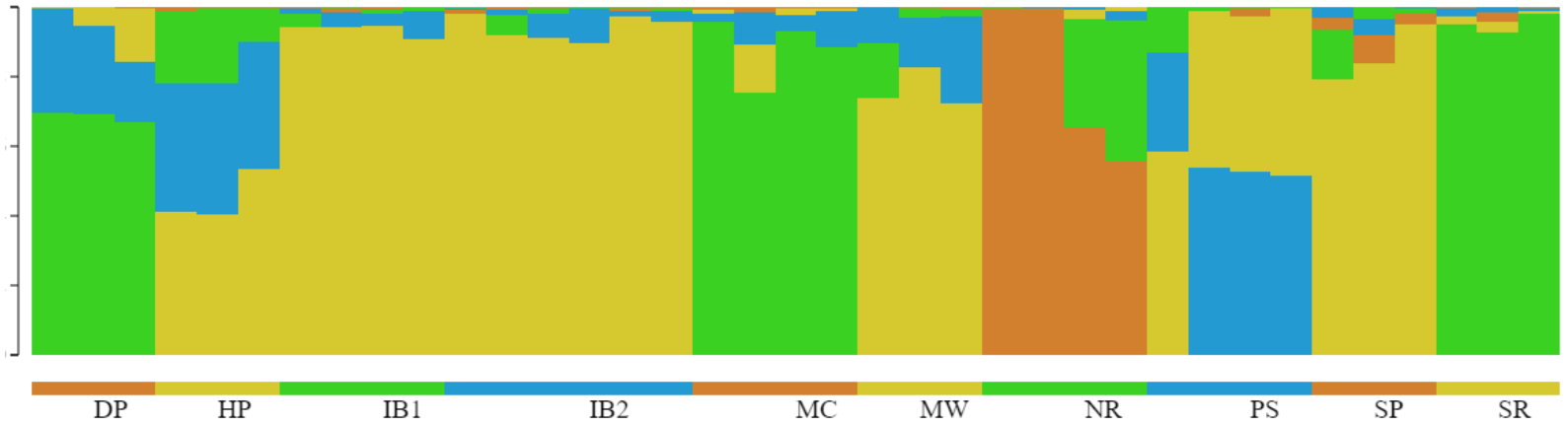

Figure 3.6. The proportion of membership coefficient for each individual in the 10 *C. coccinea* populations for the inferred clusters when K = 4 using the population data set.

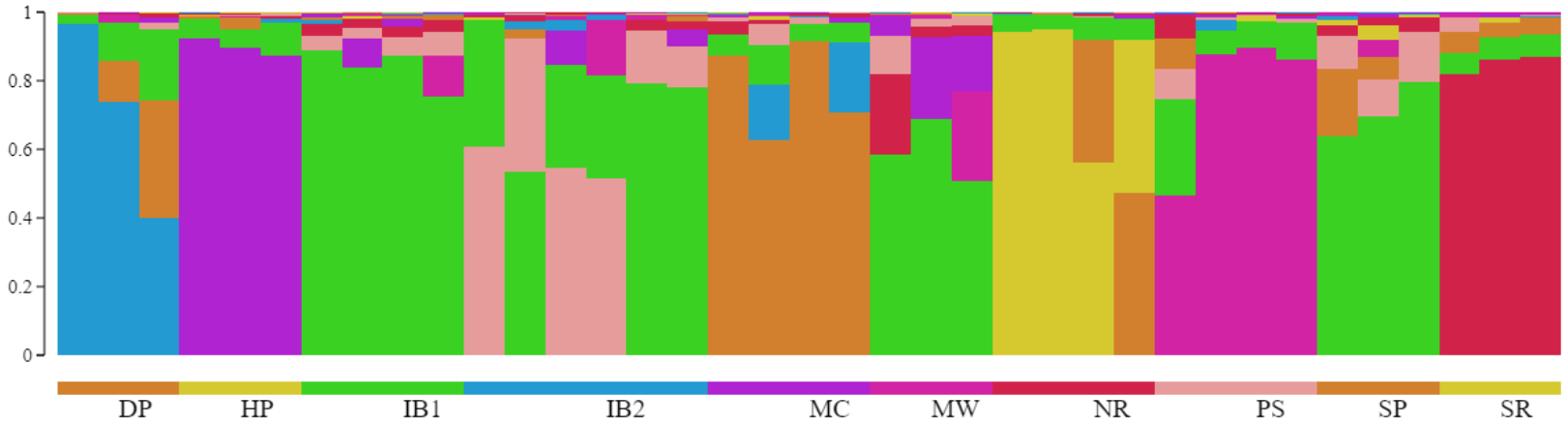

Figure 3.7. The proportion of membership coefficient for each individual in the 10 *C. coccinea* populations for the inferred clusters when K = 8 using the population data set.

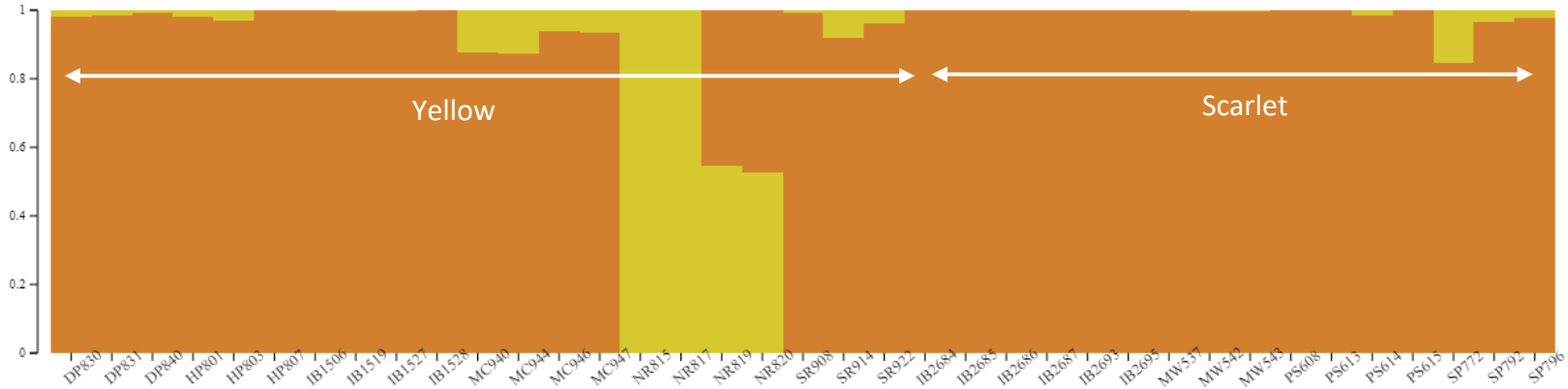

Figure 3.12. The proportion of membership coefficient for each individual in the two *C. coccinea* color morphs for the inferred clusters when K = 2 using the color-morph data set. Sample labels on the X-axis begin with collection site IDs.

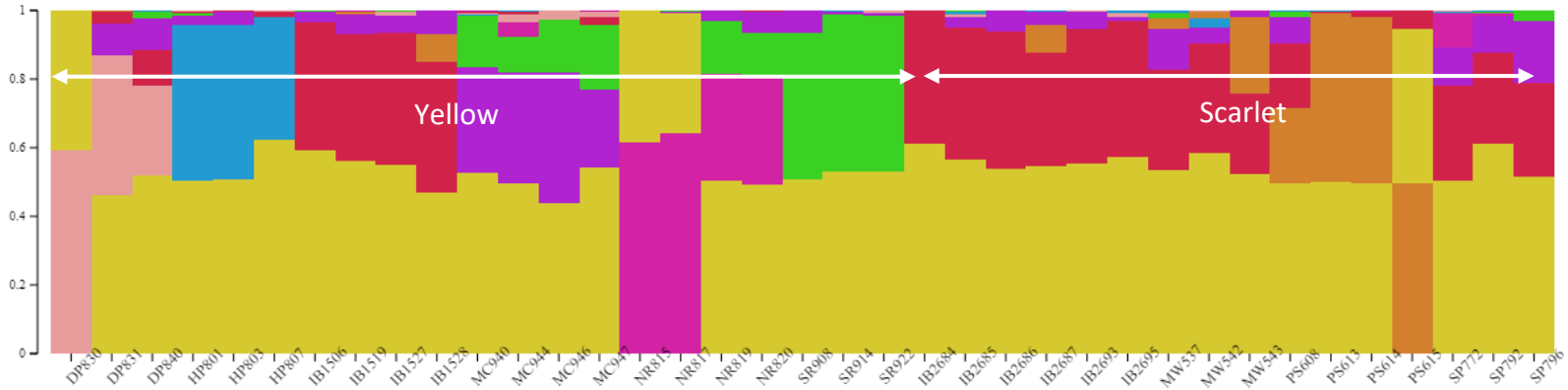

Figure 3.13. The proportion of membership coefficient for each individual in the two *C. coccinea* color morphs for the inferred clusters when K = 8 using the color-morph data set. Sample labels on the X-axis begin with collection site IDs.

DAPC minimizes the variation within groups and maximize the variation among groups (Figure 3.14). Results of DAPC showed that samples within populations were tightly clustered whereas several populations (DP, HP, MC, NR, PS, and SR) were well differentiated from each other. Four populations, IB1, IB2, MW, and SP, clustered together, suggesting genetic similarity among these populations. As in the other analyses, DAPC clustering did not group populations by bract color.

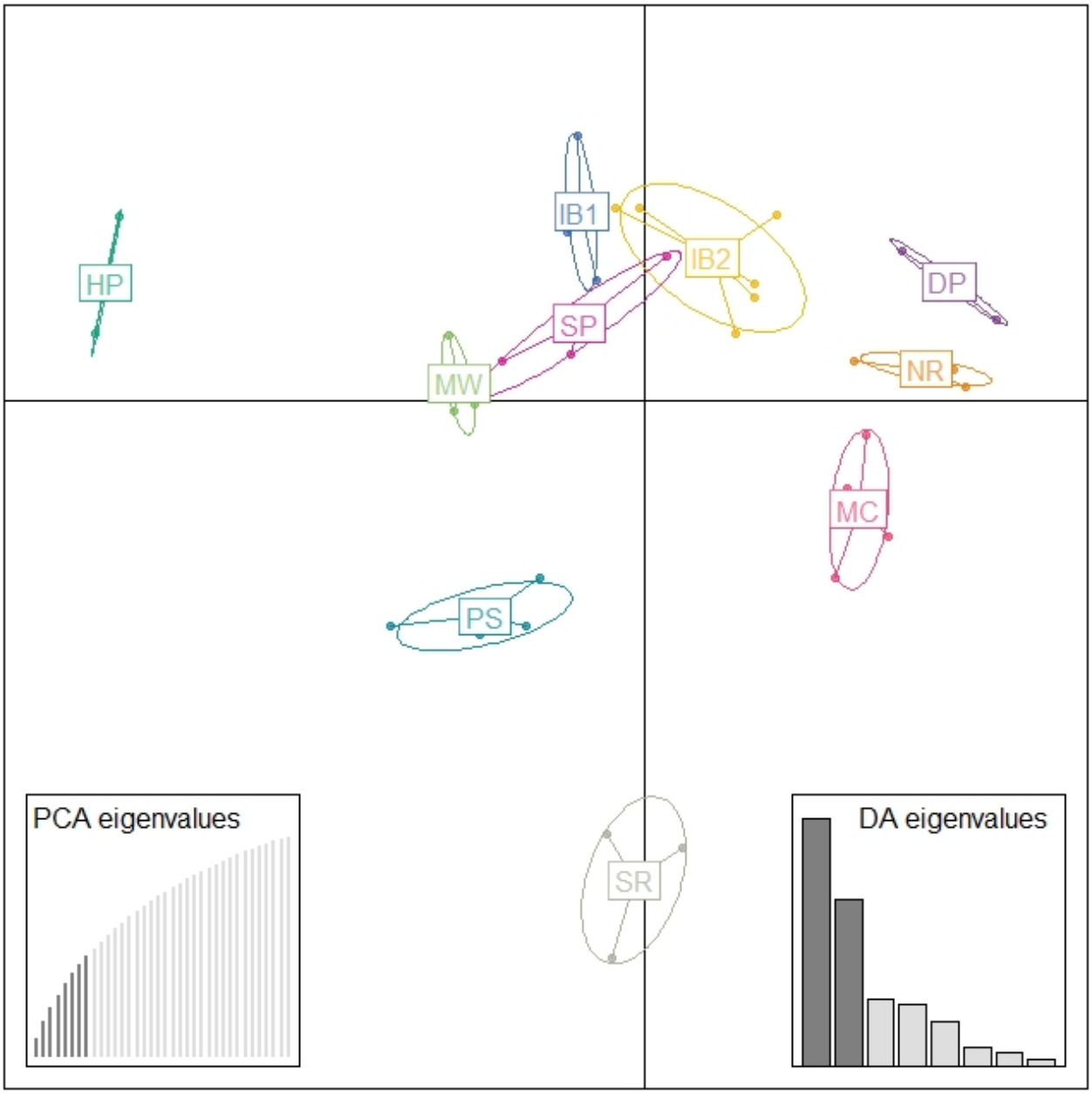

Figure 3.14. Results of discriminant analysis of principal components (DAPC). PCA eigenvalues and DA eigenvalues are shown in the inset.

PCoA did not identify strong patterns of genetic relationship between samples (Figure 3.15). The percentage of the variation explained by the first three axes were 7.27%, 6.66%, and 6.15%. PCoA results showed similar, albeit weak, patterns to the results of Structure. NR samples were genetically distinct from other samples. Hierarchical structure of the dataset revealed that 0 % variation was partitioned between color morphs whereas 83% of the variation was partitioned within populations and 17% was partitioned among populations. The results of the Mantel test, Rxy = 0.291 (P = 0.112), suggested a nonsignificant positive correlation between geographic and genetic distances (Figure 3.16, Appendix M).

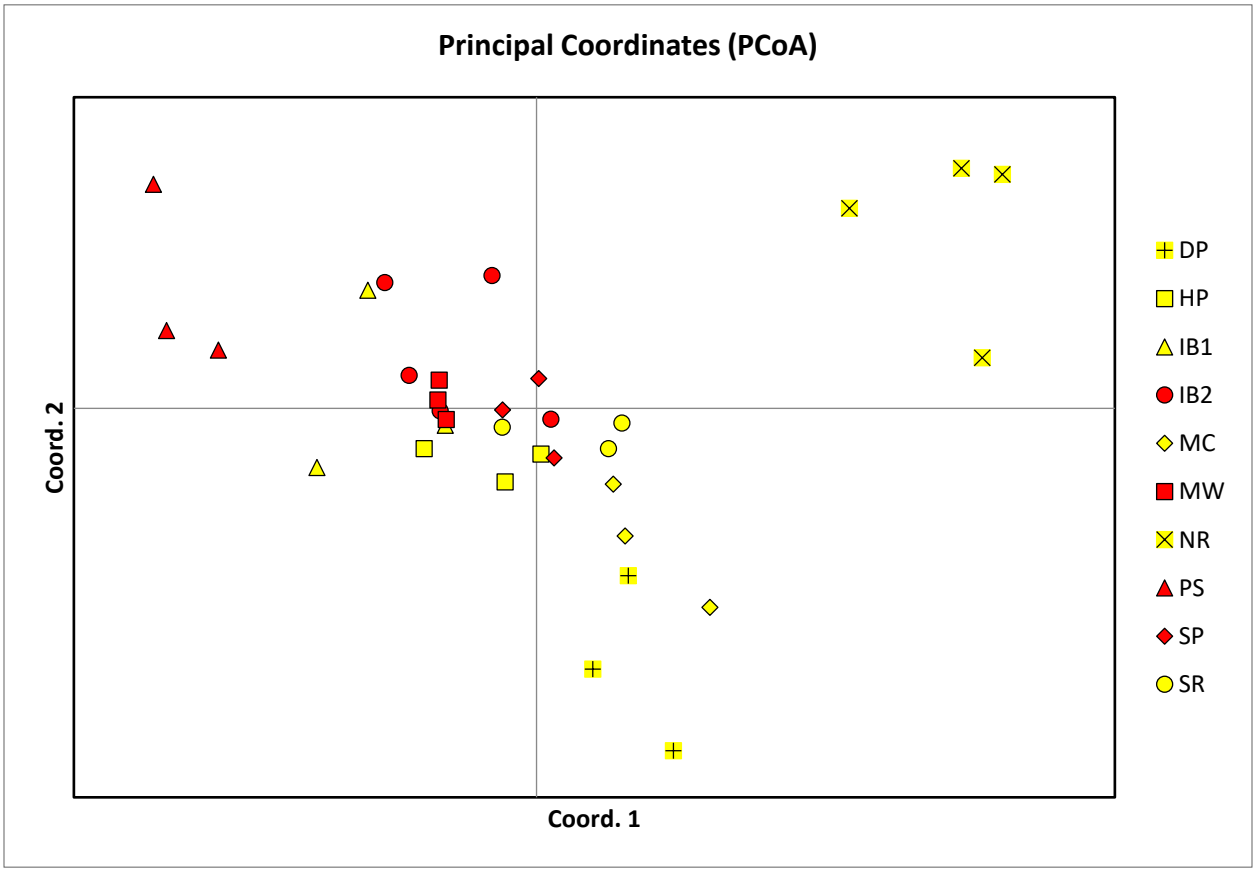

Figure 3.15. Principal Coordinates Analysis (PCoA) results using the population data set, excluding four samples missing more than 90 SNPs. Colors of the markers represent the predominant color of the population.

Comparison of posterior probabilities of scenarios using *DIYABC* identified the null hypothesis scenario as the optimal scenario among the scenarios, 1, 2, and 3 (Figure 3.17, Appendix N). The posterior probability of scenario 1 was significantly different from scenarios 2 and 3 because the 95% confidence intervals did not overlap. In the second comparison, scenario 1 was identified as the optimal scenario compared to scenario 4 (Figure 3.18, Appendix O). The posterior probability of scenario 1 was not significantly different from scenario 4 because the 95% confidence intervals overlapped.

3.4 Discussion

I undertook a ddRADseq study to investigate processes underlying the distribution of yellow and scarlet bract color morphs in *Castilleja coccinea* (Scarlet Indian Paintbrush) in the Midwestern US. I targeted this region because here populations are predominantly one color or the other. Yellow populations are common but scarlet ones also occur, and their distribution has no evident ecological or evolutionary explanation. By examining patterns of genetic structure and differentiation among populations and color morphs I hoped an explanatory mechanism would emerge.

The paired-end, double digest Illumina reads yielded 958 SNP loci that were variable among populations and 5532 that were variable between color morphs. Table XXIII shows the number of sites retained at each filtering step. The final numbers of SNP loci are low compared to many other ddRADseq studies, which typically have tens of thousands of SNPs (Sobel and Streisfeld, 2015; Munshi-South et al., 2016; Trumbo et al., 2016; Gaughran et al., 2017; Shih et

al., 2018), but see (Funk et al., 2016; Çilingir et al., 2017; Johnson et al., 2017). This may be due in part to issues of DNA quality and a relatively low number of reads for some samples (10 individuals with less than a million reads were removed from the study). However, the low number of variable SNP loci discovered in this study may be due to low genetic variability in *C. coccinea* at least in this part of their range. Less than 0.3% of the nucleotide sites (958 of 268,735 sites) were variable across populations. Genetic variation could be reduced in these study populations because of recent range expansion and associated genetic bottlenecks or founder effects (Schwaegerle and Schaal, 1979; Friar et al., 2000; Landergott et al., 2001; Hassel et al., 2005).

Table XXIII. The numbers of SNPs retained after applying each filter

Filter	SNPs retained	
	Population data set	Color-morph data set
After running *gstacks*, including both fixed and variant sites	25,692,045	25,692,045
Variant sites with minor allele frequency >0.01	197,231	197,301
Heterozygosity <0.5	166,379	166,393
Genotyped in 75% of individuals	2,492	18,617
Single SNP per RAD-tag	958	5,532

Despite having relatively few SNP markers, the markers provided sufficient resolution to detect genetic differentiation and structure across the region. Pairwise F_{ST} and Nei's *D* values were significant for all comparisons. While the Structure analysis showed mixed ancestry for some individuals, some populations comprised clear genetic clusters (eg. IB1, IB2 and SP, Figure 3.6). The DAPC analysis also grouped IB1, IB2 and SP, together with MW, but the other populations were genetically differentiated from each other and from the central cluster (Figure 3.10).

The first question I wanted to address was whether the yellow and scarlet bract color morphs were independent evolutionary lineages. Scarlet bract color is most common in unglaciated parts of the species' range, while the yellow bract is more common in areas of the Midwest that were glaciated. It is possible that scarlet and yellow populations experienced distinct histories of refugial isolation and subsequent post-glacial colonization. Gene flow among populations following northward expansion might be low due to the scattered and isolated distribution of *Castilleja coccinea* populations or because pollinator preferences for scarlet or yellow flowers result in assortative mating. In such a scenario, yellow individuals and populations would form one genetic cluster and scarlet individuals and populations would form a separate genetic cluster. This situation corresponds to scenario 2 in the DIYABC analyses (Figure 3.4).

None of my analyses indicated that yellow and scarlet bract colors were independently evolving lineages, or that yellow and red populations formed separate genetic clusters. When yellow and scarlet morphs were compared either as populations or as individuals, I did not find

genetic similarity within the same color or differentiation between colors. High pairwise F_{ST} values were found in comparisons between two yellow populations (eg. NR vs. DP), and between scarlet and yellow populations (e.g. PS vs DP) (Table XXII). Structure analysis indicated that yellow individuals and scarlet individuals did not form two distinct genetic clusters (Figures 3.6, 3.7, 3.12, and 3.13). Rather, many individuals shared ancestry and were highly admixed regardless of the bract color. DAPC clustered four populations (IB1, IB2, SP, and MW) with different bract colors. Other populations were genetically differentiated. Results of DIYABC further support this. Independent evolution of yellow lineages and scarlet lineages (scenario 2) was less likely than simultaneous population divergence from one ancestral population (scenario 1, the best supported scenario). Finally, the results of AMOVA found the highest genetic variation within populations (83%), 17% genetic variation among populations, and no genetic variation partitioned to between bract color morphs.

Once bract color was eliminated as a factor explaining genetic structure in the Midwestern *C. coccinea*, I tested for other geographic patterns, including isolation by distance and post-glacial range expansion. I found that the populations were not genetically differentiated based on geographic distance; the Mantel test showed a nonsignificant positive correlation between the geographic and genetic distances. Small geographic distance was not necessarily associated small genetic distance, as shown in the low F_{ST} value observed between IB1 and MW (Figure 3.1, Table XXII).

The late Wisconsin glacial episode covered my sampling region with ice until 14,000 years ago (Gleason, 1922; Colman et al., 1994). Post-glacial range expansion occurred

northward or northeastward from southern and western refugia (Gleason, 1922). In addition, Lake Michigan water level was higher 4,500 years ago (Larsen, 1985; Thompson and Baedke, 1997), inundating my near Lake sites (IB1, IB2, MW, and PS, Figure 3.1), so these populations were established even more recently than more inland populations. Post-glacial migration and recolonization can cause reduced genetic diversity through a series of founder effects and genetic bottlenecks (Lewis and Crawford, 1995; Broyles, 1998). I did not find a strong signal of the northward or northeastward movement of post-glacial range expansion. Northern populations did not show reduced genetic diversity with respect to the number of private alleles and percent polymorphic loci compared to the southern populations. Results of DIYABC also argue against post-glacial expansion, with scenarios 3 and 4 depicting possible post-glacial expansion having lower support than scenario 1. I also did not find that the near Lake populations had reduced genetic diversity or other evidence of recent founder effects. I did not observe a pattern of reduced private alleles and number of polymorphic loci in northern populations or near Lake populations. IB2 and NR are both northern populations in this study. IB2 had the highest number of polymorphic loci despite being near Lake Michigan and NR had the lowest number of polymorphic loci although it was located more inland.

My last question was whether the genetic structure of the Midwestern *C. coccinea* was the result of population isolation and genetic drift. To conclude that neutral processes are responsible, all potential hypotheses for natural selection must be tested. Only a few studies report indirect evidence for neutral processes underlying the spatial distribution and maintenance of floral color polymorphism (Keasar et al., 2016; Wang et al., 2016). I found some indirect evidence of genetic drift for *C. coccinea* populations in this study. High F_{ST} values were

observed in pairwise comparisons involving populations that showed low genetic diversity (DP, HP, and NR). While isolation and genetic drift may be operating in this region, it fails to explain the distribution of bract colors. For example, population isolation would likely not result in yellow and scarlet populations being adjacent to each other, as is the case of IB1 and IB2, which are only about 500 m apart.

A hand-pollination experiment at these two sites, IB1 and IB2, may offer a possible explanation that was not addressed in the current study (Kim et al., 2019). This experiment was conducted using crosses within and between IB1 and IB2 and revealed reproductive trade-offs. The scarlet morph had higher reproductive output (seed set) when hand pollinated, while the yellow morph had higher fitness when pollinators were excluded, and thus a reproductive assurance advantage. Recolonization from refugia during post-glacial expansion might have favored the yellow morph if pollinators were limited. The younger populations near Lake Michigan shoreline could have been established when pollinators were more abundant, giving the scarlet morph an advantage at these sites.

In conclusion, I report that bract colors fail to explain the patterns of population structure and genetic diversity observed in *C. coccinea* populations in this study. The distribution of bract colors was not associated with the population structure or patterns of genetic differentiation. I did not find neutral genetic differentiation between the color morphs, which suggests gene flow between the color morphs was not limited between the color morphs. The results further indicate that geographic distance or evolutionary history scenarios also fail to explain the apparent population structure and genetic diversity. Further investigation is needed to evaluate the role of

reproductive trade-offs in the distributions and maintenance of bract color polymorphism in *C. coccinea*.

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4. INHERITANCE OF BRACT COLOR IN *CASTILLEJA COCCINEA*

4.1 Introduction

Angiosperms display a wide range of floral colors, and in many species floral colors vary intraspecifically (Wright, 1943; Brown and Clegg, 1984; Stanton, 1987; Wolfe, 1993; Niovi Jones and Reithel, 2001). Intraspecific floral color polymorphism may be observed between populations (Miller, 1981; Streisfeld and Kohn, 2005; Schemske and Bierzychudek, 2007; Arista et al., 2013) or within populations (Levin and Brack, 1995; Gigord et al., 2001; Irwin and Strauss, 2005; Eckhart et al., 2006; Takahashi et al., 2015). Environmental factors such as temperature, light intensity, and UV-B exposure may affect intensity of floral colors (Ben-Tal and King, 1997), and explain variation in floral colors between populations. Microscale environmental differences could be responsible for within population polymorphism. Alternatively, floral colors may be genetically determined and be expressed quite consistently in different environments (Whibley et al., 2006).

Floral colors result from three groups of color pigments, betalains, carotenoids, and anthocyanins (Grotewold, 2006). Betalains are responsible for yellow to orange and red to violet colors. Betalains are only found in the order Caryophyllales and do not co-occur with anthocyanins. Anthocyanins are found in the majority of vascular plants, and cause orange, red, purple, and blue floral colors. Carotenoids are synthesized and stored in plastids and are present in all plants for protection from photo-oxidative damage and production of phytohormone abscisic acid (Cazzonelli, 2011). Further, carotenoids are substrates for synthesizing the

phytohormone abscisic acid (Grotewold, 2006). As floral pigments, carotenoids produce colors ranging from yellow to orange, but when carotenoids and anthocyanins are both present, orange-red, bronze, and brown colors are displayed (Forkmann, 1991). These color pigments can be expressed in petals as well as bracts (Stewart et al., 1969).

Castilleja coccinea, scarlet Indian paintbrush, is a hemiparasitic forb, native to the U.S.A. Its range extends from Maine to Minnesota, and south to Kansas through Oklahoma through Florida. The green-colored flowers are surrounded by colorful bracts. In *C. coccinea*, the most prevalent bract color across its range is scarlet (orange-red), but bract colors ranging from yellow to scarlet are also observed. In the Midwestern region of United States, both scarlet and yellow plants occur, but in each population greater than 90% of individuals exhibit the same bract color (Braum, 2014). The alternative bract color plants do occur at low frequency, and they are not spatially separated; I have observed two individuals 10 cm apart with different yellow and scarlet bract color. Results from a common garden experiment indicated that bract color was primarily a heritable trait and not heavily influenced by environmental factors (Braum, 2014), but the mode of inheritance has not been investigated.

Color pigments are most accurately characterized using high-performance liquid chromatography (HPLC), work that has not been done in *C. coccinea.* Anthocyanins have been visually assessed without using HPLC in *Castilleja hispida* and *Castilleja levisecta* (Haan et al., 2018). To the best of my knowledge, the only study of pigments in *C. coccinea* was conducted nearly a century ago, using methods available at time (Weatherby, 1922). Weatherby first boiled petals in water or alcohol, then added a drop of acid or ammonia. He reported observing an

anthocyanin reaction for scarlet bract color and plastid pigment, presumably carotenoids, reaction for yellow bract color. Although limited, these studies make it is reasonable to assume that the range of bract colors, yellow to scarlet, in *C. coccinea* are due to carotenoids and anthocyanins, since anthocyanins are quite common in plants and carotenoids are universal (Grotewold, 2006).

The goal of my study was to investigate inheritance patterns of bract colors in *C. coccinea*. I conducted a hand-pollination experiment among yellow, orange, and scarlet bract colored individuals. I varied bract colors of pollen donors (paternal plants) and recipients (maternal plants) to investigate transmission patterns of parental bract colors to offspring. Seeds resulting from hand-pollinations were collected, sown, and grown until offspring plants flowered. I assessed the resulting offspring bract colors to infer the mode of inheritance for bract colors.

4.2 Methods

Seeds were collected by Anna Braum from natural populations in the summer of 2012 and sown in 2013 for a common garden experiment at Chicago Botanic Garden (Braum, 2014). In late May 2014, I selected a total of 26 individuals, 19 yellow, 3 scarlet, and 4 orange, from 8 populations for a hand-pollination experiment. I moved 26 potted plants to a growth chamber at Chicago Botanic Garden to exclude animal pollinators. Bract colors of scarlet and orange individuals were assessed using a Royal Horticultural Society (RHS) Colour Chart (Royal Horticultural Society, London, England). Bract colors of yellow individuals were less ambiguous

to determine than distinguishing between scarlet and orange, so 11 of 19 yellow individuals were assessed using the RHS Colour chart and the rest were recorded as yellow via visual assessment. A total of 26 crosses were made between same bract colors and different bract colors, including self-pollination (Table 1). Flowers on the same plant that received different treatments were marked with different colored threads. In early June 2014, I collected fruits and stored seeds at room temperature. In May 2015, seeds from 7 maternal plants were transferred to Chicago Botanic Garden for cold stratification and germination. Seedlings were kept in a warm greenhouse for two months and were vernalized for three months at 1.7 °C. The plants were then kept in the warm greenhouse until they flowered in March 2016. I assessed the bract colors of the offspring using the RHS Colour Chart. I converted RHS values to four color groups. RHS values ranging from 2 – 13 were "yellow", 14 – 23 were "yellow-orange", 24 – 29 were "orange", and 30 – 33 were "scarlet." Crosses from different individuals were assigned to one of the ten "cross types" to search for patterns in bract color transmission to the offspring generation. The ten cross types were 1) yellow recipient and yellow pollen donor; 2) yellow recipient and scarlet pollen donor 3) yellow recipient and orange pollen donor; 4) yellow self-pollination; 5) scarlet recipient and scarlet pollen donor; 6) scarlet recipient and yellow pollen donor; 7) scarlet self-pollination; 8) orange recipient and yellow pollen donor; 9) orange recipient and scarlet pollen donor; and 10) orange self-pollination.

4.3 Results

Excluding one "yellow x yellow" cross that did not yield any offspring, other crosses yielded a total of 315 offspring that survived and flowered by the time of bract color assessment

(Table XXIV). I pooled offspring bract color data by cross types. Offspring belonged to one of the four color groups, yellow, scarlet, orange, and yellow-orange. I observed an unambiguous pattern that crosses involving yellow individuals (yellow x yellow, yellow x scarlet, yellow x orange, scarlet x yellow, and orange x yellow) produced yellow offspring. The only exception was yellow self-pollination where one offspring (of 68) showed orange bract color while the other offspring showed yellow bract color. In crosses involving scarlet and orange (scarlet x scarlet, scarlet self- pollination, orange x scarlet, and orange self-pollination), offspring showed a range from scarlet to orange bracts, but no yellow offspring were observed. The only yellow-orange offspring were produced by the orange self-pollination cross.

Table XXIV. Number of offspring from ten cross types and their bract colors

Cross type	N	Total Offspring	Yellow	Scarlet	Orange	Yellow-orange
Yellow x Yellow	7	74	74	0	0	0
Yellow x Scarlet	3	28	28	0	0	0
Yellow x Orange	3	30	30	0	0	0
Yellow self-pollination	5	68	67	0	1	0
Scarlet x Scarlet	1	26	0	7	19	0
Scarlet x Yellow	2	25	25	0	0	0
Scarlet self-pollination	1	2	0	2	0	0
Orange x Yellow	1	26	26	0	0	0
Orange x Scarlet	1	12	0	10	2	0
Orange self-pollination	1	24	0	0	20	4
Total	25	315	250	19	42	4

Cross type= bract color of the recipient x bract color of the pollen donor, N= number of crosses, and Yellow, Scarlet, Orange, and Yellow-orange= the four color groups and shows the number of offspring.

4.4 Discussion

The results of this hand-pollination experiment suggest that bract color of *Castilleja coccinea* is likely primarily controlled by multiple alleles at a single locus and follows an autosomal dominant mode of inheritance with yellow dominant over scarlet and orange. In several other species, floral color follows simple Mendelian inheritance and is controlled by a

single locus with two alleles. Examples include purple and white flower colors of *Parthenium argentatum* (Estilai, 1984), crimson and bright pink flower colors of *Trifolium incarnatum* (Mosjidis, 2000), purple and pink flower colors of *Ipomoea purpurea* (Zufall and Rausher, 2003), and blue and white flower colors of *Pontederia cordata* (Gettys and Wofford, 2007). Since the pigments responsible for yellow to scarlet bract colors remain unknown, it is difficult to suggest what type of mutation in which gene would cause intraspecific variation of bract colors in this species. In the following paragraphs, I discuss which color pigments could be responsible for the three color morphs of *C. coccinea*. I also consider the possibilities of loss of anthocyanins vs. gain of anthocyanins.

Pelargonidins, cyanidins, and delphinidins are three basic anthocyanins responsible for different flower colors. The scarlet color of *C. coccinea* is likely due to pelargonidins that give orange/brick red colors (Tanaka and Brugliera, 2013). Cyanidins give magenta/red colors as in *Phlox drummondii* (Hopkins and Rausher, 2011) whereas delphinidins give blue/violet colors as in *Antirrhinum kelloggii* (Ishiguro et al., 2012; Tanaka and Brugliera, 2013). Although astaxanthin, a type of carotenoids, can also produce orange-red colors, it is found in only few plants like *Adonis aestivalis* and *A. annua* (Tanaka et al., 2008).

Carotenoids are color pigments responsible for yellow flower colors of many species such as *Brassica napus* (Zhang et al., 2015), *Calendula officinalis* (Pintea et al., 2000), and *Mimulus aurantiacus* (Streisfeld and Kohn, 2005). In addition, chalcone and aurones can create yellow flower colors. Inactivity of an enzyme chalcone isomerase in the early stage of the anthocyanin biosynthesis pathway causes pale yellow colored chalcone to accumulate, which

results in yellow flowers of *Callistephus chinensis* (Kuhn et al., 1978), *Dianthus caryophyllus* (Forkmann and Dangelmayr, 1980), and *Cyclamen persicum* (Miyajima et al., 1991). Bright yellow flowers of *Antirrhinum majus*, *Dahlia variabilis*, *Cosmos bipinnatus*, and *Limonium* are produced by aurones, a class of flavonoids that anthocyanins and chalcones also belong to (Martin and Gerats, 1993; Ono et al., 2006; Tanaka et al., 2008). While chalcone and aurones produce pale yellow and brighter yellow flowers, respectively, only carotenoids, especially xanthophylls, can give rise to a range of yellow colors from pale yellow to deep yellow that are observed in *C. coccinea* (Tanaka et al., 2008).

The orange bract color of *C. coccinea* may be caused by presence of both anthocyanins and carotenoids. In *Gentiana lutea*, comparison of total carotenoid contents and the total anthocyanin contents between yellow and orange flowers revealed that anthocyanins were only found in orange flowers while carotenoids were present in both yellow and orange flowers at similar levels (Berman et al., 2016). Thus, it is possible that orange bract color of *C. coccinea* is due to a higher amount of anthocyanins combined with carotenoids as in *Chrysanthemum morifolium*, *Gerbera jamesonii*, and *Zinnia elegans* (Kishimoto et al., 2007). On the other hand, orange flowers can also be produced by higher concentration of carotenoids or higher concentration of carotenoids with more red color (Kishimoto et al., 2007).

The bract color shift in *C. coccinea* could have been from scarlet to yellow or from yellow to scarlet. Loss of anthocyanins would change bract colors from scarlet to yellow. Three transcription factors, R2R3 MYB, bHLH, and WDR, regulate the anthocyanin biosynthetic pathway (Wessinger and Rausher, 2012). Mutations at R2R3 MYB cause loss of anthocyanins in

floral tissues only (Wessinger and Rausher, 2012). Unlike R2R3 MYB, bHLH and WDR are not tissue-specific, so mutations at bHLH and WDR would cause loss of anthocyanin pigments in floral tissues and other flavonoids in vegetative tissues (Wessinger and Rausher, 2012). Such mutations have deleterious effects because flavonoids are essential for plant developmental processes (Taylor and Grotewold, 2005). Studies have found mutations at R2R3 MYB were responsible for the transition from anthocyanin pigmented to non-pigmented flowers in *Petunia integrifolia* and *Antirrhinum majus* (Sobel and Streisfeld, 2013).

Gain of anthocyanins could have caused a transition from yellow (carotenoids only) to scarlet. Smith and Goldberg (2015) found that gains of anthocyanins were favored over losses of anthocyanins in the floral color evolution of four clades (Antirrhineae, Iochrominae, Loeselieae, Quamoclit). In many plants, the anthocyanin biosynthetic pathway produces other flavonoids in vegetative tissues, even when floral tissues do not display anthocyanin pigments (Sobel and Streisfeld, 2013). Thus, the gain of anthocyanins does not necessarily mean generating the anthocyanin biosynthetic pathway. In *Phlox drummondii*, anthocyanins increase in the floral tissue as a result of a cis-regulatory mutation. In *Mimulus aurantiacus*, a *cis*-regulatory mutation in an existing R2R3 MYB gene caused the gain of anthocyanin pigments in floral tissue (Sobel and Streisfeld, 2013).

To summarize, the hand-pollination study described here suggests that bract color of *C. coccinea* is controlled by multiple alleles at a single locus. Bract colors that range from pale yellow to scarlet are likely due to anthocyanins and carotenoids, but future research using HPLC could identify the color pigments present in yellow, orange, and scarlet morphs. Sequencing

color pigment gene fragments from bracts (Berman et al., 2016) would allow further investigation into genetic basis of bract color polymorphism in *C. coccinea*. Analysis of SNP markers, similar to the restriction site-associated DNA sequencing (RADseq) study described in chapter 3, could reveal genomic regions that segregate with bract color and eventually lead to identification of the mutations that control bract color in this species.

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APPENDICES

A

Table IV. Sample size for fruit set comparison between self-pollination and outcrossing.

	Yellow population		Scarlet population	
Treatment	SP	SS	SP	SS
No. individuals	3	2	3	3
No. flowers	22	11	43	32

B

Table VI. Sample size for seed set comparison between self-pollination and outcrossing.

	Yellow population		Scarlet population	
Treatment	SP	SS	SP	SS
No. individuals	3	2	3	3
No. fruits	16	9	14	10

C

Table VIII. Sample size for fruit set comparison in the open control between the two populations.

	OC Yellow	OC Scarlet
No. individuals	3	3
No. flowers	31	58

D

Table IX. Sample size for seed set comparison in the open control between the two populations.

	OC Yellow	OC Scarlet
No. individuals	2	2
No. fruits	6	10

E

Table X. Sample size for fruit set comparison between bagged, no hand pollination and self-pollination.

	Yellow population		Scarlet population	
Treatment	BN	SP	BN	SP
No. individuals	3	3	3	3
No. flowers	31	22	33	43

F

Table XI. Sample size for seed set comparison between bagged, no hand pollination and self-pollination.

	Yellow population		Scarlet population	
Treatment	BN	SP	BN	SP
No. individuals	3	3	3	3
No. fruits	13	16	14	14

G

Table XIV. Sample size for fruit set comparison in inter-population and inter-morph crosses.

	Yellow population				Scarlet population			
Treatment	SS	DS	SD	DD	SS	DS	SD	DD
No. individuals	2	3	3	3	3	3	3	3
No. flowers	11	40	29	35	32	34	31	39

H

Table XV. Sample size for seed set comparison in inter-population and inter-morph crosses.

	Yellow population				Scarlet population			
Treatment	SS	DS	SD	DD	SS	DS	SD	DD
No. individuals	2	3	2	2	3	3	3	3
No. fruits	9	12	6	13	10	10	26	26

I

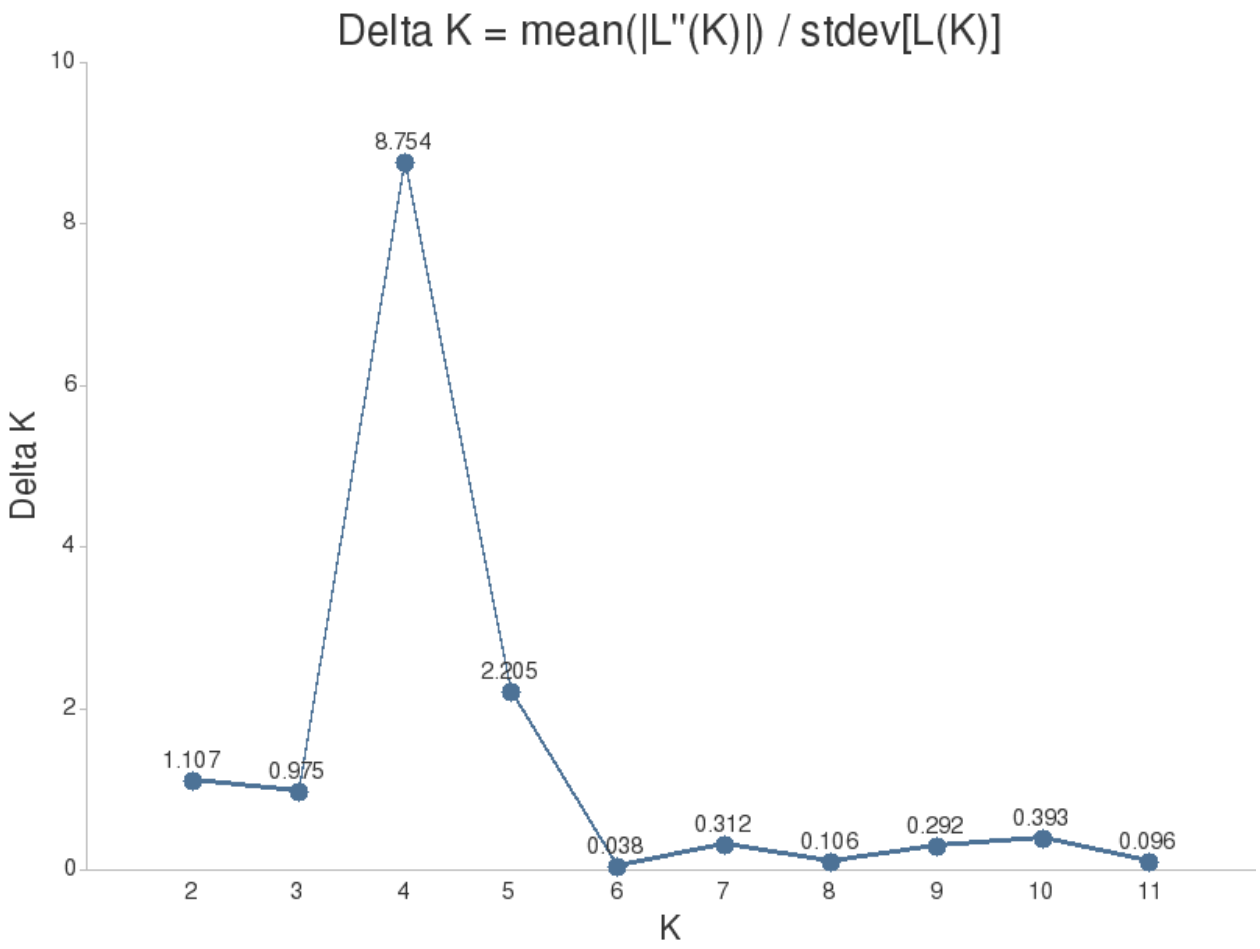

Figure 3.8. A plot of delta *K* values (Evanno et al. 2005) for *K* from the results of STRUCTURE analysis (Pritchard et al., 2000) of *Castilleja coccinea* individuals using the population data set

J

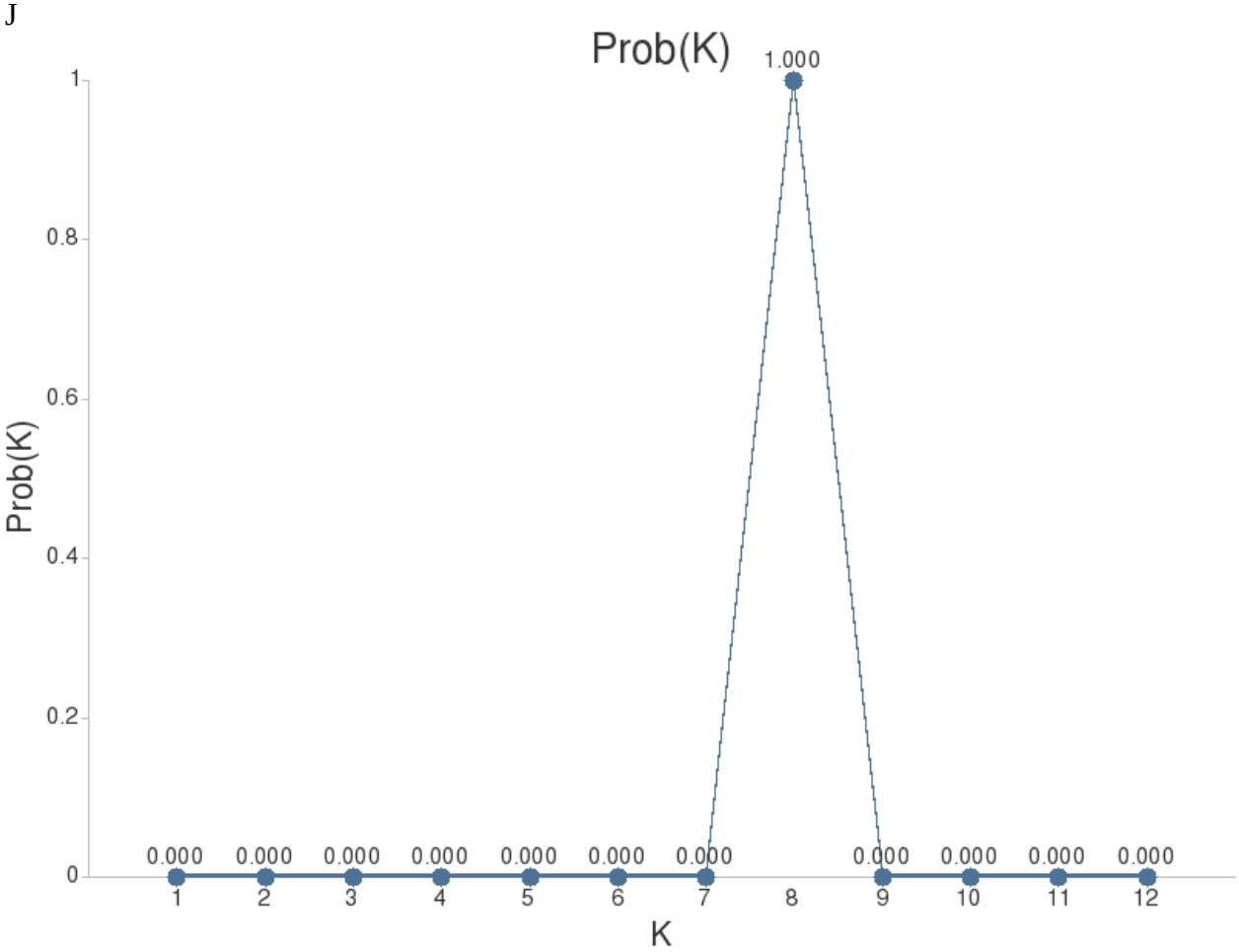

Figure 3.9. A plot of probability of *K* and *K* values in STRUCTURE analysis (Pritchard et al., 2000) of *Castilleja coccinea* using the population data set.

K

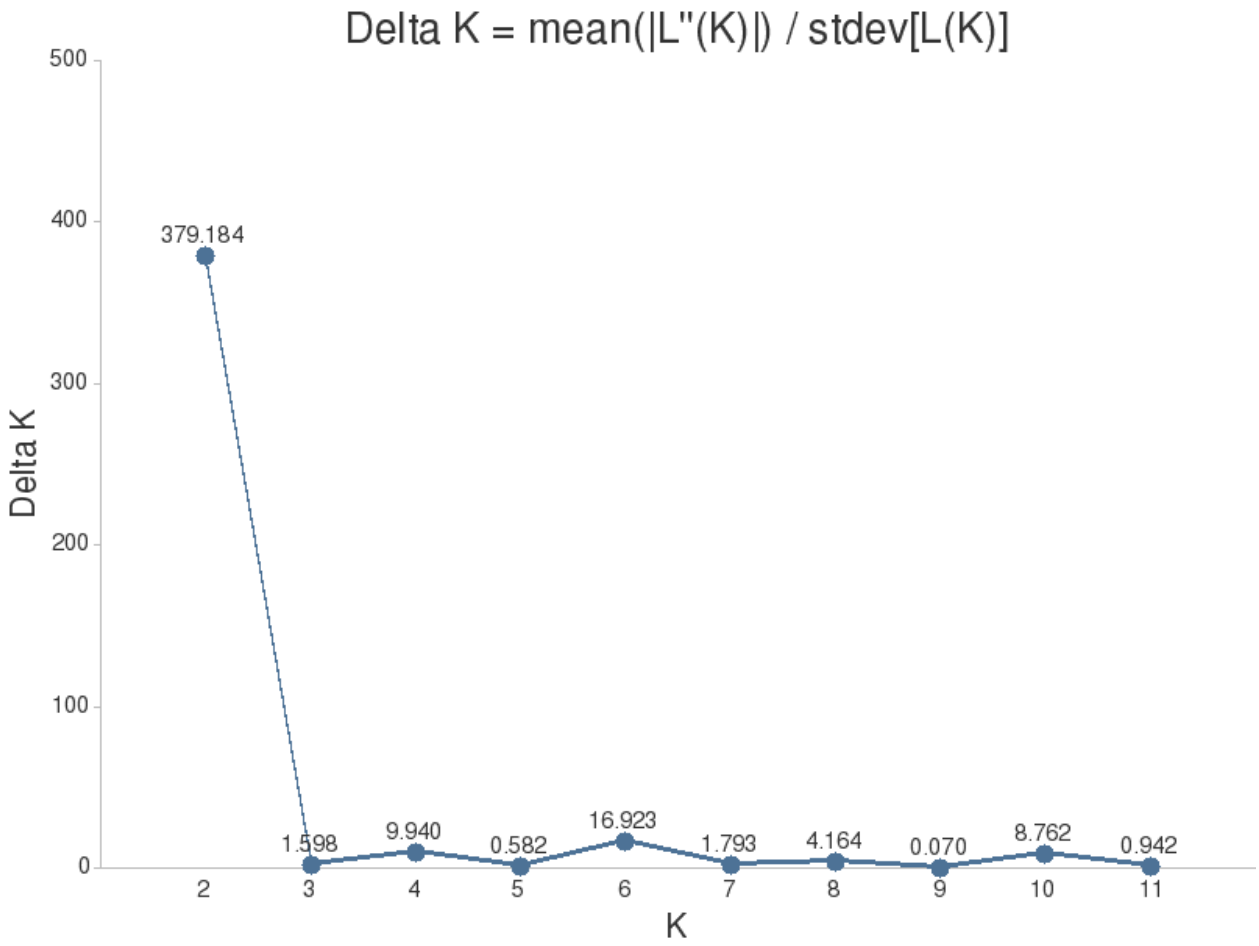

Figure 3.10. A plot of delta *K* values (Evanno et al. 2005) for *K* from the results of STRUCTURE analysis (Pritchard et al., 2000) of *Castilleja coccinea* individuals using the color-morphs data set

L

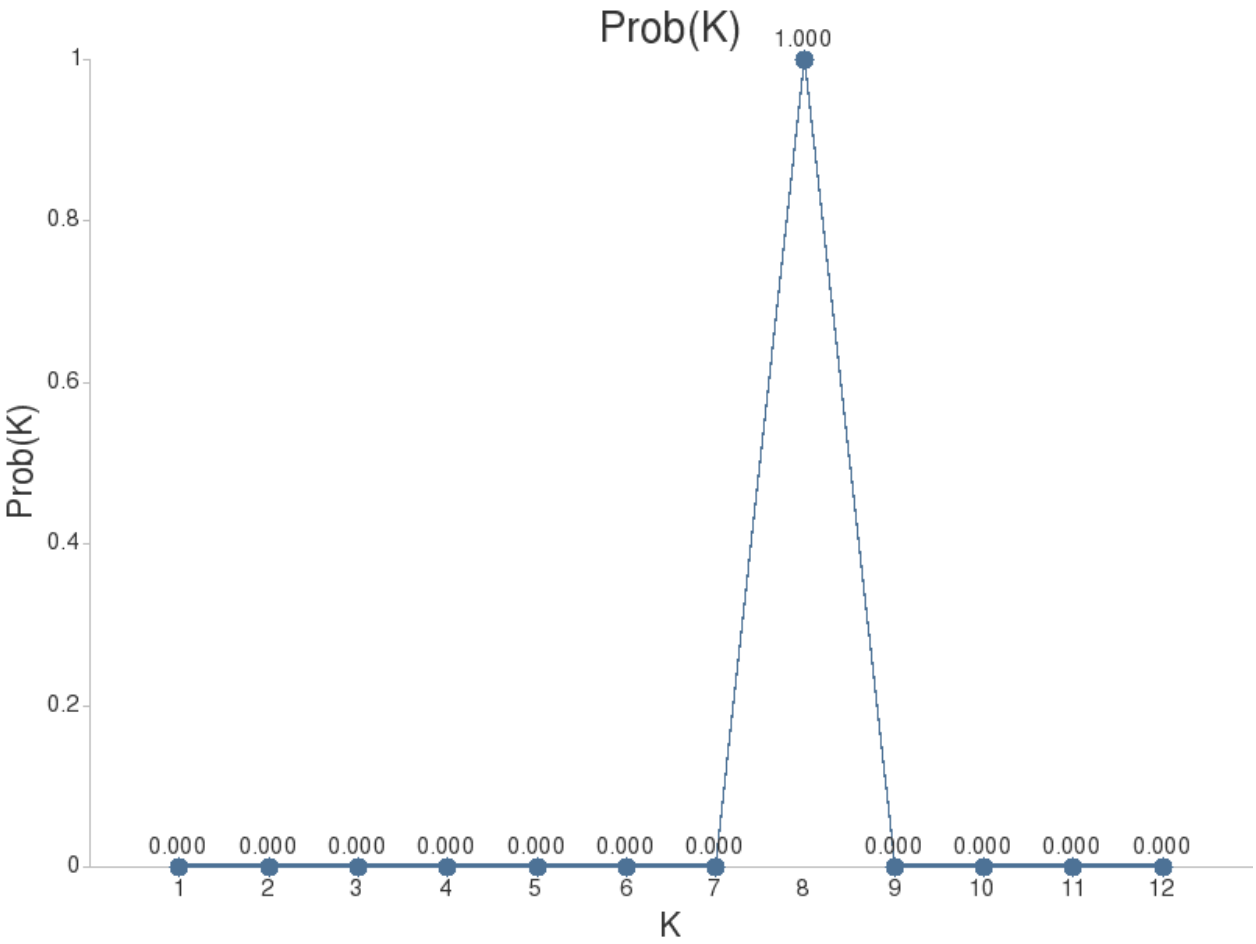

Figure 3.11. A plot of probability of *K* and *K* values in STRUCTURE analysis (Pritchard et al., 2000) of *Castilleja coccinea* using the color-morph data set.

M

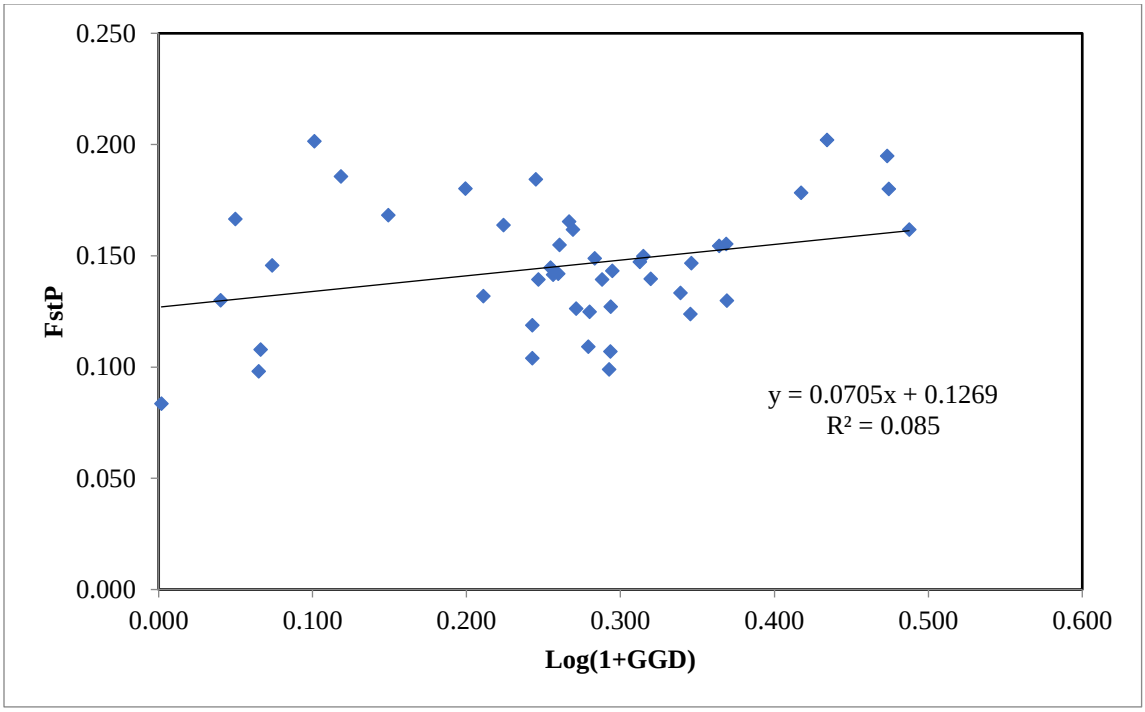

Figure 3.16. A plot of pairwise F_{ST} and log transformed geographic distances from the results of Mantel test

N

Figure 3.17. Results of first comparison which compared posterior probabilities of scenarios 1, 2, and 3 using logistic regression in DIYABC

O

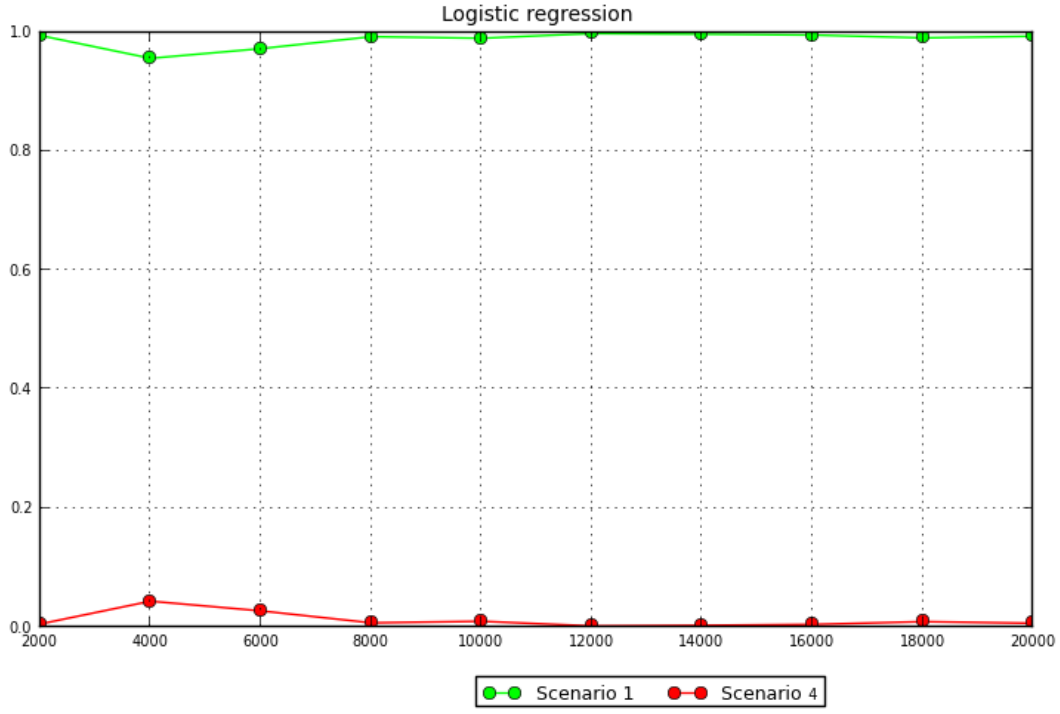

Figure 3.18. Results of second comparison which compared posterior probabilities of scenarios 1 and 4 using logistic regression in DIYABC

P

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