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Evolution of Dioecy in Echinocereus coccineus: Relative Influence of Pollinators, Resources and Elevation over Multiple Spatial Scales

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UNIVERSITY OF MIAMI

EVOLUTION OF DIOECY IN *ECHINOCEREUS COCCINEUS*: RELATIVE INFLUENCE OF POLLINATORS, RESOURCES AND ELEVATION OVER MULTIPLE SPATIAL SCALES

By

Summer Ann Scobell

A DISSERTATION

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EVOLUTION OF DIOECY IN *ECHINOCEREUS COCCINEUS*: RELATIVE INFLUENCE OF POLLINATORS, RESOURCES AND ELEVATION OVER MULTIPLE SPATIAL SCALES

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"Why have separate sexes?" is a fundamental biological question that has been investigated intensively since Darwin first proposed two alternative hypotheses: Separate sexes evolve 1) to avoid the detrimental effects of self-fertilization or 2) to improve the allocation of scarce resources to each sex's reproductive function. In animal-pollinated plants these two hypotheses have been invoked to explain why: 1) dioecy is strongly associated with small, generalized pollinators. Dioecy is hypothesized to evolve because this type of pollinator produces high rates of self-fertilization, and separation of the sexes avoids this inbreeding. It has also been hypothesized that: 2) dioecy may evolve primarily because this allows plants to optimize the different ways resources are used for the dissemination (male) and collection (female) of pollen. I concurrently tested these two hypotheses using *Echinocereus coccineus* (Cactaceae), a perennial tetraploid (2n = 44) cactus that blooms throughout the mountains of the southwestern United States. It has a variable mating system across its range: populations in the high-elevation center are hermaphroditic, while populations at the low-elevation edges of the range are dioecious. Both pollinators (hummingbirds vs. bees) and amount of resources (rainfall) varied across this range. This allowed me to test the relative influence of pollinator type and resource gradients on the evolution of dioecy. I found that, as predicted, when specialized

pollinators (hummingbirds) were excluded from plants and only generalized pollinators (bees) visited, the selfing rate of plants increased 238%. This combination of high selfing rates and high levels of inbreeding depression found in *E. coccineus* populations could select for dioecy, even in the absence of resource reallocation by the plants.

Besides maintaining high outcrossing rates within populations, hummingbirds may also maintain high levels of gene flow among *E. coccineus* populations: Synchronously blooming populations whose phenologies coincided with the hummingbird migration through this area had lower F_{ST} values (i.e. less genetic divergence) than populations with asynchronous phenologies, or those that bloomed after the hummingbird migration had passed by. Population inbreeding (F_{IS}) was not correlated with pollinator visit rates. Instead, it was negatively correlated with elevation. This could indicate that inbred progeny are selected against at higher rates in cold, high-elevation environments. These population genetic data suggest that the frequency of dioecious populations should be negatively correlated with both hummingbird abundance and elevation. Structural equation models of the effects of hummingbird abundance, elevation and annual rainfall (resources) on dioecy across the range of *E. coccineus* supported this hypothesis. Across all models, elevation, rainfall, and hummingbird abundance were consistently negatively correlated with dioecy; meaning that dioecy is more likely to be found as each of these variables decline. Only models with hummingbird abundance as a direct predictor of dioecy significantly fit the data. Models with only resources as a predictor of dioecy did not fit the data. This allowed me to reject hypothesis 2 for the evolution of dioecy: In this system resource reallocation does not appear to be important because there is no correlation between resources and dioecy.

Darwin's first hypothesis is supported: as hummingbird abundance declines (and selfing rates increase), the incidence of dioecious populations increases.

Finally, to determine how changes in pollinator type may be globally influencing the evolution of dioecy, I conducted a literature survey of change in pollinator types along elevation gradients and compared these to studies of dioecy along elevation gradients. Across studies the trend was clear: specialized insect pollinators decline with elevation, leaving only generalized pollinators (usually flies) at the highest elevations. Along with this pattern, proportion of dioecious species in communities (or proportion of single-sex mutants in a population) increases with elevation. In cases of vertebrate-pollinated plants (e.g. those pollinated by birds and/or bats), dioecy is predicted to evolve at lower elevations because these specialized pollinators often occur at middle to high elevations, leaving lower elevations to generalized pollinators. The *E. coccineus* study system fits this prediction. I propose that elevation may be useful as a worldwide predictor of dioecy due to its effects on pollinator community composition and amount of inbreeding depression expressed in plant populations as elevation increases.

The research presented in this dissertation represents the first study to concurrently test the inbreeding avoidance and resource allocation hypotheses for the evolution of dioecy. It was also the first to show that a change in pollinators across the range of an ancestrally hermaphroditic plant species could cause sufficient selection on hermaphrodite individuals to promote the selection for separate sexes and eventually allow the evolution of dioecy in populations. Predictions formulated by this study will be used in future work to test how changes in pollinators along elevation gradients may influence the evolution of dioecy around the world.

DEDICATION

To
my wonderful parents,
Beverley and Henry Scobell
for giving me a sense of adventure,
the freedom to explore
and, most of all,
your love.

To
my sister
Sunny Scobell
for being the best sister
and an even better
colleague.

To
my grandparents
Everett and Ede Workman
for starting it all by
teaching me to
love being
outdoors.

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CHAPTER ONE

INTRODUCTION

During the advent of agriculture in human civilization it was discovered that some plants have separate sexes. Mesopotamians in the 9th century B.C. learned that shaking the staminate branches from a male date palm over the female flowers would improve the yield of their date crop (Baker 1983). However, it was not until late in the 17th century that scientific exploration of plant mating systems would begin. The first experiments demonstrating the male and female function of dioecious species were made by Rudolf Camerarius in 1694 (Baker 1983). However, the idea plants had sex was not widely accepted until after 1735, when Linnaeus devised his system of classifying seed plants based on the "sexual relations of flowers." In this classification, he recognized four different mating systems in seed plants: hermaphroditic, polygamous, monoecious, and dioecious (Baker 1983; Sakai and Weller1999). Because male and female parts of flowers are often morphologically similar across related taxa, these categories proved useful as Linnaeus organized the diversity of plant life. Charles Darwin, through systematic observations and experiments, explored the nature of plant mating systems in depth in *The Different Forms of Flowers on Plants of the Same Species* (Darwin 1877). Darwin pointedly asked why "hermaphrodite plants should ever have been rendered dioecious?" He hypothesized hermaphroditism should be selected over dioecy because in the hermaphroditic state plants could ensure seed set through self-fertilization even if mates or pollinators were rare or absent (i.e. Darwin's reproductive assurance hypothesis (Darwin 1876)). Darwin therefore stated he found evolution of separate sexes in plants to be "a curious and obscure problem" (Darwin 1877). He proposed two hypotheses to explain how dioecy could evolve in spite of the "problem" of reproductive assurance: 1) Dioecy evolves because it allows more efficient partitioning of scarce resources or 2) because separate sexes avoid the detrimental effects of uniparental inbreeding. Darwin's own experiments found more evidence supporting 1), the resource partitioning hypothesis (hereafter called the resource allocation hypothesis; Darwin 1877), but the debate was far from over. These hypotheses have been investigated for over a century by evolutionary biologists. While much has been discovered, many of these discoveries acted only to add complexities to the puzzle of how sex evolves in plants. Many of these complexities still remain to be resolved. One of the main questions that remains is: What is the impetus for the evolution of dioecy? In other words, what ecological and genetic factors promote the initial selection for separate sexes over hermaphrodites (Webb 1999)?

Much research has been focused on investigating ecological factors that are correlated with evolution of dioecy (e.g. growth form, pollinator type, habitat type, distribution and abundance of populations and/or individuals; Bawa and Opler 1975; Bawa 1980; Thompson and Brunet 1990; Charlesworth 1993; Bawa 1994; Renner and Ricklefs 1995). The goal of these studies was often to find support for the researcher's favored Darwinian hypothesis (1 or 2 above), but despite tremendous effort, the causation behind these correlations remains elusive. Additionally, the factor or set of factors that promote the initial selection for sexual dimorphism (the first step in the evolution of dioecy) are still not well understood (for review see Sakai and Weller 1999; Webb 1999).

To understand possible casual relationships among changes in ecological factors and evolution of dioecy across the Angiosperms, a detailed phylogeny is necessary

(Donohue 1989; Weller and Sakai 1999; Sakai and Weller 1999). Recent work using phylogenetically corrected datasets of Angiosperms, their mating systems and ecological data has revealed six ecological correlates were significant after taking relatedness into account: tropical distribution, woody growth form, small inconspicuous flowers, small inflorescences, abiotic pollination, and fleshy fruit (Vamosi et al. 2003). Further work indicated dioecious plant lineages that possessed more of these traits in combination had higher evolutionary success (more species relative to hermaphroditic sister groups), and dioecious lineages with tropical distribution and fleshy fruits had the highest evolutionary success (Vamosi and Vamosi 2004). These analyses tell us which traits or trait combinations are associated with dioecy and diversification and maintenance of dioecious lineages. Vamosi and Vamosi (2004) hypothesize, however, that association of these traits with dioecy exists because these traits help to keep the dioecious lineages from extinction once they are formed, as opposed to being associated with dioecy because they act to promote the evolution of dioecy. This still leaves open the tantalizing question: What changes in a plant's environment promote initial evolution of dioecy in hermaphroditic plant species? To answer this question I will reexamine Darwin's two hypotheses for the evolution of dioecy in greater detail.

The idea that dioecy evolves primarily as a result of selection against inbreeding (Hypothesis 2; Darwin 1877) is now termed the inbreeding avoidance hypothesis (Lloyd 1975). According to this hypothesis, in populations with high levels of inbreeding depression (i.e. loss of fitness in selfed progeny due to expression of recessive deleterious alleles, hereafter IBD or δ) and a high selfing rate (s), if loss of fitness of hermaphrodite plants due to self-fertilization is greater than half of their seed fitness (specifically when:

$\delta * s > 0.5$), then any female mutants that arise in the population will spread. Spread can occur due solely to genetic advantage of outcrossing females over selfed hermaphrodites (Lloyd 1975; Charlesworth 1999).

This hypothesis has garnered much theoretical and empirical support (Thomson and Barrett 1981; Kohn 1988; Sakai et al. 1989; Schultz and Ganders 1996), but it has been questioned because its mechanism seems paradoxical: The very processes that select for dioecy through outcrossing advantage could also select against it. If selfing rate is high, deleterious recessive alleles that contribute to inbreeding depression are often purged from the population, and the population remains hermaphroditic (Charlesworth et al. 1990). Also, if there is any genetic variation in the population that allows an increase in the hermaphrodite's outcrossing rates, this will also reduce or eliminate the outcrossing advantage of females (Schultz and Ganders 1996).

Selective interference is one genetic mechanism that could maintain the high levels of inbreeding depression necessary to select for dioecy in plant populations (Lande et al. 1994). At high rates of genomic mutation to recessive lethal alleles, selfing rates must exceed a certain threshold in order for purging to occur; otherwise, all selfed seeds die. In their study of *Bidens sandvicensis*, Schultz and Ganders (1996) found a high inbreeding depression (0.94) in the hermaphrodites of this gynodioecious species despite moderate levels of selfing (0.25-0.5). They hypothesized selective interference could be maintaining the high level of IBD found in this species. In a recent literature survey of inbreeding in adult trees and their seeds, Scofield and Schultz (2006) found nearly all populations of adult trees had an inbreeding coefficient near or less than zero, regardless of the rate of inbreeding in seeds they produced. This implies essentially all selfed seeds die (IBD ~1) before reaching reproductive maturity in long-lived perennial plants (Scofield and Schultz 2006). Under these conditions, there would be no purging of deleterious recessive alleles, and levels of inbreeding depression in populations would remain consistently high.

Darwin's first hypothesis for the evolution of dioecy, the resource allocation hypothesis, is usually presented as an alternative to the inbreeding avoidance hypothesis. The resource allocation hypothesis states that separate sexes evolve because resources (e.g. water, nutrients) needed for optimal sexual function are scarce, and can be allocated more efficiently in unisexual individuals. The resource allocation hypothesis has been invoked in studies showing resource limitation in dry habitats drives disruptive selection. This type of selection favors single-sex plants that are able to segregate themselves among habitat niches more effectively than hermaphrodites (e.g. females in low-lying areas with more moisture for producing fruits and seeds, males in higher areas better suited for dispersing pollen; Freeman 1980; Freeman et al. 1993; Freeman et al. 1997). However, as I will discuss next, evolution of dioecy is a complex process and distribution of resources can affect not only the plants themselves, but also distribution and abundance of pollinator species as well as the interactions among plants and their pollinators.

Darwin's hypotheses for the evolution of dioecy have been debated for over a century, but as evidence has accumulated a synthesis emerged: Many recent studies are invoking a combination of both the resource allocation and inbreeding avoidance hypotheses to explain why dioecy has evolved in their systems (Barrett 1992; Sakai et al. 1997; Case and Barrett 2004). Theoretical models for the invasion of single-sexed plants into cosexual (hermaphroditic or monoecious) populations now incorporate both the amount of inbreeding and resource allocation (Charlesworth 1999). Both factors therefore need to be considered when determining how dioecy evolves in natural plant populations.

Another important consideration when studying the evolution of dioecy is that there are multiple ways to transition from cosexuality to dioecy. Five evolutionary pathways from cosexuality (hermaphroditic or monoecious) to sexual dimorphism (or often to full dioecy) have been discovered (for review see Charlesworth 1999 and Webb 1999). These can be subdivided into three categories: The first category involves invasion of a hermaphroditic population by individuals with a sterility mutation that silences the expression of one of the sexes (gynodioecy and androdioecy pathways). The second category involves selection acting on an existing dimorphism to gradually select sexual morphs in the population that are more male or female than hermaphroditic (heterostyly and heterodichogamy pathways). The third category – from monoecy through paradioecy to dioecy – combines traits from the first two categories, with both sterility mutations and gradual changes in the amount of sexual dimorphism playing a role in the transition from cosexuality to dioecy. Each of these pathways is described in greater detail below.

Gynodioecy involves the production and maintenance of individuals with a male-sterility mutation (females) within cosexual (hermaphroditic or monoecious) populations while androdioecy involves individuals with female-sterility mutations (males) being produced and maintained in a cosexual population. Gynodioecy is by far the more prevalent of these two categories for two reasons: First, because the cytoplasm and its contents are passed on in the ovule, but not in the pollen, male-sterility can be accomplished by mutations to either nuclear and/or cytoplasmic genes that control sex expression, whereas female-sterility can only be passed on through nuclear genes. Second, female plants produce only outcrossed offspring. If cosexual plants are losing fitness through production of lower-quality selfed offspring, female plants will be able to out-compete cosexuals because females produce no self-pollinated offspring. If females are also able to reallocate resources previously used for male function, they will spread even more quickly through hermaphroditic populations (Charlesworth 1999). Since pollen of male individuals must still compete with pollen from cosexual plants in order to produce outcrossed seeds, upon elimination of female function there would have to be a two-fold increase in pollen production in order for males to compete with hermaphrodites (Lloyd 1975; Charlesworth and Charlesworth 1978a). Therefore there is a significant disadvantage to being male in a cosexual population, subsequently androdioecy is extremely rare. Both inbreeding avoidance and resource allocation have been theorized to be important in selection toward dioecy in these evolutionary pathways (Lloyd 1975 and 1982; Charlesworth 1999).

When selection acts on a pre-existing dimorphism such as reciprocal dichogamy (separation of sexual functions in time) or reciprocal herkogamy (separation of sexual parts in space), there is a more gradual progression toward dioecy as morphs that are incrementally more “male” or “female” out-compete hermaphrodites. The reasons why this progression occurs are not well understood (Webb 1999). It has been postulated that loss of self-incompatibility and/or an increase in the levels of self-fertilization could select for separation of the sexes (Charlesworth 1989).

In the monoecy-paradioecy-dioecy pathway both sterility mutations and gradual changes in the ratio of male to female flowers take place. In monoecious species, individual flowers are already male-sterile or female-sterile with both types of flowers found on each individual plant in relatively equal ratios. This path toward dioecy begins when selection favors individuals that have larger ratios of female to male flowers. Charlesworth and Charlesworth (1978b) modeled this transition and found plants with fewer male flowers could invade a monoecious population if there was an increase in seed fitness due to reallocation of resources formerly used by male flowers, and if there was a reduction in the rate of self-fertilization. Eventually, when female-biased plants predominate, male-biased plants will have an advantage over monoecious plants with a balanced sex ratio and males will start to invade, leading over time to a fully dioecious population.

Gynodioecy is the most-studied among the pathways to dioecy listed above and also appears to be the most prevalent pathway to dioecy among flowering plants (Webb 1999; Weiblen et al. 2000). It is also the pathway that the focal species of this dissertation (*Echinocereus coccineus*), appears to have followed (Ferguson 1989; Hoffman 1992). For these reasons, I will go into greater detail in explaining the hermaphroditism – gynodioecy – dioecy pathway.

The invasion of females into a hermaphrodite population (gynodioecy) is the first step. Evolution of strict dioecy requires female-sterile mutants (males) invade, and hermaphrodites are completely eliminated (Charlesworth and Charlesworth 1978a; Schultz 1994; Charlesworth 1999). For full dioecy to remain evolutionarily stable both sterility loci must be linked and one sex must be completely recessive to the other

(Charlesworth and Charlesworth 1978a; Schultz 1994). Therefore, evolution of dioecy by this pathway requires a high initial inbreeding depression followed by maintenance of high inbreeding depression levels despite continued high rates of selfing (Schultz and Ganders 1996). As stated above, high IBD can be maintained by selective interference. Another possible factor that has also been implicated in producing and maintaining high levels of IBD and high selfing rates is polyploidy (genome duplication).

The work of Miller and Venable (2000) indicates genome duplication (polyploidy) is one process that can cause both high selfing rates and consequent expression of high levels of inbreeding depression in plant populations. In twelve genera included in their study, polyploidy is associated with the break down genetic self-incompatibility systems, subjecting newly polyploid plants to initially high levels of selfing and IBD (When previously heterozygous deleterious recessive alleles are expressed in the homozygous condition). Polyploidy might also help to maintain high levels of IBD at equilibrium in plant populations. Empirical and theoretical results indicate equilibrium levels of IBD can be very high in polyploids (Dewey 1969; Johnston and Schoen 1994; Ronfort 1999). Inbreeding depression can even increase with increased selfing at particular dominance coefficients (Ronfort 1999). This is surprising because it is opposite to the genetic response to selfing in diploids (i.e. IBD decreasing with increased selfing as deleterious alleles are purged; Lande et al. 1994).

Selfing is also predicted to increase in polyploids that are formed from self-compatible progenitors (Stebbins 1971; Grant 1981; Lande and Schemske 1985; Schemske and Lande 1985). Cook and Soltis (1999; 2000) tested this hypothesis by comparing outcrossing rates (t) of a polyploid species (*Tragopogon mirus* (Asteraceae)) with one of its diploid progenitors (*T. dubius*) under both natural conditions (Cook and Soltis 1999) and using artificial arrays of plants with known genotypes to maximize ability to detect outcrossing events (Cook and Soltis 2000). In natural populations, polyploids had higher outcrossing rates; in artificial arrays they had lower outcrossing rates. Higher outcrossing in the polyploid under natural conditions may be due to pollinator behavior. Polyploids have larger flowering heads, are purple and yellow instead of just yellow, and stay open longer each day than diploids. If different types of pollinators are attracted to the field vs. the artificial arrays, differences in floral display traits might lead to greater outcrossing rates in the natural polyploid populations. These studies did not measure the effect of pollinator behavior on selfing rate (Cook and Soltis 1999; 2000). As mentioned above, inbreeding depression could also be higher in the polyploid than the diploid and this could be selecting for higher outcrossing in the natural polyploid populations (Dewey 1969; Johnston and Schoen 1994; Ronfort 1999). However, IBD was not measured in this study. The recent origin of this polyploid species (*T. mirus*) may also help explain lack of consistency in outcrossing rates across the natural and artificial array treatments. Its parents are native to Europe and western Asia and were introduced into North America less than 100 years ago, with the polyploid forming in the early 20th century (Ownbey 1950). Although this was enough time for polyploidy to arise multiple times within this genus (Novak et al. 1991; Soltis et al. 1995), it might not be enough time for the mating system to change and become stabilized across all populations (Cook and Soltis 2000). The results of this study have intriguing implications for the study of evolution of mating systems in polyploids: Selfing rates can be highly variable between populations, and therefore both pollinators and inbreeding depression need to be measured across multiple populations to determine how differences in these variables will affect plant fitness.

What this implies for evolution of dioecy is high levels of inbreeding depression can be maintained in plant populations through several mechanisms; with polyploidy playing an important yet relatively unexplored role in this process (Charlesworth 2001). If all populations of a species experience large amounts of inbreeding depression, then ecological factors that cause differences in selfing rates between these populations can determine whether there is selection for dioecy.

Biologists studying evolution of plant mating systems have reported intriguing associations between certain pollinator types and dioecy: Most animal-pollinated dioecious species are pollinated by small, generalist insect pollinators (e.g. solitary bees and flies), and only very few dioecious species (only 4 genera, Renner and Ricklefs 1995) are predominantly pollinated by specialized pollinators (e.g., birds and bats; Bawa and Opler 1975; Bawa 1980; Lloyd 1982; Sobrevilla and Arroyo 1982; Delph 1990; Charlesworth 1993; but see Renner and Feil 1993; Bawa 1994). For example, in his review of the evolution of dioecy in flowering plants, Bawa (1980) found examples from four different taxonomic groups where, with one exception, all hermaphroditic species were bird-pollinated and all dioecious species were pollinated by insects. However, in their review of evolution of dioecy in flowering plants, Renner and Ricklefs (1995) pointed out the mechanism for how a shift in pollinator type within one plant species could select for dioecy is not well understood. Other authors have proposed a shift in pollinators may increase self-fertilization rates of plants, which in turn may promote selection for dioecy (Bawa and Opler 1975; Bawa 1980; Bawa 1994; Lloyd 1982; Sobrevilla and Arroyo 1982; Delph 1990).

This hypothesis stems from information gathered by pollination biologists that all floral visitors are not equally effective agents for fertilizing ovules (e.g. Schemske and Horvitz 1984). There are two ways pollinators can directly influence fertilization success and therefore evolution of plant mating systems: by the quantity of pollen carried (total grains delivered) and its quality (self-pollen or outcrossed pollen). Unless the plant has evolved a system to prevent self-fertilization (e.g., genetic self-incompatibility), in the process of collecting floral rewards almost all pollinators will cause some self-fertilization, either within the same flower or between flowers on the same plant. The size, morphology and behavior of each different species of pollinator could cause variation in amount of self-pollen distributed within one plant species. The fitness cost of self-pollination will then depend on the amounts of self-fertilization and inbreeding depression (IBD) self-fertilized plants express. If the fitness toll is high enough in hermaphrodite individuals within a population, selection can favor evolution of separate sexes and eventually dioecy in populations that have experienced a change in pollinators.

It has been known since the earliest pollination studies that pollinators can vary over the geographic range of one plant species (Mueller 1883). In a widespread plant species, the available pollinator pool can change with latitude (Eckert 2002; Brunet and Sweet 2006), altitude (Cruden 1972; Pellmyr 1986; Delph 1990; Galen et al. 1991; Webb and Kelly 1993; Scobell 1999), and/or time of day (Cruden 1972; Pellmyr 1986; Fleming et al. 1996; Herrera 2000). Three recent studies have explored effects of different types of pollinators on selfing rates or subsequent seedling fitness (the outcome of self-fertilization and inbreeding depression) in hermaphroditic plant populations (Herrera 2000; Eckert 2002; Brunet and Sweet 2006). All of the above studies found differences in the pollinator assemblage over their species' range.

Herrera's study, with careful experimental manipulation of the pollinator assemblage blocked by plant, was able to find significant differences in how different assemblages (small bees, flies and butterflies vs. large bees) affected fitness of progeny. In this case, Herrera found the former group produced seedlings that were more fit (higher fruit and seed set and better survival in adverse germination conditions) than those produced by the latter. He reasoned because butterflies and small bees visited at higher rates and flew more often between plants, a higher outcrossing rate might be causing increased fitness in the offspring produced by this group. Without separate tests of how each group of pollinators affects the outcrossing rate of these plants, however, the exact impact of different assemblages of pollinators on plant fitness is difficult to assess.

Eckert's (2002) study looked for correlation between selfing rates and pollinator assemblages over 15 populations of a widespread plant species *Decodon verticillatus* (Lythraceae): 10 northern populations in Ontario and Michigan and 5 southern populations in Georgia and Florida. The pollinators in these populations varied, with northern populations being predominantly visited by honey bees, bumblebees and carpenter bees and southern populations visited by bumblebees, carpenter bees and butterflies. Eckert predicted southern populations might be more outcrossed than northern populations because butterflies flew longer distances between foraging bouts and moved more frequently between inflorescences. When northern and southern populations were compared however, the selfing rates were nearly identical. Without experiments like Herrera’s that carefully manipulated pollinators, with each treatment blocked by plant, Eckert could not determine the reason for lack of differences in selfing rates between northern and southern populations.

Brunet and Sweet (2006) measured plant population size, plant density, floral display size, herkogamy, pollinator abundance and outcrossing rates in eight populations of *Aquilegia coerulea* (Ranunculaceae) spread across Colorado, Utah and Arizona. Pollinators of this columbine included hawkmoths, bumblebees, solitary bees, syrphid flies and other muscidae. They found outcrossing rates were significantly increased by increasing hawkmoth abundance but not by any other type of pollinator. Outcrossing rates also increased with decreasing floral display size, but were not significantly correlated with any other independent variable measured. Together hawkmoth abundance and floral display size explained 87% of the variation in outcrossing rates. The authors concluded that it was behavior of the hawkmoths at these plants (due to their preference for visiting female-phase flowers first in this dichogamous species) that caused increased outcrossing rates.

What is needed now is a combination of the above protocols and experimental techniques. First to assess how different pollinators can affect the selfing rate of a population, then to take a broad geographic perspective on the influence of different pollinator assemblages on selfing rates of plant populations throughout the range of each plant species. Finally, because both selfing rate and resource allocation have been found to be important in evolution of dioecy, effects of both changes in pollinators and resources should be examined for direct and indirect influences on evolution of dioecy.

Echinocereus coccineus (Cactaceae, Cactoideae) is an excellent model system to test hypotheses about effects of pollinator changes and resource conditions on evolution of dioecy. This species is a polyploid cactus with a chromosome number of 2n = 44 (Cota and Philbrick 1994). It possesses contemporaneously both hermaphroditic and dioecious populations and possibly gynodioecious populations as well. *Echinocereus coccineus* flowers appear specialized to hummingbirds (large, red and tubular, with high-sucrose nectar; Cota 1993), but are also heavily visited and pollinated by bees (Scobell 1999). These two floral visitor types vary greatly in their pollinating behavior, and based on my previous research (Scobell 1999; Scobell 2002) and the studies of others (Waser 1982; England et al. 2001; Williams et al. 2001), I hypothesize pollination by bees alone will result in higher selfing rates than those produced when hummingbirds are also present. Distribution and abundance of pollinator types varies across the geographic range of the plant: Both hummingbirds and bees visit flowers in the center of the geographic range, in Rocky Mountain and Sierra Madre mountain ranges, but in drier, lower altitude areas to the east and west of these ranges, hummingbirds become rare or absent. Dioecious populations appear to be associated with areas of low hummingbird abundance, low elevation, and low rainfall. This system was used to ask the following questions about evolution of dioecy in *Echinocereus coccineus*:

> 1) Do the predominant pollinator types (hummingbirds and bees) produce different selfing rates in *E. coccineus* plants?
>
> 2) What is the relationship of visit rates of pollinators to selfing rates of populations they visit over the geographic range of *E. coccineus*?

3) Is there an association between absence of hummingbirds and presence of dioecious populations in this species?

4) Is there a direct association of dioecy with dry areas or is this association indirectly caused by lack of hummingbirds in dry areas?

Questions 1 and 2 are addressed in Chapter 2 of this study. The goal was to determine relative effects of hummingbirds vs. bees on plant mating system (selfing rates and inbreeding depression) and population genetic parameters (adult inbreeding coefficients (F_{IS}) and genetic structure among populations (F_{ST})). To accomplish this, I first used pollinator exclusion experiments, followed by microsatellite analysis of parents and progeny to determine if flowers pollinated by an assemblage of hummingbirds and bees (Open pollination treatment) were more outcrossed than flowers pollinated by bees alone (Caged pollination treatment). I then compared visit rates of hummingbirds and bees to population-level F_{IS} values for seven populations (five hermaphroditic and two dioecious) across the range of *E. coccineus* to determine if higher visit rates of bees were associated with increased population levels of inbreeding in the adult generation. I would predict this if bees are producing higher selfing rates and there is uniform selection against inbred progeny across the geographic range of *E. coccineus*. I also examined the correlation between elevation and F_{IS} to see if increasing habitat harshness (in terms of decreasing temperatures) with elevation had an effect on population levels of inbreeding. Finally, I examined F_{ST} values among seven populations to estimate how hummingbirds might be mediating gene flow over large distances. If hummingbirds are carrying genes over long distances among populations, this will also increase the outcrossing rate and may possibly be the reason why hermaphroditic populations are maintained in the center of the geographic range of *E. coccineus*, which coincides with the center of the hummingbird migration corridor (Williamson 2001).

The third chapter aims to answer Questions 3 and 4. These questions distinguish between the two predominant hypotheses for the evolution of dioecy: The inbreeding avoidance hypothesis and the resource allocation hypothesis. A Geographic Information System (ArcView 3.3) was used to produce a map of all known (through herbarium data and personal observations) hermaphroditic and dioecious populations of *E. coccineus*. Maps of hummingbird abundance, annual rainfall, and elevation were layered with this map in ArcView. Data on each of these variables were then extracted from every *E. coccineus* population in the compiled map. This dataset was then tested for direct and indirect associations of hummingbirds (assumed to be negatively associated with inbreeding), annual rainfall (a surrogate for available resources), and elevation (a factor known to affect both hummingbird abundance and amount of annual rainfall) using structural equation modeling. Three structural models were tested to determine which model explained most of the variance in the data: 1) A model with resources alone having a direct effect on dioecy, 2) a model with hummingbird abundance having a direct effect on dioecy, and 3) a model with both hummingbird abundance and rainfall directly affecting dioecy. Determining which of the three models is most supported by the data will help me to determine if resource distribution or inbreeding avoidance, or both, are driving the evolution of dioecy in this system.

In the fourth chapter, I review the dioecy literature to show there has been, on a global scale, a consistent association between small generalist pollinators and evolution of dioecy, especially along elevation gradients. I hypothesize because conditions often become harsher at high (and sometimes low) elevations, generalist pollinators – which can use a broader variety of resources and are therefore less limited by declining abundances of plant species – may be more common. I predict dioecy should be more likely to be found at high (or low) elevation margins of species' ranges if generalist pollinators are more prevalent in these areas and if these pollinators consistently produce high selfing rates (a recurrent assumption in the dioecy literature). I also update estimates of dioecy on oceanic islands compiled by Baker and Cox (1984). Their study examined the association of maximum island elevation, latitude and percent of the total flora that is dioecious. I discuss results of this update in relation to the combined effects of elevation and latitude on distribution of pollinators, selfing rates, and levels of inbreeding depression maintained in plant populations. Possible effects of each of these variables on evolution and distribution of dioecy throughout the world are then discussed.

The goal of this study was to determine, across a variety of scales (plant, population, species-range, and global) ways in which distribution of different types of pollinators (e.g., small, generalist vs. large, specialist) contribute to evolution of dioecy. In addition, I examined effects of edaphic conditions (e.g., rainfall, elevation, UV radiation) on both pollinator distribution and evolution of dioecy in plants. This type of study is necessary to not only determine ways natural changes in pollinators across the range of a plant species will affect plant fitness and possibly select for a change in mating systems, but also to start building databases of species interactions and their outcomes. These databases can then be used to better determine ways human-induced changes in distribution of plants and pollinators may affect persistence of these important interactions – and the species themselves.

CHAPTER TWO

GEOGRAPHIC VARIATION IN POLLINATORS, OUTCROSSING, AND THE EVOLUTION OF DIOECY IN *ECHINOCEREUS COCCINEUS* (CACTACEAE)

A fundamental question in biology is: Why have separate sexes? In animal-pollinated plants, it has been hypothesized that dioecy evolves in certain areas (e.g., deserts, islands, mountain-tops) because marginal habitats cannot support specialized pollinators, and generalist pollinators (e.g. small bees, flies, beetles) that can occupy these habitats have behaviors that cause them to predominantly self-fertilize plants. Evolution of separate sexes therefore allows plants in these areas to avoid detrimental effects of inbreeding brought about by generalist pollinators (the inbreeding avoidance hypothesis). Under this hypothesis, separate sexes will be selected when both the selfing rate (s) and the amount of inbreeding depression (IBD = δ) in selfed seeds are high (specifically when: $\delta * s > 0.5$). Thus, if a hermaphroditic population had a high level of IBD and then underwent a shift in pollinators, from a specialized pollinator that provided a high level of outcrossing to a generalized pollinator that produced a high level of selfing, this could select for separate sexes in this population, and if these conditions persisted, could eventually produce a dioecious population. The goal of this chapter is to test this hypothesis in *Echinocereus coccineus* (Cactaceae, Cactoideae), a cactus with hummingbird-syndrome flowers that varies in the type of pollinator present (hummingbirds vs. bees) and its mating system (hermaphroditic vs. dioecious) across the plant's geographic range.

The great majority of zoophilous, dioecious species are pollinated by small, generalist insect pollinators (e.g. small bees, flies and beetles), and only very few (in four genera, Renner and Ricklefs 1995) dioecious species are predominantly pollinated by specialized pollinators (e.g., birds, bats and other specialized mutualists; Bawa and Opler 1975; Bawa 1980; Lloyd 1982; Sobrevilla and Arroyo 1982; Delph 1990; Charlesworth 1993; but see Renner and Feil 1993; Bawa 1994). In a review of the evolution of dioecy in flowering plants, Bawa (1980) found examples from four taxonomic groups where, with one exception, all hermaphroditic species were bird-pollinated and all dioecious species were insect-pollinated.

Despite these intriguing correlations, the causal mechanisms behind the association between generalist pollinators and dioecy have yet to be explored. Three causal hypotheses could help explain these patterns: 1) Changes in pollinator type across the range of a plant species may select directly for evolution of dioecy by increasing the amount of self-fertilization and decreasing hermaphrodite fitness relative to unisexual plants. 2) A change in an environmental factor (e.g. water availability) across the range of a species may drive selection toward dioecy if unisexuals allocate reproductive resources more efficiently than hermaphrodites when resources are scarce. Because amount of rainfall can influence pollinators and dioecy, concluding pollinator type alone selects for dioecy could be in error. 3) Both pollinator distribution and resource abundance may be selecting for evolution of dioecy by the combined impact of inferior pollinator service and low resource availability.

Hypothesis 1 is often termed the inbreeding avoidance hypothesis (Lloyd 1975). This hypothesis states mutations that eliminate self-fertilization will be selected in a hermaphrodite population if inbreeding depression (relative loss of fitness due to selfing) and selfing rate are both sufficiently high. Specifically, any completely dominant nuclear mutation that produces a female plant by causing male sterility will increase in a population when loss of fitness in hermaphrodite plants due to self-fertilization is greater than half of seed fitness (Lloyd 1975). Mutations in hermaphrodites that produce male plants by causing female sterility can spread more easily as female frequency increases. In one experimental study in a natural plant population, Schultz and Ganders (1996) found support for this hypothesis by showing selfing rates and inbreeding depression levels in *Bidens sandvicensis* (Asteraceae) were high enough to select for dioecy.

An alternative hypothesis (Hypothesis 2) for the evolution of dioecy was proposed by Freeman et al. (1980) and states lack of rainfall produces resource limitation and directly promotes disruptive selection and niche segregation of male and female plants. Therefore, dioecy could be selected in arid regions in absence of any pollinator effects. They cited several plant species in the resource-poor intermountain western United States they believed followed this route to dioecy (Freeman et al., 1980). However, they asserted resources more often selected for dioecy in this fashion if the species' ancestors were monoecious (Freeman et al. 1997).

Evidence supporting Hypothesis 3 includes studies showing pollinator distribution and abundance often follows patterns determined by climatic variables. In a study in the mountains of Mexico, Cruden (1972) found hummingbirds were more effective pollinators at high elevations because of high incidence of rainfall in these areas. Rainfall gradients could therefore indirectly select for dioecy through their influence on pollinator distributions and abundances (Weller and Sakai 1990; Barrett 1992; Weller et al. 1995; Soltis et al. 1996; Sakai et al. 1997; Weller et al 1998). The proposed mechanism in these systems is that conditions are no longer favorable for effective pollinators when plants invade drier areas. Plants in these areas are left with less-effective pollinators. The behavior of these pollinators (e.g. restricted foraging areas, generalized foraging patterns among many plant species) may increase the amount of self-fertilization in these populations, thus selecting for dioecy.

Despite widespread acceptance of the hypothesis that changes in pollinator effectiveness may select for dioecy, it has not been tested empirically. This is probably due to lack of a suitable model system. Previous studies of dioecy have compared dioecious species to hermaphroditic relatives (Bawa and Opler 1975; Bawa 1980). In these systems, however, it was impossible to determine the composition of the pollinator community as dioecy was evolving, leaving open the question of how pollinators initially influenced evolution of their plant communities. The assumption that current ecological factors remain similar to what they were when the sister species diverged could lead to spurious conclusions. This is because both pollinator community composition and plant population dynamics respond to changes in resources that may have changed greatly since the time of divergence. Interactions among plants, pollinators, and resources are often complex, with both direct and indirect effects from pollinators and resources selecting, in complex and often conflicting ways, on plant traits (Schemske and Horvitz 1988; Iriondo et al. 2003). In order to determine ways in which interacting factors influence selection for dioecy, this hypothesis must be tested in a system in which populations are actively evolving, or have very recently evolved dioecy, in order to determine if pollinators, resources, or both are selecting for this mating system.

Echinocereus coccineus (Cactaceae; Cactoideae) is an excellent model system to test hypotheses about effects of pollinator changes and resource conditions on evolution of dioecy. It concurrently possesses hermaphroditic, dioecious and possibly gynodioecious populations. Populations described as “possibly gynodioecious” are found in northwestern areas of the species range and have female-to-hermaphrodite phenotypic ratios that are less than 1:1 (S. Scobell, unpublished data). These populations have not yet had their mating systems experimentally verified by crossing experiments and thus are labeled “possibly gynodioecious”.

Echinocereus coccineus flowers appear to be specialized for hummingbirds but are also pollinated by bees. These two kinds of floral visitors vary greatly in their pollinating behavior, and, based on previous research (Scobell 1999; England et al. 2001; Scobell and Scott 2002), I hypothesized pollination by bees alone will result in a higher selfing rate than that produced when hummingbirds are also present. Distribution and abundance of pollinator types vary across the geographic range of the plant. Both hummingbirds and bees visit flowers in the center of *E. coccineus'* geographic range (which runs north-south along the continental divide in the Rocky Mountains and the Sierra Madre Mountains). In drier, lower altitude areas to the east and west of this range, hummingbirds become rare or absent. Distribution of dioecious populations appears to be associated with areas of low hummingbird abundance, low elevation, and low rainfall (Figure 1.1).

Levels of inbreeding depression are likely to be high (or to have been high initially) in *E. coccineus* because it is a self-compatible descendent of self-incompatible (obligately outcrossing) progenitors (Taylor 1985, Scobell 1999). Taylor in his Kew Monograph of the genus states “Almost without exception flowers of *Echinocereus* species appear to be self-incompatible”. To my knowledge, my crossing experiments on two hermaphroditic *E. coccineus* populations are the first to demonstrate self-compatibility within this genus (Scobell 1999).

This change in self-compatibility may have been brought about by a change in ploidy. *E. coccineus* is tetraploid, (2n =44, Cota and Philbrick 1994), whereas most other species within *Echinocereus* (Taylor 1985) are diploid (2n =22, Cota and Philbrick 1994). Miller and Venable (2000) proposed that polyploidy is an important trigger for the evolution of dioecy. They hypothesized polyploidy disrupts genetic self-incompatibility systems and exposes new polyploid lineages to high levels of inbreeding depression brought on by build-up of recessive deleterious mutations while self-incompatible (but see Mable 2004). The combined effects of increased selfing and IBD could then select for separation of the sexes. Miller and Venable (2000) found an association between polyploidy and gender dimorphism in 12 genera (including *Echinocereus*, based on data from Scobell 1999) involving at least 20 independent evolutionary events.

In order to empirically determine if changes in pollinator type across the range of *E. coccineus* have selected for evolution of dioecy, I conducted pollinator exclusion experiments (treatments with and without hummingbirds as pollinators) followed by microsatellite analysis of progeny arrays. These analyses allowed us to determine if pollination by bees alone produces higher selfing rates than pollination by both hummingbirds and bees. Estimates of population-level inbreeding depression were also derived from the microsatellite data. If both selfing rate and inbreeding depression are high (i.e. if $s * \delta > 0.5$), then conditions that select for dioecy exist (Lloyd, 1975).

To determine the genetic structure of populations (and the possible influence of hummingbird pollination on pollen-mediated gene flow among populations), I calculated pairwise F_{ST} values for seven populations of *E. coccineus*. I then calculated number of migrants (*N*m) from pairwise F_{ST} values. If there is a pattern of higher rates of gene flow (e.g. higher *N*m) along the hummingbird migration corridor, then hummingbirds might be helping to maintain hermaphroditic populations in the center of the range of *E. coccineus* by carrying genes among populations via pollen. In absence of hummingbirds, the genetic neighborhood may get much smaller. Research on *Echinocereus* indicates gene flow via seed dispersal is carried out predominantly by small mammals (Jackrabbits (*Lepus californicus*), Cottontails (*Sylvilagus auduboni*) and Woodrats (*Neotoma sp*.) and Harvester Ants (*Pogonomyrmex sp.*) and most dispersal is within-population (Lozano and Reid 1982). This could increase inbreeding and add to the selection for dioecy if most plants within a population are related.

Finally, F_{IS} values from adult plants in seven populations spanning the geographic range of *E. coccineus* were compared to visit rates of hummingbirds and bees within these populations. In absence of inbreeding depression, F_{IS} will increase as the selfing rate increases (Scofield and Schultz 2006). Therefore, if bees consistently self-pollinate plants, then populations where they are the main pollinator may show higher F_{IS} values. Alternatively, if inbreeding depression varies with habitat harshness as has been shown in *Mimulus* (Scrophulariaceae) (Dudash 2005), then F_{IS} may decrease with elevation because very few inbred progeny can survive to adulthood under the harsh, cold conditions at high elevations (Scofield and Schultz 2006).

METHODS

Study Species: *Echinocereus coccineus* is a long-lived perennial cactus that blooms throughout much of the southwestern United States into northern Mexico. It has a variable mating system with hermaphroditic populations located in the center of the range and dimorphic populations occupying eastern and western edges (Figure 1.1). Five dimorphic populations have been tested thus far and all are functionally dioecious (Hoffman 1992 (study sites shown with yellow triangles, Figure 1.1), Powell 1995 (study site shown with a red circle, Figure 1.1), Scobell unpublished data (study sites shown with blue stars, Figure 1.1)). Some plants in these populations possess flowers that appear hermaphroditic (fully developed anthers, stigma and ovules) but only produce functional pollen (functionally male). No seed was produced on these plants from either hand self-fertilization or hand cross-fertilization at any of the five sites. Plants that appear female, with stigma and ovules fully developed, but with stunted styles, anthers and no pollen, produce only seeds (functionally female). In functionally hermaphroditic populations, all individuals produce viable pollen and ovules (see Figure 1.4). In two hermaphroditic populations tested with hand-pollination experiments in the Chiricahua Mountains (LPAZ and BPAZ, white stars in Figure 1.1), fruit set and seeds/fruit were statistically similar across hand-selfed, hand-outcrossed, autogamous, and natural pollination treatments (Scobell 1999).

Hermaphroditic populations of *E. coccineus* show none of the signs of self-incompatibility that are said to characterize the genus *Echinocereus*, and they possess sufficient overlap of anthers and stigmas in these populations for autogamy (Taylor 1985, Scobell 1999). Interestingly, herkogamy varies with elevation with a higher degree of anther/stigma overlap at high elevations (Scobell 1999). The effect of herkogamy on levels of autogamous mating across elevations was not tested.

E. coccineus plants can form large mounds of 20 - 500 short (5-40 cm) cylindrical stems, but the average for plants included in this study is 19 ± 2 stems (Zimmerman 2003; S. Scobell, unpublished data). Despite the large size individual plants can attain, there are no reports of vegetative reproduction in this species (Taylor 1985; Zimmerman 2003). Flowers are large, averaging 7 cm long and 3 cm wide (Scobell 1999). This is large enough to accommodate the entire head of a hummingbird or whole body of large bees such as bumble bees (Scobell 1999). Plants average nine flowers per plant (range 0-70), but with a flower life of up to eight days, multiple flowers are often open at one time on each plant (Scobell 1999). Populations included in this study all had > 30 plants within a 100 m x 100 m area. There is ample opportunity, therefore, for pollinators to mediate autogamy, geitonogamy, and/or xenogamy while pollinating these populations.

Echinocereus coccineus is part of the Triglochidiatus group (Taylor 1985). Members of this group are characterized as cacti that produce large, red, tubular flowers with abundant nectar. These floral traits correspond to hummingbird-syndrome traits (sensu Faegri and van der Pijl 1971) these traits have been suggested to be adaptations for the attraction and retention of hummingbirds as pollinators (Grant 1967; Cota 1993). Previous research has indicated that hummingbirds are the most common pollinators in the center of the range of this species and are replaced by bees at the periphery of its range (Hoffman 1992, Scobell 2002, Scobell and Schultz 2005). Powell (1995) found few hummingbirds but numerous bees pollinating dioecious *E. coccineus* plants in an experiment conducted outside the greenhouses at Sul Ross State University in Alpine, Texas. He also said that hummingbirds are rare across most populations he knew to be dioecious in Texas (A. Powell, personal communication). Variation in pollinator taxa throughout the range of *E. coccineus* allows us to test hypotheses concerning the effects of geographic variation in pollinator type on its mating system.

Pollinator Visitation Rates: To determine visitation rates of hummingbirds and bees to flowers, pollinator observations were carried out in nine *E. coccineus* populations (see Figure 1.3). Every attempt was made to spread observations out evenly across the entire daylight period, from dawn until dusk. Visits were recorded only if floral visitors contacted anthers and/or stigma during the visit. Visitor species and sex were recorded for hummingbirds, bee visitation rates were recorded in broader categories: small bees (Halictids), medium bees (*Lasioglossum* sp.), honey bees (*Apis melifera*), bumblebees (*Bombus* sp.), and carpenter bees (*Xylocopa* sp). For the purposes of this paper, all bee visits were lumped together to give an overall visit rate for bees. This was done because small bees heavily dominated the visit rate observations for insects, and all types of bees were able to penetrate the mesh in the pollinator exclusion experiment (see Pollinator Exclusion Experiments section below, and Pollinator Visitation Rates in Results). Visitation rates were estimated using the standardized protocol suggested by Kearns and Inouye (1993, p. 342): The number of flowers visited per pollinator was divided by total number of flowers watched per 10-minute period to express our dependent variable, visitation rates, in visits/flower/10 min.

Populations and dates of observation included: Long Park, Chiricahua Mountains, AZ (LPAZ, May 23-25, 2002; May 25-28, 2003) Barfoot Park, Chiricahua Mountains, AZ (BPAZ, May 20-22, 2002), Pinaleno Mountains, AZ (PNAZ, May 18-21, 2002; May

29-30, 2003), Santa Catalina Mountains, Tucson, AZ (SCAZ, May 20-26, 2001), and Magdalena, New Mexico (MGNM, June 11-14, 2001; June 12-15, 2003). In addition to sites where hummingbird exclusion experiments were carried out, additional visitation rate observations were made in four other populations (HUAZ, April 16-18, 2003; HCAZ, May 3-5, 2003; LMTX, April 8-11, 2002 and GHNM, April 14-17, 2002 – see Figure 1.1 for population locations) throughout the range of *E. coccineus.* This was done in order to document geographic variation in pollinator visitation rates and to compare these rates, using linear regression (SYSTAT 2004), to inbreeding (F_{IS}) in each population. Additional data on characteristics of sites used for visit rate observations (e.g. population size and geometry, plant density) are contained in Appendix 1.

Differences in the dependent variable, hummingbird and bee visit rates (visits/flower/10 minutes), across all sites and between hermaphroditic and dioecious populations were investigated with a Factorial ANOVA using R statistical software (R Development Core Team 2008). Differences in the variances of visit rates between hummingbirds and bees among sites were investigated using a test of equality of the coefficients of variation (Lewontin 1966).

Pollinator Exclusion Experiments: The first step in examining the hypothesis that a change in pollinators could select for dioecy involved hummingbird exclusion experiments. In this experiment, a block of half the flowers on each plant were arbitrarily selected and covered prior to bud anthesis in 1.5 cm poultry mesh. This mesh allowed all species of bees (even bumble bees) to enter but excluded hummingbirds. The other half of the flowers on each plant were left open and were pollinated predominantly by hummingbirds and bees (see Figure 1.2). Cages were left in place until all fruits had ripened. The goal of this study was to determine if pollination by only small bees increases the selfing rates of flowers more than a pollinator assemblage including hummingbirds and bees. This experiment attempted to mimic the natural geographic variation of pollinators across the range of *E. coccineus* populations: both hummingbirds and bees pollinate in the center of the range, but bees are the predominant pollinators at the eastern and western range margins.

This experiment was carried out in five populations over three years (see Figure 1.1 for site locations and Appendix 1 for detailed descriptions of each site). The study populations included: Long Park, Chiricahua Mountains, AZ (LPAZ, n = 15 plants in 2000, 30 plants in 2002, 41 plants in 2003), Barfoot Park, Chiricahua Mountains, AZ (BPAZ, n = 20 plants in 2002), Pinaleno Mountains, AZ (PNAZ, n = 20 plants in 2003), Santa Catalina Mountains, Tucson, AZ (SCAZ, n = 9 plants in 2001), and Magdalena, New Mexico (MGNM, n = 5 plants in 2000, 15 plants in 2001).

In all caged populations, hummingbirds were excluded from touching sexual parts of flowers within the cages, whereas all species of bees entered cages unhindered and visited at rates similar to those outside the cages (Scobell, unpublished data). Two flowers per plant were randomly selected and encased in bridal veil mesh when in bud to completely exclude pollinators. Because these plants can autogamously self (Scobell 1999), this produced a self-pollinated treatment to compare to Caged and Open treatments. In one population (MGNM) two flowers per plant were randomly selected and hand-outcrossed from 2 different flowers coming from 2 different randomly selected plants at least 10 m away. This created the OUTX treatment seen in Figure 1.5 for MGNM. The dependent variable in this experiment will be the number of fruits and seeds produced per flower from each treatment, as well as the number of seeds produced per fruit. The units of the dependent variables are described below.

Cages were left in place until fruits were collected approximately four weeks later. All fruits produced by each treatment were counted, the seeds from 2-4 fruits per plant were counted and average number of seeds/fruit was recorded. Treated flowers that produced no fruits were also included in this analysis, because this reflects the amount of fruit abortion per treatment, this dependent variable is called fruits/flower/plant. Since 1-4 fruits per treatment were counted for each treatment on each plant, seed set was calculated as the mean amount of seeds produced per treatment. This dependent variable, called seeds/fruit/plant also included zero values to reflect the effect of fruit abortion on seed set. Seed set was also calculated for only the subset of flowers that produced fruits to reflect the differences in investment in seeds in each treatment after the plant has invested in producing fruit. This dependent variable is called seeds/fruit. Differences among the means of the treatment groups were investigated with a one-way ANOVA followed by a Tukey multiple comparison post-hoc test to look for differences among pairs of treatment groups (Zar 1999). Some populations were not reached in time to beat frugivores to the fruits. For this reason fruit set and seed set are only reported for populations that had little or no frugivore damage.

In the University of Florida greenhouse 100 seeds/fruit from 2-4 fruits per treatment (Caged, Open and Selfed) were germinated in separate Petri dishes on #1 Whatman filter paper moistened daily with DI water. Populations included the MGNM 2001, HCAZ 2003, PNAZ 2003, ZNUT 2003 and LPAZ 2002 and 2003 populations. However, germination rates were so poor, only the LPAZ 2003 population had a sufficient sample size in all treatment groups for analysis. The dependent variable, percent germination of the LPAZ 2003 population, was arcsine transformed for analysis. Differences among means in percent germination were then investigated with a one-way ANOVA followed by a Tukey multiple comparison post-hoc test. For the seeds collected in 2003 from the Long Park, AZ population, at least 40 sprouted seeds per treatment were retained for subsequent genetic analysis.

Estimating IBD from Fruit Set, Seed Set and Germination in Caged and Open Treatments: In order to estimate amount of inbreeding depression in plant populations, one usually calculates the relative fitness of selfed to outcrossed progeny (e.g. $1-w_s/w_o$). This test was conducted in MGNM in 2001. Unfortunately, the fruit set data for this population was lost (see Lost Data section above), and the seeds from this population failed to germinate, leaving us only with the estimate of relative fitness from seed set of this MGNM population, instead of cumulative reduction in fitness of selfed offspring across all three early-life stages (i.e. fruit set, seed set and germination). This estimate of IBD from seed set was $\delta = 0.30$.

We can infer δ, however, from the differences in fitness of our two caging treatments from the LPAZ 2003 population using a method similar to Equation 2 from Schultz and Ganders (1996). In this paper the authors were comparing the relative fitness of females and hermaphrodites in the gynodioecious plant *Bidens sandvicensis* (Asteraceae). The equation for this ratio is:

$$(3)\quad \frac{w_f}{w_{eh}} = \frac{w_{of}\left(1 - s_f \delta_f\right)}{w_{oh}\left(1 - s_{eh}\delta_h\right)}$$

where w_f and w_{eh} are the fitness of females and hermaphrodites, w_{of} and w_{oh} are the fitness of fully outcrossed progeny of females and hermaphrodites, s_f and s_{eh} are the effective selfing rate of females and of experimentally outcrossed progeny of hermaphrodites and δ_f and δ_h are the inbreeding depression of females and hermaphrodites.

If I am instead comparing the fitness of flowers from our Caged and Open treatments, this equation becomes:

$$(4)\quad \frac{w_a}{w_c} = \frac{(1 - s_a\delta\ )}{(1 - s_c\delta\ )}$$

where w_a and w_c are the fitness of Open and Caged treatments, s_a and s_c are the effective selfing rate of Open and Caged treatments, and δ_a and δ_c are the inbreeding depression of Open and Caged treatments. The terms w_{of} and w_{oh} are removed from this equation based on the assumption that fitness of outcrossed ovules in both of my experimental treatments is the same. I made this assumption because both treatments were carried out on each individual plant included in this experiment.

When I solve equation 4 for δ (S. Schultz, personal communication) the calculations from this paper can be used to estimate the amount of inbreeding depression from our caging treatments. The following equations were used to estimate fitness of outcrossed and selfed seeds from the Caged and Open treatments:

$$(5)\quad \frac{w_a}{w_c} = \frac{s_a w_s + (1 - s_a)w_o}{s_c w_s + (1 - s_c)w_o}$$

When I solve equation 5 for the fitness of self and outcrossed ovules I find:

$$(6)\quad w_s = \left(\frac{1}{s_a - s_c}\right)(w_a - w_c + s_a w_c - s_c w_a)$$

$$(7)\quad w_o = \left(\frac{1}{s_a - s_c}\right)(s_a w_c - s_c w_a)$$

The mean fitness values from fruit set, seed set and germination for each treatment were inserted into equations 6 and 7 to solve for w_s and w_o. The equation for calculating inbreeding depression ($\delta = 1 - w_s / w_o$) was then used to give the amount of IBD expressed by each life stage in the LPAZ 2003 population.

Microsatellite Analyses: Prior to the initiation of this study, no microsatellite primers had been developed for the Cactaceae. Primer development for *E. coccineus* took place in collaboration with M. Arakaki and P. Soltis at the Laboratory of Molecular Systematics and Evolutionary Genetics of the Florida Museum of Natural History at University of Florida. Initial DNA extraction for primer development took place in the lab of Dr. Colin Hughes at the University of Miami.

Microsatellite Primer Development: DNA was extracted from 10 g of young stem tissue from the Great Sand Dunes National Park, CO (GSCO, Figure 1.1) population. The method used was a modification of the Organelle Pellet Method developed by Wallace specifically for DNA extraction from cacti (Wallace and Forquer 1995). The modifications were made by Alongi (1997) for DNA extraction from *Echinocereus triglochidiatus* var. *arizonicus*, a closely-related congener of *E. coccineus*.

Following DNA extraction, a genomic library was constructed with the CA/GT repeat pattern. DNA was digested with Sau3AI, and fragments ranging from 400 to 1500 base pairs in length were retrieved, purified, and ligated with T4 and Sau3I. Sau-L-A and Sau-L-B reverse primers were used to amplify the fragments. The PCR fragments were denatured and hybridized for the CA repeat with the matrix VECTREX Avidin-D. The hybridized DNA was amplified with Sau3AI primer and cloned into the *E. coli* vector using the TA-TOPO cloning kit. DNA was inserted into bacterial plasmids in TOP 10 using One-shot™ *E. coli* (Invitrogen) and was hybridized with a $(CA)_n$ probe. Clones were detected using chemiluminescence. Positive clones (18) were identified, separated, and purified with Sephadex columns. The plasmids were sequenced with the DTCS Quick Start kit (Beckman Coulter) using T7 and M13 universal primers and a Beckman Coulter sequencer.

Primers were developed for 18 sequences of microsatellite repeats using Oligo 4.0 Program and BioEdit. Primer length ranged from 15 bp to 25 bp. The melting temperature for the upper and lower primers was set at 60° C, and melting temperature of all primers was matched within 2° C. Duplex and hairpin formations for the upper and lower primers were kept below a value of 7 kcal/mol. Total product size was kept within a 200-250 bp range. The primers were checked for amplification abilities, and DNA fragments were separated with 2% agarose gel and 6% acrylamide gel. For the PCR amplification reaction, 10 µl reaction mixtures were created using 1 µl DNA, 1 µl dNTP, 1 µl of each primer, 0.2 µl Taq DNA polymerase, 5.8 µl water, and 1 µl Taq buffer.

Analysis of Microsatellite Repeat Length Patterns: *E. coccineus* is a tetraploid cactus and the three primer sets used in this analysis (A, B and Q) each exhibited up to four alleles in an individual, which is characteristic of tetraploids. Loci amplified by primer sets A and B exhibited characteristics of disomic inheritance (e.g., display fixed heterozygosity in the form of two sets of long and short alleles). For example, locus B showed sets of allele size ranges from 103 to 111 and 141 to 161. Both of these regions were amplified by primer set B, so they are labeled Locus B1 and Locus B2, respectively. These two products were treated as separate loci in this analysis, each with disomic inheritance. Locus A showed similar patterns, and it was therefore split into Locus A1 and Locus A2. Locus Q did not exhibit this pattern and instead showed both balanced (Peaks were of equal height – coded as $Q_1Q_1Q_2Q_2$ – where Q_1 and Q_2 are the respective allele lengths for each peak.) and unbalanced (Peaks were of different heights – coded as $Q_1Q_1Q_1Q_2$ or $Q_1Q_2Q_2Q_2$ depending on which length of peak was highest) heterozygotes characteristic of tetrasomic inheritance; therefore, it was analyzed separately. Sometimes the second peak (the longer allele of the two) would be slightly lower than the first. This occurs because longer allele lengths do not amplify as strongly in PCR as shorter ones. The second peak had to be less than half the height of the first for the individual to be considered an unbalanced heterozygote – otherwise they were counted as a balanced heterozygote. All samples had to produce peaks on the electropherograms that were well-defined and of high intensity (Dye Signal over 2000) to be included in the analysis.

Loci A1, A2, B1 and B2 were analyzed using Ritland's MLTR program. This program analyzes mating system parameters for diploid data and was used to calculate the single- and multi-locus outcrossing rates of each treatment, the parental estimate of Wright's inbreeding coefficient (F_{IS}), and allele and gene frequencies (Ritland 2002). Variances of these estimates and their standard errors were determined by bootstrapping with 1000 bootstrap replicates. Due to lower numbers of seed produced and lower germination percentages (See Figure 1.5), fewer seedlings were available for analysis in the Caged treatment than the Open treatment in the disomic data (Table 1.3). To determine what affects this imbalance had on the results of our selfing rate analysis, the sample size was evened by removing individuals until each maternal plant had the same number of Caged and Open progeny scored. Each individual also did not amplify all 4 loci equally; therefore a third dataset was analyzed consisting of only individuals that amplified all 4 loci. Differences in the dependent variable, selfing rate, between the Open and Caged treatment groups were tested in Excel with a t-test.

Locus Q was analyzed using a multi-allelic extension (Murawski et al. 1994) of the TETRAT progeny testing program designed by Ritland (1990) for autotetraploids called MLTET. This program was used to calculate selfing rates of each treatment as well as single-locus outcrossing rates, multi-locus outcrossing rates, gene frequencies, and parental inbreeding coefficients (Ritland 1990). Variances of these estimates were determined by bootstrapping with 1000 bootstrap replicates. Differences in the dependent variable, selfing rate, between the Open and Caged treatments were tested in Excel with a t-test.

Estimates of Selfing Rates from Microsatellite Analysis: Budget constraints limited microsatellite analysis to progeny from the LPAZ 2003 caging experiment. Therefore, only these data are presented here. This site is located on a southeast-facing basalt rock outcrop at 3000 m elevation in the Chiricahua Mountains (LPAZ in Figure 1.1). More than 500 plants grow along a 600 m transect along the top of this ridge, making it one of the larger populations included in this study (Appendix 1).

A total of 100 seeds from two fruits per plant per treatment (Selfed, Caged and Open) were germinated in the greenhouse and 25 seedlings from each maternal plant were randomly selected for subsequent microsatellite analyses. Twenty-seven of the original 41 plants included in this experiment germinated sufficient seeds from both treatments for analysis. Maternal genotypes for each experimental plant were derived from DNA extracted from 1 cm^2 of young stem tissue.

Estimates of Inbreeding Depression Based on Changes in the Inbreeding Coefficient: The magnitude of inbreeding depression present in each population was estimated using the method of Ritland (1990). This method compares Wright's inbreeding coefficient (*F*) between the parental and seed generations. Wright's inbreeding coefficient directly measures excess in homozygosity due to selfing in a population (Wright 1969). If seeds with higher levels of inbreeding (more homozygous individuals) are being selected against (e.g., are suffering from inbreeding depression), then *F* should decrease between the seedling and parent generation. If adult inbreeding coefficients are assumed to be constant over generations (i.e. if populations are assumed to be at inbreeding equilibrium), then only one generation is needed to estimate inbreeding depression. Relative fitness of selfed seeds can be estimated from progeny arrays such as the one produced for this study. If selfing rate of the population is known, then the relative fitness (estimated inbreeding depression) of selfed individuals with disomic inheritance is given by the following equation:

$$(1)\quad \delta = \frac{(s-2F)-Fs}{s(1-F)}$$

For tetrasomic inheritance, the equation for inbreeding depression can be derived from the equilibrium equations for *F* in Murawski et al. (1994). This equation then becomes:

$$(2)\quad \delta = \frac{F(8-5s)-3s}{s(-3-5F)}$$

Estimates of maternal (*F*) as well as selfing rate (*s*) estimates were obtained by analyzing data from the microsatellite analysis of parental tissue samples and their respective progeny arrays (Table 1.3) from the Long Park, AZ (LPAZ 2003) population using MLTR and MLTET programs as explained above. These estimates were then inserted into equations (1) and (2) respectively to estimate inbreeding depression given disomic or tetrasomic inheritance. The selfing rate from the Caged treatment was inserted for (*s*) in these equations to test the hypothesis that selfing rates produced when only bees pollinate produces conditions that can select for dioecy (e.g. $s * \delta > 0.5$). This estimate may underestimate the level of inbreeding depression in this population because the assumption of inbreeding equilibrium between adult generations may be violated. This estimate might also be low because it is based on the selfing rate in seedlings, the secondary selfing rate, not the selfing rate in zygotes (Schultz and Ganders 1996). Thus this may underestimate IBD because many inbred zygotes die before the seedling stage.

Population-Level Analysis: One gram of young stem tissue was taken from 20 – 60 adult plants per population from seven populations (BBTX, CCAZ, GSCO, HCAZ, LPAZ, MGNM AND PNAZ, see Figure 1.1) and stored in the field in a Dewar flask of liquid nitrogen. These were transferred to a -80 freezer and stored until they were used for microsatellite analysis. The program Fstat (Goudet 2001) was used to determine population genetic parameters for these sites (F_{IS}, F_{ST} - see Tables 4 and 5; Allele frequencies and number of private alleles – see Appendix 3; genetic diversity estimates – see Appendix 4; and Nei's statistics of genetic diversity – see Appendix 5). The Mantel test and multiple regression analyses were also carried out in Fstat (Goudet 2001). Regression analyses of F_{IS} on hummingbird and bee visit rates were carried out in Excel.

Lost Data: Due to unforeseen circumstances (car theft) some data (MGNM 2003 visit rate data, some LPAZ 2003 insect visit rate data, and fruit set data from hand-selfed and hand-outcrossed treatments in the MGNM 2001 caging experiment) were lost.

RESULTS

Pollinator Visitation Rates: *Overview of Pollinator Observations*: Pollinators of *E. coccineus* included a wide variety of hummingbird and insect species. The most common hummingbird species that visited *E. coccineus* was the Broad-tailed hummingbird (*Selasphorus platycercus*). These birds are resident in Sonora and Chihuahua south to the southern border of Mexico. Every spring they migrate north early (February –March) and by summer (June-July) are found across much of the southwestern U.S. – from Arizona and New Mexico north to Idaho and Wyoming (Williamson 2001). These hummingbirds visited seven out of ten populations surveyed in this study and were present in 45% of the sessions where a visitor was recorded. They were not observed to visit any of the three dioecious populations surveyed (BBTX, LMTX, or HUAZ in Figure 1.1) during 170 ten-minute observation sessions carried out at these sites.

Magnificent hummingbirds (*Eugenes fulgens*) are larger birds that migrate into the sky island mountain ranges of west Texas, southern New Mexico and Arizona in March. They stay through the summer, “traplining” their way through sometimes widely separated populations to forage (Powers 1996), and depart in September for their wintering grounds in south and central Mexico (Williamson 2001). Magnificents were found visiting flowers of *E. coccineus* in five out of ten hermaphroditic populations studied. Magnificents were also not observed in any dioecious population over the course of this study (170 ten-minute observations).

Dioecious populations did have visiting hummingbirds. Anna’s hummingbirds (*Calypte anna*) were present in only 1 dioecious population, the Hualapai Mountains

(HUAZ – Figure 1.1). Anna’s hummingbirds are resident in Arizona and California and are not known to migrate (Williamson 2001). Another rare hummingbird that is resident over much of its range is the Broad-billed hummingbird (*Cynanthus latirostris*). Rarely these birds will migrate into west Texas (Williamson 2001). The only sighting of this hummingbird in this study is a one-time visit to the dioecious *E. coccineus* population in Lizard Mountain, TX (LMTX – Figure 1.1).

Black-chinned hummingbirds (*Archilochus alexandri*) were the main hummingbird pollinator in the dioecious LMTX population. They are permanent residents in coastal southern Texas, but many migrate north (some all the way to southern Canada) starting in early March (Williamson 2001). These hummingbirds visited this low-elevation (1450 m) population at high rates (approximately 1 visit/flower every 33 minutes). They also visited a hermaphroditic population further north at a rate of 1 visit/flower every 24 minutes (Gila Hot Springs, NM – GHNM – Figure 1.1). Because many hummingbirds found in dioecious populations are resident in these areas and the hummingbird species found in hermaphroditic populations are more migratory, the different species of hummingbirds may be mediating gene flow in very different ways within and among *E. coccineus* populations.

Insects are frequent visitors to most *E. coccineu*s populations, and come from taxonomically diverse groups such as butterflies, flies, bees, beetles, and ants (Scobell 1999; Scobell and Scott 2002), but small sweat bees from the Halictid family make up the majority of visitors in most *E. coccineus* populations. Halictid bees (mostly from the genera *Dialictus* and *Lasioglossum*) were found in all populations except the Hualapai Mountains (HUAZ – Figure 1.1) where no insect visitors were observed in over 100 ten-minute observation periods. These small insects often swarm newly-opened flowers, gathering as much pollen as they carry, seldom visiting more than two flowers in a row (Scobell 1999). Most visits by bees recorded in >500 hours of observing pollination of *E. coccineus* were visits within one flower or between a few flowers on one plant (Scobell 1999 and S. Scobell, unpublished data). It is for this reason that I hypothesize that if pollination is carried out primarily by these small bees, selfing rates of plants will increase.

Honeybees (*Apis melifera*), bumblebees (*Bombus sp*.) and carpenter bees (*Xylocopa californica*), although observed visiting *E. coccineus* plants in the Chiricahua mountains in 1996 and 1997 (Scobell 1999) were rare (only two incidences of bumblebee visits recorded in two populations - LPAZ and MGNM) or absent (*Apis* and *Xylocopa*) in observations from 2000 - 2003. For this reason I lump all bees into a single category and assume that most of the pollination by bees across populations is by small Halictid bees.

Butterflies of various species (Swallowtails – *Papilio multicaudatus* and *Battus philenor*, a Lycaenid Blue (unknown sp.), and a Sulfur Butterfly (unknown sp.)) were seen only occasionally, and visited populations at varying elevations and geographic locations. They may be poor pollinators because they rarely touch sexual parts of the flowers when feeding (Scobell 1999).

Flies (Muscidae and Syrphidae) were rare in most populations, with high numbers only showing up in one population (PNAZ – 32% of observation periods had flies). Flies are likely to be poor pollinators because they rarely touch the sexual parts of the flower, unless they are eating pollen off the stigma (Scobell, personal observation).

Pollinator Visitation Rates: *Visitation Rates of Hummingbirds vs. Bees*: Overall, there were significant differences between the mean 10-minute hummingbird and bee visitation rates across all sites, with the grand mean of bee visit rates being higher than the grand mean visit rate of hummingbirds (grand mean bee visits/fl/10 min = 0.181, grand mean hummingbird visits/fl/10 min = 0.092, Factorial ANOVA, $F_{1, 873} = 4.46$, p=0.035). Hummingbirds were more consistent pollinators than bees across these nine populations, with the variance in bee visit rates being significantly higher than the variance of hummingbird visit rates across sites (Bee VR $s^2 = 0.93$, Hummingbird VR $s^2 = 0.05$, $F_{8, 8} = 17.26$, p<0.0005).

To test the hypothesis that range-wide biogeographic changes in pollinator type, from hummingbirds to bees, is responsible for the evolution of dioecy in *E. coccineus*, I compared the mean 10-minute visit rates of hummingbirds and bees in each type of population. The prediction that follows from this hypothesis is; hummingbirds will have higher visit rates than bees in hermaphroditic populations and bees will have higher visit rates than hummingbirds in dioecious populations (Assuming pollinator distributions have remained relatively stable as dioecy has evolved in this species). This prediction was not consistently supported by this study (but see Chapter 3 herein for analyses supporting this hypothesis). Hummingbirds visited significantly more often than bees in our focal hermaphroditic experimental population: LPAZ 2003 (0.08 visits/flower/10 min for hummingbirds vs. 0.004 visits/flower/10 min for bees, $t_{2, 21}= 0.98$, p=0.02) (Figure 1.3). Hummingbirds also had significantly higher visitation rates in one other hermaphroditic population (HCAZ). Bees however, had significantly higher visitation rates than hummingbirds in three hermaphroditic populations (SCAZ, MGNM and

GHNM), but these populations also had high hummingbird visit rates. One dioecious population (HUAZ) was entirely lacking any type of bee pollinator and also had one of the lowest hummingbird visit rates. Overall, the visitation rates of hummingbirds and bees in dioecious populations (LMTX and HUAZ) differed significantly from those in hermaphroditic populations (Factorial ANOVA, $F_{1,873} = 4.43$, $p=0.036$), but our predicted trend was not supported. With this low sample size of two dioecious populations and seven hermaphroditic populations it is not surprising that I did not find a trend in this highly variable data. More measurements of visit rates from both types of population across the range of *E. coccineus* are needed to determine if there is a trend for hummingbird visit rates to be high in hermaphroditic populations and bee visit rates to be high in dioecious populations.

Pollinator Exclusion Experiments: *Fruit set, Seed set and Germination*. In many cases the presence of hummingbirds in the pollinator assemblage increased the number of fruits produced per flower, seeds produced per plant and % germination over that produced by only bee-pollination or self-pollination (Figures 1.4 and 1.5).

The differences in fruit set were significant at three sites (BPAZ, PNAZ and LPAZ) and across two years (2000 and 2003) within one site (LPAZ). When the effect of treatment is considered over all sites, the Open pollination treatment had significantly increased fruit set compared to the hummingbird-exclusion treatment (Factorial ANOVA, $F_{1,126} = 31.6$, $p<0.001$). This is a first indication that addition of hummingbirds as pollinators increases the fitness of hermaphrodites.

Seed set also differed significantly among treatments in the LPAZ 2003, MGNM and PNAZ populations, with addition of hummingbirds increasing seed set well over

100% in all populations (247.1 $\pm$ 28 SE seeds/fruit with hummingbirds vs. 115.1 $\pm$ 26 SE seeds/fruit with bees alone in LPAZ 2003 (N = 41mean seed set values/treatment); 278.5 $\pm$ 56 SE seeds/fruit with hummingbirds vs. 74.5 $\pm$ 28 SE with bees alone in MGNM (N = 20 mean seed set values/treatment); 157.6 $\pm$ 72 SE seeds/fruit with hummingbirds vs. 32.8 $\pm$ 28 seeds/fruit with bees alone in PNAZ (N = 21 mean seed set values/treatment)). The differences across all treatments were significant in all populations in a one-way ANOVA ($F_{2,120}$= 15.88, p < 0.0001 for LPAZ 2003; $F_{4,108}$= 8.16, p < 0.0001 for MGNM; $F_{2,57}$= 9.79, p = 0.0002 for PNAZ). The LPAZ 2000 population only had two treatments (Open and Caged), but still showed significant differences between the treatment groups (Open = 217.2 $\pm$ 49 SE, Caged = 76.7 $\pm$ 26 SE, $t_{2,14}$= 0.98, p = 0.02). In Figure 1.5 it can be seen that there were significant differences across all populations in all post-hoc tests between the Caged and Open treatment groups using a Tukey multiple comparison test (Zar 1999). In addition, in all populations where both treatments were carried out, bee pollination in the Caged treatment produced no more seeds than the Self-pollination treatment. Hummingbird pollination again is shown to improve plant fitness over that of bee pollination. In the population that received the most thorough set of pollination treatments (MGNM: selfed, outcrossed, caged, open and natural pollination) only the Caged vs. Open, Natural vs. Self and Natural vs. Caged treatments differed significantly. This indicates excluding hummingbirds had a significant effect on seed set, but the treatment (putting up cages) did not significantly reduce seed set (Open vs. Natural = NS). There also was no significant difference between the selfed treatment and the outcrossed treatment. This may indicate that *E. coccineus* requires more than two outcrossed pollen donors (the method used in this study) to produce significantly higher levels of seed set.

When only fruits producing seed were included in calculation of the average values for seed set (i.e. seeds/fruit instead of seeds/flower), there were no significant differences among the groups ($F_{2,65}= 2.68$, $p = 0.07$ for LPAZ 2003 (n = 11 Selfed, 23 Caged, 34 Open); $F_{3,34}= 1.03$, $p = 0.39$ for MGNM; $t_{2,14} = 0.88$, $p = 0.162$ for PNAZ). This seems to indicate that once a plant invests in a fruit, the investment in seeds is similar regardless of treatment.

Poorer quality seed production through bee- and self-pollination can also seen in the germination rate data. Although I attempted to germinate seed from six populations and LPAZ across two years (2002, 2003), only LPAZ 2003 germinated sufficient seeds to test for differences among treatments. There was no significant difference in the germination rates of Caged vs. Selfed treatments in a post-hoc Tukey multiple comparison test (Caged vs. Selfed = $q_{65,3} = 1.10$, $p >0.05$). There was a significant treatment effect in LPAZ 2003 in a one-way ANOVA ($F_{2,65} = 5.97$, $p = 0.004$), as well as significant differences among all treatments except Caged vs. Selfed in post-hoc Tukey multiple comparisons (Means ± SE: Open = 36.3 ±7%., Caged = 19.7 ±7%, Selfed = 11.7.±7%: Tukey multiple comparison test: Open vs Caged = $q_{65,3} = 3.89$, $p <0.025$, Open vs. Selfed = $q_{65,3} = 4.00$, $p <0.025$). The analysis of germination rate data echoes that of fruit- and seed-set data; bee pollination (Caged treatment) is similar to self-pollination and both of these treatments germinated significantly fewer seeds than the treatment with hummingbird pollination (Open treatment).

Estimating IBD from Fruit Set, Seed Set and Germination in Caged and Open Treatments: In order to determine the amount of inbreeding depression expressed in the LPAZ *E. coccineus* population I used equations modified from Schultz and Ganders (1996). The mean fitness values from fruit set ($w_a = 0.53$, $w_c = 0.26$ fruits/flower), seed set ($w_a = 279$, $w_c = 115$ seeds/fruit) and germination ($w_a = 0.37$, $w_c = 0.20$ percent germination) for each treatment were inserted into equations 6 and 7 to solve for w_s and w_o. The equation ($\delta = 1 - w_s / w_o$) was then used to give the amount of inbreeding depression expressed by each life stage. When differences in cumulative fitness (the product of fruit set, seed set and germination) are calculated (Husband and Schemske 1997), total estimated inbreeding depression for this population is $\delta_{est} = 0.99$. This estimate is very different from the one obtained by microsatellite analysis ($\delta = 0.17$, see below), and it is clear now why this difference exists: The estimate from our microsatellite data was not calculated using the primary selfing rate (selfing rate upon fertilization), but the secondary selfing rate in seedlings after selection had already removed many selfed progeny (Lande et al. 1994). I can calculate the primary selfing rate using the equation below from Schultz and Ganders (1996):

$$(8) \qquad r = \frac{r^*}{r^* \delta^* + \left(1 - \delta^*\right)}$$

In this equation r^* is the secondary selfing rate after selection (in our case 0.77 for the Caged treatment, see Table 1.3) and δ^* the amount of IBD expressed prior to this stage ($\delta_{est} = 0.99$). When these values are entered into equation (8) above, this gives a primary selfing rate of $r = 1.0$ for the Caged treatment. If I then plug the primary selfing rate back into equation (1) I find my new estimate for IBD from microsatellite data to be $\delta = 0.50$. Altogether, these analyses demonstrate that IBD is likely to be between 0.50 and 0.99

within this treatment in the LPAZ population. This, combined with a primary selfing rate of 1.0 from the Caged treatment, demonstrates that the conditions to select for separate sexes would exist in this population if only bees were pollinating these plants (e.g. $r * \delta$ will range from 0.50 - 0.99 when only bees pollinate LPAZ plants).

Under these conditions of extremely high IBD ($\delta = 0.99$), hummingbird pollination with its high outcrossing rate, provides a crucial respite from the loss of fitness due to selfing. The primary selfing rate for the Open treatment calculated from the disomic data is $r = 0.96$. When this value is put into equation (1) I get an adjusted IBD from microsatellite data of $\delta = 0.41$. With an IBD of 0.41 and a primary selfing rate of 0.96 selection for dioecy is not possible (e.g. $r * \delta = 0.40$ with both hummingbirds and bees pollinating), but this population appears precariously balanced near the tipping point for the selection for separate sexes, making outcrossing that hummingbirds provide important in maintaining the hermaphroditic mating system of this population.

<u>Microsatellite Analysis and Results of Microsatellite Primer Development:</u> A total of 18 possible microsatellite sequences were isolated and genotyped. Of the 18 possible sequences containing microsatellites, 10 sequences contained sufficient coverage of the sequence to create primers (see Appendix 2). These 10 primers were then tested on 20 DNA samples from three *E. coccineus* populations. Of these, three primer sets yielded sufficient variation and repeatability for use as genetic markers. These three primers and their features are shown in Table 1.1.

Using the primers for these three loci, a total of 1200 samples from 10 populations of *E. coccineus* were genotyped. Due to low intensity of peaks or inability to score alleles, many samples were dropped; 579 individual samples from 29-31 maternal plants and their progeny from the Long Park population (LPAZ) remained and were used for this analysis. Results for number of alleles scored at each locus and total number of alleles scored from the microsatellite analysis of *E. coccineus* for the Long Park population caging experiment are shown in Table 1.2. The population-level analysis included 263 samples from seven populations. The F_{IS} values from these populations are shown in Table 1.4, the F_{ST} values in Table 1.5. Results of these analyses are discussed below.

Estimates of Selfing Rates and Inbreeding Depression from Microsatellites: Both disomic and tetrasomic loci show that without hummingbirds, bee pollination produces a significant increase in selfing rates in *Echinocereus coccineus* (Table 1.3). For the disomic data, both the single-locus (t_s) and multi-locus estimates for outcrossing rates (t_m) in the Caged treatment were significantly lower than those for the Open treatment ($t_{2,25} = 3.51$, $p = 0.0017$ for singlelocus outcrossing rate; $t_{2,25} = 3.35$, $p = 0.0025$ for multilocus outcrossing rate). This indicates, as predicted, when hummingbirds are excluded, selfing rates ($s = 1\text{-}t$) increase, but are these rates high enough to select for dioecy in this population? The multilocus selfing rate increased 238% when only bees pollinate. The parental fixation index (F_{IS}) was positive (0.215), indicating a deficiency of heterozygous gametes compared with random expectations. When selfing rates (s) produced under bee pollination and fixation indices (F_{IS}) are inserted into Equation (1), estimated inbreeding depression (δ) for this population is found to be $\delta = 0.17$. When this value is used to calculate Lloyd's (1982) criterion for invasion of unisexual populations by females ($s * \delta > 0.5$), I find that $s * \delta = 0.13$, which is low, and less than what is needed to allow invasion of unisexual females. However, as I established above, these values were calculated with the secondary selfing rate produced by assaying progeny after selection had taken place. When the primary selfing rate, and IBD estimated from early life stages are used instead, I find that $r * \delta > 0.5$, meaning that the criteria for the invasion of unisexual mutants would be met in the LPAZ population if only bees were pollinating these plants.

When equal sample sizes from all parental plants are selected from both treatments, the difference in selfing rates between the two groups decreases, the standard errors increase, and the two groups are no longer significantly different (Caged $t_m = 0.579 \pm 0.235$; Caged $t_s = 0.438 \pm 0.172$; Open $t_m = 0.807 \pm 0.115$; Open $t_s = 0.784 \pm 0.129$; $t_{2,19} = 0.76$, $p = 0.46$ for singlelocus outcrossing rate; $t_{2,19} = 1.47$, $p = 0.15$ for multilocus outcrossing rate). This reduction in the significance of this difference is probably due to a loss of statistical power through a reduction in sample size. With all data included there are 321-454 progeny per allele from which to estimate these parameters. In order to equalize the number of samples per maternal family for each treatment, over 100 progeny had to be removed. The new sample size of this analysis is 183-251 progeny per allele. It is interesting to note that there is no change in the relationship between these two treatments, Open pollination still has a higher outcrossing rate than the Caged treatment. Reducing sample size just increases the estimate of the outcrossing rate and the variance of both groups.

The results are similar if sample size is reduced in order to include only progeny that amplified all five loci, except the single locus outcrossing rate continues to show a significant difference. Again, the difference between the two groups decreases, the standard errors increase, and the two groups are no longer significantly different in their multilocus outcrossing rates (Caged $t_m = 0.496 \pm 0.253$; Open $t_m = 0.829 \pm 0.072$; Open $t_s = 0.705 \pm 0.092$; $t_{2,15} = 1.23$, $p = 0.23$). However, even with the reduction in sample size, there is still a significant difference in the single locus outcrossing rate between these two treatments (Caged $t_s = 0.319 \pm 0.164$; Open $t_s = 0.705 \pm 0.092$; $t_{2,15} = 2.11$, $p = 0.05$). In order to only include loci that amplified all 5 alleles for each treatment, over 100 progeny had to be removed. The new sample size of this analysis is 120 progeny per allele. As in the previous analysis, Open pollination still has a higher outcrossing rate than the Caged treatment. Reducing sample size again increases the estimate of the outcrossing rate and its variance in both groups.

The single tetrasomic locus tells a similar story with hummingbird exclusion significantly increasing selfing rates ($t_{2,17} = 3.72$, $p = 0.001$ for single locus outcrossing rates). The F_{IS} value ($F_{IS} = 0.363$) was similar to the disomic value, and when this F value and the selfing rate produced by bee pollination are inserted into Equation 2, it gives an inbreeding depression of $\delta = 0.29$. When I use this value to estimate whether bee pollination could select for dioecy in this population, I find that $s * \delta = 0.25$, a result higher than that produced by the disomic loci, but still insufficient to select for dioecy. When these values are adjusted, as done above, the primary selfing rate of this population becomes 1.0 and the IBD becomes $\delta = 0.39$. The product of the primary selfing rate and the new estimate of IBD is then ($1.0 * 0.39 = 0.39$), which is still too low to allow unisexual mutants to invade, but as shown in the disomic case above, this estimate is probably still an underestimate, and if IBD is closer to the estimate from fruit set, seed set and germination (0.99) there would be strong selection for the evolution of dioecy in this population.

Even these new estimates only encompass early-acting inbreeding depression. In plants inbreeding depression can be expressed across the entire lifetime of the plant, from zygote to the mature plant's own ability to produce seeds, though IBD is often strongest in early life stages (Husband and Schemske 1996). If late-acting IBD also plays a role, then the selection for separate sexes with bee pollination would be even stronger.

Population-Level Analysis: *Genetic Diversity*: Only the disomic data (Locus sets A1, A2 and B1, B2) were used to calculate genetic diversity values, because the alleles in these loci behave like separate diploid loci, so they can be analyzed with a program like Fstat (Goudet 2001) that calculates population genetic parameters for diploid species. This analysis revealed that there were a total of 62 alleles resolved across seven populations and four loci (Appendix 3). Many populations had private alleles (31 total, mean = 4.4 per population). Most alleles within these populations were polymorphic (Average proportion of polymorphic loci was $P = 0.98$). The average number of alleles per locus (*AP)* was high 15.5 (1.21), with MGNM having the highest number of alleles per locus. The overall genetic diversity of the species was also relatively high, H_t=0.56. The population with the highest genetic diversity was HCAZ (H_t=0.56), BBTX was the lowest (H_t=0.23).

Population-Level Analysis: *Genetic Structure*: Mean observed heterozygosities (H_o) were lower than the expected heterozygosities (H_e) for all populations. This indicates that a moderate deficiency of heterozygotes exists in the species as a whole. The mean F_{IS} was 0.313 over all populations. This is similar to Nei's (1987) estimator for F_{IS}, $G_{\text{IS}} = 0.398$ (Appendix 5). These positive inbreeding coefficients suggest that *E. coccineus* is undergoing some degree of inbreeding, population subdivision or both.

Hummingbird and Bee Visit Rates vs. F_{IS}: I conducted this analysis to determine whether hummingbird and bee visit rates could be influencing population inbreeding values (F_{IS}) across the geographic range of *E. coccineus*. Populations of *E. coccineus* cover a wide range of values for parental inbreeding coefficients (Table 1.4). I found no relationship, however, with either type of pollinator's visit rates and F_{IS} within populations ($r^2 = 0.21$, $P = 0.35$ for hummingbird visit rate; $r^2 = 0.12$, $P = 0.48$ for bee visit rate). There is an intriguing pattern in the data, however, when F_{IS} is plotted against site elevation (Figure 1.6). The highest inbreeding values come from two sites with the lowest elevations: Big Bend, TX (1450 m) at 0.542 and Cave Creek, AZ (1750 m) at 0.580. The lowest inbreeding value (0.001) comes from the highest elevation site: Magdalena, NM (3150 m). This is a significant linear relationship ($r^2 = 0.56$, $p = 0.05$), which suggests that elevation might be affecting levels of inbreeding that persist to the adult stage in these populations. The estimate of F_{IS} averaged across all loci was 0.398 using Nei's (1987) coefficient of gene differentiation, G_{IS} as computed in Fstat (Goudet 2001).

<u>Population-Level Analysis</u>: *Isolation by Distance and Geographic Patterns of Gene Flow*: The pattern of isolation by distance for the seven genotyped populations (Table 1.5 and Figure 1.7) was tested using the Mantel test in Fstat (Goudet 2001). Using F_{ST} is sometimes not recommended when analyzing microsatellite data, because microsatellites mutate in a stepwise manner, and at a higher rate than F_{ST} calculations assume (Wright 1978). This lowers the expectation of F_{ST}, which could lead to a bias in inferences drawn from this statistic. The statistic that is often suggested is R_{ST} which is

independent of mutation rate, and assumes a stepwise mutation model (Nei 1987), but both F_{ST} and R_{ST} are used widely in the literature of population genetics. Balloux and Goudet (2002) studied the performance of both statistics in a simulation study and came to the following conclusions: When populations are highly structured (Nm = 0.1) or sample size is small, R_{ST} does best. When populations are weakly structured (Nm = 10) F_{ST} does best. Since most of our values for Nm are greater than 1 (Table 1.5), I feel that use of F_{ST} is valid.

There are also statistical issues when translating F_{ST} into Nm. Whitlock and McCauley (1999) mention several assumptions of this transformation that are often violated in studies of natural systems (e.g. No selection, no mutation, all populations contribute equally to the migrant pool). However, they conclude their paper by saying that estimates of Nm can be useful if accuracy is not of great concern. The purpose of using Nm in this work is to take a first look at how genes might be flowing among *E. coccineus* populations, and therefore the general pattern is more important than the absolute values of the number of migrants.

When all populations are included in the analysis, the relationship between F_{ST} and distance is not significant ($r^2 = 0.10$, $p = 0.16$). However, if the high F_{ST} value between Cave Creek, AZ and the Huachuca Mountains, AZ is treated as an outlier and removed, the relationship of F_{ST} to distance becomes significant ($r^2 = 0.38$, $p = 0.003$). The reason for the large value ($F_{ST} = 0.27$) of this outlier may lie in the flowering phenology of these populations (see Table 1.4). *E. coccineus* populations at Cave Creek (1750 m) begin blooming in late March, reach peak bloom around mid-April and are finished blooming by early May (Scobell 1999). Populations at 2100 m in the Huachuca

Mountains do not begin blooming until early May and are not at peak bloom until mid- or late May, thus there is little chance of pollen being directly transferred between these two populations.

There are other intriguing patterns in this data; the Great Sand Dunes National Park, CO (GSCO) population is the most geographically distant population, and the large genetic distances (and low values of *N*m) between this population and each of the others reflects this. Note, though, that populations that are blooming near the time that GSCO blooms (LPAZ, PNAZ and MGNM) have lower F_{ST} values (and thus higher *N*m) than those that bloom earlier (LMTX, CCAZ and HCAZ).

Long Park, AZ (LPAZ) and Cave Creek, AZ (CCAZ) are the closest in geographic distance, but CCAZ is more genetically similar to the Lizard Mountain, TX (LMTX) population that is blooming at the same time. In fact, if differences in the timing of peak flowering are plotted against F_{ST}, I find that there is a significant linear relationship between time of peak bloom and genetic distance (Figure 1.8). These patterns suggest that elevation, which affects the timing of flowering in this species through its effect on temperature, has a large influence on the way genes are distributed in these populations. In fact, when differences in elevation (in meters) and time of bloom of each pair of populations are added to the analysis, I find that the percent of the variance in F_{ST} explained increases ($r^2 = 0.53$, $p = 0.006$).

DISCUSSION

Dioecy is hypothesized to have been selected in *E. coccineus* because this plant underwent a shift in the predominant pollinator type from hummingbirds to bees. This hypothesis rests partially on the assumption that in the absence of hummingbirds, self-pollination increases significantly when this plant is pollinated by bees alone. There are several reasons why I believed that hummingbirds would produce a higher outcrossing rate than bees. Bees require fewer floral visits per foraging bout at *E. coccineus* flowers because of the abundant pollen (approximately 600 stamens, Hoffman 1992) and nectar rewards (35 mg sugar/flower/day, 10x the average hummingbird syndrome flower, Scobell 1999). Most visits by bees recorded in >500 hours of observing pollination of *E. coccineus* were visits within one flower or between a few flowers on one plant (S. Scobell 1999 and Scobell, unpublished data). Conversely, since the daily energy expenditure of broad-tailed hummingbirds is approximately 23.3 kJ/day, they had to visit >40 flowers per day to meet their energy requirements (Montgomerie and Gass 1981; Scobell 1999). In his observations on pollination of *Delphinium nelsonii* in Colorado, Waser (1982) found that broad-tailed hummingbirds carried pollen 50-150% longer distances between plants than halictid bees. He estimated that the number of plants in the genetic neighborhood (Wright 1969) would increase nearly eight-fold if broad-tailed hummingbirds were the sole pollinator compared to halictid bees (Waser 1982).

The contribution of hummingbirds to plant fitness in *E. coccineus* is first demonstrated by the increase in fruit set, seed set and germination rates produced by hummingbird pollination (Open vs. Caged treatment). By comparing both the fruit and seed set values from both treatments in our caging experiment, I can see that addition of hummingbirds to the pollinator pool increases plant fitness by 60% (as measured by seed set). Across all populations (Figure 1.4), addition of hummingbirds increases fitness at fruit set by 30% and without hummingbirds, germination decreased by 17%. Thus, if hummingbirds are not present, plant fitness will decrease even without taking the selfing rates produced into account. Higher selfing rates produced by less efficient pollinators will decrease plant fitness even further, if inbreeding depression affects the selfed seeds.

I found support for the assumption that loss of hummingbirds, and reliance on bees as pollinators, increases plant selfing rates in one hermaphroditic population of *E. coccineus* (LPAZ). In this population, selfing rates increased by 238% when hummingbirds were excluded from pollination by the caging treatment. High selfing rates produced by bee pollination did not seem to produce inbreeding depression estimates high enough to select for dioecy in this population when using Ritland's (1990) estimators for population inbreeding. There are two possible reasons for this, the estimation method itself and the timing of when the progeny were assayed for microsatellites.

Ritland (1990) cautions that estimating levels of inbreeding based on only the parental *F* values and the seedling selfing rates (which is the method I used) can underestimate the levels of inbreeding depression in populations because it does not take into account all of the inbred individuals of the parental generation lost to selection before the surviving parents were genotyped. In their review of inbreeding values in short and long-lived plants Scofield and Schultz (2006) caution that estimates of inbreeding depression often have large amounts of error associated with their measurement.

The values obtained from the microsatellite data were also likely to underestimate the actual level of selfing because these values were actually the secondary selfing rate (selfing rate after selection). Since selection against inbred progeny was found to be very strong in this species (early-acting IBD = 0.99), correcting our selfing rate estimates to reflect the primary selfing rate (selfing rate in zygotes) was an important step to accurately predicting if the conditions for selecting for dioecy were present in this population. When the primary selfing rate was calculated, this produced estimates of selfing and IBD that indicated if bees were the only pollinator in the Long Park, AZ population, there would be strong selection for separate sexes. This new estimate still leaves out the effects of late-acting IBD (e.g. growth to maturity and seed production of progeny), so even our high estimate of IBD could still be an underestimate.

In order to extrapolate the findings from the LPAZ population, it is important to note that F_{IS} in this population is relatively low (around 0.3). At the higher levels of F_{IS} (0.580, 0.344) found in hermaphroditic populations at Cave Creek and the Pinaleno Mountains, AZ, even the secondary selfing rate produced by bees in this experiment (s = 0.77), if maintained, would produce high levels of inbreeding depression (according to Equation 1). This high selfing rate and high inbreeding depression would be sufficient to select for dioecy (under the conditions of ($s * \delta > 0.5$)). High levels of F_{IS} are also associated with both dioecious populations included in this study: The Great Sand Dunes NP, CO population and the Lizard Mountain, TX population. This reinforces the supposition that high levels of population inbreeding, along with high levels of self-pollination, could have helped to promote dioecy in these areas.

Purging of recessive mutations that produce the deleterious effects of IBD is supposed to increase with increasing levels of selfing (Husband and Schemske 1996), meaning that it should be very difficult to maintain the both the high level of selfing and the high levels of IBD long enough for both females and males to invade and for the genes encoding each sex to become linked. However, if the levels of inbreeding depression in a population are very high (near 1) as they are in the LPAZ populations, then most selfed seeds will die and little purging will occur (i.e. selective interference; Lande et al. 1994). This phenomenon may be what allowed the evolution of dioecy in *E. coccineus*. If IBD is very high, and deleterious mutations are difficult to purge, then a switch from pollinators that promote high outcrossing rates (e.g. hummingbirds) to ones that promote high selfing rates (e.g. small bees) could shift the balance from a system that favors hermaphrodite plants to one that favors dioecy.

Other experiments that have examined effects of pollinator assemblages on selfing rates (e.g., England et al. 2001; Eckert 2002; Brunet and Sweet 2006) have found different results depending on types of pollinators investigated and the experimental methods employed. Eckert (2002) examined selfing rates in *Decodon verticillatus* in 10 populations in Michigan and 5 in Florida. Michigan populations were predominantly bee-pollinated, but Florida populations contained both butterflies and bees. No significant differences in selfing rates were found between Michigan and Florida populations. Eckert (2002) hypothesized butterflies might carry small amounts of pollen compared to bees and therefore would not produce a change in outcrossing in Florida populations.

In an Australian study similar to ours, England et al. (2001) hypothesized introduced honeybees were causing increases in selfing rates in a predominantly bird-pollinated shrub *Grevillea macleayana.* They compared outcrossing rates produced on inflorescences subjected to Open pollination (birds + bees) to those produced when birds were excluded from inflorescences by poultry mesh. Bee pollination (bird exclusion treatment) had significantly higher selfing rates (1-2 times higher in 2 out of 3 populations) than Open pollination (birds + bees) treatments. High selfing rates produced by bee pollination (as high as 0.9), when combined with even moderate inbreeding depression, would be sufficient to select for male sterility in these populations, although this was not investigated in the England et al. (2001) study.

Across the geographic range of *E. coccineus*, do conditions exist that would promote selection for dioecy? Visitation rates of hummingbirds were higher than those of bees in two of the more centrally-located hermaphroditic populations (compare site names from Figure 1.3 to map in Figure 1.1), and bee visitation was higher in two other hermaphroditic populations situated closer to the dioecious edges of the range. These trends reinforce conclusions of Scobell and Schultz (2005); that dioecy is negatively associated with hummingbird abundance. From this data I predicted that dioecious populations will have low or no hummingbird visitation, but this is not supported by our visitation rate data. In these two dioecious populations, hummingbird visits were either the only kind recorded (HUAZ) or were not significantly different from bee visitation rates (LMTX). One trend that was apparent from the visit rate data is that bee visitation rates vary much more across the range of *E. coccineus* than hummingbird visit rates. If bees are more consistent pollinators at the edges of the range, or visit at rates high enough to leave little pollen for hummingbirds when they visit, this could also push populations toward dioecy. What is most likely however, is that links between pollinators and dioecy are not as clear-cut as only looking at how hummingbirds or bees dominate the visit rates of populations within the time span of one study. Visitation rate data for hummingbirds and bees are needed from more populations, over a longer time span, in order to clarify how visitation rates of pollinators relate to the distribution of mating systems across the range of *E. coccineus*.

If bees produce higher selfing rates than hummingbirds, then I might see the results of this in F_{IS} values of populations with different visitation rates of these two pollinator types. Effect of hummingbird and bee visit rates on the population inbreeding values (F_{IS}) was also equivocal, however, with populations with high hummingbird visit rates (> 0.1 visit/fl/10 min) showing both the highest (CCAZ – 0.580) and the lowest (MGNM-0.001) population inbreeding levels. The one dioecious population included in this analysis (LMTX) did show a very high F_{IS} value (0.542) along with a high bee visit rate (> 0.2 visits/fl/10 min). This was countered, however, by the MGNM population, which had a higher bee visit rate, but no evidence of inbreeding in the adult population ($F_{IS} = 0.001$). This evidence suggests there are probably other factors producing the pattern seen in F_{IS} values across these seven populations.

One interesting possibility suggested by the population inbreeding (F_{IS}) data is that elevation could be influencing the amount of inbred progeny that make it into the adult generation. One way this could occur is through stronger selection against inbred progeny at high elevations. Climatic variables (temperature, precipitation, UV radiation, seasonality, and disturbance) often become more extreme at high elevations (Lomolino

2001). More extreme climate conditions, and more extreme fluctuations in climate seen at high elevations, would then select against homozygous individuals carrying recessive deleterious alleles. Individuals with similar levels of homozygosity could survive, however, in a more benign environment (Ritland 1990, Dudash 2005). This might be why I see no evidence of inbreeding in the adult plants at the Magdalena, NM site, which is above treeline at 3150 m, but see high levels of inbreeding in the more benign, low-elevation sites of Cave Creek, AZ and Lizard Mountain, TX.

Population F_{ST} and Nm values seem to show a system of populations well connected through gene flow, sometimes in spite of large geographic distances. The influence of phenology was found to often produce higher separation in F_{ST} values than distance. The gene flow signature across large distances (Table 1.5) when populations are blooming at the same time (Figure 1.8), may point to the influence of the long-distance migration of hummingbirds, which coincides with the bloom time of *E. coccineus*. Many of the hummingbird species that are the most common visitors to *E. coccineus* populations migrate from south to north starting in the early spring. Both Broad-tailed (*Selasphorus platycercus*) and Black-chinned hummingbirds (*Archilochus alexandri*) are migrating north as claret-cup populations are blooming (Williamson 2001). I know that these pollinators visit these populations in high numbers, and hummingbirds have been shown to carry pollen moderate distances within populations (> 10 m: Waser 1982; > 7 m: Campbell 1991). If pollen can remain viable for long periods of time (> 6 hours; Singer and Sazima 2000) the migration of hummingbirds might have a strong influence on the way genes flow through this system. To draw these kinds of conclusions definitively, however, I will need to increase our sampling of populations.

CONCLUSIONS

The unique opportunity represented by *E. coccineus* to study evolution of dioecy in an animal-pollinated plant has provided answers to questions that were not answerable in other correlative or observation-based studies. Because *E. coccineus* has both hermaphroditic and dioecious populations, this species was used to associate specific ecological changes (pollinator type, elevation, and phenology) with evolution of dioecy. I found that lack of hummingbird pollination, and the subsequent increase in selfing rates can produce conditions that promote selection for dioecy.

The elevation of a population also seems to have a strong influence on selection for dioecy through its influence on F_{IS} values and phenology of flowering: Populations that are at similar elevations often flower at the same time, and I found these populations are more likely to exchange genes. High-elevation populations were found to carry a lower genetic load in this species and will therefore not suffer as much inbreeding depression upon selfing. Both of these conditions would make dioecy less likely to evolve, and this correlates well with the broad geographic pattern seen in *E. coccineus* populations. Populations in the high-elevation center of the range through which hummingbirds are migrating are hermaphroditic, and populations in the lower-elevation margins of the range where hummingbirds are less common are dioecious.

This is the first experimental test of the effects of different pollinator types on selfing rates in a dioecious plant species and the first to show that a switch in pollinator type could produce conditions sufficient to select for the evolution of dioecy in the absence of any effect of resources.

Table 1.1: Characteristics of microsatellites and primers developed for amplifying microsatellite repeat regions in *Echinocereus coccineus*.

Primer Name	*Product Length*	*Repeat Length*	*Number of Repeats*	*Primer Sequence*
A-forward	210	122	61	TGAGAAGCGGTTCCTACCTT
A-reverse				CATCTAAGAATTTTGTGAGGAATAACA
B-forward	124	38	19	TGGGATCACAAACTATCAAC
B-reverse				AGCGATTGAAGATATTGAGC
Q-forward	174	64	32	AAACTAAGCCTGACCACATA
Q-reverse				GATTTTGTGAGTGTAAACATAAGTT

Table 1.2: Characteristics of five microsatellite loci found in *Echinocereus coccineus* from the Long Park, AZ 2003 caging experiment.

Locus Name	*Disomic or Tetrasomic*	*# of Alleles*	*Total # of Progeny Scored*	*# of Caged Progeny Scored*	*# of Open Progeny Scored*
A1	Disomic	8	329	120	271
A2	Disomic	12	329	122	268
B1	Disomic	4	454	185	332
B2	Disomic	22	321	129	245
Q	Tetrasomic	25	220	133	112

<u>Table 1.3:</u> Outcrossing Rates for Caging Experiment: Multilocus (t_m) and single-locus (t_s) outcrossing rate estimates for treatments with flowers receiving hummingbird and bee pollination (Open treatment) and bee pollination (Caged treatment) for Long Park, Arizona, in 2003. N_{prog} is the total number of progeny scored for each treatment. N_{mat} is the number of maternal plants sampled per treatment. N_{flow} is the number of flowers sampled. N per flower is the average number of progeny scored per flower. Standard errors of the mean are in parentheses. Table 1.3A contains data from Locus sets A and B that have a disomic inheritance pattern. Table 1.3B contains data from Locus Q that displays a tetrasomic inheritance pattern.

1.3A: Disomic Data

Treatment	N_{prog}	N_{mat}	N_{flow}	***N per flower***	t_m	t_s
Open	403	31	32	5.0 (0.65)	0.771 (0.06)	0.680 (0.06)
Caged	176	29	15	7.0 (1.53)	0.227 (0.14)	0.181 (0.13)

1.3B: Tetrasomic Data

Treatment	N_{prog}	N_{mat}	N_{flow}	***N per flower***	t_s
Open	164	18	22	6.7 (0.67)	0.568 (0.10)
Caged	133	21	14	7.9 (1.55)	0.151 (0.13)

Table 1.4: Population Statistics and Inbreeding Coefficients for Seven *E. coccineus* Populations: Herm stands for hermaphroditic population. Populations are arranged from most southern to most northern.

Population	*Acronym in Fig. 1*	*Dioecious or Herm?*	*Time of Peak Bloom*	*Elevation of site*	F_{IS}
Big Bend National Park, TX	BBTX	Dioecious	Early April	1450 m	0.542
Huachuca Mts., AZ	HCAZ	Herm.	Mid May	2100 m	0.120
Cave Creek, Chiricahua Mts., AZ	CCAZ	Herm.	Early April	1750 m	0.580
Long Park, Chiricahua Mts., AZ	LPAZ	Herm.	Mid June	2800 m	0.247
Pinaleno Mts., AZ	PNAZ	Herm.	Mid May	2650 m	0.344
Magdalena Mts., NM	MGNM	Herm.	Early June	3150 m	0.001
Great Sand Dunes NP, CO	GSCO	Dioecious	Late June	2600 m	0.355

<u>Table 1.5:</u> Distance and Pairwise F_{ST} and N*m* Matrix for Seven *E. coccineus* Populations: Distance in linear kilometers is above the diagonal, F_{ST} and (N*m*) between pairs of populations is below the diagonal. Populations are arranged from most southern to the most northern.

SITE	***BBTX***	***HCAZ***	***CCAZ***	***LPAZ***	***PNAZ***	***MGNM***	**GSCO**
BBTX		716	636	644	724	660	965
HCAZ	0.195 (1.0)		113	121	145	402	821
CCAZ	0.008 (30.6)	0.270 (0.70)		8	105	298	740
LPAZ	0.069 (3.4)	0.022 (10.9)	0.072 (3.2)		97	306	692
PNAZ	0.024 (10.1)	0.077 (3.0)	0.050 (4.7)	0.028 (8.6)		290	692
MGNM	0.091 (2.5)	0.024 (10.2)	0.123 (1.8)	0.015 (16.2)	0.025 (9.8)		434
GSCO	0.178 (1.2)	0.154 (1.4)	0.185 (1.1)	0.123 (1.8)	0.135 (1.6)	0.136 (1.6)	

Figure 1.1: Distribution of dimorphic (dioecious or gynodioecious)(red squares) and hermaphroditic (green circles) *E. coccineus* populations in the Southwestern United States superimposed upon a map of hummingbird (broad-tail *Selasphorus platycercus* and black-chinned *Archilochus alexandri*) distribution and abundance obtained from the Audubon Breeding Bird Survey website (http://www.mbr-pwrc.usgs.gov/bbs/bbs.html). Abundance values indicate the average number of hummingbirds seen in 2.5 hours of observation. Acronyms in boxes are *E. coccineus* sites names, explained further in the text. Other shapes are locations of experiments designed to test the breeding system of each population. Stars indicate tests done by Scobell (blue stars – confirmed dioecious site, white stars – confirmed hermaphroditic and self-compatible site). Yellow triangles are populations confirmed dioecious by Hoffman (1992), and the red circle is one population confirmed dioecious by Powell (1991).

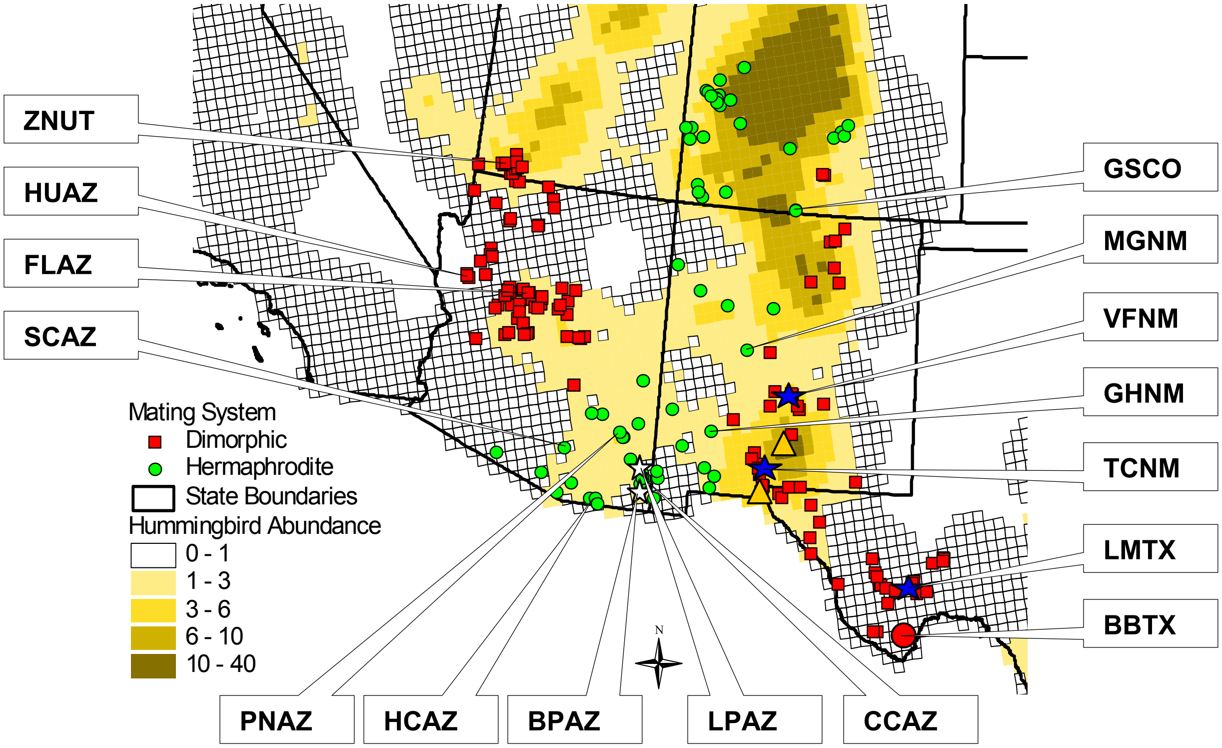


Figure 1.2: Cage constructed to exclude hummingbirds from pollinating flowers.

Figure 1.3: Hummingbird (black bars) and bee (white bars) visit rates in hermaphroditic and dioecious populations of *E. coccineus*. Dioecious sites of *E. coccineus*, HUAZ and LMTX, are outlined. Treatments within each site were compared using independent-samples t-tests. Significant differences (p<0.05) in visit rates between hummingbirds and bees are indicated by an asterisk (*). Populations are arranged from lowest to highest bee visit rate. Population locations are shown in Figure 1.1.

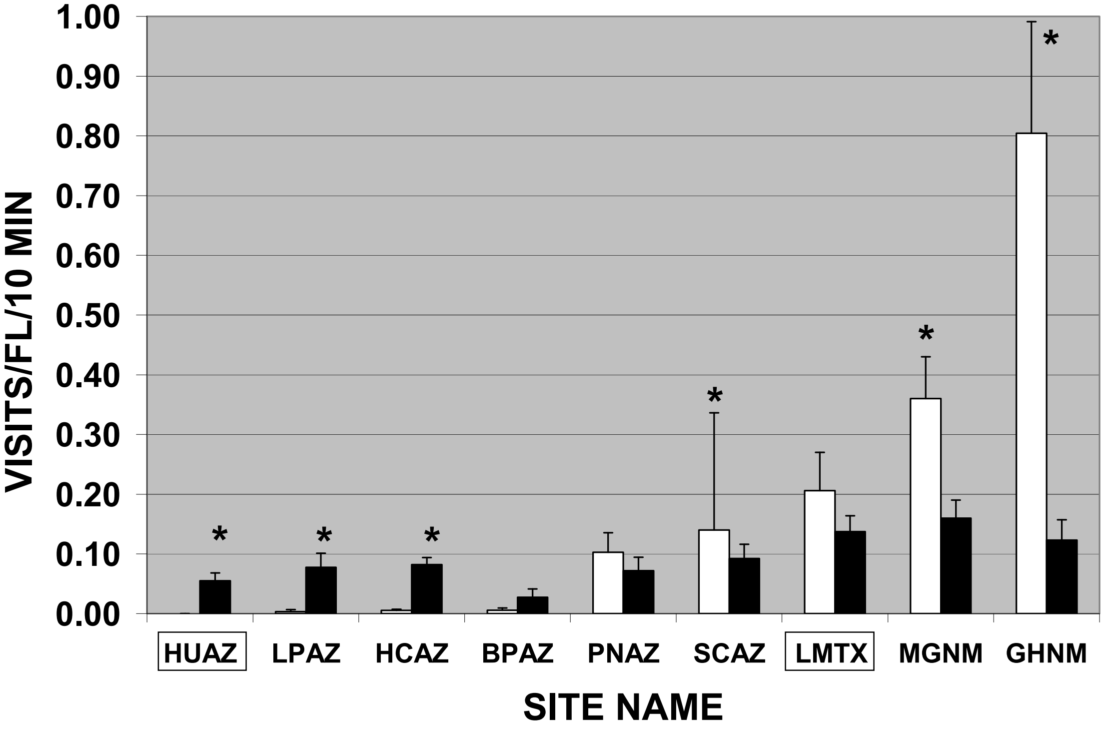


Figure 1.4: Average number of fruits produced per flower on half-caged plants. White bars are Open-pollinated flowers. Cross-hatched bars indicate Caged flowers. Sites are listed in order of increasing latitude. Treatments within each site were compared using paired t-tests. Significant differences ($p<0.05$) are indicated by an asterisk (*).

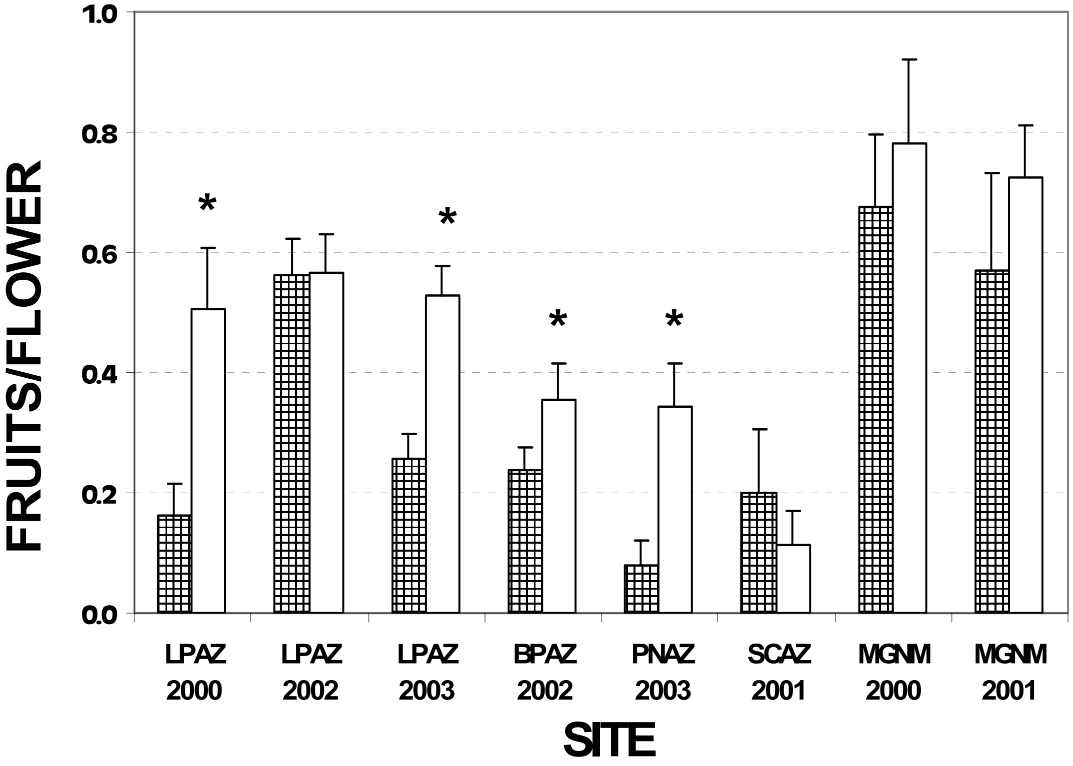


Figure 1.5: Average number of seeds produced per flower per treatment per plant for four *E. coccineus* populations. Significant differences among treatments within populations are denoted with different lowercase letters. In the MGNM population, only Natural vs. Self, Natural vs. Caged and Caged vs. Open comparisons were significantly different after Bonferroni correction. See Methods for description of treatments. Population acronyms are explained in Appendix 1. Population locations are shown in Figure 1.1.

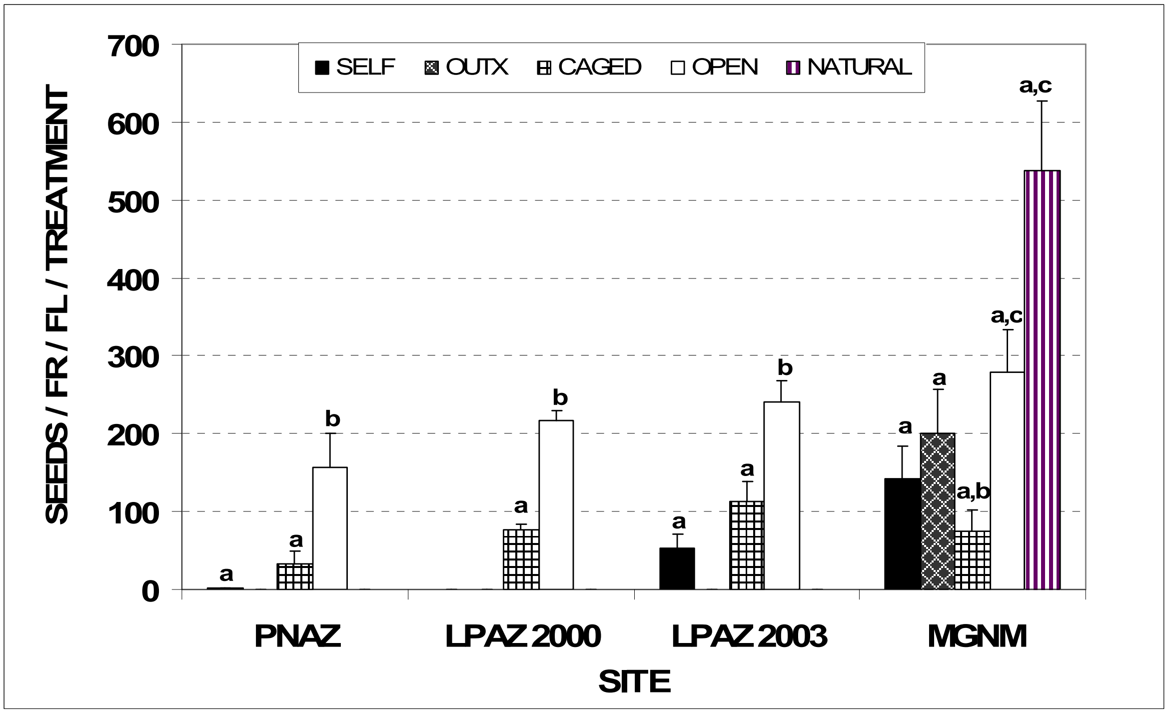


Figure 1.6: Regression of F_{IS} on site elevation. Line indicates the best-fit regression to the data.

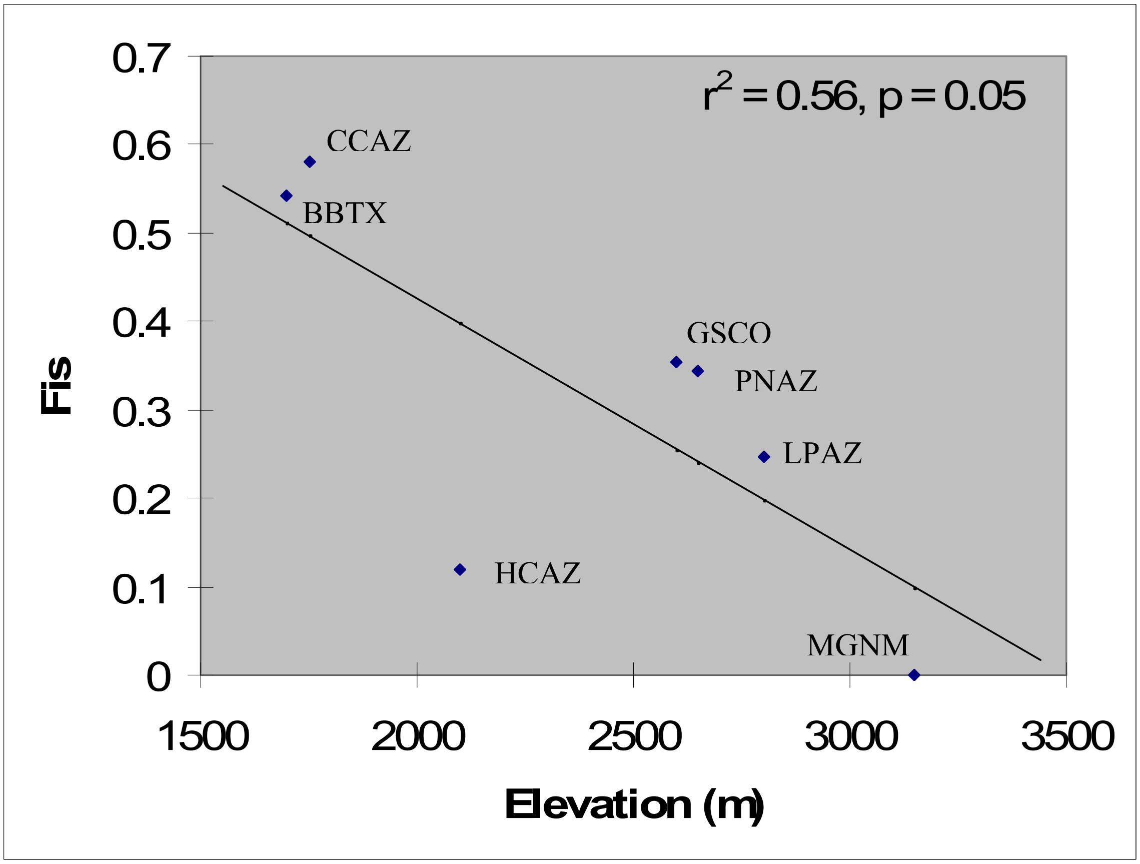


Figure 1.7: Relationship between genetic distance and geographic distance in pairs of *E. coccineus* populations. The larger, white point represents the F_{ST} between the Cave Creek (CCAZ) and Huachuca Mountains (HCAZ) populations, which was treated as an outlier for the Mantel Test.

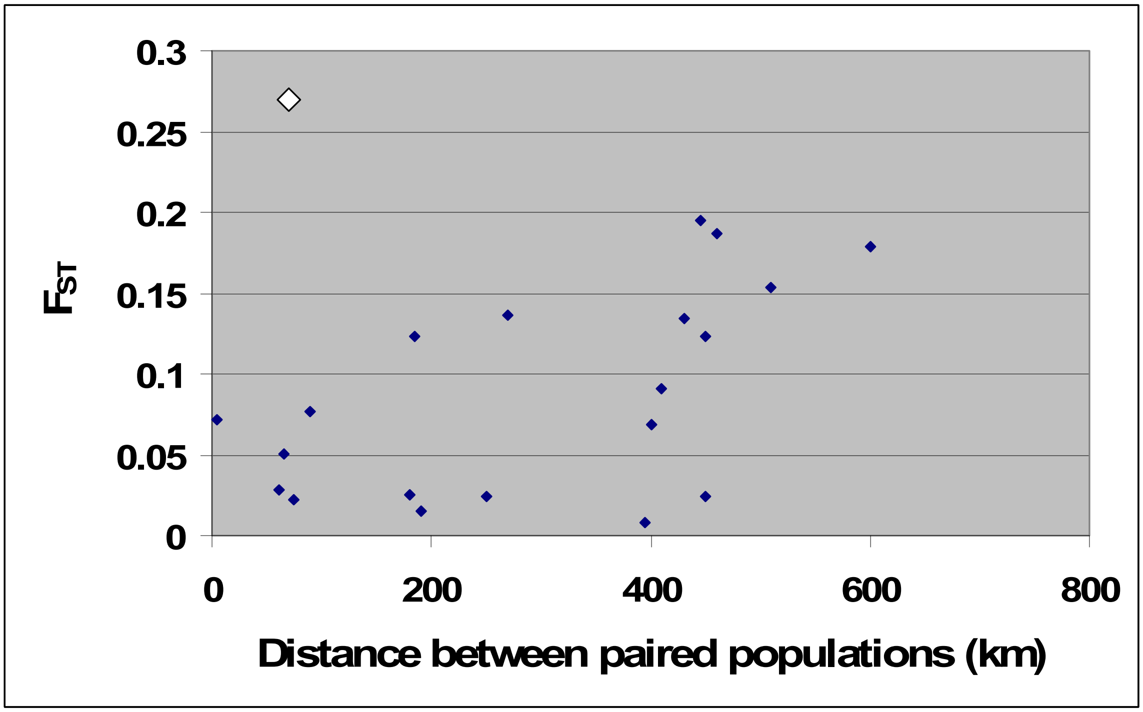
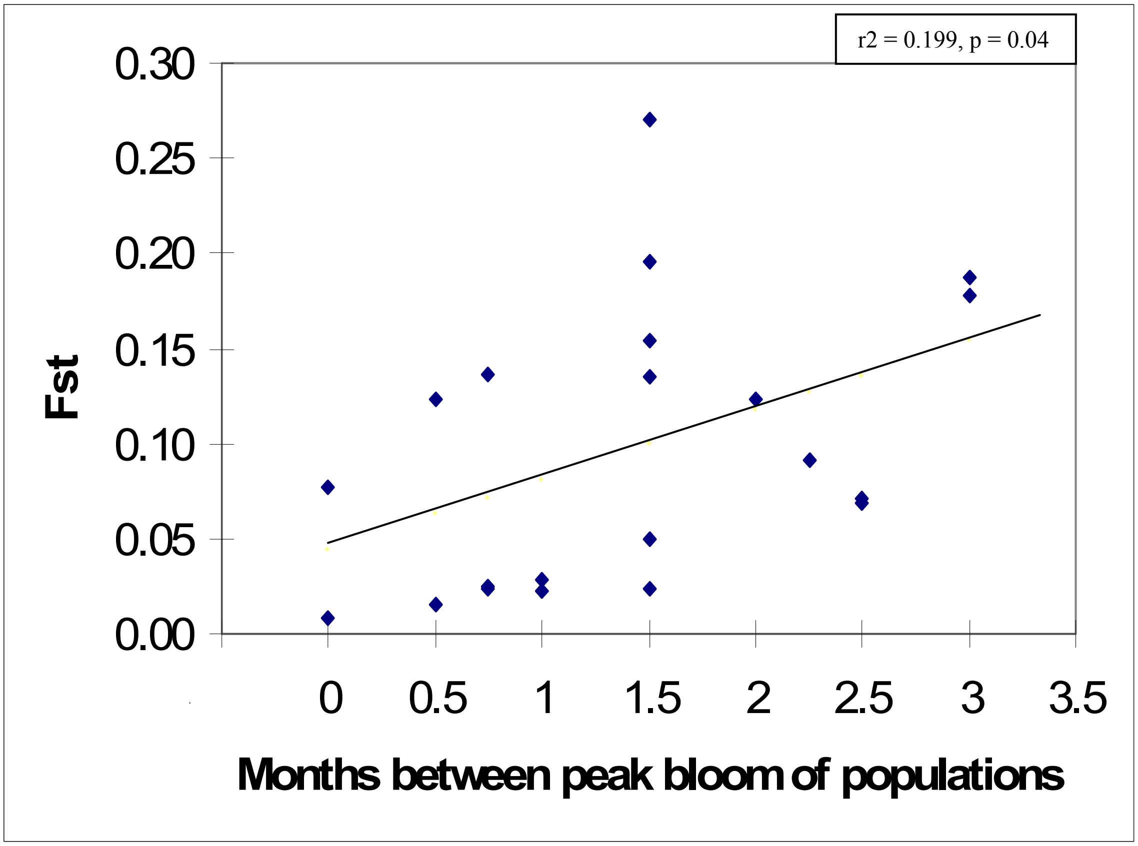


Figure 1.8: Relationship between genetic distance (F_{ST}) and differences in timing of peak flowering among pairs of *E. coccineus* populations.



CHAPTER THREE

GIS AND PATH ANALYSIS: EXAMINING ASSOCIATIONS BETWEEN THE BIRDS, THE BEES AND PLANT SEX IN *ECHINOCEREUS COCCINEUS* (CACTACEAE)

Previously published as Scobell and Schultz (2005)

Most flowering plants require animal pollinators to carry out mating between individuals. However, very few empirical studies have focused on effects of the behavioral ecology of pollinators on plant mating system evolution. Dioecy is a mating system consisting of separate male and female plants. When it occurs in animal-pollinated species, dioecy may be brought about by a change in pollinators. In many plant families an association between mating system and pollinator specialization has been reported: Species with specialized pollinators tend to be hermaphroditic and species with small, generalist pollinators tend to be dioecious (Bawa 1980; Delph 1990; but see Renner and Feil 1993). In his review of the evolution of dioecy in flowering plants, for example, Bawa (1980) found examples from four taxonomic groups where, with one exception, all of the hermaphroditic species were bird-pollinated and all of the dioecious species were insect-pollinated. How a shift in pollinator type within one plant species could select for dioecy is not well understood (Renner and Ricklefs 1995). Many authors have proposed that certain types of pollinators produce higher self-fertilization rates when visiting flowers. This higher self-fertilization rate then in turn may select for dioecy to avoid selfing (Lloyd 1982; Bawa 1994; Schultz and Ganders 1996).

The specific genetic conditions that favor evolution of dioecy in plant populations are outlined by Lloyd’s outcrossing advantage hypothesis (Lloyd 1975). This hypothesis states in a hermaphrodite population, if inbreeding depression (relative loss of fitness due to selfing) and selfing rate are both sufficiently high, mutations that eliminate self-fertilization will be selected. Specifically, any completely dominant nuclear mutation that produces a female plant by causing male sterility will increase in a population where loss of fitness in hermaphrodite plants due to self-fertilization is greater than one-half of seed fitness (Lloyd 1975). Linked modifier mutations in hermaphrodites that produce male plants by causing female sterility can then spread more easily through the population as female frequency increases. Thus, I would expect that if the pollinator assemblage of an outcrossing hermaphrodite population were to change such that selfing increased, and inbreeding depression was high in the population, then this could result in evolution of dioecy if the requisite mutational variation occurs in the population.

Despite its widespread use as an explanation for the evolution of dioecy in animal-pollinated plants, this hypothesis has not been tested empirically. This is probably due to lack of a suitable model species in which populations vary in mating system from purely hermaphrodite to completely dioecious. Most previous studies of dioecy have compared dioecious species to hermaphroditic relatives, and have looked for both biotic and abiotic correlates of dioecy (Bawa 1980). In these systems, however, it is impossible to estimate how interactions with pollinators may have selected for dioecy because it is unknown what the pollinator community composition was at the time when dioecy was initially evolving.

Both pollinator community composition and plant population dynamics respond to changes in resources (Abrams 1995) and interactions among plants, pollinators, and resources can be complex (Schemske and Horvitz 1988; Iriondo et al. 2003). Pollinator distribution and abundance often follow patterns determined by climatic variables. In a study in the mountains of Mexico, Cruden (1972) found that hummingbirds were more effective pollinators at high elevations due to high incidence of rainfall in these areas. Rainfall gradients could indirectly select for dioecy through their influence on pollinator distribution and abundance (Weller et al. 1995; Sakai et al. 1997; Soltis et al. 1996). The proposed mechanism in these systems is when plants invade drier areas, conditions are no longer favorable for effective pollinators; plants are then left with inferior pollinators that may increase the amount of self-fertilization in these populations, and selection favors separation of the sexual functions. An association between rainfall and dioecy has been noted in several studies, but whether it acts directly upon the fitness of the gender morphs or indirectly through its influence on pollinator distributions is still in question. A competing hypothesis proposed by Freeman et al. (1980) states that lack of rainfall produces resource limitation and directly promotes disruptive selection and the resulting niche segregation of male and female plants. Therefore, dioecy could be selected in the absence of any effects of pollinators. This paper describes the first study to jointly test the outcrossing advantage and resource limitation hypotheses within a geographical context in which evolution of dioecy is incipient.

Echinocereus coccineus is an excellent model system to test hypotheses about the effects of pollinator changes and edaphic conditions on the evolution of dioecy. It possesses contemporaneously both hermaphroditic and dioecious populations and possibly gynodioecious populations as well. *Echinocereus coccineus* flowers appear specialized to hummingbirds but are also pollinated by bees. These two types of floral visitors vary greatly in their pollinating behavior, and based on previous research

(Scobell 1999; England et al. 2001; Scobell and Scott 2002) we hypothesize that pollination by bees alone will result in a higher selfing rate than that produced when hummingbirds are also present. Distribution and abundance of these pollinator types varies across the geographic range of the plant: Both hummingbirds and bees visit flowers in the center of the geographic range, in the areas along the Rocky Mountain/Sierra Madre migration corridor. In the drier, lower altitude areas to the east and west of this range, hummingbirds become rare or absent. Distribution of dioecious populations appears to be associated with areas of low hummingbird abundance, low elevation, and low rainfall (see Figure 2.1). Hypotheses we test in this paper include:

1. Evolution of dioecy is pollinator-driven: Hummingbirds produce a higher outcrossing rate than bees; therefore, hermaphrodite populations are maintained only in populations with high hummingbird abundance. In populations lacking hummingbirds, dioecy is selected for by the higher selfing rates bee pollination produces.
2. Evolution of dioecy is resource-driven: In arid areas dioecy is selected for because of improved resource allocation of separate male and female plants.
3. Evolution of dioecy is driven by a combination of these factors: In the Southwestern United States, both rainfall and pollinator distributions vary with elevation. Pollinators and resources may both be involved in the selection for dioecy in this species.

We used structural equation modeling (SEM) to test these hypotheses. SEM is a statistical technique for solving simultaneous linear equations that combines traditional path analysis with factor analysis (Joreskog and Sorbom 1982). Causal graphs of hypothesized relationships among the data are produced, and then statistically tested, using a chi-square goodness of fit test to select the hypothesis that best describes the data (Shipley 2000).

Several studies have used structural equation modeling (or its predecessor, path analysis; Wright 1934) to evaluate the direct and indirect influences of pollinators and/or edaphic factors on plant demographics and fitness (Schemske and Horvitz 1984; Mitchell 1992; Iriondo et al. 2003). Schemske and Horvitz (1984) used path analysis to unravel the complex interactions among pollinators, herbivores, and ant guards and their effects on the number of mature fruits produced by *Calathea ovandensis* (Marantaceae). Iriondo et al. (2003) used structural equation modeling to determine which factors influenced seed production on two different soil types in an endangered plant *Erodium paularense* (Geraniaceae). Mitchell et al. (1992) used path analysis to determine which factors influenced hummingbird approach rate and probes/flower and how these variables influenced fruit set in *Ipomopsis aggregata* (Polemoniaceae). Each of these studies was able to explore causal networks among variables and assess their relative influence on the dependent variable (measures of plant fitness) more thoroughly than through regular linear regression or multiple regression techniques.

METHODS

<u>Study System</u>: *Echinocereus coccineus* has both hermaphroditic populations (Scobell 1999) and dimorphic populations (Hoffman 1992; Powell 1995) (See Figure 2.1). All dimorphic populations that have been tested with hand-pollination crosses between morphs are functionally dioecious (one by Hoffman (1992), one by Powell

(1995), and three by Scobell (unpublished data -TCNM, VFNM, and ALTX – Figure 2.1)). We found anecdotal evidence of gynodioecy in three other dimorphic populations observed before and after seed set (Hualupai Mt, AZ (HUAZ), Zion National Park (ZNUT), and Flagstaff, AZ (FLAZ) (See Figure 2.1)). In these populations, females are present and some of the hermaphrodite morphs produced seed (1/10 of hermaphrodites in HUAZ, 7/10 in ZNUT and 9/10 in FLAZ, S. Scobell, unpublished data). Herein, all dimorphic populations that have been determined experimentally to be dioecious will be termed dioecious. All other untested dimorphic populations will be termed dimorphic. Further breeding system experiments on northwestern populations of this species are needed to determine the functional mating system of these populations.

Echinocereus coccineus is part of the Triglochidiatus group (Taylor 1985). Members of this group are characterized as cacti that produce large, red, tubular flowers with abundant sucrose-rich nectar. These floral traits are termed hummingbird-syndrome traits (Grant 1967). In previous research, we observed four species of hummingbirds visiting this cactus in the Chiricahua Mountains of southern Arizona (Scobell and Scott 2002). The broad-tailed hummingbird (*Selasphorus platycercus*) was the predominant pollinator at all other sites observed, except in the Hualapai Mountains (HUAZ, Figure 2.1) where Anna’s hummingbird (*Calypte anna*) was the sole pollinator (S. Scobell, unpublished data). However, in a survey of two dioecious populations in central New Mexico, Hoffman (1992) found only small halictid bees visiting flowers. Michael Powell observed that hummingbirds were rare in *E. coccineus* populations he knew to be dioecious in Texas (A. M. Powell, personal communication).

The hypothesis that variation in pollinator type produces variation in selfing rate rests on the assumption that pollinator types vary in amount of self-pollen they bring to each flower. There are several reasons why we believe this to be the case in this study. Bees require fewer floral visits per foraging bout at *E. coccineus* flowers because of the abundant pollen (approximately 600 stamens, Hoffman 1992) and nectar rewards (35 mg sugar/flower/day, 10x the average hummingbird syndrome flower, Scobell 1999). Most visits by bees recorded in >500 hours of observing pollination of *E. coccineus* are visits within one flower or between a few flowers on one plant (S. Scobell, unpublished data). Only under conditions of severe pollen depletion at the end of flowering do small bees make more trips between plants. Conversely, since the daily energy expenditure of broad-tailed hummingbirds is approximately 23.3 kJ/day, they have to visit >40 flowers per day to meet their energy requirements (Montgomerie and Gass 1981). In his observations on pollination of *Delphinium nelsonii* in Colorado, Waser (1982) found that broad-tailed hummingbirds carried pollen 50-150% longer distances between plants than halictid bees. He estimated that number of plants in the genetic neighborhood (Wright 1969) would increase nearly eight-fold if broad-tailed hummingbirds were the sole pollinator compared to halictid bees (Waser 1982).

<u>Geographic Information System Mapping and Database Compilation</u>: Data on hermaphroditic and dimorphic populations were compiled into a database of more than 300 herbarium records. Plants were considered to be from the same population if they were within 1 km of each other, producing a sample size of 108 populations. All populations were then mapped into a GIS data layer in ArcView 3.3. Populations represented by a herbarium specimen containing female flowers were considered

dimorphic. If a flower containing pollen (male or hermaphrodite) was collected within a 30 km radius of a female flower, the population of origin of this flower was considered dimorphic as well. These assumptions about the mating system of populations may be overestimating dioecy, but they at least represent populations where selection for dioecy has begun. All herbarium specimen flowers containing pollen that were outside of this 30 km range were considered to come from hermaphroditic populations. These data were coded as 0=hermaphroditic and 1=dimorphic. The data layer created by this process was overlain with a data layer of maps of distribution and abundance of broad-tailed and black-chinned hummingbirds. These are the two main species that are present when *E. coccineus* populations are in bloom. These maps are available from the Audubon Society Breeding Bird Survey Database (http://www.mbr-pwrc.usgs.gov/bbs/bbs.html). Mean annual rainfall, in cm, was obtained for each population from the USGS National Atlas Database (http://www.nationalatlas.gov/prismm.html).

Structural Equation Modeling: In order to thoroughly test the hypotheses, associations among the data obtained from the GIS were analyzed using structural equation modeling (SEM). SEM allows for comparison of different causal models (Figure 2.2) using chi-square goodness of fit tests as well as indices such as CFI (Bentler's comparative fit index) and TLI (Tucker-Lewis index). A non-significant chi-square result indicates that the hypothesized model is a good fit to the data (Hayduk 1987). The CFI and TLI fit indices should be over 0.9 as a rule of thumb to prevent Type II errors (Bentler and Bonnett 1980). Analyses were carried out with Mplus (Muthen 2002), a free software package that is capable of analyzing a structural equation model that contains categorical dependent variables such as dioecy versus hermaphroditism.

RESULTS

The alternative causal models presented below (Figure 2.2) represent three possible scenarios for how elevation, precipitation, and hummingbird abundance may be directly and indirectly influencing the evolution of dioecy in *E. coccineus*. In the first model (Figure 2.2a) we are testing the hypothesis that only hummingbird abundance is directly affecting the evolution of dioecy in this species. Hummingbird abundance is directly affected by elevation and precipitation, which only indirectly influence the evolution of dioecy. This model is consistent with our data, and has the second strongest support out of the three models (χ^2= 4.22, df= 2, P=0.11, CFI = 0.962, TLI =0.906). The negative value for the effect of hummingbirds on dioecy is consistent with the hypothesis that dioecy is associated with areas of lower hummingbird abundance. Elevation is positively associated with precipitation and hummingbird abundance, but the overall indirect effect of elevation on dioecy is negative (-0.17), indicating that, overall, dioecy is negatively correlated with elevation. Surprisingly, precipitation appears to be negatively affecting hummingbird abundance, which seems to contrast with the results of Cruden (1972). Possible reasons for this negative correlation will be covered in the discussion.

When this model is compared to the second model (Figure 2.2b), it is apparent that a model with precipitation as the only direct cause of dioecy has a much poorer fit to the data (χ^2= 10.90, df =1, P=0.001, CFI = 0.832, TLI =0.159). The significant chi-square value makes it clear that this model does not fit the data. The value of the path coefficient from precipitation to dioecy is also not significantly different from zero. This indicates that precipitation alone is a poor predictor of dioecy in this species.

The third possibility, that rainfall and hummingbirds are both directly influencing evolution of dioecy, is supported by the data (χ^2= 2.52, df= 1, P=0.11, CFI = 0.974, TLI =0.871). The indirect negative effect of elevation is stronger in this model (-0.27), again indicating that dioecy is associated with lower elevations, but the path from precipitation to dioecy is not significantly different from zero (95% CI = -0.11 to 0.069), and the improvement of this model over the first model (Figure 2.2a) is not significant (χ^2= 1.70, df= 1, P >0.05). This indicates that the direct effect of precipitation on the occurrence of dioecy is negligible if it exists at all. Overall, including the direct effect of precipitation on dioecy does strengthen the model somewhat (although not significantly) to produce a model that explains 29% of the incidence of dioecy.

All three models have portions of the variation in dioecy left unexplained. Future work on this system will use exploratory SEM (Shipley 1997) to determine whether including variables such as aspect, temperature, and geologic substrate would improve the explanatory power of these models.

DISCUSSION

There are many advantages to using structural equation models to answer questions about causal relationships between interacting variables. If properly applied, this technique can be used to choose the model that best fits the data by comparing differences in the model's chi-square statistics (Hayduk 1987). This is not the same thing as proving causality or stating that the selected model is the only causal model that explains the data, but causal models that do not explain the data can be rejected. This is an improvement over conventional path analysis in which models cannot be statistically tested but can be compared based only on the amount of the variation left unexplained by each model (Sokal and Rolf 1995).

In our case, we can reject model (b) (Figure 2.2b), the hypothesis that rainfall is directly affecting the evolution of dioecy, based on the lack of fit of the model to the data. In this system it appears that a model that includes only direct influence of rainfall as a predictor of dioecy does not adequately explain the distribution of dioecious populations. The effect of rainfall on the presence of dioecious populations is not significantly different from zero. Rejection of this model suggests that Freeman's resource allocation hypothesis for the evolution of dioecy is incorrect for *E. coccineus* (Freeman et al. 1980).

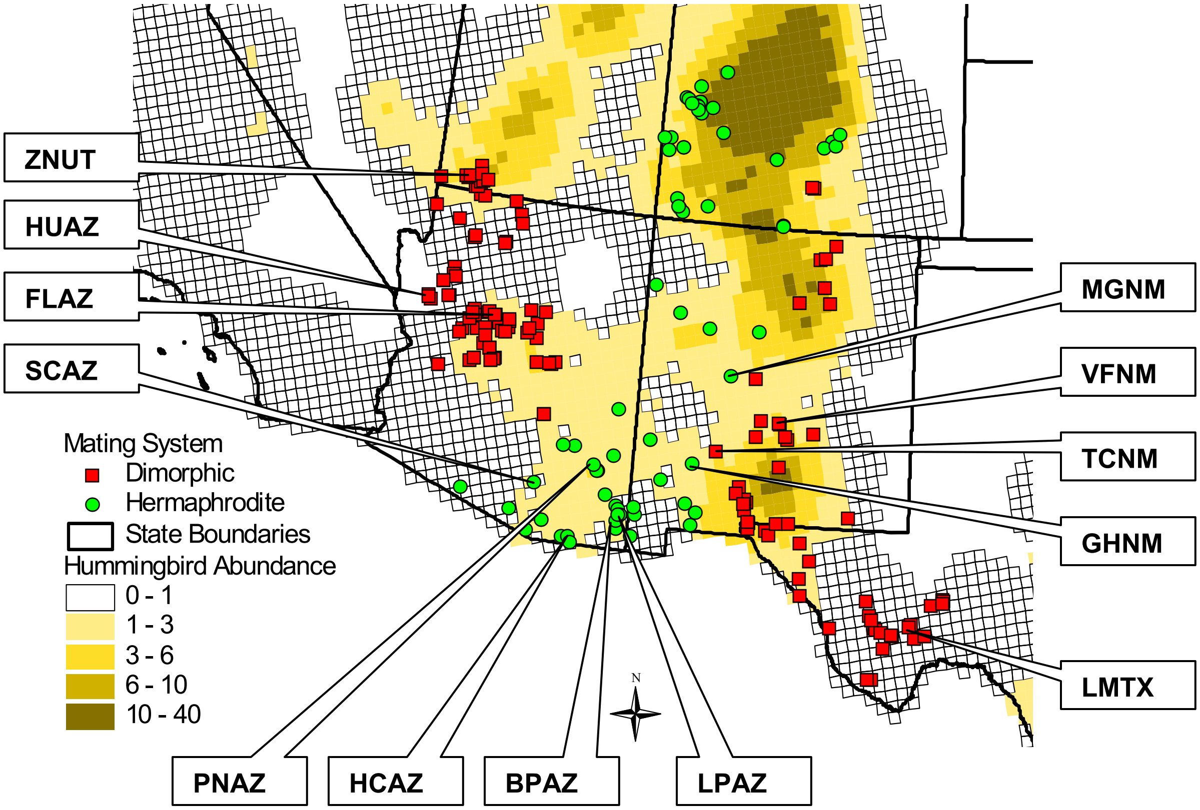
It is interesting to compare models (a) and (c), which were both found to be consistent with the patterns in our data. In both models there is a strong negative effect of hummingbird abundance on the presence of dioecious populations. In other words, dioecious populations are more likely to be found in areas with low hummingbird abundance. When precipitation is included in the model as a direct effect, it has a non-significant effect on model fit. However, since precipitation has a strong negative effect on hummingbirds, the total effect (sum of direct and indirect paths from precipitation to dioecy) of precipitation on dioecy is strong (-0.49), though still less than the direct effect of hummingbirds on dioecy (-0.56). Even though the trend is as predicted by Freeman et al. (1980) with a negative association between precipitation and dioecy, the effect of precipitation is smaller than the effect of hummingbirds. Ultimately, addition of this variable to the model does not produce a significantly better model.

The strong negative relationship between annual precipitation and hummingbird abundance is at first confusing, considering that, on average, both hummingbird abundance and precipitation are shown to increase slightly with elevation, and the GIS shows the highest abundance of hummingbirds at the highest elevations. However, since the data for rainfall and hummingbird abundance were gathered only in places where the cactus populations exist, the natural history of the cactus changes the distribution of the data. These cacti prefer to grow on the drier southeastern slopes of the Sky Islands (The isolated, small mountain ranges of the Southwestern U.S. that are south of the Rockies in the U.S. and north of the Sierra Madre Mountains of Mexico). Therefore data were not gathered in this study for areas with precipitation levels higher than the tolerance level of this cactus. The negative correlation is produced when high elevation populations with high hummingbird abundance have low precipitation values because this cactus prefers to grow on the drier southeastern sides of the Sky Islands. Overall, however, the indirect effect of elevation on dioecy across all models is negative, indicating that dioecy is more often found in low-elevation environments.

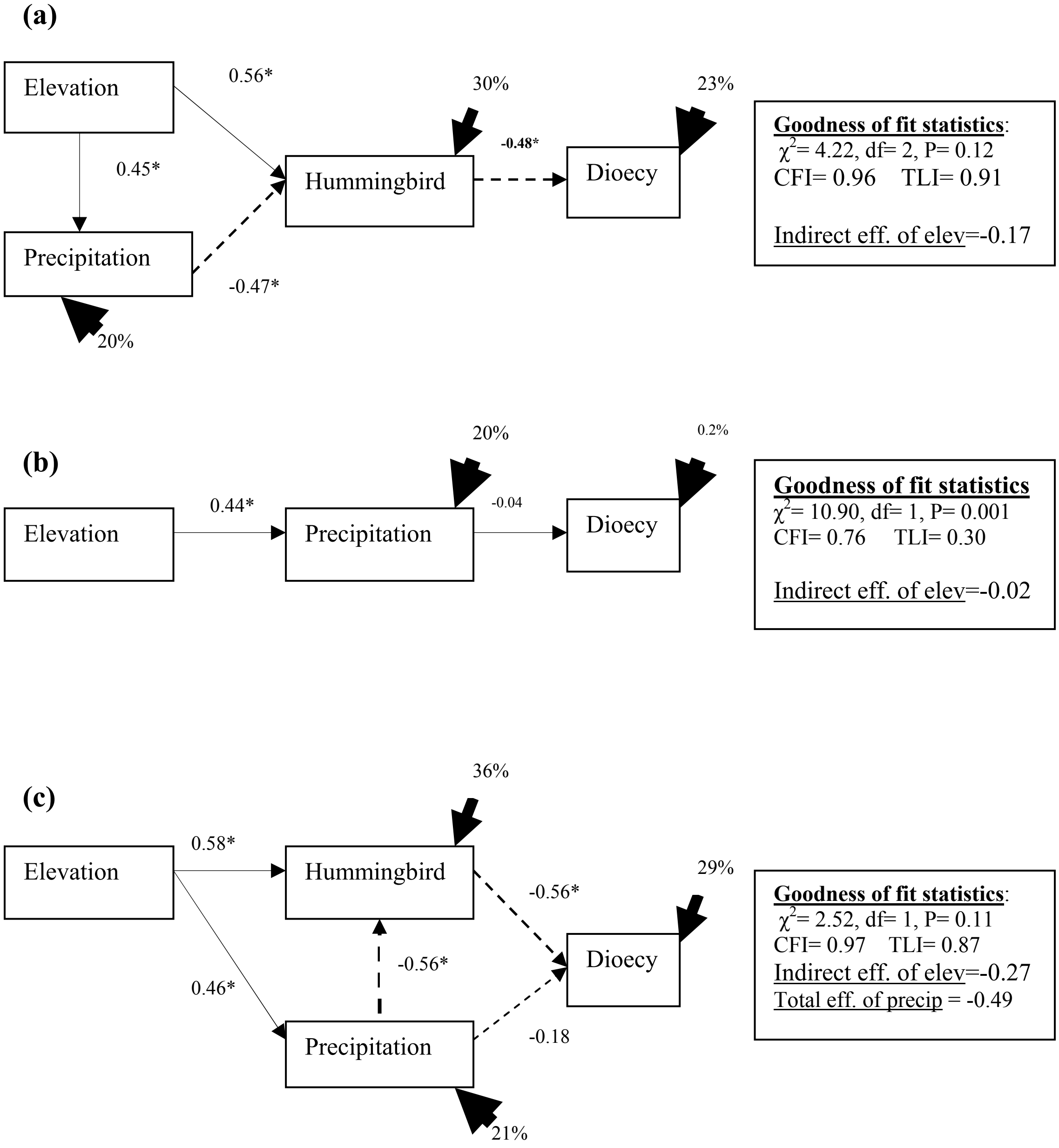
The findings of these models support the hypothesis that pollinator behavior may be selecting for the evolution of dioecy in this system. To test this hypothesis further, experiments where plants were caged to produce bee-pollinated and hummingbird + bee-pollinated treatment groups were carried out in six populations in 2000-2003. Microsatellite data were then used to determine that bees produced higher selfing rates than hummingbirds.

The combination of GIS and SEM in this paper in conjunction with the incipient evolution of dioecy has allowed the first combined test of both the inbreeding avoidance hypothesis and the resource allocation hypothesis for the evolution of dioecy. In this system the inbreeding avoidance hypothesis for the evolution of dioecy is most strongly supported. Future work will focus on the effects of hummingbird pollination on gene flow within and between populations of *E. coccineus* across the Madrean Archipelago. The results of this research may have implications for the conservation of many plant species that depend on hummingbirds for pollination.

Figure 2.1: Distribution of dimorphic (dioecious or gynodioecious-red squares) and hermaphroditic (green circles) *E. coccineus* populations in the Southwestern United States superimposed upon a map of hummingbird (broad-tail *Selasphorus platycercus* and black-chinned *Archilochus alexandri*) distribution and abundance obtained from the Audubon Breeding Bird Survey website (http://www.mbr-pwrc.usgs.gov/bbs/bbs.html). Abundance values indicate the average number of hummingbirds seen in 2.5 hours of observation. Acronyms in boxes are *E. coccineus* site names, explained further in the text.



<u>Figure 2.2</u>: Alternative structural models for factors affecting the evolution of dioecy in *E. coccineus*. Positive paths are shown with solid arrows, negative paths with dashed arrows. Arrow widths are proportional to magnitude of the path coefficients. Asterisks denote paths that are significantly different from zero. Arrows not originating in a variable indicate the amount of variation explained by each variable in the model. Indirect eff. of elev. is the total indirect effect of elevation on the distribution of dioecious populations. Total eff. of precip. in (c) is the sum of the direct and indirect pathways from precipitation to dioecy. (a) Hypothesis 1: Evolution of dioecy is pollinator-driven. (b) Hypothesis 2: Evolution of dioecy is resource-driven. (c) Hypothesis 3: Evolution of dioecy is driven by both pollinators and resources.



CHAPTER FOUR

ELEVATION AS AN INDIRECT FACTOR SELECTING FOR THE EVOLUTION OF DIOECY IN PLANTS: A GLOBAL PERSPECTIVE.

Studies of dioecy along elevation gradients have found an intriguing biogeographic pattern; proportion of dioecious species in a community, or proportion of single-sex mutants in a population, increases with elevation. This change in plant mating systems has been attributed to changes in pollinator communities – from specialized to generalized – as elevation increases. Such a change in pollinator community is hypothesized to select for dioecy because generalized pollinators are assumed to increase selfing rates, but a thorough test of this hypothesis is still lacking. To test this hypothesis, I need to know how pollinator assemblages change along elevation gradients and whether generalist pollinators produce selfing rates sufficient to select for dioecy. Biologists have long used comparative studies of plant/pollinator interactions along elevation gradients to understand how changing mutualistic interactions may shape both communities (e.g., Muller 1883). Mountains are favorable places to study spatial variation in species interactions because climate, vegetation, and animal species vary over short distances. In addition, confounding factors such as day length and seasonality are controlled within each range, making elevation studies both more practical and more parsimonious than studies of how interactions change over large latitudinal gradients (Peterson et al. 1997, Hodkinson 2005). In this paper I investigate ways in which changes in biotic and abiotic factors along elevation gradients may be an important and mostly overlooked driver for the evolution of dioecy in plant populations.

Research into the biological phenomenon of dioecy and the various evolutionary pathways that can lead to this breeding system have fueled fruitful research programs and produced lively and productive debates (for a review, see Geber et al. 1999). I propose that with molecular genetics techniques, GIS technology, and bioinformatics databases, research in dioecy is on the cusp of a new paradigm – one that views the evolution of dioecy in a geographic mosaic context (sensu Thompson 1994; 2005).

A good research question for this new paradigm is one that has been bugging (pun intended) dioecy researchers for decades: Do small, generalized pollinators select for the evolution of dioecy in zoophilous plants? The answer falls into two camps: (1) Pollination by generalized pollinators promotes dioecy (Bawa and Opler 1975; Bawa 1980; Lloyd 1982; Sobrevilla and Arroyo et al.1982; Bawa 1994; Charlesworth 1999) or (2) there is no correlation between generalized pollinators and dioecy (Renner and Feil 1993).

I contend that resolution of this debate can be found by viewing the interaction of the plant and pollinator communities at the appropriate scale and in the right context. That is, as abiotic conditions change across the range of a plant species, both quantity (fruit and/or seed set) and quality (outcrossing) of pollination need to be measured in order to determine how changes in pollinator communities contribute to the evolution of dioecy. To my knowledge, studies of this type have not yet been done. I summarize here what is known from community-level surveys of plant-pollinator interactions along elevation gradients (Table 3.1), and the ways in which changes in pollinator community composition have been hypothesized to select for dioecy within and among plant species along these gradients. I also discuss a method for testing whether changes in the pollinator community can select for dioecy using microsatellite analysis of plant progeny arrays produced by specialized vs. generalized pollinators. I predict under conditions of decreased outcrossing brought about by loss of effective specialized pollinators at high elevations, hermaphroditic populations of plants will be susceptible to invasion by unisexual mutants, and if such selective conditions persist, dioecy will be favored.

Though the majority of plant species are insect-pollinated (Crepet 1983), vertebrate pollinators can produce strong selective effects on plants they visit (e.g., Helversen 1993; Anderson 2003; Newstrom and Robertson 2005). Hummingbird and bat species richness declines with elevation (Stiles 1981; Remsen et al. 1986; Graham 1990), but substantial numbers still pollinate plants in tropical highlands (e.g., 15 species of hummingbirds and one species of bat in Venezuela; Fleming et al. 2004), and bats and hummingbirds pollinate plants at elevations over 3400 m in the Andes of Peru and Bolivia (Graham 1990; Kromer et al. 2006). These volant vertebrates are often important pollinators because they can visit montane flowers under cold and wet conditions that deter insect pollinators (Cruden 1972; Berry and Calvo 1989).

In addition to producing higher quantities of seeds, vertebrate pollinators increase the quality of seeds by increasing outcrossing rates. The outcrossing rate in *Echinocereus coccineus* (Cactaceae) populations, for example, increased by 238% when hummingbirds and bees pollinated these plants, compared to flowers pollinated solely by bees (see discussion below). Similarly, in two Australian populations of *Grevillea macleayana* (Proteaceae), visits by honeyeaters (Meliphagidae) increased outcrossing rate nearly two-fold over pollination by bees (England et al. 2001). In the trioecious, self-compatible cactus *Pachycereus pringlei*, bats are highly efficient outcrossers; four cactus populations covering a wide range of bat abundances showed outcrossing rates near 1.0 (Molina-Freaner et al. 2003). I would predict from these studies that plants visited by vertebrate pollinators are less likely to evolve dioecy than those visited only by insects because the high outcrossing rates produced by these pollinators preclude the evolution of dioecy. This prediction is borne out by broad surveys of dioecy in Angiosperms that show fewer than four bird- or bat-pollinated genera (out of approximately 750 possible genera with bird or bat pollination) contain dioecious species (Bawa and Opler 1975; Bawa 1980; Renner and Ricklefs 1995).

Various researchers studying breeding system evolution in plants have hypothesized changes in type or loss of pollinators across elevation gradients have led to changes in the breeding system of plants (Delph 1990; Pailler et al. 1998; Humeau et al. 1999), but none have thus far empirically tested the assumption behind this hypothesis: that a switch from specialized to generalized pollinators increases selfing rates sufficiently to select for the evolution of dioecy. After reviewing the available evidence from studies that have examined the evolution of dioecy across elevation gradients, I will present a method that I have used to test this assumption in *Echinocereus coccineus*.

Following the logical progression of the ways elevational changes in pollinator communities may select for dioecy, I predict that the abundance and diversity of all types of pollinators will decline with elevation (especially specialized insect pollinators), and that small, generalist insects (e.g. flies, beetles and small bees) will be the most common pollinators at high elevations (With the caveat that generalist pollinators may also predominate in the lowest-elevations areas when the base of mountains are rooted in dry deserts). In areas with volant vertebrate pollinators, however, these will be important pollinators of plant species that can attract and utilize them as pollen vectors regardless of elevation. I also predict that elevational changes in pollinator distributions, abundances, and efficiencies will result in increased selfing rates in hermaphroditic, self-compatible plants in areas with predominantly generalist pollinators. This will select for an increase in single-sex mutants so that in plants with labile sex expression (e.g., subdioecy, gynodioecy, or leaky dioecy) the proportion of separate sexes will increase with increasing (and sometimes decreasing) elevation. As a result, dioecious populations will be more likely to occur at the highest (and sometimes lowest) elevations. Finally, I predict that populations of hermaphroditic plant species with adaptations to vertebrate pollination can be invaded by single-sex mutations only in areas where their vertebrate pollinators are rare or absent. I have summarized these predictions in Tables 2 and 3 and will refer to them throughout the text as I present evidence from my own studies and the literature that support or refute each prediction.

This is only the second broad survey of associations between elevation and dioecy. Baker and Cox (1984) were interested in why dioecy seemed to be highest on islands such as Hawaii and New Zealand. They surveyed the literature of percent dioecy on oceanic islands (e.g., those not physically connected to the mainland since the evolution of angiosperms) and found a strong multivariate relationship (82% of variation explained) between maximum island elevation, latitude, and percent dioecious species; high-elevation islands close to the equator have the highest percentage of dioecy. Baker and Cox (1984) attributed this pattern to colonization from mainland sources as opposed to *in situ* evolution of dioecy, but they did not test if the ancestral state of the colonist was dioecious.

I offer an alternative hypothesis: The number of dioecious species on islands (and elsewhere) increases as elevation increases and latitude decreases because of the combined effects of generalized pollinators increasing selfing rates at high elevations and higher amounts of inbreeding depression being produced and expressed in high-elevation, low-latitude populations. I predict that there is an increase in the amount of inbreeding depression along this gradient for two reasons: (1) increased mutation rates brought about by increased UV radiation (which is highest in tropical mountains (Sullivan et al. 1992)) and (2) increased selection against inbred progeny in high-elevation areas of tropical mountains that have higher rates of transpiration than their low-elevation counterparts (Leuschner 2000; Dudash 2005). In light of this prediction, I reexamine the associations between elevation, latitude, and percent dioecy first discussed by Baker and Cox (1984), updating estimates of percent dioecy where possible and including new island groups.

METHODS

Studies of pollinator/plant communities that spanned large (usually over 1000 m) elevation ranges were surveyed for the types of pollinators that were most common (i.e., had the highest visit rate) at each elevation. Data on changes in pollinator and plant communities with elevation for each community-level study were included and are summarized in Table 3.1. Pollinator types are classified into functional groups (sensu Fenster et al. 2004) because I am interested in pollinators that produce equivalent results in terms of quantity and quality of pollination, and that is what this classification system seeks to do. Because the shape, scent, flowering time, etc. of a flower can also influence the functional group of floral visitors that are effective pollinators, and because small, generalized flowers are often associated with dioecy, data on the predominant floral syndrome of each community is also included in Table 3.1 (Fenster et al. 2004). Categorization of flower types into pollinator syndromes follows Faegri and van der Pijl (1971).

The terms generalization and specialization have had multiple interpretations in the ecological and evolutionary literature relating to species interactions (Waser and Chittka 1996; Waser 2006). The use of these terms in the dioecy literature does not exactly match either of the currently accepted definitions of these terms. The term generalized, when used for pollination interactions as defined by Armbruster (2006), are those pollinators that are evolving or have evolved to be good at gathering resources from many different plant species. This is contrasted with ecological generalization which only requires that each pollinator interacts with many plant species, but makes no assumptions about evolutionary trajectories (Armbruster 2006). Evolutionary and ecological specialization both imply that the pollinators interact with a limited number of species, with evolutionary specialization requiring that the pollinators have adaptations to visit and gather resources from one or a few related plant species (Armbruster 2006).

In the dioecy literature these two definitions (i.e. evolutionary and ecological generalization) seem to be muddied with generalist pollinators (e.g., small, solitary bees, flies and beetles; Bawa 1980; Lloyd 1982; Delph 1990) seeming to refer both to pollinators that are not evolutionarily specialized for any particular species and those that visit many different plant species. The main implication from the use of the terms generalist and specialist in these papers seems to be that pollinators separate into groups that are more likely to inbreed plants (these are labeled generalist pollinators) and those that are more likely to outcross plants (labeled specialist pollinators). There is some evidence to support the assumption that generalist pollinators produce higher levels of inbreeding (England 2001; Scobell and Schultz 2005; Chapter 2 herein), but this is definitely not always the case. I have continued here with this loose application of these terms to maintain continuity with the dioecy literature, but I acknowledge that a better understanding of the level of inbreeding produced by pollinators labeled “generalist” and “specialist” is needed before this assumption can be validated. I therefore classify flies, beetles, butterflies and some bees (usually small, short-tongued, non-social bees) as generalized pollinators and long-tongued social bees (e.g., bumblebees and honeybees), birds, and bats as specialized pollinators. More empirical tests on the effectiveness of pollinator functional groups are greatly needed to clarify whether the assumption of generalist pollinators producing higher rates of inbreeding holds across different species and environments.

Studies on evolution of dioecy that hypothesized, but did not test, the association between pollinators and mating systems along elevation gradients were not included in Table 3.1. They are included in Results and Discussion, however, to provide a framework for, and to further stimulate research on the influence of elevation on dioecy.

Data on maximum island elevation, latitude, and percent dioecy on islands by Baker and Cox (1984) were updated with data from Table 3 in Sakai and Weller (1999) and information gleaned from the literature. Relationships among maximum island elevation, latitude, and percent dioecy in the flora were analyzed using linear regression and stepwise multiple regression in SYSTAT (2004).

RESULTS

Community-Level Studies of Pollinators: There are a growing number of community-level surveys of plant/pollinator interactions where all possible interacting species have been carefully documented over an entire flowering season (e.g., Herrera 1988; Olesen et al. 2006 and references therein). This type of survey is necessary to document the range of possible selective factors (e.g., pollinator type, frequency of visits, flower constancy, competition with other plants for pollinators) operating on plant traits important to pollination (e.g., floral syndrome, flowering time and mating system). Studies that compare plant/pollinator communities along elevation gradients are less common, but they allow us to determine the ways these interacting communities change with variation in climatic gradients produced by elevation (e.g., temperature, precipitation, solar radiation), and in turn, the ways changes in plant/pollinator communities correlate with the evolution of dioecy.

A good method for estimating the impact of changing pollinator communities on the evolution of dioecy, therefore, would be to compare communities of plants and pollinators across a wide elevational gradient within the same mountain range, preferably with the same aspect, as this can influence species composition of pollinator/plant communities as well (Squeo et al. 1993). However, studies that document the ways different pollinator types influence the fitness of plants are very rare (but see Schemske and Horvitz 1984 and England et al. 2001). Therefore, a surrogate for a direct fitness measure is needed.

The studies summarized in Table 3.1 recorded visitation rates of each functional group of pollinators to flowers at different elevations. Visitation rates need to be supplemented with estimates of fitness gains produced by each pollinator type for both male and female function to truly assess how each type affects plant fitness (Kearns and Inouye 1993) but this was not done in these studies. However, visitation rates are one of the most widely utilized surrogates for fitness in the pollination literature, and it is a better estimate of relative contribution to fitness from each pollinator type than mere presence/absence counts (Primack and Inouye 1993; Olesen and Jordano 2002). The studies included here incorporated some comparisons among communities along elevation gradients, and Arroyo et al. (1982; 1985), Primack and Inouye (1993), and Kearns (1992) used the same methodologies to assess visitation rates.

M.T.K. Arroyo and her colleagues conducted a community-level study of plant/pollinator interactions along a 2200-3600 m elevation gradient in the Andes of central Chile (33^{o} S) (Arroyo et al. 1982; 1985). They documented pollinator visitation rates for 137 plant species throughout the entire flowering season (October 1981-March 1982). Bee species richness and abundance dropped off sharply with increasing elevation (from 70% of the pollinator pool at the lowest elevations to less than 20% at highest elevations). Butterfly and fly species richness, in contrast, declined slowly with increasing elevation. Consequently, a higher percentage of plants were pollinated by flies and, to a lesser extent, butterflies at high elevations compared to low elevations (Arroyo et al. 1982). In similar plant/pollinator surveys conducted at both lower (18^{o} S) and higher (50^{o} S) latitude populations in Chile, flies again had the highest visit rates at the highest elevation (Arroyo and Squeo 1990 and Squeo unpublished data). Along this latitudinal gradient, all pollinator visitation rates consistently declined with increasing elevation and with increasing latitude, with the highest elevation populations at 50^{o} S having visit rates near zero (Arroyo and Squeo 1990, figure 3). Arroyo et al. (1982) concluded that the lower visit rate of insects at high elevations could be attributed to two factors: 1) lower insect abundance relative to plant abundance and 2) lower activity levels in the insects present.

Primack and Inouye (1993) reviewed studies of pollinator/plant interactions from sites at different elevations throughout the world that used the same experimental protocol as Arroyo et al. (1982). Although studies along an elevation gradient within a single area are preferable (in order to avoid confounding changes in biotic and abiotic factors due to elevation with those due to changes in latitude), these studies can still tell us if there is a widespread trend in pollinators with elevation. In these studies, bees were the most common pollinators at lower-elevation sites in Massachusetts, Colorado, and South Africa (Table 3.1). At higher-elevation sites in New Hampshire, Colorado, Australia, and New Zealand, flies were the most common pollinators. Other pollination ecology studies along elevation gradients that did not document visit rates have also found similar variation in visitor assemblages (Muller 1883; Mani 1962; Delph 1990). In the Himalayas, bees are more common at low elevations, whereas flies and butterflies are the only pollinator types at high elevations (Mani 1962). Reflecting this, bee-pollinated plants were not usually found above 4000 m; instead, fly- and butterfly-pollinated plants predominated. A similar trend was seen from an extensive survey of the pollinators of plants in the European Alps by Muller (1883).

In the only community-level survey of pollinators in New Zealand, Primack (1978; 1983) found that flies were the most common pollinators at all sites across an elevation gradient of 1100 – 1800 m. Generalized bees, beetles, and butterflies were increasingly less common as elevation increased. The proportions of these pollinator types changed with elevation (Table 3.1). At lower elevations (1100 m), flies made up approximately 50% of pollinators, bees 19%, beetles 19%, and butterflies 13%. At higher elevations, flies made up 81% of pollinators, bees 11%, beetles 4%, and butterflies 3%. Primack (1983) hypothesized lack of floral specialization in the New Zealand flora might be due to an overall lack of specialized pollinators and the need for each plant species to accommodate visitors from a wide variety of insect orders.

Vertebrate pollinators were not mentioned in this study, so their contribution to pollination along elevation gradients is still unknown. However, honeyeaters are active pollinators in New Zealand winters when insect pollinators are rare, so there is a possibility that they may visit colder, high-elevation sites (Newstrom and Robertson 2005). It is believed that bird-dominated plant communities were once more widespread in New Zealand (Newstrom and Robertson 2005), but now most pollinating birds have been extirpated from the mountainous areas of the North and South Islands, so testing this hypothesis may need to wait for efforts to restore these lost pollinators.

Although Australia has more than 100 species of native bees, many plants are predominantly pollinated by diverse assemblages of flies. Across a gradient from 1860-2040 m in the Snowy Mountains, Inouye and Pyke (1988) recorded visit rates of all pollinators visiting plants flowering from December 1983 to March 1984. They found that flies pollinated more than twice the number of flowers as bees across this gradient and comprised almost three times the number of floral visitors per flower species. They reported that visitation rates were negatively affected by both decreasing temperature and increasing wind speed, two variables that are correlated with increasing altitude. They attributed the predominance of short, white flowers in Australian montane plant communities to the abundance of fly pollinators in this area and contrasted this with other bee-rich pollinator faunas in montane Colorado (Moldenke and Lincoln 1979) and New England (McCall 1986) that show a much more diverse array of floral colors and floral lengths.

A switch from insect to bird- or bat-pollination at higher elevations has been attributed to the ability of vertebrates to withstand cooler temperatures and inclement conditions at high elevations owing to their endothermic metabolism (Blake 1959; Cruden 1972; Stevens 1976). The proportion of plant species pollinated by hummingbirds in the Andes of Chile at 33^{o} S was highest at high elevations (Arroyo et al. 1982). Though few community-level surveys like Arroyo's (1982) have included vertebrate pollinators, there is strong indirect evidence that birds can exert strong selective pressures on plants at higher elevations through the elevational distribution of floral syndromes.

Several groups of plants show a shift in floral syndrome from bee-adapted forms at low elevations to bird-adapted forms at high elevations. These include *Palicourea* (Rubiaceae), a bird-adapted genus which has two-thirds of its species growing only in the mountains of Central and South America (Taylor 1997). Within the Melastomataceae, a nectarless family primarily adapted for buzz-pollination, there are several hummingbird-adapted species at high elevations that have re-evolved nectar production (Stein and Tobe 1989; Renner 1989). In New Guinea, species of *Rhododendron* (Ericaceae) occur along elevation gradients that extend above 3000 m. Adaptations in floral syndrome (red corolla and long floral tubes) that are attractive to honeyeaters (Meliphagidae) predominate at the highest elevations (Stevens 1976). At low elevations most species (66%) have bee-adapted traits. Kay and Schemske (2003) compared 11 species of hummingbird- and bee-syndrome *Costus* (Costaceae) species along elevation gradients in Central and South America. All *Costus* species above 800 m displayed a hummingbird syndrome, and bee syndrome flowers were found only at or near sea level.

Pollinator communities along an elevation gradient in the Chiricahua Mountains of southeastern Arizona, U.S.A., at first seem to contradict the trend of birds replacing bees at high elevations (Scobell and Scott 2002). When viewed at the proper temporal and spatial scales, however, they fit well with this prediction (Scobell and Schultz 2005).

Scobell and Scott (Figure 1; 2002) found little variation in the composition of the pollinator community with elevation within a single mountain range. In the Chiricahuas, *Echinocereus coccineus* (Cactaceae) occurs along an elevation gradient from 1500 - 2800 m on rocky basalt outcrops. The most common pollinators were small halictid bees (*Dialictus sp*. and *Lasioglossum sp*.) and broad-tailed hummingbirds (*Selasphorus platycercus*). Both types of pollinators were present at all elevations and both bee and hummingbird visitation rates increased with elevation. However, they did find a trend in pollinators with elevation across the geographic range of *E. coccineus* (an elevation range of 900-3300 m; Scobell and Scott unpublished data). Hummingbird abundances decline at low elevation (< 1500 m) near the eastern and western range limits of this cactus. Coincident with this is a rise in the number of dioecious populations in this species at low elevation, which I discuss further in the next section.

Testing the Assumptions: Pollinator Changes Selecting for the Evolution of Dioecy in *Echinocereus coccineus*: Scobell's study of incipient evolution of dioecy in

Echinocereus coccineus is one of the first to test the assumption that changes in pollinators with elevation are sufficient to select for dioecy (Scobell 1999; Scobell and Schultz 2005; Scobell et al. in prep.). *E. coccineus* has both hermaphroditic and dioecious populations arranged in an intriguing pattern across the range of this species (see Scobell and Schultz 2005: Figure 4). Hermaphroditic populations occupy high-elevation areas along the Rocky Mountain-Sierra Madre corridor in the Southwestern United States and Mexico. Hummingbirds migrate along this corridor and have higher abundances in high-elevation areas than in low-elevation areas (Grant and Grant 1965; Scobell and Schultz 2005). *E. coccineus* is pollinated by a wide variety of insects, but small solitary bees of the genera *Dialictus* and *Lasioglossum* (Halictidae) have visit rates 2 to 10 times higher than those of other insect pollinators, and their visit rates increase with elevation (Scobell 1999). In absence of hummingbirds, these small bees are the most common pollinators, and their pollination behavior (extended periods within flowers, few trips between flowers) led us to believe that loss of hummingbirds would increase selfing rates in this system and select for dioecy (S. Scobell, unpublished data). Dioecy was found to be negatively correlated with hummingbird abundance and was not associated with drier areas (low annual rainfall) when models of these hypotheses were compared using structural equation modeling (Scobell and Schultz 2005). These results support the hypothesis that a change in pollinator type (from hummingbirds to solitary bees) may be selecting for the evolution of dioecy in this system (Prediction 4, Table 3.3).

This result was further supported by experimentally testing the assumption that bees produce higher selfing rates than hummingbirds (Prediction 1, Table 3.3). To test this assumption, microsatellite analysis of progeny arrays produced inside (bees only) and outside (hummingbird + bee pollination) of mesh cages was carried out on 41 plants in one hermaphroditic population (Scobell et al. in prep, Chapter 2 herein). Results of this analysis indicated that selfing rates increased 238% with bee pollination compared to pollination by both hummingbirds and bees (hummingbird + bee, $s = 0.229$; bees only, $s = 0.773$, $p<0.0001$). Inbreeding depression estimated from fruit set, seed set and germination rates was extremely high ($\delta = 0.99$), and this combined with high selfing rates produced by bee pollination produced conditions that would select for separate sexes in this population if bees were the sole pollinator. Further studies documenting selfing rates and inbreeding depression values in populations of hermaphrodites closer to the borders of dioecious and hermaphroditic populations are under way. I predict I will see higher levels of selfing caused by higher levels of bee pollination as well as higher inbreeding depression in these hermaphrodite populations. I believe studies such as ours that test Prediction 1 (Table 3.3) – that selfing rates will increase with elevation-associated changes in pollinators – are necessary to provide a causal link between changes in pollinator type and the evolution of dioecy.

In summary, abundance and diversity of pollinator species tend to decrease with increasing elevation in community-level studies of plant/pollinator interactions. In addition, in high (and sometimes low) elevation populations, pollinator faunas show an increasing tendency toward generalized pollinators. These pollinators have been shown, in some cases, to increase the selfing rates of plants. From this I predict that higher rates of sexual dimorphism and dioecy will be found at the high and (sometimes) low margins of plant species' elevational ranges, particularly in populations that experience shifts in pollinator type. The following studies of trends in sexual dimorphism with elevation lend support for this prediction.

Response of Plant Communities to Changes in Pollinators with Elevation – Mating Systems: Several studies have found changes in plant mating systems over elevation gradients (Sobrevilla and Arroyo 1982; Arroyo and Squeo 1990; Delph 1990; Weller et al. 1990; Sakai et al. 1995a; Humeau et al. 1999; Humeau et al. 2000; Renner and Won 2001; Scobell and Schultz 2005). Researchers hypothesized these changes were caused by lower visitation rates of pollinators at high elevations and/or lack of specialized pollinators (Lloyd 1982; Arroyo and Squeo 1990; Delph 1990; Weller et al. 1990; Sakai and Weller 1999; but see Renner and Won 2001). These studies showed that declining numbers of specialized pollinators can lead to increased reliance on less specialized insects for pollination in high-elevation plant populations. Several studies also found lower visitation rates of all floral visitors at high elevations (Arroyo et al 1982; Arroyo and Squeo 1990). Lower visitation rates and generalist pollinators could then result in higher selfing rates and/or lower fitness in populations at high elevations. Loss of fitness in hermaphrodite progeny because of the combined effects of high selfing rates and inbreeding depression (loss of fitness in selfed progeny because of increased homozygosity) is often the first step in the evolution of separate sexes (Lloyd 1975; Sakai and Weller 1999).

Arroyo and Squeo (1990) conducted a community-level study of the relationship between pollination and plant mating systems in the Chilean Patagonia region of South America (50^{o} S, 73^{o} W). They surveyed four populations (with six to nine sites per population along an elevation gradient from 700 m to 1500 m) for pollinators and plant mating systems. This is a harsh habitat with mean summer temperatures below 10^{o} C and a growing season of less than 2.5 months. Winds often approach gale-force at the upper elevation populations located near an extensive ice field, creating an increasing gradient of physiologically harsh, arid conditions for plants and challenging flight conditions for pollinators. Accordingly, pollinator visit rates declined as elevation increased and were lowest at sites nearest the ice field. Flies were the most common pollinators at the highest elevations and visited over one-third of plant species in this community. Vertebrate pollinators were completely absent. They also noted the scarce pollinators that did visit plants tended to concentrate on single large flowering individuals. They hypothesized this pollinator behavior could potentially cause high selfing rates.

Along the elevation transects at each site, Arroyo and Squeo (1990) measured amounts of sexual dimorphism and dioecy in each of four life forms (annual herbs, perennial herbs, woody herbs, shrubs) at each site and across the population as a whole. For all populations, the percent of sexual dimorphism (number dimorphic out of total number of plant species in each community) increased with elevation, from approximately 10% at low elevations to 20% at high elevations. Within each life form, percent dioecy also consistently increased with increasing elevation, indicating that all life forms exhibit increased selection for dioecy with increasing elevation. Percent sexual dimorphism also increased across life form classes (lowest in annual herbs, highest in shrubs) consistently within each elevation, which seems to indicate longer-lived species are experiencing stronger selection for dioecy at each elevation. Arroyo and Squeo (1990) found a strong negative correlation between visitation intensity (percent of 10-minute observation periods on plants for which visits were observed) and dioecy across this gradient ($y = -1.55x + 11.55$; $P < 0.005$), which suggests that poor pollinator service at high elevations could be responsible. This trend was also found at a 1600-1800 m site at 33° S latitude near the Arroyo et al. (1982) elevation transects. Woody plants showed higher percentages of dioecy (annual herbs 0%, perennial herbs 2%, shrubs 17%, and trees 57%; Arroyo and Uslar 1993).

In the 50° S Patagonian populations, two indicators of habitat quality were also negatively correlated with dioecy – species richness ($y = -0.17x + 31.14$, $P < 0.001$) and p.p.m. available nitrogen ($y = -1.29x + 32.48$, $P < 0.005$), which indicates that declining habitat quality may also select for dioecy. Arroyo and Squeo (1990) also found a strong increase in the number of self-incompatible (SI) hermaphrodite plants with elevation in these populations. When dioecy and SI hermaphrodites are added together, the percent xenogamy increased from 32% at low elevations to 51% in high alpine populations. These results of higher xenogamy under harsher biotic and abiotic conditions run counter to the prediction that pollinator limitation selects for self-compatibility (Lloyd 1965; Wyatt 1983). Arroyo countered that this prediction was put forth by researchers studying short-lived, self-compatible annuals and offered a contrasting hypothesis for long-lived perennial species that make up a large part of this Patagonian plant community and other high-alpine environments (Arroyo and Squeo 1990; Arroyo and Uslar 1993). Citing the work of Ledig (1986), she pointed out that since all germ lines in plants come from vegetative cell lines, mutations will accumulate in proportion to plant size, and this large genetic load should favor xenogamy. Arroyo and Squeo (1990) concluded that high selfing rates, caused by poor pollinator service, higher inbreeding depression in long-lived plants, and low seedling survival in this harsh environment, could all be operating to produce the patterns of dioecy and xenogamy seen in high alpine areas of Chilean Patagonia.

The predominance of small, generalist insects in the pollinator fauna of New Zealand has been hypothesized to be one of the reasons for the high percentages of dioecy found there (23% of genera; Webb et al. 1999). New Zealand lacks native social bees, which are presumed to be efficient outcrossers (Primack 1978). However, other types of pollinators do exist (e.g., three species of honeyeaters (Melphagidae); Anderson 2003) or did exist until recently extirpated by human disturbance (e.g., the flower-visiting bat *Mystacina robusta* (Mystacinidae); Lord 1991; 40% - 50% of the bird species on the North and South Islands; Holdaway 1989). Honeyeaters have been shown to increase fruit set over insect-only pollination treatments in small, restored islands off the coast of the North Island of New Zealand (Anderson 2003). However, a difference in outcrossing rates was not found when comparing five populations of *Metrosideros excelsa* (Myrtaceae) containing different proportions of honeyeaters to insects (Schmidt-Adam et al. 2000).

Some care, therefore, needs to be taken when labeling New Zealand pollinators as generalists or specialists because small, solitary, short-tongued, native bees may be efficient outcrossers for some plant species (Webb 1994; Newstrom and Robertson 2005; Robertson et al. 2005), and birds and bats tend to visit a wide variety of flower shapes and sizes (Lord 1991; Anderson 2003). Direct comparisons of outcrossing rates produced by different functional groups of pollinators are needed to determine which pollinator groups may be selecting for dioecy.

In a review of gender dimorphism in New Zealand, Webb et al. (1999) found that the incidence of dioecy was higher than previously reported (23% of genera dioecious). This estimate is one of the highest recorded incidences of dioecy for any geographical region. Like Arroyo and Squeo (1990), Webb et al. (1999) found that dioecy was correlated with woodiness but indicated that it was not correlated with pollination mode. However, they drew this conclusion only from associations between insect- or wind-pollination and dioecy and did not compare incidence of dioecy associated with different types of insect pollinators. Because dioecy was equally likely to evolve through wind or insect pollination, it seems that in most cases generalist insects in New Zealand may not provide any better outcrossing advantage than random dispersal of pollen by the wind (but see Webb 1994 and Newstrom and Robertson 2005). The authors concluded that the high incidence of dioecy is related to the generalist pollinator fauna of this mountainous island group. This review did not investigate changes in percent dioecy with elevation. However, in an earlier study of the Apioid Umbelliferae of New Zealand (a predominantly (88%) sexually dimorphic group in NZ that is monomorphic elsewhere), Webb (1979) hinted that gender might vary with elevation and this variation might be related to pollinator availability.

Delph (1990) conducted a study of evolution of gender dimorphism along an elevation gradient in the New Zealand genus *Hebe* (Scrophulariaceae). She suggested that differences in feeding behavior of pollinators at high elevations (i.e., restricted foraging in beetles and flies) led to higher selfing rates, which in turn selected for increasing sexual dimorphism with elevation within this genus. She observed that bees predominated in low-elevation populations of *Hebe,* whereas beetles and flies predominated at higher elevations (> 1000 m). Associated with this trend was a strong positive correlation between frequency of females and elevation ($r=0.827$, $P < 0.001$). This result of changes in pollinators with elevation echoed that of Primack (1983), who found that in *Hebe* alone, native bees were common (22% of pollinator species) at low elevations (1100 m) but were completely absent above 1600 m. Flies increased from 66% to 80% of pollinator species on *Hebe* across this gradient; beetles increased from 3% to 9% across this gradient. Delph (1990) hypothesized that beetles may self-pollinate plants more often because they rarely travel between plants; flies may self-pollinate more because they are less discriminating in their food sources than bees; and that this change in the types and behavior of pollinators has led to the pattern of dimorphism found across this range.

New Zealand seems ripe for future study of biotic and abiotic changes along elevation gradients that may promote the evolution of dioecy. The increasing reliance on generalized pollinators with elevation found by Primack (1978; 1983) and Delph (1990), the possibility that some "generalist" small bees may be efficient outcrossers (Delph 1990; Webb 1994; Newstrom and Robertson 2005), and elevational changes in the amount of sexual dimorphism found in *Hebe* (Delph 1990), all seem to indicate that the story behind why New Zealand has the highest proportions of dioecy in the world might be more complex and interesting than previously imagined.

Hawaii also supports one of the highest percentages of dioecious species (14.7%), and at least 12 lineages of plants have evolved dioecy after colonizing these islands (Sakai et al. 1995b). Dioecy is significantly associated with endemism (16% of endemic species are dioecious) and occurs more frequently on older islands (20% of endemics on

Kauai (the oldest island) are dioecious, 13% of endemics on Hawaii (the youngest island) are dioecious; Sakai et al. 1995a). The majority of dioecious species in Hawaii are woody (139/144 sp) which is not entirely surprising considering that two-thirds of all angiosperms in Hawaii are woody. Within woody species, there is a significant association between dimorphism and elevation ($\chi^2 = 19.1$, $P=0.004$), with greater than expected proportions of dimorphic species found in lowland (15 – 2000 m) and lowland-montane (500-2700 m) categories (Sakai et al. 1995a).

Although the elevation categories of Sakai et al. (1995a) are wide and overlapping, I may still be able to draw some comparisons between this work and the other studies included here by just comparing the overall trend along the gradient. Unfortunately, there have been no systematic community-level studies of pollinators along elevation gradients in the Hawaiian Islands, so I can only guess how pollinators might change along this gradient. Like New Zealand, Hawaii has no native bumblebees and few Lepidoptera (two species of butterfly and six rare species of hawkmoths), so the majority of plants may have to rely on generalized pollinators in these islands. The general associations between pollinators and dioecy in Hawaii mirror what has been found in other studies included in this review: All but one bird-pollinated plant species (N = 150) are hermaphroditic, whereas most dioecious plants have small white or green flowers that are pollinated by small, generalized pollinators. All dioecious herbs are insect-pollinated, and none are bird-pollinated (Sakai et al. 1995b). Sakai et al. (1995b) cautioned that these correlations may be overly influenced by a few species-rich dioecious lineages and called for further phylogenetic studies of the evolution of dioecy in Hawaii across all families.

Many studies have stressed the importance of considering phylogeny when making causal assumptions about ecological correlates of dioecy (Donoghue 1989; Soltis et al.1996; Charlesworth 1999; Sakai and Weller 1999; Weller and Sakai 1999; Vamosi et al. 2003). Without phylogenetic analysis, you cannot assume the directionality of the transition to dioecy (e.g. hermaphroditic to dioecious or dioecious to hermaphroditic). The number of independent origins of dioecy can also be over-estimated, and the hypothesized order of acquisition of traits associated with dioecy (e.g., high elevation → generalist pollination → inbreeding → dioecy) may be wrong (Donoghue 1989; Sakai and Weller 1999).

When Soltis et al. (1996) created a phylogeny from a combined molecular and morphological dataset for Hawaiian *Schiedea* and *Alsinidendron*, they were trying to determine what may have caused the high diversity of mating systems (including dioecy) in these genera. Ranging from obligately autogamous to fully dioecious species, the genus *Schiedea* has the highest diversity of mating systems of all of the native angiosperm lineages in Hawaii. The most parsimonious tree showed a single shift for these lineages into dry habitats, followed by several shifts to dioecy (Soltis et al. 1996; Sakai et al. 2006). Dry habitats were thought to select for dioecy in *Schiedea* because these areas have low numbers of insect pollinators, high selfing rates and high levels of inbreeding depression (see Table 1 in Sakai et al. 2006). Unfortunately, resolution in this phylogeny was too low to determine the exact order of events. Given the high percentage of dioecy in the Hawaiian flora and the interesting biotic and abiotic associations with dioecy found within *Schiedea* and across all angiosperm families in this archipelago, further studies along elevation gradients in Hawaii are warranted.

On La Reunion Island, strict dioecy is more common at high elevations and leaky dioecy is more common at low elevations across plant communities over the 3000 m elevational range of this island (Pailler et al. 1998; Humeau et al. 1999; Humeau et al. 2000). Leaky in this context means that plants that appear to be male produce varying amounts of seed. In *Dombeya ciliata* (Sterculiaceae), a cryptically dioecious species, high-elevation populations were strictly dioecious, whereas low-elevation populations showed leaky dioecy. Humeau et al. (1999) hypothesized that changes from specialized pollinators (birds, butterflies, and hawkmoths) at low elevations to generalist bee pollinators at high elevation might be the reason for this change.

This associated increase in frequency of dioecy with elevation on La Reunion and other islands led these authors to question the commonly stated reasons for elevated percentages of dioecy in other island floras (Humeau et al. 1999). As outlined above, the highest levels of dioecy in floras investigated thus far occur on islands such as Hawaii and New Zealand (Sakai and Weller 1999 and references therein). This could be caused by selection for outcrossing in small, colonizing populations or as an indirect correlate with fleshy fruits that are more likely to have long-distance dispersal (Thomson and Brunet 1990). Humeau et al. (1999), however, hypothesize another possible reason for this may be the mountainous nature of these islands and the selective force of changes in pollinators along elevation gradients. They cited the work of Baker and Cox (1984), who found that the maximum elevation and latitude of oceanic islands explained 82% of the variance in dioecy. Baker and Cox (1984) attributed this pattern mainly to the amount of dioecy in the mainland source flora for oceanic islands instead of *in situ* evolution of dioecy. Humeau et al. (1999) called for future work on evolution of dioecy across elevation gradients on mountainous oceanic islands to consider their alternative hypothesis.

Dioecy and Elevation on Oceanic Islands – Updating Baker and Cox: In the 24 years since Baker and Cox (1984) compiled their data, few updates have been added to their survey of the percent dioecy in the floras of the oceanic islands. Data from Hawaii (Sakai et al. 1995a and 1995b), La Reunion (Humeau et al. 1999), and the Juan Fernandez Islands (Bernardello et al. 2001) have been updated to reflect more thorough surveys of dioecy in these islands. I have added Puerto Rico and the Virgin Islands (Flores and Schemske 1984) as well as the estimates of dioecy in the British Isles by Kay and Stevens (1986) that were in a review of the geographic distribution of dioecy by Sakai and Weller (1999). In a cited reference search of Baker and Cox (1984) in the ISI Web of Science Index, and other searches including each island cited in their paper as well as the key words dioecy, mating systems, or breeding systems, only one reference added a new island system to this database – the Ogasawara Islands of Japan (Abe 2006). When these updates are included in the survey of dioecy on islands, the strong multivariate relationship that Baker and Cox (1984) found (82% of variation in dioecy explained) among maximum island elevation, latitude, and percent dioecy in the flora is reduced, but still significant (multiple r^2 in reanalysis = 0.28; P = 0.03; Table 3.5A). The reason for the reduction in the strength of this three-way relationship is the updated estimates of dioecy. A reduction in the high original estimate for Hawaii (from 28% to 15%) and an increase in the estimate for La Reunion (from 4% to 15%) has flattened the distribution of points considerably (compare Figures 1A and1B).

This reduction in strength of the multiple regression does not mean that our original hypothesis is rejected (e.g., that changes in biotic and abiotic conditions along elevation gradients promote evolution of dioecy), but it might mean something is missing. When these data are smoothed with a DWLS smoothing function (SYSTAT 2004), I can see two interesting trends: (1) The highest values of dioecy are found in high-elevation, low-latitude islands, and (2) low-latitude islands appear to have higher values of dioecy than high-latitude islands of similar elevations.

When a stepwise multiple regression is applied to these data, it can be seen elevation explains the majority of the variance in dioecy in these island systems ($r^{2 =}$ 0.22, P = 0.02; Table 3.5B). However, if changes in pollinators across elevation gradients were the only driver selecting for dioecy, I would expect to find the highest values of dioecy on islands with the highest elevations with latitude having very little effect. The trends in these data suggest that something else is contributing to the high percentages of dioecy in low-latitude islands. The possible causation behind these trends and the predictions I derive from them are discussed below.

DISCUSSION

I have presented four predictions concerning the effect of elevation on the distribution of pollinator types (Table 3.2) and four predictions about subsequent effects of differences in pollinator types on the distribution of mating systems (Table 3.3). Predicted trends in pollinator distributions are mainly supported by the data, but much of the support for predictions of the distribution of mating systems is still anecdotal. I will discuss the effects of elevation on distributions of pollinators first, followed by discussion of how these changes may affect the global distribution of dioecy in plants.

Distribution and Abundance of Pollinators along Elevation Gradients (Table 3.2): Prediction 1 proposes that the abundance and diversity of all pollinators will decline as elevation increases. Declines in total abundance of pollinators as elevation increases have been documented in several studies, and the reasons for these declines are complex (Arroyo et al. 1982; Inouye and Pyke 1988; McCall and Primack 1992). However, there are still too few studies that have documented the abundance of pollinators at the community level across elevation gradients to determine if this prediction is supported based solely on pollinator community data. I therefore will bolster Prediction 1 with an in-depth survey of studies that provide additional information on why this prediction should be supported. This support is based on how changes in the physical environment (e.g. area and climate) drive changes in the plant communities that pollinators rely on, and how these changes in the physical environment and flora have driven changes in other insect species as well as pollinators along elevation gradients.

In a review intended to focus future research in montane ecosystems, Lomolino (2001) outlined four biogeographic explanations for changes in species diversity with elevation: 1) Area: Areas decrease with increasing elevation, and general predictions for species-area relationships (numbers of species decline as area declines; MacArthur and Wilson 1967) can be applied to life zones as elevation increases. 2) Climate: Climatic variables (temperature, precipitation, UV radiation, seasonality, and disturbance) covary in a non-random fashion along elevation gradients, and productivity should peak in areas where this covariation produces minimal environmental stress to species – usually in the lowlands or at intermediate elevations. 3) Isolation: Isolation of high-elevation communities should cause rates of immigration to decrease and rates of extinction to increase as elevation increases. These factors in conjunction with increasing speciation rates with increasing isolation and decreasing effects of human disturbance with elevation predict a peak in diversity at intermediate elevations (the mid-domain effect: Colwell and Hurt 1994; Colwell and Lees 2000; Colwell et al. 2004; McCain 2004). 4) Exchange: Biotic exchange along transition zones between juxtaposed communities should cause a local peak in species density at the transition. At highest elevations, the mixing provided from higher communities will be minimal, producing lower increases in density at high elevations. These four explanations lead to predictions of a general trend of lower species density as elevation increases, with the possibility of higher species density at mid-elevations (a mid-elevation peak) and at transition zones between different communities (Lomolino 2001).

A probable reason for lower pollinator abundances at high elevations is the decrease in the density and diversity of plants they rely on for food and shelter with increasing elevation. Plant species diversity peaks at mid-elevations and declines toward high elevations across many mountain ranges: Arizona (peak at 1250 m, range 730 m - 2766 m; Whittaker and Niering 1975), China (peak at 1200 m, range 470-3080 m; Zhao et al. 2005), Nepal (peak at 2000 m, range 1000-5000 m; Vetaas and Grytnes 2002), and Fennoscandia (peak at 400 m, range 250-1525 m; Bruun et al. 2006). Gentry (1988) did not find a mid-elevation peak in the Neotropical Andes (range 1540-3010 m), but instead found a significant linear decrease in number of species with elevation across 11 upland sites. Although he did not measure lowland sites, Gentry (1988) hypothesized that because the average lowland value for Amazonian species diversity is higher than the values at the lowest end of the measured gradient, the trend in plant species abundance is probably linear across the entire elevation gradient. Most of these studies attributed declines in plant species richness to isolation of high mountain environments and/or climatic variables. Vetaas and Grytnes (2002) had an alternative hypothesis for the mid-elevation peak. They hypothesized that the most recent glaciation has limited the number of species in the highest elevations in Nepal and that this area is being slowly recolonized.

These trends of decreasing plant diversity cause an inevitable feedback response in the pollinators that rely on them. In a review of surveys of insect diversity with altitude, Hodkinson (2005) found that species richness trends with elevation were highly variable and depended on the breadth and type of insect groups studied and the sampling effort of the study. Despite this variability, approximately 60% of the studies (N = 12 studies) found a mid-elevation peak or an overall decline in insect species richness with altitude. Gagne (1979) found a mid-elevation peak in insect diversity along a 2400 m gradient in an *Acacia/ Metrosideros* community in Hawaii. McCoy (1990) also found a mid-elevation peak in insect diversity along a 1600 m gradient in open field communities in the southeastern United States. Wolda (1987), however, found that insect species richness declined with elevation along a 2100 m gradient in tropical forests of Panama. Both Wolda (1987) and McCoy (1990) cautioned that mid-altitude peaks could be an artifact of short-term sampling and human disturbance at lower elevations, suggesting that the true, undisturbed trend would be a monotonically decreasing trend in insect species with increasing elevation.

Hodkinson (2005) meticulously outlined how the direct effects of the physical characteristics of mountains (e.g., decreasing temperature, increasing UV radiation, decreasing partial pressure of oxygen, increasing precipitation, turbulence, and wind speed as elevation increases) affect insect populations and how these populations are further affected by changes in the plant communities brought about by these factors. When faced with low temperatures, low oxygen availability and shorter growing seasons of their host plants, insects living at high elevations often reduce the number of instars, the number of generations per year, and the number of eggs per clutch (Hodkinson 2005). Flight conditions also deteriorate as elevation increases, and volant insect species must compensate for this in the short term by greater stroke amplitude of each wingbeat and in the long term by evolving longer wings (Dillon et al. 2006).

Predictions 2 and 3 (Table 3.2) state that as elevation increases, specialist pollinators will decline and small generalist pollinating insects will be the most common pollinators. Types of pollinators in communities were found to change in predictable ways along elevation gradients included in this study. Bees were the most common pollinators at lower elevations in four out of the six studies surveyed, and at higher elevations flies were the most common pollinators in all studies surveyed (Table 3.1). In a study of bee species diversity along an elevation transect in southern Tasmania, coastal (10 m - 100 m; 58 bee species) and mid-elevation areas (200-350 m; 56 bee species) had fairly high bee species richness, but high-elevation areas (720-1000 m; 16 bee species) showed low numbers of bee species (Hingston 1998). Hingston (1998) attributed this decline to fewer Tasmanian bees being adapted to colder temperatures. During the winter months at high elevations one particularly cold-adapted species (*Lasioglossum pertribuarium*) makes up almost the entire bee assemblage.

In Colorado, Kearns (1992) found that flies made up an increasing proportion of the pollinator fauna at high altitudes. While overall insect abundance decreased with elevation (from 296 ± 61 individuals/week at low elevations to 120 ± 22 individuals/ week at high elevations), fly abundance did not. She attributed the success of flies at high elevations to their larval habitat preferences and low energy requirements relative to bees, as well as their lack of nest-provisioning behavior. Flies require wet habitats for nesting which are abundant in areas of snowmelt or near streams at high elevations whereas solitary bees prefer dry nest sites that are rarer at high elevations. Bees must also expend much of their energy on nest provisioning. Flies do not provision their young. They use all the food they collect for their own energy needs and thus have more time to thermoregulate inside flowers, allowing them a better energy budget than bees at high elevations (Kearns 1992 and references therein).

The preference of bees for drier sites and flies for wetter sites was also found by Devoto et al. (2005) in their study of plant-pollinator interaction networks along a rainfall (400-2800 mm annual precipitation) and altitude (700-1020 m) gradient in Argentina. Again, the percent of the pollinator fauna made up by flies increased with elevation and the percent of the visiting fauna made up by bees decreased with elevation. Devoto et al. (2005) concluded that because the temperature variation across this elevation gradient was small compared to differences in precipitation, variation in types of visitors in these communities reflected differences in precipitation with bees preferring drier low-elevation areas and flies preferring wetter high-elevation areas. While not all flies are generalists (see discussion of highly specialized flies in South Africa in Johnson and Steiner 2003) and not all bees are specialists (see discussion of generalist pollen-collecting bees in Minckley and Roulston 2006), these broad, consistent trends with elevation in these functional groups tend to show that specialist pollinators will decline and generalist pollinators will predominate at high elevations.

This trend is not found in areas where volant vertebrate pollinators (birds and bats) are present (Prediction 4, Table 3.1). Because of their large energy requirements these pollinators usually visit flower species that offer large nectar rewards, therefore the number of species that can support this type of pollinator is smaller than those that can support insect pollinators. For this reason birds and bats are often considered specialist pollinators (Thompson 1994). These pollinators often become important to plants at high elevations, especially under cold or wet conditions, where they are more reliable pollinators than bees (Cruden 1972). Variation in bird and bat abundance with elevation is best explained by the amount of precipitation. Species richness in both groups is positively correlated with annual rainfall (Fleming et al. 2004), and it declines with increasing elevation. Hummingbird diversity in Costa Rica and elsewhere also displays a mid-elevation peak because of the high diversification of trochilines in montane habitats (Stiles 1981; 2004; Bleiweiss 1998). This decline in species numbers at high elevations may be explained by the increasing energetic costs of flight as the partial pressure of oxygen and ambient temperatures decline with altitude (Altshuler and Dudley 2006). Like insects (Dillon et al. 2006), hummingbirds can compensate for the lower air pressure at high elevation both in the short term by increasing wing amplitude and in the long term by evolving a larger wing to body size ratio. These adaptations are countered by the energetic costs of increasing wingbeat amplitude and greater loss of body heat as body size decreases (Altshuler and Dudley 2006). There is physiological evidence that birds can adapt more efficiently to hypoxic environments than mammals (although the mammals measured were terrestrial rather than volant; reviewed in Altshuler and Dudley 2006), and this may explain why the species diversity of hummingbirds drops off more slowly than bats as elevation increases (Fleming et al. 2004: Figure 2).

The limits imposed by volant vertebrate physiology thus predict that both types of pollinators will decline in areas with low moisture (often found in low-elevation environments), low temperatures and low air pressures (highest elevation environments). In areas lacking vertebrate pollinators, plant species adapted to these functional groups may be left with inferior pollinators. If these inferior pollinators produce high selfing rates, and this in turn selects for dioecy, this would support Prediction 4 (Table 3.3).

This was found to be the case in the study of *E. coccineus* along elevation gradients in the southwestern United States (Scobell and Scott 2002; Scobell and Schultz 2005). Hummingbird visitation produces the majority of the seed set at high elevations (Scobell et al. in prep.), but in arid areas at low elevations, hummingbirds become rare or absent (Scobell and Schultz 2005). Halictid bees are ubiquitous throughout the range of *E. coccineus* and visit these plants at high rates, often removing all pollen within a few hours (Scobell and Scott 2002). The effect of loss of hummingbirds and reliance on halictid bees on selfing rates and evolution of dioecy in populations of *E. coccineus* is discussed below.

Effects of Changes in Pollinator Distribution and Abundance with Elevation on Evolution of Plant Mating Systems (Table 3.3): Declines in the quantity and quality of pollinators with increased elevation have been hypothesized to be a causal factor explaining an increase in floral dimorphism and evolution of dioecy (Delph 1990; Arroyo and Squeo 1990; Humeau et al. 1999; 2000). This hypothesis frequently is offered as an alternative after rejection of another hypothesis, such as the resource allocation hypothesis (i.e. Dioecy evolves in drier areas in order to partition scarce resources more efficiently; Soltis et al. 1996; Case and Barrett 2004). Even with multiple studies putting forth this hypothesis and some evidence supporting Predictions 2 and 3 (Table 3.3), the assumption behind these predictions, i.e. a switch to a different type of pollinator can produce a selfing rate high enough to select for dioecy, has yet to be empirically tested (but see Scobell et al. in prep).

A few studies have found differences in selfing rates produced by different types of pollinators (e.g., England et al. 2001; Eckert 2002; Brunet and Sweet 2006), but included neither an elevation component nor were they related to the evolution of dioecy. Eckert (2002) and Brunet and Sweet (2006) used natural variation in pollinators across the geographic range of two plant species to document how changes in pollinator type might influence selfing rates of the plants they visited. Eckert (2002) examined selfing rates in *Decodon verticillatus* in ten populations in Michigan and five in Florida. Michigan populations were predominantly bee-pollinated, but Florida populations contained both butterflies and bees. Allozyme analysis of progeny arrays revealed no significant difference in selfing rates between Michigan and Florida populations. Eckert hypothesized that butterflies might carry only small amounts of pollen compared to bees and therefore did not produce a change in outcrossing rates in Florida populations. Brunet and Sweet (2006) did find that geographic variation in pollinators produced differences in selfing rates, with western areas of the range of *Aquilegia coerulea* having higher hawkmoth abundance and plants in eastern areas being pollinated primarily by bees. Hawkmoth abundance was positively correlated with outcrossing rate (P = 0.005) but there was no relationship between outcrossing rate and abundance of syrphid or muscid flies, bumblebees, or solitary bees. They attributed the effects of hawkmoths on outcrossing rates to their behavior of visiting female-phase flowers first, whereas bees visit male-phase flowers first, which tends to increase within-plant selfing (Brunet and Sweet 2006).

In an Australian study similar to Scobell et al. (in prep), England et al. (2001) hypothesized that pollination by introduced honeybees caused increases in selfing rates in the predominantly bird-pollinated shrub *Grevillea macleayana* (Proteaceae). England et al. (2001) directly tested how different pollinator types influence selfing rates by conducting pollinator exclusion experiments. They compared outcrossing rates produced on inflorescences subjected to Open pollination (birds + bees) to those produced when birds were excluded from inflorescences by poultry mesh and found that bee pollination (bird exclusion treatment) had significantly higher selfing rates than Open pollination (birds + bees) treatments. The high selfing rates produced by bee pollination (up to 0.9), when combined with even moderate inbreeding depression, would be sufficient to select for male sterility in these Australian populations, but no attempt was made to link this study to the evolution of dioecy.

This result is similar to ours in that bees produce a selfing rate in *E. coccineus* significantly higher than that produced by hummingbirds (s = 0.77 by bees, s = 0.23 by hummingbirds and bees; Scobell et al. in prep). The high estimates of IBD (δ = 0.99) from fruit set, seed set, and germination rates of *E. coccineus* means that the high rate of selfing produced by bee pollination creates conditions that could theoretically select for dioecy (i.e. $\delta * s = 0.76$ which is greater than the 0.5 cutoff for invasion of females; Lloyd 1982). Conditions for the evolution of dioecy have evidently been met multiple times in *E. coccineus*, because hermaphrodite populations are surrounded and outnumbered by dioecious populations in this species (Scobell and Schultz 2005). If environmental stress (e.g., water stress) was promoting the evolution of dioecy in this species, I would expect to find dioecious populations in areas with low precipitation, but the paths for precipitation in our structural equation models of this system were consistently non-significant (Scobell and Schultz 2005). Since hummingbird abundance was strongly and negatively correlated with dioecy in each of our path models, I predict that higher selfing rates occur as elevation decreases and hummingbird abundance declines toward the eastern and western edges of this species' range. This fits in well with Prediction 4 (Table 3.3), which states that dioecy will evolve in lineages adapted for vertebrate pollination only in areas where their specialized pollinators are absent.

These studies show that a change in pollinator type can increase selfing rates. However, to select for dioecy, inbreeding depression must also be quite high initially and remain high until dioecy is established. This can occur if there are high deleterious mutation rates (Lande et al. 1994). The only estimates for plant mutation rates come from a study of mutation accumulation across ten generations of *Arabidopsis thaliana* by Schultz et al. (1999). They found that the mutation rate of this plant (U=0.1) was orders of magnitude higher than those found for *Drosophila* (U=0.02), *C. elegans* (U=0.003) and *E. coli* (U=0.0002). In addition, unlike animals, plants do not produce a separate germ line, and thus somatic mutations can accumulate throughout the plant's lifetime and continue to contribute to the germ line as the plant grows. This leads to a prediction that large perennial species (hereafter large plants) have higher mutation rates per generation than small annual species (hereafter small plants) and that long-lived species accumulate more mutations over their lifetime than short-lived species (Klekowski 1988).

Scofield and Schultz (2006) tested this theory by comparing inbreeding coefficients of adults to selfing rates of progeny in large and small plants. The logic behind this comparison is that if there is little effect of inbreeding depression (i.e., the deleterious mutation rate is not high), then the inbreeding coefficient of parents will increase as the selfing rate of the population increases because there are few deleterious alleles expressed to reduce the fitness of selfed progeny relative to outcrossed progeny. This was found to be the case in small plants where inbreeding coefficients in parents increased as selfing rates in progeny increased. Large plants, however, showed no response in parental inbreeding coefficients to an increase in selfing rates, indicating that selfed progeny do not survive to adulthood and thus do not contribute to increasing the inbreeding coefficients of the adult generation. Mutation rates calculated from their data indicated that large plants had rates 80% greater than small plants (Scofield and Schultz 2006).

These results help explain why, in many surveys, percent dioecy in woody species is higher than that of herbaceous species (Bawa 1980; Sakai et al. 1995b). It does not explain why the proportion of woody dioecious species increases with elevation (Arroyo and Squeo 1990) or why there are multivariate interactions among latitude, elevation, and percent dioecious species in island floras (Baker and Cox 1984 and Figure 3.1C herein).

To explain these patterns, I propose two possible factors that could increase inbreeding depression in a way that is consistent with these trends in the evolution of dioecy: 1) Increasing UV radiation as elevation increases and latitude decreases creates higher mutation rates; and 2) increasing habitat harshness along this same gradient produces stronger selection against inbred progeny.

Effects of Increasing UV Radiation and Habitat Harshness to Explain the Distribution of Dioecy: The damaging effects of UV radiation on DNA are known lead to irreparable harm to living cells and tissues (e.g. cells destroyed due to sunburn, DNA damage leading to mutation). High-elevation mountain environments have higher incident levels of UV radiation because lower aerosol loads and low moisture lead to greater atmospheric penetration of these harmful rays. In addition, because of the decreasing thickness of atmospheric ozone and the angle of incoming UV rays from the sun, more UV radiation penetrates the atmosphere at the equator and less penetrates toward the poles (Caldwell et al. 1980). Tropical mountains therefore receive the highest amounts of UV radiation in the world, and these levels can be six times greater than levels at arctic latitudes (Caldwell et al. 1980).

In order for UV radiation to inflict DNA damage in plants, it must have access to the DNA and circumvent their natural DNA repair enzymes. Most plant tissues accumulate UV-absorbing pigments in response to increased UV, effectively protecting their DNA from damage. However, pollen, especially when germinating, can be highly sensitive to UV damage. Most DNA damage inflicted by UV radiation can be repaired with specialized enzymes called photolyases. Low temperatures can slow the action of these enzymes so that plants in cold, high-elevation environments may suffer from an unfavorable balance between DNA damage and repair. There is also some evidence in *Arabidopsis* that UV damage can also directly affect DNA repair mechanisms leading to an increased tendency to mutate in subsequent generations after UV exposure. Finally, mutations are known to accumulate within perennial plants when exposed to elevated levels of UV radiation, producing larger effects in subsequent years (Caldwell et al. 1998 and Caldwell et al. 2003 and references therein). Higher levels of DNA damage with increasing UV exposure and lower levels of DNA repair in cold, high-elevation environments could significantly increase the amount of inbreeding depression in high-elevation, low-latitude regions. This could partially explain why higher percentages of dioecy are found in high-elevation, low-latitude islands such as Hawaii and La Reunion (Baker and Cox 1984; Humeau et al. 1999; Sakai and Weller 1999).

In addition to the direct effects of increased mutation rates on inbreeding depression, some inbreeding depression may be context-specific with inbred progeny having lower survival rates in harsher climates (Dudash et al. 2005). Tropical mountain climates tend to have higher levels of potential transpiration (Ep) and are more arid than previously thought; these levels can be 50% to 90% higher in tropical mountains than mid-latitude mountains (Leuschner 2000). The additional selective pressure of these more arid environments against inbred progeny in tropical environments may also contribute to the higher percentages of dioecy found in these areas.

CONCLUSIONS

Pollinator communities along elevation gradients change in ways that may promote the evolution of dioecy by increasing selfing rates as elevation increases. In addition, inbreeding depression may increase with elevation because of effects of increased mutation load with higher UV radiation and increased expression of inbreeding depression in harsher environmental conditions. These two factors when taken together may help to explain and possibly predict phenomena such as increases in the percent of dioecious species with elevation, increase in the proportion of woody dioecious species with elevation, why dioecy is more common in woody than herbaceous species and perhaps even the global distribution of dioecious species.

The associations outlined here are intriguing but still need more empirical work. Additional surveys of pollinators and percentages of dioecy along elevation gradients are needed to determine the generality of trends I have described. In order to draw solid conclusions about causation behind the effects of elevation on the evolution of dioecy, I suggest the following approach: (1) Use standard methods to assess elevational changes in the abundance of pollinators and their relative importance to the plant community (Kearns and Inouye 1993; Primack and Inouye 1993). (2) Conduct empirical tests of pollinator effectiveness that use pollinator exclusion cages and microsatellite or allozyme analysis of gene flow whenever possible to determine how each type of pollinator functional group influences plant selfing rates (e.g. Scobell et al. in prep). (3) Map distributions of dioecious species and populations into a GIS database and use multivariate data analysis and phylogenetic correction (e.g., structural equation modeling sensu Scobell and Schultz 2005; BIOCLIM and PCA sensu Vaughton and Ramsey 2004;

'GeoPhyloBuilder' extension for ArcGIS sensu Kidd and Ritchie 2006) to determine how climatic variables, pollinator distributions and phylogenetic histories influence the frequency of dioecy along the gradient. The ultimate goal of this line of research would be to produce a global database of the geographic distributions of dioecious species and their hermaphroditic relatives in order to determine more precisely the importance of elevation as an indirect factor producing the worldwide distribution of dioecy in animal-pollinated plants.

Table 3.1: Distribution of common pollinator types (those with the highest visit rates) and common floral syndromes (most-visited type of flower) visited in studies of pollinator communities visiting across whole communities of plants along elevation gradients. (N/A: values not listed or not included in this study; n.s.: no significant difference found).

Study Location(s)	***Elevation Range***	***Taxa studied***	***Predominant Pollinator type***	***Predominant floral syndrome visited***	***Reference***
Chile, Andes Mountains	Low (2200-2600 m) Mid (2700-3100 m) High (3200-3600 m)	Plants: 137 sp. Bees: 52 sp Flies: 69 sp. Butterflies: 16 sp.	Low: Bees Mid: Flies High: Flies and butterflies	Low: Bee flowers Mid: Fly flowers High: Fly and butterfly flowers	Arroyo et al. (1982)
USA, Massachusetts, New Hampshire, Presidential Range	Low (lowland meadow) High (alpine tundra)	Plants:124 lowland 35 alpine Bees: N/A Flies: N/A Butterflies: N/A	Low: Bees High: Flies	Low: Bee flowers (yellow and purple) Mid: Bee flowers (yellow)	McCall and Primack(1992)
South Africa, Cape Province, Jonkershoek State Forest	Low (325- 935 m)	Plants: 92 sp. Bees: N/A Flies: N/A Butterflies: N/A Beetles: N/A	Low: Bees	Low: n.s.	McCall and Primack(1992)
USA, Colorado, Rocky Mountains	Mid (2800 m) High (3500 m)	Plants:30 sp. Bees: N/A Flies: N/A Butterflies: N/A	Mid: Bees High: Flies	Mid: N/A High: N/A	Inouye and Pyke (1988)
Australia, New South Wales, Snowy Mountains	Mid (1860-1920 m) High (1940-2040 m)	Plants: 62 sp. Bees: 27 sp. Flies: 60 sp. Butterflies: 3 Beetles: N/A	Mid: Flies High: Flies	Mid: Bee/Fly (white/yellow with short corolla tube) High: Bee/Fly (white/yellow with short corolla tube)	Inouye and Pyke (1988)
New Zealand, South Island, Cass Hill, Arthur’s Pass NP, Mount Cook NP, Craigburn Mts.	Low (1100 m) High (1600-1800 m)	Plants: 109 sp. Bees: 22 sp. Flies: 77 sp. Butterflies: 64 sp. Beetles: 20 sp.	Low: Flies High: Flies	Low: generalized flowers High: generalized flowers	Primack 1978 and Primack 1983

Table 3.2: Predictions of pollinator distribution and abundance with increasing elevation and the studies supporting or not supporting these predictions.

Prediction	***Supported?***	***References***
1. The abundance and diversity of all types of pollinators will decline.	Yes	Arroyo et al. 1982, McCall and Primack 1992, Inouye and Pyke 1988
2. The abundance and diversity of specialized insect pollinators will decline.	Yes	Arroyo et al. 1982, McCall and Primack 1992, Inouye and Pyke 1988
3. Small, generalist insects will be the most common pollinator at high elevations.	Yes	Arroyo et al. 1982, McCall and Primack 1992, Inouye and Pyke 1988
	No	Scobell and Scott 2002
4. In areas that contain volant vertebrate pollinators, these will be important pollinators of plant species that can attract and utilize them as pollen vectors.	Yes	Scobell and Scott 2002, Scobell and Schultz 2005

Table 3.3: Predictions of effects of changes in pollinator distribution and abundance with increasing elevation on plant mating systems and the studies supporting or not supporting these predictions.

Prediction	***Supported?***	***References***
1. Selfing rates in hermaphroditic, self-compatible plants will increase at the elevational extremes of a species' range.	Yes	Scobell et al. in prep. – as elevation decreases.
2. In plants with labile sex expression (e.g., subdioecy, gynodioecy or leaky dioecy), the proportion of separate sexes will increase with increasing elevation.	Yes	Delph 1990; Humeau et al. 1999 and 2000
	No?	Webb 1979 – anecdotal; Weller and Sakai 1990 – dry environments?
3. Dioecious populations will be more likely to be found in areas containing small generalist pollinators.	Yes	Arroyo and Squeo 1990; Delph 1990
4. Populations of hermaphroditic plant species adapted to vertebrate pollination will only be invaded by single-sex mutants in areas where their vertebrate pollinators are rare or absent	Yes	Scobell and Schultz 2005

Table 3.4: Updated information on percent of island floras containing a dioecious breeding system. Nearby continental (or island) source indicates the nearest possible source of plant migrants to the oceanic islands, computed in Google Earth by straight-line distance. Number of Species is the total plant species included in each survey of dioecy. Original % dioecy came from the review by Baker and Cox (1984); sources for the Updated % dioecy are cited in the Update column.

Location	*Nearby continental (or island) source*	*# of Species*	*Original % dioecy*	*Updated % dioecy*	*Final % dioecy*	*Latitude*	*Maximum Elevation*	*Original Citation*	*Update*
Aldabra	Africa, (Madagascar)	171	4		4	9	8	Baker and Cox 1984	
Azores	Portugal, Africa	391	2		2	36	2350	Baker and Cox 1984	
Bermuda	Virginia	136	4		4	32	76	Baker and Cox 1984	
Bikini Atoll	Australia	44	2		2	11	12	Baker and Cox 1984	
British Isles	France	1377	-	4	4	50	1343	Kay and Stevens 1986	
Chagos Arch.	India	71	1		1	6	15	Baker and Cox 1984	
Easter	Chile	30	0		0	27	460	Baker and Cox 1984	
Galapagos	Ecuador	439	3		3	1	1707	Baker and Cox 1984	
Guadalupe	Mexico	116	3		3	28	1298	Baker and Cox 1984	
Guam	Australia, China	279	13		13	13	406	Baker and Cox 1984	
Hawaii	California	1467	28	15	15	18	4208	Baker and Cox 1984	Sakai et al.1995a
Iceland	(Greenland)	472	3		3	63	2119	Baker and Cox 1984	
Juan Fernandez	Chile	141	11	9	9	33	1319	Baker and Cox 1984	Bernardello et al. 2001
La Reunion	Africa, (Madagascar)	838	4	15	15	20	3070	Baker and Cox 1984	Humeau et al. 1999
Leeward Islands	America (Hawaii)	40	0		0	22	176	Baker and Cox 1984	
Mauritius	Africa (Reunion)	682	11		11	20	828	Baker and Cox 1984	
New Zealand	Australia	1800	13		13	34	3754	Baker and Cox 1984	
Norfolk Island	Australia	153	12		12	28	319	Baker and Cox 1984	
Ogasawara	Korea (Japan)	269	-	13	13	24	453	Abe 2006	
Puerto Rico and Virgin Islands	United States	2037	-	6	6	17	1339	Flores and Schemske 1984	
Samoa	Australia	539	17		17	13	1857	Baker and Cox 1984	
San Clemente	California	221	2		2	32	600	Baker and Cox 1984	
San Nicolas	California	92	6		6	33	276	Baker and Cox 1984	
Seychelles	Africa, (Madagascar)	237	8		8	4	905	Baker and Cox 1984	
Tonga	Australia	404	16		16	18	1033	Baker and Cox 1984	

Table 3.5: Updated regression analysis of relationships among maximum island elevation, island latitude, and percent dioecy in island floras. 5A: Multiple linear regression of maximum island elevation and island latitude on dioecy. 5B: Stepwise multiple regression of maximum island elevation and island latitude on dioecy.

5A.

Source	*Sum of Squares*	*df*	*F*	*‘P’*
Regression	0.078	1	6.13	0.02
Residual	0.282	22		

5B.

Effect	*df*	*F*	*'P'*
Elevation	1	6.126	0.022
Latitude	1	1.838	0.190

<u>Figure 3.1</u>: Updated distribution of dioecy on oceanic islands (Figure 3.1A), compared to original data from Baker and Cox (1982) (Figure 3.1B). Numbering of islands on graph 1A corresponds to those of Baker and Cox (1984). Additional islands included in this analysis are: (23) British Isles (24) Puerto Rico and the Virgin Islands (25) Ogasawara Islands, Japan. Figure 3.1C is the same data as Figure 3.1A with a DWLS smoothing function applied. This function fits a line through a set of points by least squares differences (SYSTAT 11 2004).

Figure 3.1A

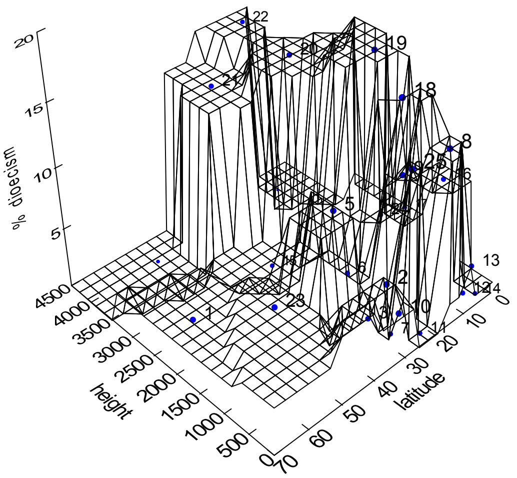


Figure 3.1B

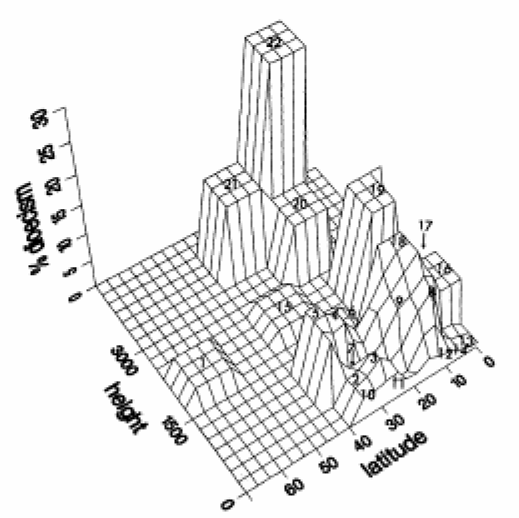


FIGURE 1. Graphical representation of percentage of dioecious angiosperms in island floras plotted against island height in meters and distance from the equator measured in degrees latitude for 22 different archipelagos or islands: (1) Iceland, (2) San Nicolas, (3) San Clemente, (4) Norfolk, (5) Juan Fernandez, (6) Guadalupe (Pacific Ocean), (7) Easter Island, (8) Guam, (9) Mauritius, (10) Bermuda, (11) Leeward Islands (Hawaii), (12) Bikini, (13) Aldabra, (14) Chagos archipelago, (15) Azores, (16) Seychelles, (17) Galápagos, (18) Tonga, (19) Samoa, (20) Réunion, (21) New Zealand, and (22) Hawaii.

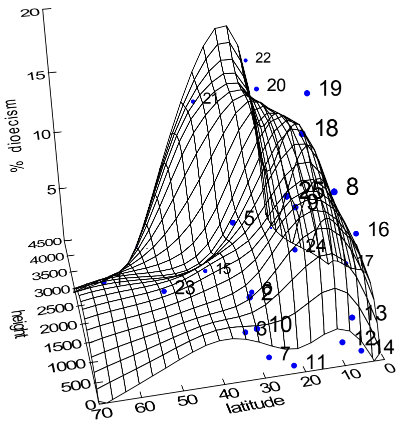


Figure 3.1C

CHAPTER FIVE

CONCLUSIONS

Out of the nearly 800 predominantly dioecious genera in the angiosperms, 69% of these are insect- (550) or vertebrate- (4) pollinated (Renner and Ricklefs 1995). It would seem therefore, that an explanation of how dioecy arises in zoophilous plants would be a priority, but while the mechanisms that promote evolution of dioecy in wind-pollinated species are fairly well understood (Darwin 1876; Kerner 1895; van der Pijl 1978; Freeman et al. 1980), the mechanisms selecting for dioecy within zoophilous plants are not (Renner and Ricklefs 1995). Small, generalized pollinators have been hypothesized to select for dioecy for decades (Bawa and Opler 1975; Bawa 1980; Lloyd 1982; Sobrevilla and Arroyo et al.1982; Charlesworth 1993; Bawa 1994; Charlesworth 1999). The assumption is these small, generalist pollinators do not travel as much between plants and/or are indiscriminant in their choices of forage plants, resulting in more self-pollination and/or less outcrossed pollination. Increases in the amount of self-pollination within populations could then select for dioecy if rates of inbreeding depression in selfed seeds are high. In spite of the preponderance of animal-pollinated dioecious plants and the number of times this hypothesis has been put forward, it has yet to be tested.

The goal of my dissertation research was to answer the question: In animal-pollinated plants, can a change in the type of pollinator, from specialized to generalized, produce sufficient selection against hermaphrodites to promote the selection of single-sex mutants and eventually dioecy? I examined this question from four different perspectives: 1) Within-population level: Does the selfing rate increase within plant populations when comparing seeds pollinated by both specialized and generalized pollinators to those pollinated only by generalized pollinators? 2) Among-population level: Are levels of inbreeding (F_{IS}) correlated with the visit rates of generalized pollinators? 3) Species-level: Across the range of a plant species, does the loss of specialized pollinators correlate with the presence of dioecy? 4) Global-community level: If pollinators commonly change from specialized to generalized with increasing elevation around the world, does this correlate with an increase in number of dioecious species with elevation?

I predicted from these questions that: 1) Selfing rates would increase when only generalized pollinators were visiting flowers. 2) Visit rates of specialized pollinators would be negatively correlated with population levels of inbreeding (F_{IS}) while visit rates of generalized pollinators would be positively correlated with inbreeding (F_{IS}). 3) The occurrence of dioecy will be negatively associated with the abundance of specialized pollinators across the range of a plant species. 4) Globally, proportions of dioecious species would increase with elevation because pollinators change from specialized to generalized along this gradient.

I tested the first three predictions using *Echinocereus coccineus* (Cactaceae), a plant that exhibits a unique distribution of mating systems: It is hermaphroditic in the center of its range and dioecious on the edges. Persistence of hermaphroditic populations in the center of the range allowed me to test predictions 1) and 2). These predictions could not have been tested within a species that is fully dioecious in that selfing is not possible when sexes are on separate individuals. The distribution of dioecious and hermaphroditic populations across the range of this species allowed me to test prediction 3) as well: To test whether resources or elevation also had an effect on the distribution of dioecious populations. Again, this was a unique opportunity because, since dioecy has not fully spread across this species, I can still ask why it has not, and why it has spread as far as it has. The usual method of comparing dioecious species to their hermaphroditic relatives brings in the confounding factor of time. In the intervening years (usually thousands to millions of years) since the split of a dioecious species from its hermaphroditic progenitor, much may have changed in the ecological and genetic factors that first promoted dioecy (e.g., pollinator shifts, amounts of inbreeding, edaphic conditions). By comparing concurrent dioecious and hermaphroditic populations within one species, I may be able to make better inferences about the conditions that promote the evolution of dioecy. Below I outline the main results of using this approach to answer questions about the evolution of dioecy in *Echinocereus coccineus.*

In Chapter 1, I used pollinator exclusion experiments to determine if the specialized pollinators of *Echinocereus coccineus* (hummingbirds) produced lower rates of selfing than generalized pollinators (e.g., small halictid bees) that visit this cactus. I found that the selfing rate increased over 200% when hummingbirds are excluded. This supported the prediction that generalized pollinators can substantially increase selfing rates in hermaphroditic populations. If this selfing rate was matched with an inbreeding depression of 0.65 or higher, the conditions to select for dioecy (in the absence of resource reallocation) would be met. In the LPAZ 2003 population, these conditions were met and exceeded after the effect of early-acting inbreeding depression was factored into my calculations. If I assume selfing rates produced by bee pollination are similar in all populations across the range of *E. coccineus*, there are several hermaphroditic populations (e.g., CCAZ and PNAZ) where dioecy would be selected for if bees were the only pollinators. The fact that these populations have high hummingbird visit rates may be what is maintaining the hermaphroditic state of these populations.

I did not find support for prediction 2). There was no relationship of either hummingbird or bee visit rates to the population-level of inbreeding in adults (F_{IS}). This may be due to the small number of populations examined in this study (N = 7 populations), but it also may be due to other factors, such as higher selection against inbred progeny in high elevation populations. There was a significant, negative relationship between population elevation and F_{IS}. This seems to indicate that inbred progeny do not survive as well at higher elevations (the F_{IS} value at the highest elevations approached zero). Since this plant is a cactus, it makes intuitive sense that it would be better-adapted to the hotter, drier, lower-elevation environments.

Other edaphic conditions also become harsher at high elevations (see discussion in Chapter 4), and this would also reduce the amount of inbred progeny populations would support. These low F_{IS} values at high elevations may also help explain the pattern of dioecious populations in *E. coccineus*. If populations at high elevations purge a large proportion of their genetic load through the elimination of inbred progeny, then they will not display the high levels of inbreeding depression upon selfing that are required to select for dioecy. This was the case in the high-elevation (2800 m) LPAZ population, which had a low F_{IS} value (0.25).

In Chapter 3 using structural equation models, I found that dioecy was negatively correlated with both elevation and hummingbird abundance across the range of this species. The accumulation of evidence seems to indicate that selection against inbred progeny, along with higher rates of outcrossing, may be working in concert to maintain hermaphroditic populations in the higher-elevation center of the range of *E. coccineus*.

Another piece of evidence that supports the assertion that hummingbirds are promoting outcrossing comes from examining the genetic distances (F_{ST} values) among the seven studied *E. coccineus* populations. First, it must be noted that, even though I found a significant effect of geographic distance on genetic distance, phenology of populations also appears very important within this species. A significant positive relationship was found between the amount of time between the peak bloom of each population and the genetic distance between populations. This indicates that populations that exhibit more overlap in their blooming cycles are more likely to exchange genes. Once I note this, I can see that some populations that bloom concurrently have lower F_{ST} values than those that don't, even though the synchronous blooming populations are farther apart in geographic distance (e.g., BBTX and CCAZ bloom at the same time and have low genetic distance ($F_{ST} = 0.01$) in spite of being 636 km apart and having different mating systems, whereas HCAZ and CCAZ are only 113 km apart and both are hermaphroditic, yet because they bloom 1.5 months apart, they have a very large genetic distance between them ($F_{ST} = 0.27$)). This seems to indicate that something is carrying the genes of this species long distances among synchronously blooming populations. The most likely scenario for this is that hummingbirds are transporting pollen over long distances as they migrate north among these populations. At the time that *E. coccineus* populations are blooming, there are twelve (four common) hummingbird species migrating through or living as residents in its range (Williamson 2001). Hummingbirds do not groom pollen off their bodies as often as bees do during foraging bouts

(Castellanos et al. 2003), so among the several thousand hummingbirds that migrate through each year (Wethington 2003), it is likely that these birds could carry substantial pollen among populations, particularly those that are blooming sequentially along the birds' migration route. (This may be another reason why the F_{ST} value between CCAZ and HCAZ is the highest I found: not only does HCAZ bloom 1.5 months later than CCAZ, but because it blooms this late, it is also situated behind the flow of genes carried north by hummingbirds as they migrate (Williamson 2001)). Rare long-distance gene dispersal events mediated by hummingbirds carrying pollen were hypothesized to be important in *Ipomopsis aggregata*, another hummingbird pollinated species (Campbell 1991). If hummingbirds are not only increasing outcrossing rates within populations (as was shown in the LPAZ population) but are also bringing in outcrossed genes from distant populations, then I should see a negative correlation between the abundance of hummingbirds and the presence of dioecious populations.

That is what I found when comparing alternative structural equation models of the factors selecting for the evolution of dioecy in *Echinocereus coccineus,* described in Chapter 3. Across the range of this cactus, both hummingbird abundance and precipitation increase with elevation. Abundance of hummingbirds was strongly negatively correlated (- 0.56) with the presence of dioecious populations across the range of *E. coccineus*. Models that included only resources (annual rainfall) having a direct effect on the distribution of dioecious populations were not supported by the data. Having both hummingbird abundance and resources as direct causal factors for dioecy did improve the fit of the model to the data, but not significantly. These models therefore support prediction 3): Dioecy is negatively correlated with the abundance of the specialized pollinators (hummingbirds) of *E. coccineus* across its range. There was no support for the resource allocation hypothesis for the evolution of dioecy in this species. This therefore lends further support to the idea that a change in the type of pollinator, from hummingbirds at the high-elevation center of the range to bees at the low-elevation edges of the range, might have been a primary causal factor for the selection for dioecy in this species.

To determine how common this pattern (change in pollinator species across an elevation gradient leading to selection for dioecy) might be across all dioecious species, I conducted a literature review. Chapter 4 outlines all available pollination studies that looked at communities of pollinators across elevation gradients and compared these to studies that examined mating systems (primarily dioecy, some gynodioecy and leaky dioecy) across elevation gradients. I found a common pattern to these studies: Pollinator communities often change across elevation gradients, from specialized pollinators at low elevations to generalist pollinators at high elevations. This does not seem to be the case, however, for areas with specialized vertebrate pollinators. These pollinators often reach very high elevations, and the plants specialized to them often occupy higher elevations than their bee-specialized relatives. Still, since the majority of zoophilous dioecious species are insect-pollinated, most dioecious species do support prediction 4): Around the globe, a larger percentage of dioecious species are found in the floras as elevation increases.

Echinocereus coccineus is an exception to this prediction because it is one of the rare examples of dioecy evolving in a vertebrate-pollinated plant species (only the fifth species of this type to be documented, S. Renner, personal communication). In the predicted manner; dioecy is evolving in areas where the pollinator community is composed primarily of generalist pollinators (in this case at low elevations).

In addition to the literature survey, I updated and reanalyzed the dataset of Baker and Cox (1984) on the relationships of maximum elevation, latitude, and percent of flora that is dioecious on oceanic islands. Using multiple linear regression, I found that the relationship was still significant, but reduced from that of Baker and Cox (1984) (from 82% of the variation in dioecy explained to 28% of the variation explained). A stepwise multiple regression revealed that elevation was the only significant predictor of dioecy, but trends with latitude were also apparent in a smoothed graph of the data. The patterns in the data led me to a different conclusion than that drawn by Baker and Cox (1984). They concluded that the distribution of dioecy on oceanic islands was primarily due to the percentages of dioecy in the mainland source flora. I concluded that in-situ evolution of dioecy may be more important than they considered. I hypothesized that increased self-pollination brought about by decreasing specialization of pollinators at high elevations along with increased inbreeding depression in plant populations (Possibly brought about by increased UV radiation, which increases with decreasing latitude and increasing elevation) are important factors selecting for dioecy on oceanic islands. This literature survey revealed that elevation gradients can shape both edaphic conditions and pollinator communities in ways that promote the evolution of dioecy as elevation increases. Elevation gradients might therefore be used in the future to predict the distribution of dioecious species around the globe.

The unique distribution of mating systems across the range of *E. coccineus* has allowed me to test several previously untested hypotheses from the dioecy literature.

This study is the first to experimentally demonstrate how changes in pollinators across the range of a zoophilous plant species could cause selection for dioecy. It is also the first to be able to concurrently test the resource allocation and inbreeding avoidance hypotheses for the evolution of dioecy. *E. coccineus* is a rare example of dioecy evolving in a vertebrate-pollinated plant species. This is also the first paper to document how hummingbirds may facilitate long-distance gene dispersal across the Sky Islands of the Southwestern United States. Finally, the literature review revealed that elevation should be considered an important indirect factor selecting for the evolution of dioecy and suggested several future directions for incorporating elevation into the global study of dioecy in flowering plants.

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APPENDICES

Appendix1A: Hermaphrodite populations.

Acronym For Site	Location	GPS Location	Elevation	Average Temp.	Approximate Dimensions	Total # plants * (est)	Plant density Pl/ m^2	Studies Conducted (N)	Study Years
HCAZ	Huachuca Mountains, AZ	31º 25’ 56.5” N, 110º 17’ 35.3” W	2100 m	19 C	150 m x 50 m	200*	0.026	CAGE (20) VR (103, 112) MSAT (20)	2003 2003 2003
CCAZ	Chiricahua Mountains, AZ	31º 52’ 30.0” N, 109º 11’ 15.0” W	1750 m	26 C	100 m x 100 m	351	0.035	MSAT (41) NAT (10)*	2002 2002
BPAZ	Chiricahua Mountains, AZ	31º 55’ 3.2” N, 109º 16’ 44.9” W	2525 m	24 C	500 m x 20 m	364	0.036	MATE (10) CAGE (20)* VR (19, 25) MSAT (20 - 20)*	1999 2002 2002 2002-‘03
LPAZ	Chiricahua Mountains, AZ	31º 53’ 9.1” N 109º 16’ 44.8” W	2800 m	23 C	500 m x 20 m	497	0.050	MATE (10) CAGE (15-30-36, 165, 250) VR (12, 21 – 2, 32) MSAT (64, 415)	1999 2000-’02-’03 2002-’03 2003
SCAZ	Santa Catalina Mountains, AZ	32º 25’ 49.8” N 110º 43’ 48.4” W	2200 m	26 C	100 m x 50 m	100*	0.020	MATE (21) CAGE (21) VR (44, 48)	2001 2001 2001
PNAZ	Pinaleno Mountains, AZ	32º 37’ 20.9” N 109º 49’ 17.0” W	2650 m	24 C	100 m x 30 m	70*	0.023	CAGE (20) VR (3, 12 - 69,74) MSAT(20-20)	2003 2002, ‘03 2001 –‘03
GHNM	Gila Hot Springs, NM	33°10'46.14"N 108°12'36.59"W	1770 m	26 C	100 m x 20 m	100*	0.050	VR (48, 75) NAT (29)*	2002 2002
MGNM	Magdalena Mountains, NM	33º 59’ 33.6” N, 107º 10’ 57.1” W	3150 m	20 C	100 m x 20 m	50*	0.025	MATE (5 – 20) CAGE (5 – 20) VR (32, 52) MSAT (48) NAT (33)	2000 – ‘01 2000 –’01 2001 2003 2002

Appendix 1B: Dioecious populations.

Acronym For Site	Location	GPS Location	Elevation	Average Temp.	Approximate Dimensions	Total # plants	Plant density Pl/ m^2	Studies Conducted	Study Years
BBTX	Big Bend National Park, TX	29° 15' 51.84" N 103° 17' 32.28" W	1700 m	27 C	60 m x 10 m	20*	0.033	MSAT (20) VR (3, 6)	2003
LMTX	Lizard Mountain, TX	30°20'4.64" N, 103°41'42.10" W	1450 m	27 C	100 m x 100 m	250*	0.025	MATE (12) VR (52,67)	2001 2002
TCNM	Truth or Consequences, NM	33° 9'45.42" N 107°18'59.24" W	1490 m	28 C	100 m x 100 m	50*	0.005	MATE (10)	2001
VFNM	Valley of Fires, NM	33°41'35.89" N, 105°55'36.56" W	1600 m	29 C	50 m x 20 m	20*	0.020	MATE (12)	2001
HUAZ	Hualapai Mountains, AZ	35° 7'27.62" N 113°54'40.96" W	1650 m	14 C	50 m x 20 m	50*	0.050	VR (100, 100) NAT (20)	2003 2003
ZNUT	Zion National Park, UT	37°25'49.05" N 113° 7'45.49" W	1775 m	26 C	100 m x 20 m	50*	0.025	NAT (21)	2003
GSCO	Great Sand Dunes Nat. Monument, CO	37°43'56.03" N 105°29'54.13" W	2600 m	24 C	100 m x 100 m	250*	0.025	MSAT (33) NAT (36)*	2001

Appendix 2: Characteristics of all attempted microsatellites and the primers developed for amplifying microsatellite repeat regions in *Echinocereus coccineus*.

Primer Name	Tm	Product Length	Flanking region	Repeat Length	# of repeats	Sequence
A-forward	72.6	211	89	122	61	TGAGAAGCGGTTCCTACCTT
A-reverse	57.4					CATCTAAGAATTTTGTGAGGAATAACA
B-forward	70.5	119	81	38	19	TCACAAACTATCAAC
B-reverse	56.0					AGCGATTGAAGATATTGAGC
C-forward	69.4	95	49	46	23	AAGCAGATGTTTATGATATGTGT
C-reverse	56.0					AAAGGGTATGCTCCATAAGA
G-forward	70.5	220	96	124	62	ATGGTTGCTTTACACACACA
G-reverse	52.0					AGCCACAAAAATATGCAATA
K-forward	72.6	231	163	68	34	AAGCTTGGATCGAGGTCTCA
K-reverse	57.3					CCGCACATTCCAAAGTTA
L-forward	72.2	235	213	22	11	GGGATTGATTTTAGTGTGTGTAAACG
L-reverse	55.3					CATTTGCATCATGTCCACCT
Q-forward	70.5	174	110	64	32	AAACTAAGCCTGACCACATA
Q-reverse	64.0					GATTTTGTGAGTGTAAACATAAGTT
R-forward	71.6	135	91	44	22	AAGGAGGGTTCTTCTTCAAC
R-reverse	60.0					CCTTTGTAGTCATTTGGNAC
T-forward	70.4	167	123	44	22	TTGCATAGGTAGCAAGAAACAA
T-reverse	57.3					GGGAGCTTGGGATCAAGTA
V-forward	72.5	288	232	56	28	TCTCCGAAAAACAGAGACAC
V-reverse	56.7					CTGCAGAAATTCGCCCTT

Appendix 3: Estimated allele frequencies in six populations of *E. coccineus*. All_W is the overall estimated allele frequency weighted for sample size. All_UW is the unweighted allele frequency. Allele frequencies within each population are in bold. Private alleles in each population are highlighted. The allele name is the number of repeats of the dinucleotide repeat motif of the microsatellites. Total number of private alleles per population is shown at the bottom of each table.

Locus: A1	Populations								
	BBTX	CCAZ	GSCO	HCAZ	LPAZ	MGNM	PNAZ	All_W	All_UW
Allele \ N	9	29	28	18	44	47	35		
p: 12	0.000	0.000	0.000	0.000	0.000	**0.011**	0.000	0.002	0.002
p: 13	0.000	0.000	0.000	**0.389**	**0.148**	**0.181**	**0.071**	0.117	0.113
p: 14	0.000	0.000	0.000	0.000	**0.045**	0.000	**0.057**	0.019	0.015
p: 15	**0.889**	**0.879**	**0.411**	**0.444**	**0.625**	**0.596**	**0.757**	0.643	0.657
p: 16	0.000	**0.086**	0.000	0.000	0.000	**0.011**	0.000	0.014	0.014
p: 17	**0.056**	0.000	0.000	**0.056**	**0.045**	0.000	**0.057**	0.026	0.031
p: 18	**0.056**	**0.034**	**0.518**	0.000	**0.125**	**0.011**	**0.029**	0.110	0.110
p: 20	0.000	0.000	**0.018**	0.000	0.000	0.000	0.000	0.002	0.003
p: 27	0.000	0.000	0.000	**0.056**	0.000	0.000	0.000	0.005	0.008
p: 41	0.000	0.000	0.000	0.000	0.000	**0.149**	0.000	0.033	0.021
p: 51	0.000	0.000	**0.018**	0.000	0.000	0.000	0.000	0.002	0.003
p: 53	0.000	0.000	0.000	0.000	0.000	**0.011**	0.000	0.002	0.002
p: 56	0.000	0.000	**0.036**	**0.028**	**0.011**	**0.032**	**0.029**	0.021	0.019
p: 58	0.000	0.000	0.000	**0.028**	0.000	0.000	0.000	0.002	0.004
# of Private Alleles	0	0	2	2	0	3	0		

Continuation of Appendix 3.

Locus: A2	Populations								
	BBTX	CCAZ	GSCO	HCAZ	LPAZ	MGNM	PNAZ	All_W	All_UW
Allele \ N	9	24	25	17	45	46	35		
p: 18	0.000	0.000	0.000	0.000	**0.011**	0.000	0.000	0.002	0.002
p: 19	**0.111**	**0.021**	0.000	**0.147**	**0.022**	**0.054**	**0.057**	0.047	0.059
p: 21	0.000	0.000	0.000	0.000	0.000	**0.033**	0.000	0.007	0.005
p: 24	0.000	0.000	0.000	0.000	**0.133**	**0.011**	0.000	0.032	0.021
p: 27	0.000	**0.063**	0.000	0.000	0.000	**0.011**	0.000	0.010	0.010
p: 30	0.000	0.000	0.000	0.000	**0.078**	0.000	0.000	0.017	0.011
p: 31	0.000	0.000	0.000	0.000	0.000	0.000	**0.014**	0.002	0.002
p: 35	**0.889**	**0.917**	**0.680**	**0.647**	**0.700**	**0.674**	**0.757**	0.731	0.752
p: 37	0.000	0.000	**0.060**	**0.059**	0.000	0.000	0.000	0.012	0.017
p: 38	0.000	0.000	**0.040**	0.000	0.000	0.000	0.000	0.005	0.006
p: 42	0.000	0.000	0.000	0.000	0.000	**0.011**	0.000	0.002	0.002
p: 43	0.000	0.000	0.000	0.000	0.000	**0.022**	0.000	0.005	0.003
p: 44	0.000	0.000	0.000	0.000	0.000	**0.011**	0.000	0.002	0.002
p: 46	0.000	0.000	0.000	0.000	0.000	0.000	**0.014**	0.002	0.002
p: 50	0.000	0.000	0.000	0.000	0.000	**0.011**	0.000	0.002	0.002
p: 53	0.000	0.000	0.000	0.000	0.000	**0.043**	0.000	0.010	0.006
p: 54	0.000	0.000	0.000	0.000	0.000	**0.011**	**0.043**	0.010	0.008
p: 60	0.000	0.000	0.000	**0.029**	**0.011**	**0.011**	**0.043**	0.015	0.013
p: 62	0.000	0.000	0.000	0.000	0.000	**0.022**	0.000	0.005	0.003
p: 66	0.000	0.000	**0.020**	0.000	**0.022**	0.000	**0.029**	0.012	0.010
p: 68	0.000	0.000	0.000	0.000	0.000	0.000	**0.043**	0.007	0.006
p: 69	0.000	0.000	0.000	0.000	0.000	**0.022**	0.000	0.005	0.003
p: 70	0.000	0.000	0.000	0.000	0.000	**0.011**	0.000	0.002	0.002
p: 71	0.000	0.000	0.000	**0.029**	0.000	0.000	0.000	0.002	0.004
p: 73	0.000	0.000	**0.080**	**0.059**	0.000	**0.022**	0.000	0.020	0.023
p: 75	0.000	0.000	0.000	0.000	**0.011**	0.000	0.000	0.002	0.002
p: 77	0.000	0.000	0.000	**0.029**	0.000	**0.022**	0.000	0.007	0.007
p: 78	0.000	0.000	0.000	0.000	**0.011**	0.000	0.000	0.002	0.002
p: 81	0.000	0.000	**0.120**	0.000	0.000	0.000	0.000	0.015	0.017
# of Pr. Al..	0	0	2	1	4	9	3		

Continuation of Appendix 3.

Locus: B1	Populations								
	BBTX	CCAZ	GSCO	HCAZ	LPAZ	MGNM	PNAZ	All_W	All_UW
Allele \ N	20	37	30	0	62	0	18		
p: 11	0.000	**0.068**	**0.117**	NA	**0.056**	NA	**0.028**	0.060	0.054
p: 13	**0.850**	**0.757**	**0.800**	NA	**0.742**	NA	**0.722**	0.766	0.774
p: 14	**0.150**	**0.176**	**0.083**	NA	**0.202**	NA	**0.250**	0.174	0.172
# of Private Alleles	0	0	0	0	0	0	0		

Locus: B2	Populations								
	BBTX	CCAZ	GSCO	HCAZ	LPAZ	MGNM	PNAZ	All_W	All_UW
N	1	20	20	0	41	0	8		
p: 29	0.000	**0.100**	**0.475**	NA	0.000	NA	0.000	0.128	0.115
p: 30	0.000	0.000	0.000	NA	**0.049**	NA	0.000	0.022	0.010
p: 33	0.000	0.000	**0.050**	NA	**0.171**	NA	0.000	0.089	0.044
p: 34	0.000	0.000	0.000	NA	**0.012**	NA	0.000	0.006	0.002
p: 35	0.000	0.000	0.000	NA	**0.049**	NA	**0.250**	0.044	0.060
p: 36	0.000	**0.075**	0.000	NA	**0.183**	NA	0.000	0.100	0.052
p: 37	0.000	**0.225**	0.000	NA	**0.305**	NA	**0.250**	0.211	0.156
p: 38	0.000	**0.200**	0.000	NA	**0.037**	NA	0.000	0.061	0.047
p: 39	0.000	**0.075**	0.000	NA	**0.000**	NA	0.000	0.017	0.015
p: 40	**1.000**	0.000	**0.075**	NA	**0.024**	NA	**0.250**	0.061	0.270
p: 41	0.000	0.000	**0.225**	NA	**0.061**	NA	**0.188**	0.094	0.095
p: 42	0.000	**0.125**	**0.100**	NA	**0.073**	NA	0.000	0.083	0.060
p: 43	0.000	0.000	0.000	NA	**0.037**	NA	0.000	0.017	0.007
p: 44	0.000	0.000	**0.075**	NA	0.000	NA	**0.063**	0.022	0.028
p: 46	0.000	**0.050**	0.000	NA	0.000	NA	0.000	0.011	0.010
p: 48	0.000	**0.150**	0.000	NA	0.000	NA	0.000	0.033	0.030
# of Private Alleles	0	2	0	0	3	0	0		
Total Private Alleles/ Pop.	0	2	4	3	7	12	3		
Mean # of Pr. All. / Pop.	0	0.5	1	0.75	1.75	3	0.75		

Appendix 4: Summary of genetic diversity estimates for each population of *E. coccineus* based on four microsatellite loci. *N* = number of individuals, *P* = proportion of polymorphic loci, *AP* = mean number of alleles per locus, *Ht* = overall gene diversity H_o = mean observed heterozygosity, H_e = mean expected heterozygosity. The All category has the sum of all individuals genotyped and the mean and standard errors for *P, AP,* H_t H_o and H_e.

Population	***N***	***P***	***AP***	H_t	H_o	H_e
BBTX	20	0.88	2.33	0.23	0.08	0.23
CCAZ	37	1.00	4.25	0.42	0.18	0.42
GSCO	30	1.00	5.00	0.54	0.36	0.54
HCAZ	20	1.00	6.50	0.62	0.30	0.62
LPAZ	62	1.00	7.25	0.58	0.44	0.58
MGNM	47	1.00	12.50	0.57	0.28	0.57
PNAZ	35	1.00	5.50	0.53	0.35	0.53
ALL	251	0.98 (0.02)	15.5 (1.21)	0.50 (0.05)	0.28 (0.05)	0.50 (0.05)

Appendix 5: Nei's statistics of genetic diversity and structure for four polymorphic loci in *E. coccineus*. *Ho* = observed proportion of heterozygotes, *Hs* = within sample gene diversity, *Ht* = overall gene diversity *Gst* = estimator of $F_{ST,}$ *Gst'*= estimator of F_{ST} independent of sample size, *Gis*= estimator of F_{IS}.

Locus Name	***Ho***	***Hs***	***Ht***	***Gst***	***Gst'***	***Gis***
A1	0.458	0.467	0.542	0.139	0.158	0.020
A2	0.365	0.418	0.430	0.028	0.032	0.128
B1	0.173	0.372	0.370	-0.004	-0.005	0.534
B2	0.243	0.799	0.898	0.111	0.135	0.696
Overall	0.310	0.514	0.560	0.082	0.095	0.398