SPATIAL GENETIC STRUCTURE AND NONRANDOM POLLINATION SUCCESS IN *Oenothera harringtonii* (ONAGRACEAE)

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☐ Pass with no revisions ☒ Pass with minor revisions

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CHAPTER ONE

LOCAL TOPOGRAPHIC HETEROGENEITY EXPLAINS FINE-SCALE SPATIAL GENETIC STRUCTURE IN *Oenothera harringtonii* (ONAGRACEAE)

Abstract

Identifying factors that shape the spatial distribution of genetic variation within and among natural populations is crucial to understanding many population- and landscape-level processes. In this study, I characterize the strength and extent of spatial genetic structure in *Oenothera harringtonii* (Onagraceae), an insect-pollinated, gravity-dispersed herb endemic to the grasslands of south-central and southeastern Colorado. I genotyped 323 individuals using microsatellite markers and utilized a combination of spatial autocorrelation techniques and landscape genetic modeling to explore how life history and landscape features influence patterns of dispersal in this species. Spatial genetic structure was consistent with theoretical expectations of isolation by distance, with genetic relatedness decreasing as a function of increasing spatial distance. However, spatial genetic structure was weak and indirect estimates of gene dispersal varied between estimates of population density, suggesting that gene flow in this system may be extensive but constrained by population density. Anisotropic analyses and landscape genetic models further indicated that dispersal at the study site is markedly directional, in this case consistent with increased dispersal along prominent slopes and a major drainage, as would be expected in a species with gravity-dispersed seeds. These findings highlight the importance of pollinator behavior and local landscape context in shaping plant dispersal processes, and demonstrate the utility of a combined approach in the study of plant dispersal.

Introduction

A central goal of landscape genetics is to infer how details of environmental context shape the microevolutionary processes that structure genetic variation both within and among populations. In the most basic sense, genetic structure arises when gene flow is nonrandom in space. Gene flow in plants is unique in that it is mediated through both the dispersal of haploid gametes via pollen and diploid embryos via seed (e.g. Ennos 1994). However, the sessile habit of plants often results in limited dispersal of their gametes and propagules, with both vectors tending to be restricted in space (Loveless and Hamrick 1984; Vekemans and Hardy 2004; Barluenga et al. 2011). Plant populations therefore frequently exhibit spatial genetic structure (SGS), or the nonrandom spatial distribution of genotypes, reflecting the joint activity of numerous biotic and abiotic factors that shape patterns of mating and dispersal (Vekemans and Hardy 2004).

At fine spatial scales, SGS is typically manifested in the presence of localized pedigree structures, creating a pattern where spatially proximate individuals are more genetically similar (Vekemans and Hardy 2004). Numerous studies have detected and characterized this pattern in plant populations, in most cases using techniques related to spatial autocorrelation (Heywood 1991; Loiselle et al. 1995; Hardy and Vekemans 1999; van Heerwaarden et al. 2010). In addition to describing the strength and extent of SGS, these studies have established links to its causal factors. For example, breeding system and growth form can be strong determinants of fine-scale SGS in plants, which is accentuated in predominantly selfing and herbaceous species (Vekemans and Hardy 2004). Differences in the mechanism of pollen and seed dispersal can also shape SGS, which tends to be stronger in animal-pollinated and gravity-dispersed species relative to their wind-pollinated and animal-dispersed counterparts (Vekemans and Hardy 2004; Luna et al.

2005). Similarly, differences in the relative dispersal distances of pollen and seed for a given species may drive patterns of SGS (Gehring and Delph 1995; Krauss et al. 2009). Spatial autocorrelation techniques have also been reconciled with traditional models of population genetics such that under the assumption of drift-dispersal equilibrium, SGS can be used to infer the spatial extent of historical gene movement (Hardy and Vekemans 1999; Fenster et al. 2003).

While these studies have yielded important insights into plant dispersal processes, they inherently assume that patterns of SGS are directionally independent. Studies of SGS based on spatial autocorrelation alone therefore neglect factors that could impose directional patterns on dispersal, which may be important for passively dispersed species growing in topographically heterogeneous environments (Born et al. 2012). This discrepancy has been addressed by extending anisotropic spatial autocorrelation techniques, originally developed to investigate directional clines in large-scale human genetic datasets, to studies of fine-scale SGS in plants (Falsetti and Sokal 1993). These techniques have been used to examine potential anisotropic dispersal associated with prevailing wind directions in wind-pollinated *Quercus lobata* (Fagaceae), but did not reveal significant directional patterns (Dutech et al. 2005; Austerlitz et al. 2007). More recently, anisotropic methods showed that that SGS in wind-pollinated and wind-dispersed *Azorella selago* (Apiaceae) was strongly associated with prevailing wind directions (Born et al. 2012). However, no association was found between SGS and local topography.

In this study, I combine spatial autocorrelation analyses with formal landscape genetic models to examine patterns of SGS in the animal-pollinated, gravity-dispersed grassland herb *Oenothera harringtonii* (Onagraceae). *Oenothera harringtonii* presents an interesting case study for plant dispersal, as it is pollinated by hawkmoths that facilitate long-distance pollen movement (Stockhouse 1976; Skogen et al. in prep.) but its seeds are dispersed via gravity, suggesting that

seed movement may be both spatially restricted and influenced by local topography. Specifically, I address the following hypotheses: (1) fine-scale SGS in *O. harringtonii* is readily detectable but relatively weak owing to presumed differences in seed and pollen movement, and (2) patterns of SGS exhibit directional trends consistent with local topographic variation.

Methods

Study species and sampling

The Colorado Springs evening primrose, *Oenothera harringtonii* W. L. Wagner, Stockh. & W. M. Klein (Onagraceae), is an annual, self-incompatible herb endemic to the grasslands of south-central and southeastern Colorado (Figure 1.1). It is pollinated primarily by hawkmoths (Sphingidae: *Hyles lineata* and *Manduca quinquemaculata*) but is also visited by a number of solitary bees from the families Apidae and Halictidae (Skogen unpublished data). Flowering occurs from late April through early June, after which capsules mature and dehisce during July and August. Seeds are thought to be primarily gravity-dispersed (Wagner et al. 1985).

This study was conducted in the Comanche National Grasslands near La Junta, CO, USA (N 37.566782, W 104.299439), managed by the United States Forest Service. The study site encompassed approximately 300 ha and is distinguished by prominent shale ridges that feed into a series of drainages in the depression they circumscribe, creating the effect of a natural amphitheater. At this site, *O. harringtonii* is found in both the flats and on the slopes of the surrounding ridges. Plants tend to occur in discrete, dense clusters on the slopes, and are more evenly distributed in the flats. Exhaustive surveys conducted over the course of three days in May 2012 located 323 reproductive individuals (Figure1.2), from which leaf tissue was collected, stored in coin envelopes and dried with silica gel for subsequent DNA extraction and

microsatellite analysis. This sampling scheme was employed to facilitate a parentage analysis and therefore excluded some vegetative individuals, but is still sufficient to characterize fine-scale SGS (Vekemans and Hardy 2004). All plants were georeferenced to a precision of ~10 cm using a Trimble GeoXH 2005 and mapped using ArcMap 10 (ESRI, Inc.).

DNA extraction and genotyping

Genomic DNA was isolated from ~1 cm^2 of silica-preserved leaf tissue following a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle 1987). All individuals were then assayed at 11 microsatellite loci described for *O. harringtonii* (Skogen et al. 2012). Microsatellite regions were amplified in a 10-μL PCR reaction containing 5 μL 2x MyTaq Mix (Bioline, Taunton, Massachusetts, USA), 3.375 μL DNA-grade H_2O, 1 μL genomic DNA, 0.25 μL of each primer, and 0.125 μL 2x BSA. Primers were labeled with WellRed D2, D3, or D4 fluorescent dye (Sigma-Proligo, St. Louis, Missouri, USA). Initial denaturation was set at 95°C for 2 min, and was followed by 30 cycles of 95°C for 50 s, 56°C for 1 min, 72°C for 1 min, ending with a final extension at 72°C for 10 min. PCR products were scored using a CEQ 8000 Genetic Analysis System with GenomeLab 400 internal size standard (Beckman Coulter, Brea, California, USA), and fragment lengths were manually verified. Genetic variation was assessed by determining the number of alleles per locus (A), effective number of alleles per locus (A_e), expected (H_e) and observed (H_o) heterozygosity, and average inbreeding (F_{IS}) using GenAlEx 6.4 (Peakall and Smouse 2006).

Isotropic spatial genetic structure

Genotypes were first examined using isotropic spatial autocorrelation analyses, wherein relative kinship coefficients were estimated between all pairs of individuals and related to the spatial distance between them. Kinship coefficients measure correlations in homologous alleles between individuals and were calculated following Loiselle et al. (1995), as this statistic is less biased in the presence of low-frequency alleles (Vekemans and Hardy 2004). To visualize SGS, average pairwise kinship coefficients (F_{ij}) were computed over a set of distance intervals (0-1, 1-5, 5-10, 10-25, 25-100, 100-500, 500-1500 and 1500-3223 m) and plotted against geographic distance in a spatial autocorrelogram, thus permitting SGS to be assessed without imposing *a priori* assumptions on its form (e.g. Fenster et al. 2003). The strength of SGS was then evaluated by regressing pairwise kinship coefficients on the natural logarithm of pairwise geographic distances (r_{ij}) and estimating the slope of the resulting relationship (b_{log}). Under isolation by distance in two-dimensional space, theory predicts that kinship will decrease approximately linearly with the logarithm of geographic distance (Rousset 1997, 2000; Hardy and Vekemans 1999); the regression approach thus investigates SGS under the assumption that it results from isolation by distance (Fenster et al. 2003). Kinship-distance regression slopes were then translated into a single metric that characterizes the strength of SGS, *Sp*, given by the expression $-b_{log}/(1 - F_{(1)})$, where $F_{(1)}$ is the average kinship coefficient between individuals in the first distance class (Vekemans and Hardy 2004). Confidence intervals for average kinship coefficients and regression slopes were obtained by jackknifing data over loci and were approximated as plus/minus twice the standard error of the mean. Statistical significance of kinship coefficients and regression slopes was determined by randomly permuting the spatial location of individuals 10 000 times and comparing observed parameter estimates to their corresponding permuted

frequency distributions under the null hypothesis of no SGS. All isotropic SGS analyses were conducted using SPAGeDi 1.3 (Hardy and Vekemans 2002).

Estimation of gene dispersal

Assuming isolation by distance in two-dimensional space, neighborhood size, given by the expression $N_b = 4\pi D_e\sigma^2$, represents the number of individuals expressing the strength of local genetic drift, where D_e is the effective population density and σ^2 is ½ the mean squared geographic distance between parent and offspring (Wright 1943; Fenster 2003). Under these assumptions, N_b can be derived from the regression of pairwise kinship coefficients on the natural logarithm of geographic distance as $N_b = -(1 - F_{(1)})/b_{log}$ (Hardy and Vekemans 1999), enabling indirect estimates of $D_e\sigma^2$. This technique is most reliable when the regression slope is calculated within the distance range of approximately $\sigma - 20\sigma$ (Rousset 1997). As σ is unknown, an iterative procedure implemented in SPAGeDi 1.3 (Hardy and Vekemans 2002) can be used to simultaneously estimate N_b and σ if D_e is known, but this procedure may be highly influenced by variation in D_e (Vekemans and Hardy 2004). Estimates of N_b and σ were therefore calculated for boundary estimates of $D_e = 0.001/m^2$ and $D_e = 0.5/m^2$ to accommodate patchiness in the distribution of flowering plants at the study site, corresponding to population-wide and within-patch estimates, respectively. Standard errors of N_b and σ estimates were obtained by jackknifing data over loci.

Anisotropic dispersal

Potential directional patterns in SGS were evaluated with an anisotropic bearing analysis, following Falsetti and Sokal (1993). This analysis was restricted to a subset of individuals at the

study site (n = 192) that are distributed in a less linear fashion and thus better conform to the assumptions of this test (Figure 1.3). Bearing analysis determines the direction of strongest correlation between a data distance matrix (K) and geographic distance matrix (D). In this study, the K matrix consists of pairwise kinship coefficients (F_{ij}) and the D matrix consists of log-transformed pairwise geographic distances ($\ln(r_{ij})$) (*sensu* Born et al. 2012). The D matrix was then translated into 36 new matrices (D_0, D_5, D_{10} … D_{175}) by multiplying each entry by the squared cosine of the angle α_{ij}, defined as the angle between a given fixed bearing ($\theta = 0°$, 5°, 10° … 175°) and a vector connecting points *i* and *j*, with $\theta = 0°$ representing the positive x-axis (due east) and $\theta = 90°$ representing the positive y-axis (due north). In effect, this transformation weights each pairwise geographic distance entry by its alignment with a particular test direction (Rosenberg and Anderson 2011; Born et al. 2012). Correlations between K and each D matrix were then evaluated with Mantel tests (Mantel 1967). Statistical significance of Mantel correlations was determined through a standard Mantel permutation test with 999 permutations. In this analytical framework, the bearing with the strongest positive correlation (θ_{MAX}) reflects the axis of maximum dispersal, and is expected to be approximately perpendicular to the bearing with the strongest negative correlation (θ_{MIN}). All anisotropic SGS analyses were conducted using PASSaGE 2.0.11.6 (Rosenberg and Anderson 2011).

Landscape genetic analysis

Potential influences of site-specific landscape features were then evaluated in a formal landscape genetic analysis, using the same set of individuals included in the bearing analysis. I considered two landscape processes hypothesized to shape spatial genetic structure at this site: isolation by distance and isolation by topography. Isolation by distance was quantified as the

natural logarithm of the geographic distance between all pairs of individuals, using the pairwise distance matrix computed by SPAGeDi. Isolation by topography was quantified using three distinct landscape variables: aspect, elevation and slope, each of which was derived into a separate pairwise similarity/dissimilarity matrix. Raster values corresponding to the coordinates of each plant were extracted from geospatial data layers resolved to ~30 m^2 (Figure S.1). Pairwise difference matrices for each landscape variable were then generated using R package ecodist (Goslee and Urban 2007). In the resulting matrices, pairwise entries are treated as cost "distances" based upon the absolute difference of a given landscape feature between two points.

Relationships between genetic data and landscape variables were modeled using a multiple regression on distance matrices (MRDM) approach (Legendre et al. 1994). While numerous techniques are available for landscape genetic analyses, MRDM offers increased statistical power and lower error rates than alternative approaches (Balkenhol et al. 2009) and provides a convenient method for comparing the relative importance of different explanatory variables (Lichstein 2007) and separating the influence of historical versus contemporary processes (Dyer et al. 2010). In this framework, pairwise distance matrices are included as terms in a linear model, here:

$$F_{ij} \sim D + A + E + S$$

where F_{ij} is a pairwise matrix of kinship coefficients, D is a pairwise matrix of log-transformed geographic distances, and A, E and S are pairwise difference matrices for aspect, elevation and slope, respectively. I developed models for two scenarios: one in which the influence of distance and topography were considered simultaneously and another in which isolation by distance was partitioned from the response data prior to fitting models with topographic variables. In the first scenario, the global model contained terms for both distance and topography, as listed above. In

the second scenario, I began by modeling kinship coefficients solely as a function of geographic distance. The residual variation of this model was then used as the response in models containing topographic predictors (c.f. Dyer et al. 2010). All predictor matrices were standardized to mean zero and unit variance so that the coefficients of model terms could be directly compared. As MRDM assumes a linear relationship between variables, predictors were examined for potential multicollinearity prior to model estimation. Pearson correlation coefficients ranged from 0.20 – 0.48 for five of the six pairs of predictor matrices, but elevation and slope were more strongly correlated ($\rho = 0.89$), suggesting that partial regression coefficients in models containing these terms may be unreliable. To ensure that multicollinearity did not confound model estimation, the variance inflation factor (*vif*) of predictor terms were estimated at each step of model selection using R package car (Fox and Weisberg 2011). I considered $vif \geq 10$ indicative of problems associated with correlated predictors (Dyer et al. 2010). Statistical significance of predictor terms and model fit were determined with 10,000 permutations of the response matrix, and terms that did not contribute significantly to model fit were removed via a backward selection procedure with a Bonferroni-corrected removal threshold of α = [0.05/number of predictors at the given step] (Legendre et al. 1994). All MRDM modeling was performed with R package ecodist.

Results

Genetic variation

All microsatellite loci were highly polymorphic, with the number of alleles per locus ranging from 7 to 38 (Table 1.1). Genetic diversity among loci was variable but consistently high, with expected heterozygosity ranging from 0.420 to 0.957. Significant departures from

Hardy-Weinberg equilibrium were observed for seven loci, all in the direction of heterozygote deficiency. However, inbreeding across all loci was low, with mean $F_{IS} = 0.044$ (Table 1.1).

Spatial genetic structure, gene dispersal and anisotropy

Isotropic SGS analysis showed that kinship decreases with the natural logarithm of geographic distance in a pattern consistent with isolation by distance (Figure1.4). Kinship coefficients decrease approximately linearly over the first four distance classes (1 – 25 m) and continue to decrease over the last four distance classes (100 – 3223 m), though less drastically so. Kinship coefficients were significantly positive at the first four distance classes (0 – 1 m, 1 – 5 m, 5 – 10 m, 10 – 25 m; $P < 0.0001$) and significantly negative at the last distance class (1500 – 3223 m; $P < 0.0001$). The kinship-distance regression slope was significantly negative ($b_{log} \pm$ SE = -0.0037 ± 0.0004; $P < 0.0001$) but explained very little of the variance in inter-individual relatedness (multilocus $R^2 = 0.009$) and indicates that observed SGS is quite weak ($Sp = 0.00374$; Table 1.2).

Indirect estimates of gene dispersal displayed extreme variation between boundaries of effective population density, increasing substantially under reduced population density. Values of σ ranged from 5.2 ± 1.6 m – 171.6 ± 13.0 m (parameter estimate ± SE) between upper and lower boundaries of D_e, corresponding to neighborhood sizes of 86 ± 16 – 370 ± 55 individuals, respectively (Table 1.2).

Bearing analysis showed that the strength of spatial autocorrelation followed a periodic function against compass heading in a pattern consistent with anisotropic dispersal, in this case favoring a general northeast-southwest orientation consistent with the orientation of slopes around the primary drainage in this portion of the study site ($\theta_{MAX} = 55°$; Figure 1.5; Table 1.2).

Correlations between K and D matrices were significantly negative between $\theta = 0°$ and $\theta = 40°$, with the strength of the negative relationship steadily decreasing within this range before becoming insignificant between $\theta = 45°$ and $\theta = 70°$. Bearing correlations were again significantly negative between $\theta = 75°$ and $\theta = 175°$, with the strength of the negative relationship steadily increasing until $\theta = 145°$, then steadily weakening until $\theta = 175°$. θ_{MAX} and θ_{MIN} were perpendicular ($\theta_{MIN} = 145°$; Table 1.2).

Landscape genetic analysis

MRDM modeling revealed significant effects of landscape features on inter-individual relatedness. Starting with a saturated model containing predictor terms for both distance and topography, the minimum adequate model was highly significant ($F = 75.46$, $P = 0.0001$, $R^2 = 0.008$) and retained terms for geographic distance and elevation after backwards elimination of insignificant predictors (Table 1.3). Variance inflation factors did not exceed 5.1 at any step of model estimation and were 1.3 in the final model, suggesting that model selection was not heavily influenced by correlations between predictor terms. Geographic distance had the largest standardized effect in the final model ($\beta_D = -0.0066$) and was ~68% more important than elevation ($\beta_E = 0.0021$) in describing inter-individual relatedness.

Consistent with the population-wide SGS analysis, geographic distance alone was a significant predictor of relatedness for the 192 individuals included in landscape genetic models ($F = 136.1$, $P = 0.0001$, $R^2 = 0.007$). The residual variation from this model was then used as the response in models limited to landscape predictors, thereby isolating the influence of topographic features after accounting for geographic distance (Dyer et al. 2010). Using the same backward elimination procedure, the minimum adequate model was again highly significant ($F = 7.83$, $P =$

0.0008, R^2 = 0.0009), although less so than the unconditioned model. In keeping with the unconditioned model, the minimum adequate conditioned model retained the elevation term, and aspect also remained significant after backwards elimination (Table 1.3). Variance inflation factors in the conditioned model were similarly low and did not suggest any problems owing to multicollinearity. Elevation had the largest effect in the final model (β_E = 0.0019) and was ~42% more important than aspect (β_A = -0.0011) in describing inter-individual relatedness conditioned on geographic distance.

Discussion

To my knowledge, this is the first study to explicitly link fine-scale topographic variation to inter-individual relatedness in a plant population. Patterns of SGS observed in this study population were consistent with a pattern of isolation by distance, as kinship coefficients among spatially proximate individuals were significantly higher than expected under the null hypothesis of random dispersal. However, observed SGS was weak, and indirect estimates of gene dispersal varied extensively with population density. Further, anisotropic bearing analysis provided strong evidence that SGS at the study site is directionally variable, suggesting isolation by distance alone cannot fully explain patterns of SGS in this system, and formal landscape genetic models confirmed the significance of local topographic variation as a driver of the observed genetic patterns. Collectively, these results suggest that pollen and seed dispersal in *O. harringtonii* operate on different spatial scales and may be strongly influenced by pollinator behavior and local topographic heterogeneity.

Spatial genetic structure and gene dispersal

Isotropic spatial autocorrelation analyses suggest that isolation by distance is largely responsible for the observed SGS in this study population. While SGS at the study site was apparent, it was relatively weak as estimated by *Sp*. Indeed, the observed value of *Sp* = 0.00374 is lower than any published value for plant species with life histories similar to *O. harringtonii* (Table 1.4), which likely reflects several aspects of the biology and autecology of this species. First, while individual plants may produce dozens of flowers throughout the growing season, individual flowers are ephemeral, lasting just 14-18 hours. Thus, over the course of an entire season, different flowers produced by a given plant will likely sample different pollen pools owing to daily variation in population-wide flowering phenology, potentially increasing season-wide mate diversity and dampening SGS (c.f. Ison 2010; Zeng et al. 2012). This would further reduce the strength of SGS if pollen were dispersed more homogeneously across the landscape than seed (e.g. Bizoux et al. 2009; Krauss et al. 2009). Second, the patchy distribution of individuals at this study site creates areas characterized by relatively high conspecific density (Figure 1.2). This is expected to increase overlap in maternal seed dispersal shadows, thereby homogenizing seed pools and reducing the strength of SGS (Hamrick and Trapnell 2011). These findings highlight the importance of pollen dispersal and conspecific density in shaping SGS, and suggest that variation in pollinator behavior and maternal reproductive output may have pronounced impacts on its strength.

This notion is further supported by indirect estimates of historical gene dispersal, which displayed more than 30-fold variation between boundary estimates of effective population density (Table 1.2). As seed dispersal in *O. harringtonii* is gravity-mediated, pollen movement likely makes the primary contribution to σ, which should be a reasonable assumption for obligate

outcrossers (Vekemans and Hardy 2004). Indirect gene dispersal estimates therefore suggest that pollen dispersal in *O. harringtonii* is highly variable but frequently spans large distances, which is consistent with prior indirect studies of pollen movement in closely related *Oenothera* species. Using fluorescent powders to infer pollinator movement and pollen dispersal in hawkmoth-pollinated *O. caespitosa* ssp. *caespitosa*, Stockhouse (1976) recorded frequent powder transfer over the scale of tens of meters (0 – 45 m) to hundreds of meters (up to 800 m). Similar results were obtained in a study of *O. c.* ssp. *macroglottis*, in which powder transfer was observed over tens of kilometers (up to 12.8 km), clearly demonstrating the potential for long-distance pollen movement under hawkmoth pollination (Stockhouse 1976; Artz et al. 2010).

Assuming that σ can be attributed mostly to pollen dispersal, indirect estimates of gene dispersal further imply that population density is likely a major determinant of realized pollen movement in this system, consistent with previous studies. Population density was shown to explain most of the variation in pollinator movement and neighborhood size in *Linanthus bicolor* (Polemoniaceae) (Schmitt 1983), with similar results having been reported in *Chamaecrista fasciculata* (Fabaceae) (Fenster 1991). My findings are therefore in agreement with a general expectation of increased pollen dispersal under lower conspecific density (e.g. Levin and Kerster 1969). Still, it must be noted that indirect estimates of historical gene dispersal based on spatial patterns in nuclear markers cannot distinguish the influence of seed and pollen movement, however safe any assumptions regarding their relative dispersion might be. These indirect estimates are therefore best interpreted as baseline expectations, and are examined more rigorously in Chapter 2.

Anisotropic dispersal and topographic heterogeneity

Bearing analysis revealed that SGS at this study site is strongly influenced by anisotropic processes, as kinship-distance regressions followed a periodic function with compass heading (Figure 1.5). This likely reflects the influence of local topographic heterogeneity. The axis of maximum dispersal was situated along a general northeast-southwest orientation ($\theta_{MAX} = 55°$), in line with the general orientation of slopes around the primary drainage in this portion of the study site (Figure 1.3). The observed directional pattern thus offers tentative support for an influence of local topography on dispersal processes in this system. This result is consistent with previous work in that significant anisotropy was detected, but the mechanisms underlying these results appear to differ. Anisotropic SGS in *Azorella selago* (Apiaceae) was attributed to prevailing winds (Born et al. 2012), whereas the directional pattern in the present study is more consistent with increased dispersal along prominent slopes or drainages. While topography has been implicated in prior studies of fine-scale SGS (Oshawa et al. 2007; He et al. 2013), this highlights a general challenge associated with autocorrelation techniques in that they only allow qualitative inferences. In cases where putative causal factors could leave the same directional signature, results of anisotropic SGS analyses should be interpreted with caution or augmented with more explicit modeling techniques.

In this study, the qualitative association suggested by the bearing analysis was strongly supported by MRDM models, which verified the importance of topographic heterogeneity as a determinant of SGS at this site. The influence of topography was further clarified by conditioning the response on geographic distance, which revealed aspect as another significant predictor of relatedness. Moreover, aspect had a negative regression coefficient in the conditioned model, indicating that smaller changes in aspect are associated with greater

relatedness. These models therefore validate the directional bias revealed in the bearing analysis, describing a more intuitive pattern of dispersal in which propagules seemingly move farther along steeper slopes of similar aspect, or more generally, up or down hills. Collectively, these results underscore the importance of local topographic heterogeneity in shaping dispersal processes and SGS in this system. From a methodological viewpoint, these models also demonstrate the value of separating the influence of historical and contemporary processes in landscape genetic analyses. Conditioning the response on geographic distance gave more nuanced insights than would have been obtained otherwise, supporting the view that historical factors should be accounted for when building landscape genetic models (Dyer et al. 2010).

Dispersal in O. harringtonii

In plants, dispersal is accomplished through the movement of pollen and seed. While the models in this study cannot distinguish between these dispersal vectors, it seems more likely that a topographic effect in this particular landscape would be manifested by shaping seed movement, especially given that seed dispersal in *O. harringtonii* is gravity-mediated. This view is further supported by seasonal climatic patterns. The population included in this study is situated in a part of Colorado subject to the North American Monsoon, which drives pronounced increases in rainfall throughout the southwestern United States during July and August (Adams and Comrie 1997; Diem et al. 2013). This timing coincides nearly perfectly with fruit maturation and dehiscence in *O. harringtonii* (Skogen unpublished data). Considering the topographic variables that remained significant in landscape genetic models along with the small size and apparent buoyancy of *O. harringtonii* seeds (Rhodes pers. obs.), it is possible that seasonal monsoon rains could aid seed dispersal in a way that would create the observed genetic patterns (i.e. promoting

seed movement along slopes of similar aspect). Hydrochory, or passive movement by water, has long been recognized as a pervasive mechanism of plant dispersal, and has been suggested as a driver of spatial pattern within plant populations (Nilsson et al. 2010). It thus seems plausible that such a process could also shape SGS in this system, but the extent to which precipitation and hydrology actually influence dispersal patterns in *O. harringtonii* remains unclear.

Conclusions

This work demonstrates the utility of combining multiple complementary approaches in the study of plant dispersal processes. I found that both isolation by distance and topography-mediated anisotropic processes are responsible for structuring genetic variation within this population of *O. harringtonii*. The presence of SGS has important implications for reproductive dynamics in this system, as SGS increases the likelihood of biparental inbreeding (Hamrick et al. 1993) as well as the potential for subsequent inbreeding depression (Heywood 1993; Nason and Ellstrand 1995). The finding that topographic variation drove patterns of SGS further suggests that landscape features may shape microevolutionary forces at much finer spatial scales than typically considered, cautioning against the dismissal of site-specific environmental details when considering dispersal and/or genetic processes. While the mechanisms by which topographic variation influences particular dispersal vectors warrant further attention, this study nonetheless provides an important point of departure for future work on the causes and consequences of fine-scale SGS in *O. harringtonii* and other plant species growing in topographically heterogeneous environments.

Table 1.1. Genetic summary statistics for n = 323 reproductive adults at Comanche National Grasslands: number of alleles per locus (*A*), effective number of alleles (A_e), expected heterozygosity (H_e), observed heterozygosity (H_o), departure from Hardy-Weinberg equilibrium (*HWE*), and average inbreeding (F_{IS}).

Locus	*A*	A_e	H_e	H_o	*HWE* †	F_{IS}
B105	16	6.5	0.845	0.829	*	0.019
D2	8	3.9	0.746	0.735	ns	0.015
C105	21	5.5	0.818	0.774	ns	0.054
D118	9	1.7	0.420	0.414	ns	0.016
D5	27	11.7	0.914	0.892	***	0.025
D102	8	3.5	0.717	0.684	***	0.046
OB2	13	6.1	0.837	0.868	ns	-0.038
OB7	7	3.5	0.718	0.621	***	0.134
D111	35	13.2	0.924	0.899	***	0.027
C126	15	5.8	0.828	0.799	ns	0.034
C106	38	23.2	0.957	0.879	**	0.081
Average	**17.9**	**7.7**	**0.793**	**0.763**	**--**	**0.044**

† Key: ns = not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 1.2. Isotropic SGS parameters, gene dispersal estimates and anisotropic SGS bearings.

Standard errors are reported for b_{log}, σ and N_b, as determined by jackknifing data over loci.

Isotropic							Anisotropic	
b_{log} †	$F_{(1)}$ ‡	*Sp*	σ (m) (D_e = 0.5)	σ (m) (D_e = 0.001)	N_b (D_e = 0.5)	N_b (D_e = 0.001)	θ_{MAX}	θ_{MIN}
-0.0035 ± 0.0004	0.054	0.00374	5.2 ± 1.6	171.6 ± 13.0	86 ± 16	370 ± 55	55°	145°

† Slope of population-wide kinship-distance regression
‡ Kinship coefficient between neighboring individuals

Table 1.3. Backward elimination procedure for landscape genetic models using multiple regression on distance matrices. Model selection is shown for a) the unconditioned model in which geographic distance and topographic variables were considered simultaneously, and b) the conditioned model in which geographic distance was accounted for prior to modeling topographic variables. Model parameters (β) are standard partial regression coefficients, and statistical significance was determined with 10000 permutations. At each step, the variable with the largest *P* value was eliminated if its probability exceeded a Bonferroni-corrected removal threshold of α = [0.05/number of predictors at the given step].

a) Unconditioned

	Step 1			Step 2			Step 3		
Predictor	β	*P*	*vif*	β	*P*	*vif*	β	*P*	*vif*
Distance	-0.0063	0.0001	1.45	-0.0066	0.0001	1.29	-0.0066	0.0001	1.29
Elevation	0.0039	0.0002	5.09	0.0038	0.0004	5.08	0.0021	0.0004	1.29
Slope	-0.0019	0.0458	4.76	-0.0019	0.0479	4.75	-	-	-
Aspect	-0.0009	0.0550	1.19	-	-	-	-	-	-

b) Conditioned

	Step 1			Step 2		
Predictor	β	*P*	*vif*	β	*P*	*vif*
Elevation	0.0037	0.0004	4.86	0.0019	0.0007	1.07
Aspect	-0.0019	0.0162	1.07	-0.0010	0.0189	1.07
Slope	-0.0019	0.0448	4.76	-	-	-

Table 1.4. Isotropic SGS parameters for plant species biologically comparable to *O. harringtonii*. All species included are self-incompatible, animal-pollinated, gravity-dispersed herbs, and data are arranged in order of decreasing *Sp* values. Reproduced from Table 2 in Hardy and Vekemans (2004).

Species	Family	F_{IS} †	$F_{(1)}$ ‡	*Sp*
Primula elatior	Primulaceae	0.233	0.162	0.02041
Ipomopsis aggregata	Polemoniaceae	0.110	0.053	0.01626
Primula veris	Primulaceae	0.324	0.026	0.01400
Centaurea jacea	Asteraceae	0.104	0.181	0.01087
Centaurea corymbosa	Asteraceae	0.028	0.050	0.01066
Lesquerella fendleri	Brassicaceae	0.016	0.042	0.00755
Arabidopsis halleri	Brassicaceae	-0.031	0.161	0.00471
Oenothera harringtonii	**Onagraceae**	**0.043**	**0.054**	**0.00374**

† Wright's inbreeding coefficient
‡ Kinship coefficient between neighboring individuals

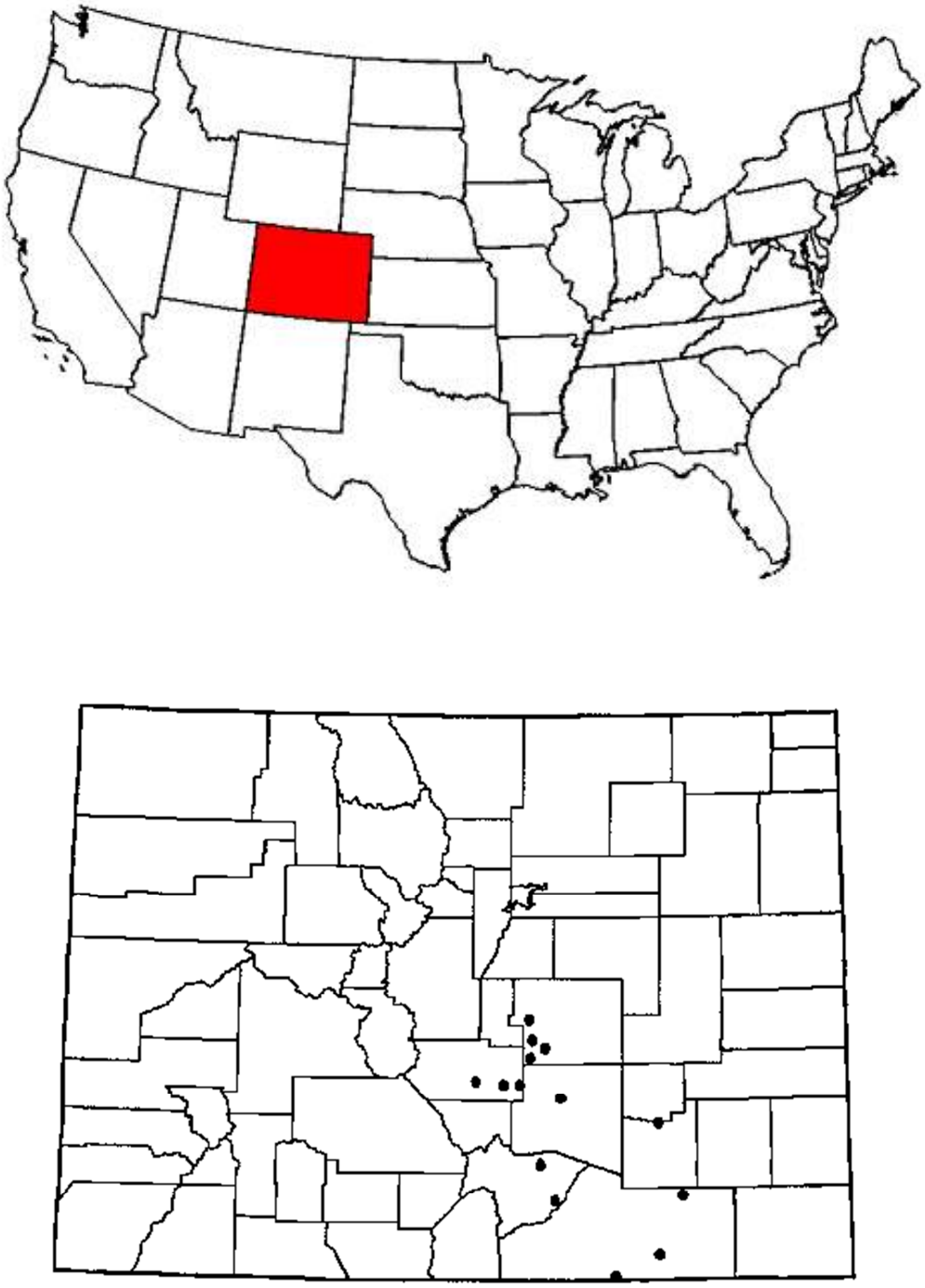

Figure 1.1. Geographic distribution of *O. harringtonii* in Colorado, USA. Adapted from Spackman et al. (1997).

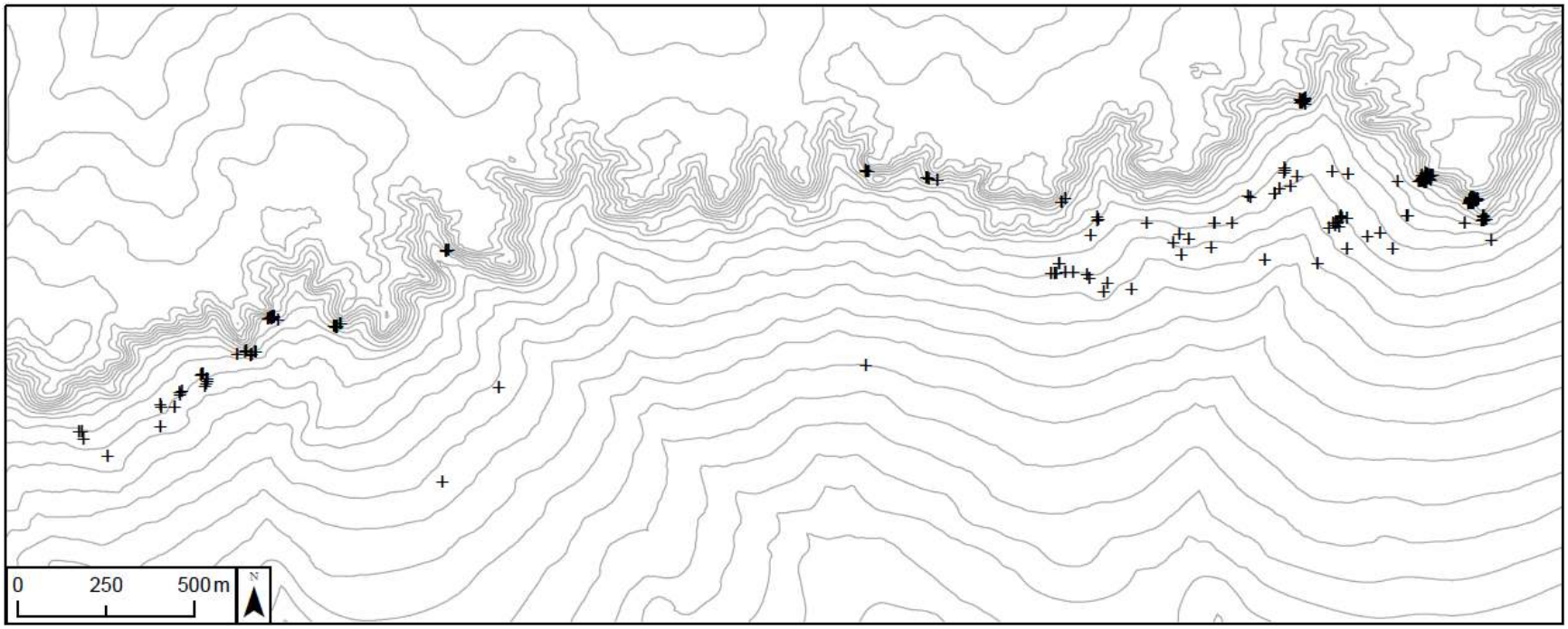

Figure 1.2. Location of sampled *O. harringtonii* individuals at Comanche National Grasslands. Contour lines convey elevation changes in 3 m increments.

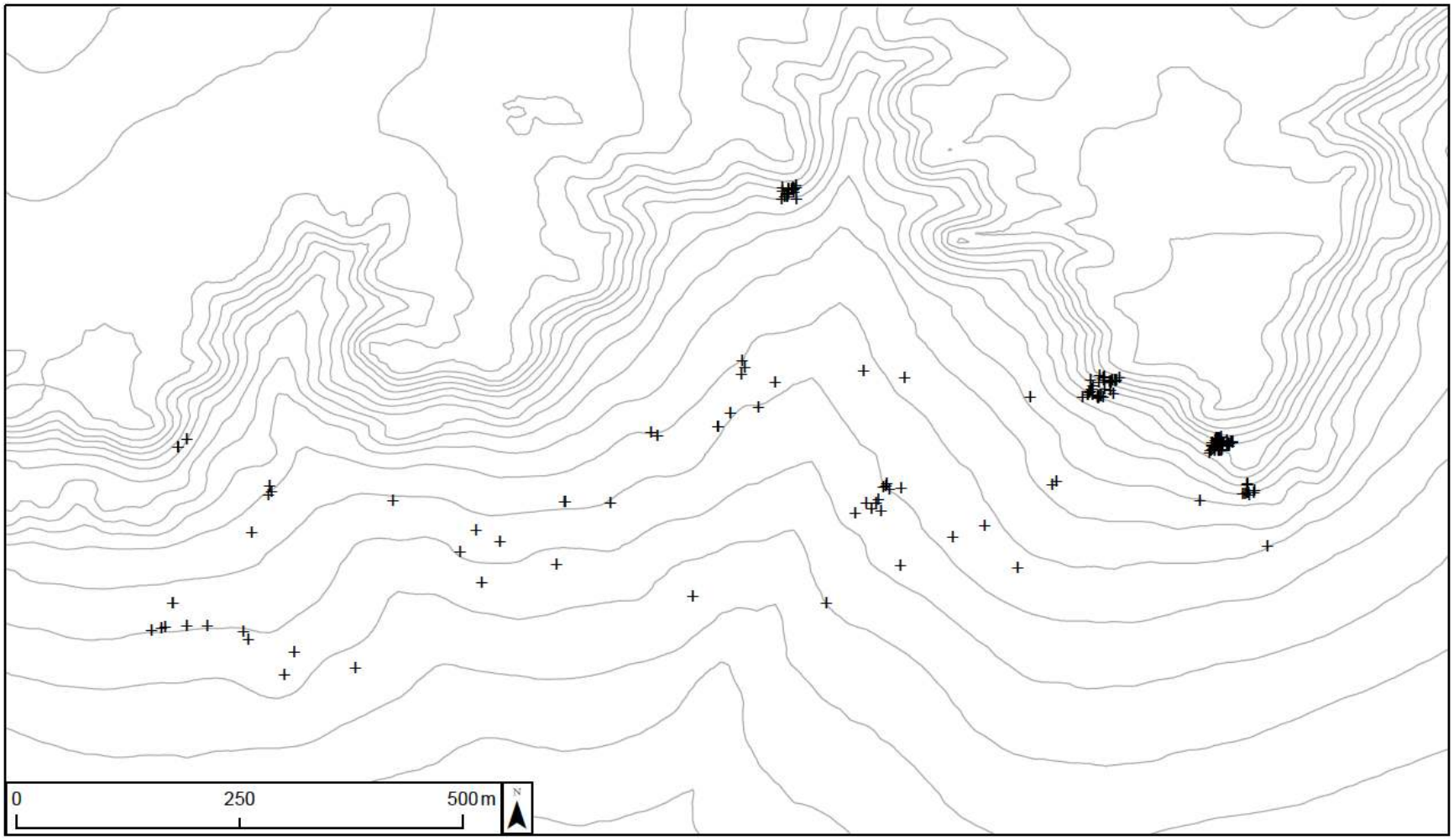

Figure 1.3. Location of *O. harringtonii* individuals included in the anisotropic SGS analysis and MRDM models.

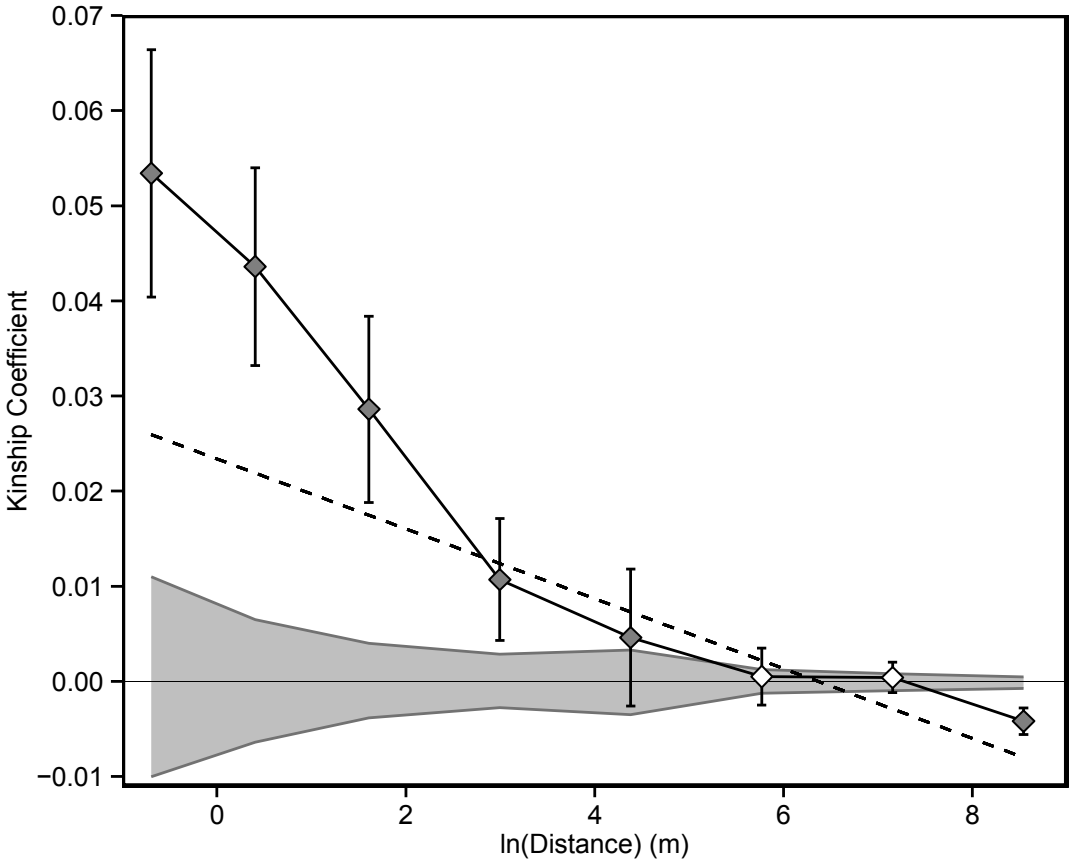

Figure 1.4. Isotropic SGS correlogram for *O. harringtonii* at Comanche National Grasslands. Average kinship coefficients for each distance class are represented by diamond symbols, with error bars spanning ± twice their standard error. The gray area portrays the 95% confidence interval about the null hypothesis of no SGS, and the dotted line illustrates the population-wide regression slope. Open symbols indicate the absence of significant differences from the null hypothesis of no SGS as determined from 10,000 permutations of individuals among spatial locations.

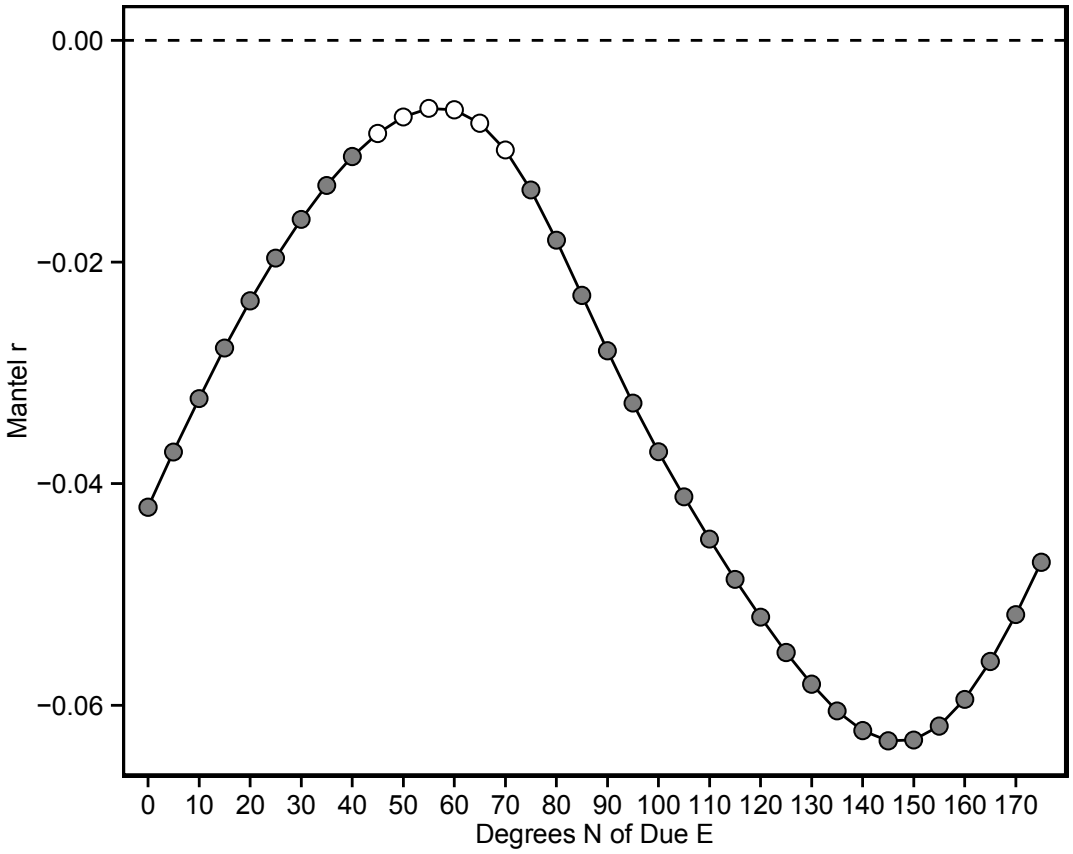

Figure 1.5. Anisotropic SGS period diagram for *O. harringtonii* at Comanche National Grasslands. Open symbols represent insignificant Mantel correlations ($P > 0.05$) as determined from 999 permutations.

CHAPTER TWO

LINKING TEMPORAL VARIATION IN POLLINATOR IDENTITY TO REPRODUCTIVE DYNAMICS AND POLLEN DISPERSAL IN *Oenothera harringtonii* (ONAGRACEAE)

Abstract

Animal pollinators exert strong influence over plant reproductive processes, but different floral visitors often vary in their effectiveness as pollinators for a given plant. Here I compare the relative influence of two functionally distinct pollinator groups on reproductive dynamics and pollen dispersal in *Oenothera harringtonii* (Onagraceae), a narrowly distributed herb whose sphingophilous flowers are visited by both nocturnal hawkmoths and diurnal bees. I combined a pollinator exclusion experiment with molecular analyses in which I genotyped 323 adults and 359 offspring to estimate mating system parameters and reconstruct pollen dispersal events through paternity analysis. Hawkmoths facilitated greater maternal fitness and pollen donor diversity than bees, consistent with differences in their morphology and behavior. However, there was no difference in the average distance of pollen movement between pollinator groups. Paternity analysis revealed a strongly leptokurtic pattern of pollen dispersal in which most pollen dispersal was spatially restricted (~10 m) but punctuated by infrequent long-distance (> 2 km) pollination events, resulting in large average pollen dispersal distances (~130 – 260 m) for an insect-pollinated herb. These findings highlight the importance of pollinator morphology and behavior in shaping pollination dynamics, underscoring the need to consider specific attributes of different pollinators, and further suggest that any changes in the relative abundance of these pollinators may have significant consequences for reproductive dynamics in this system.

Introduction

Mating plays a central role in the dynamics of natural populations, as patterns of mating have immediate consequences for individual fitness as well as the transmission and distribution of genes through time and space. In flowering plants, reproduction is shaped by a suite of ecological, morphological, demographic and genetic factors that influence the movement and fate of pollen (Barrett and Eckert 1990). This often involves interactions with animal pollinators (Eriksson and Bremer 1992; Kearns et al. 1998; Ollerton et al. 2011), as the immobile habit of plants necessitates dependable agents of pollen dispersal (Harder and Barrett 1996; Barrett 2003). Indeed, some 88% of angiosperm species rely on animals for the pollination services they provide (Ollerton et al. 2011). However, a common observation of flowering plants is that they attract highly diverse pollinator assemblages (Ollerton 1996; Waser et al. 1996). Moreover, these assemblages often consist of multiple functional groups that differ in their relative effectiveness as pollinators of a given plant species (Herrera 1987; Wilson and Thomson 1991; Armbruster et al. 2000; Fenster et al. 2004; Ollerton et al. 2006). Quantifying variation in the effectiveness of different floral visitors is therefore crucial to understanding the ecology and evolution of plant-pollinator interactions (Schemske and Horvitz 1984; Frick et al. 2013).

The pollination literature is replete with studies documenting the relative effectiveness of different floral visitors. These studies have demonstrated numerous functional consequences of diverse pollinator assemblages, for example variation in pollen transfer and seed set (Jennersten and Morse 1991; Fleming et al. 1996; Groman and Pellmyr 1999; Young 2002) as well as fluctuations in outcrossing rates and pollen dispersal (Young 2002; Barthelmess et al. 2006; Brunet and Sweet 2006; Brunet and Holmquist 2009). These patterns are generally thought to reflect underlying variation in pollinator morphology and behavior (e.g. Wilson and Thomson

1991; Young et al. 2007). For example, the mechanical fit between flower and pollinator can have pronounced impacts on pollination dynamics (Muchhala 2007), as can pollinator grooming behavior and the distances over which pollinators forage for floral rewards (Thomson 1986; Brunet and Holmquist 2009). Accordingly, specific attributes of different pollinators warrant attention when considering their relative influence in a given system, and may come to bear heavily on realized reproductive dynamics (Young et al. 2007).

One limitation of such studies is that they tend to focus on single aspects of plant reproduction, namely the influence of distinct pollinators on seed set. While this is a crucial component of plant reproduction, it neglects finer details of mating dynamics and gene flow processes that may vary with pollinator identity and have significant population- and range-wide consequences. For example, the diversity of pollen donors represented in seed crops may have important implications for offspring performance by increasing the likelihood of favorable gene combinations (Yasui 1998; Whittingham and Dunn 2006; Karron et al. 2012) and shaping the genetic relatedness of siblings (Ritland 1989). Similarly, the spatial extent of pollen-mediated gene flow will shape population genetic structure (Wright 1931), genetic neighborhood sizes (Wright 1946; Levin and Kerster 1968) and the extent of inbreeding (Nason and Ellstrand 1995; Griffin and Eckert 2003; Zhao et al. 2009; Hirao 2010), but is rarely partitioned among different functional groups of pollinators (but see Young 2002; Barthelmess et al. 2006). More comprehensive and nuanced insights may therefore be gained by combining multiple approaches to dissect plant-pollinator interactions (Karron et al. 2012).

Here, I separate the influence of two functionally distinct pollinator guilds and compare their relative impacts on fitness, mating dynamics and pollen dispersal in *Oenothera harringtonii* (Onagraceae), an annual, self-incompatible herb whose flowers are visited by both nocturnal

hawkmoths and diurnal bees. This dichotomy in pollinator identity constitutes an important functional distinction in this system. These pollinator groups differ in their time of activity, morphology and foraging behavior, and are therefore likely to differ in their effectiveness as pollinators for *O. harringtonii*. As large-bodied, nectar-foraging animals, hawkmoths are expected be effective pollinators for tubular or trumpet-shaped flowers that produce abundant nectar and have floral tubes similar in length to moth probosces (Raguso et al. 1996). Further, hawkmoths are known long-distance dispersers (Stockhouse 1973; 1976) and do not groom while foraging (Brunet and Holmquist 2009). In contrast, bees are much smaller-bodied than hawkmoths and are known to groom while they forage primarily for pollen (Hargreaves et al. 2009). With these morphological and behavioral differences in mind, I expect that hawkmoths are more effective agents of reproduction and pollen dispersal for *O. harringtonii*. Specifically, I tested three hypotheses: (1) hawkmoths facilitate greater maternal fitness than solitary bees, (2) hawkmoths alleviate inbreeding (mating among relatives) and increase mate diversity (number of pollen donors) relative to solitary bees, and (3) hawkmoths transport pollen over greater distances than solitary bees. To evaluate these hypotheses, I combined a selective pollinator exclusion experiment with molecular analyses in which genetic markers were used to estimate mating system parameters and reconstruct individual pollination events.

Methods

Study species and sampling

The Colorado Springs evening primrose, *Oenothera harringtonii* W. L. Wagner, Stockh. & W. M. Klein (Onagraceae), is an annual, self-incompatible herb endemic to the grasslands of south-central and southeastern Colorado (Wagner et al. 1985). Its flowers open shortly after

sunset and exhibit many characteristics of sphingophily, among them a broad, white corolla, long, narrow floral tube with abundant nectar, and heavy, sweet fragrance dominated by oceminoids and linaloids (Baker 1961; Fægri and van der Pijl 1971; Wagner et al. 1985; Skogen unpublished data; Figure 2.1). While a given plant may produce many flowers over the course of the flowering season (late April through early June), individual flowers open for just a single night. Stigmas remain receptive and available for pollination until the following morning, at which point flowers turn pink and wilt in the mid-morning sunlight. Hawkmoths (Sphingidae) are the primary floral visitors to *O. harringtonii*, and typically appear shortly after flowers begin to open in the evening. Solitary bees from the families Apidae and Halictidae have also been observed visiting in the morning to collect pollen before floral senescence (Skogen unpublished data). Fertilized ovaries develop a single loculicidal capsule that matures and dehisces during July and August, releasing up to 100 gravity-dispersed seeds.

This study was conducted in the Comanche National Grasslands (USDA Forest Service) near La Junta, CO, USA (N 37.566782, W 104.299439), in a grassland landscape encompassing approximately 300 ha. Exhaustive surveys conducted in May 2012 located 323 potentially co-flowering individuals (Figure 2.2), from which leaf tissue was collected, stored in coin envelopes and dried with silica gel for subsequent DNA extraction and microsatellite analysis. All plants were georeferenced to a precision of ~10 cm using a Trimble GeoXH 2005 and mapped using ArcMap 10 (ESRI, Inc.).

Pollinator observation

In this system, it has been shown that floral visits occurring between dusk and dawn are dominated by hawkmoths while bees dominate those occurring after dawn (Skogen unpublished

data). To verify this pattern, pollinator observations were conducted concurrently with a pollinator exclusion experiment. A total of 29 observation hours were completed by six observers between May 14th and 17th, 2012 (12 morning observation periods from 7:30 to 8:30 and 17 evening observations from 19:30 to 20:30). During observation periods, each observer watched a discrete group of plants in which all visits to *O. harringtonii* flowers were recorded, noting the identity of the visitor and the number of flowers visited. Floral visitors were then classified into one of two functionally distinct pollinator groups (*sensu* Fenster et al. 2004), hawkmoth or solitary bee, and the abundance of each group was calculated as the average number of visits per flower per hour. Differences in the relative abundance of each pollinator group during morning and evening observation periods were assessed with non-parametric Wilcoxon rank-sum tests in R 2.15.0 (R Development Core Team 2012).

Pollinator exclusion

A pollinator exclusion experiment was conducted at the David's Canyon site during three consecutive nights from 14 – 16 May, 2012. Three exclusion treatments were applied to a total of 214 flowers on 83 different plants: night exclusion (NE) day exclusion (DE), and open-pollinated control (C). For the NE treatment, flowers buds likely to open that evening were bagged prior to 18:00 and bags were removed by 7:00 the following morning, before diurnal pollinators were active. For the DE treatment, flowers remained open until 7:00 the following morning and were then bagged before diurnal pollinators were active. New, clean bags were used for all exclusions to avoid unintentional pollen transfer, and all exclusion bags were removed once flowers had fully senesced. For the control treatment, flowers were left unmanipulated and were available to both nocturnal and diurnal pollinators. A total of 55 flowers received the NE treatment, 65

flowers received the DE treatment, and 94 flowers received the C treatment. Sixteen maternal plants received all three treatments. The ovaries of each treatment flower were tied off with colored electrical wire to prevent seed loss as the fruits matured, as capsules dehisce upon maturation. Each treatment had a unique wire color, and wires were also secured to each flower's subtending leaf to facilitate relocation. Mature fruits were collected from maternal plants in August 2012 and stored in coin envelopes at rom temperature.

Fruits were then processed to determine whether maternal fitness differed between pollination exclusion treatments. In *O. harringtonii*, capsule length is strongly and positively correlated with the number of viable seeds in closed, open-pollinated fruits (Pearson $r = 0.72$; $P < 0.0001$; Figure 2.3). Maternal fitness was therefore defined as the length of mature capsules, which circumvents the confounding effect of capsules that dehisced and released seeds prior to being collected. One-way ANOVA was used to evaluate the impact of pollinator exclusion on maternal fitness, and differences in maternal fitness between exclusion treatments were assessed with a post-hoc Tukey HSD test in R (R Development Core Team 2012).

DNA extraction and genotyping

DNA isolation and genotyping of parental plants were detailed in Chapter 1, and comprise all maternal plants and putative pollen donors included in the parentage study. To obtain DNA from offspring (seeds), embryos and cotyledons were excised from mature seeds after soaking them between two pieces of wet filter paper in a sealed petri dish for 48 h. Offspring tissue was then dried overnight with silica gel, after which DNA was isolated following the same CTAB protocol used for parental plants, modified from Doyle and Doyle

(1987). DNA was obtained from up to eight seeds per treatment fruit from 16 maternal plants that received all three exclusion treatments, which yielded 359 offspring samples.

Simulations of parentage analyses were then conducted in CERVUS 3.03 (Marshall et al. 1998; Kalinowski et al. 2007) to determine the optimal combination of microsatellite markers with which to genotype offspring. Simulations were based on observed allele frequencies in the parental dataset and were parameterized as follows: 10,000 simulated offspring, 350 candidate fathers, 95% of candidate fathers sampled, genotyping error rate of 1%, and a minimum of six typed loci. After repeating these simulations with different combinations of the microsatellite markers described in Skogen et al. (2012), a set of seven markers was identified that maximized the resolving power of simulated paternity tests (Table S.1); offspring were therefore assayed at these loci. Microsatellite regions were amplified in two 10-μL multiplex PCR reactions containing 5 μL 2x MyTaq Mix (Bioline, Taunton, Massachusetts, USA), 1.875 μL DNA-grade H_2O, 1 μL genomic DNA, 0.125 μL of each of two blue primers, 0.25 μL of each green primer, 0.5 μL of each black primer and 0.125 μL 2x BSA. Thermal conditions for multiplex PCR reactions were the same as those used for parental plants, and PCR products were scored and fragment lengths verified following the same procedure described in the previous chapter. Genetic variation was assessed by estimating the following summary statistics separately for parents and offspring: number of alleles per locus (A), effective number of alleles (A_e), expected (H_e) and observed (H_o) heterozygosity, and average inbreeding (F_{IS}) using GenAlEx 6.4 (Peakall and Smouse 2006). Single- and multilocus estimates of exclusionary power were obtained using CERVUS 3.03 (Marshall et al. 1998; Kalinowski et al. 2007).

Estimation of mating system parameters

Offspring genotypes were first analyzed to determine whether mating system dynamics differ between pollinator exclusion treatments. Two mating system parameters were estimated using the maximum-likelihood methods implemented in MLTR 3.3 (Ritland 2002): biparental inbreeding ($s_b = t_m - t_s$; the prevalence of mating among relatives) and correlated paternity (r_p; the probability that pairs of offspring from a given fruit were fathered by the same pollen donor). The number of effective pollen donors per family (N_{ep}) was estimated as the reciprocal of r_p (Smouse et al. 2001; Buehler et al. 2012). Families were defined as the group of seeds from each pollinator exclusion treatment per maternal line, such that each maternal line had three families, and statistical confidence for parameter estimates was determined with 1,000 bootstraps of individuals within families. Two of the 16 maternal lines were excluded from the mating system analysis owing to unbalanced sample sizes between treatments. Differences in mating system parameters between exclusion treatments were assessed through pairwise comparisons of bootstrap estimates, following Eckert and Barrett (1994). A significant difference between two treatments was accepted if 97.5% (two-tailed test, $\alpha = 0.05$) of the differences between randomly paired bootstrap estimates were greater or less than zero (*sensu* Steenhuisen et al. 2012). This technique was also used to determine whether biparental inbreeding estimates differed significantly from zero (one-tailed test, $\alpha = 0.05$).

Paternity assignment and pollen dispersal

Seed paternities were resolved using the maximum-likelihood method implemented in CERVUS 3.0.3 (Marshall et al. 1998; Kalinowski et al. 2007). To identify the most likely father, CERVUS first calculates the natural logarithm of the likelihood-odds ratio (LOD) for each

candidate father. A positive LOD indicates that an individual is more likely to be the father than one randomly selected from the population, whereas a negative LOD score indicates the opposite. The candidate male with the highest LOD score is the most likely father. CERVUS then evaluates statistical support for parentage assignments by measuring the difference in LOD scores (Delta) between the most likely and second-most likely fathers and comparing this value to a critical value (the Delta criterion) that is determined by simulation and reflects user-defined confidence requirements. For example, simulations can identify the value of Delta at which 95% of Delta scores exceeding that value come from the distribution of Delta scores for true parents. In this case, any candidate father with a Delta score greater than this critical value would be assigned paternity with 95% confidence.

Paternity analyses were conducted using the same simulation parameters previously described, with relaxed and strict confidence levels set at 80% and 95%, respectively. After resolving seed paternities, effective pollen dispersal distances were calculated as the straight-line distance between georeferenced maternal plants and most-likely fathers, following Buehler et al. (2012). Inter-parent distances were derived from a pairwise distance matrix calculated using SPAGeDi 1.3 (Hardy and Vekemans 2002). One-way ANOVA was used to evaluate the impact of pollinator exclusion on pollen dispersal distance, and realized pollen dispersal distances were transformed as $log_{10}(x + 1)$ prior to analysis to stabilize variances and improve homoscedasticity.

Results

Pollinator observation

Hyles lineata (Sphingidae) was the only observed hawkmoth visitor to *O. harringtonii* flowers during the study period, and solitary bees from the following four genera were also

observed: *Agapostemon*, *Anthophora*, *Halictus* and *Lasioglossium*. Hawkmoths were more frequent visitors (2.35 ± 0.50 visits per flower per hour, mean ± SE; Figure 2.4) than were solitary bees (0.13 ± 0.08 visits per flower per hour) during evening observations ($W = 275$, $P < 0.0001$). Conversely, solitary bees were more abundant (1.11 ± 0.40 visits per flower per hour) than hawkmoths (0.08 ± 0.03 visits per flower per hour) during morning observations ($W = 24.5$, $P = 0.004$).

Pollinator exclusion and maternal fitness

I recovered 84 open-pollinated control fruits, 58 day-excluded fruits and 51 night-excluded fruits from the pollinator exclusion experiment. Pollinator exclusion had significant effects on maternal fitness (one-way ANOVA; $F_{2,190} = 6.47$, $P = 0.0019$), and maternal fitness (measured as fruit length) was significantly reduced when nocturnal pollinators were excluded relative to both open-pollinated and day-excluded flowers (Tukey HSD; $P_{C\text{-}NE} = 0.001$, $P_{DE\text{-}NE} = 0.04$; Figure 2.5). Maternal fitness of day-excluded flowers was intermediate but did not differ from the control treatment (Tukey HSD; $P_{C\text{-}DE} = 0.62$). Using the regression of seed count against fruit length (Figure 2.3), observed differences in fruit length translate to a fitness consequence of approximately 27 - 33% in terms of seed set for bee-pollinated flowers relative to hawkmoth-pollinated and open-pollinated flowers, respectively.

Genetic variation

All microsatellite loci displayed considerable polymorphism in both adults and offspring, with the number of alleles per locus ranging from 8 to 38 in parental plants and from 5 to 30 in the offspring (Table 2.1). Twenty-eight alleles were private to the parents, and there were no

private alleles in the offspring. Genetic diversity was similarly variable in both groups, with expected heterozygosity ranging from 0.429 to 0.957 in adults and from 0.386 to 0.949 in the offspring (Table 2.1). Inbreeding was minimal across loci in both groups, with mean $F_{IS} = 0.028$ and 0.020 for adults and offspring, respectively (Table 2.1). These markers yielded a combined parent-pair exclusionary power of 0.99999984, indicating high resolving power for paternity analysis.

Mating system dynamics

Biparental inbreeding was minimal and did not differ significantly from zero in open-pollinated progeny arrays ($s_b = 0.024$, $P = 0.111$; Table 2.2). Day-excluded progeny arrays were weakly but significantly inbred ($s_b = 0.037$, $P = 0.029$; Table 2.2), as were night-excluded progeny arrays ($s_b = 0.069$, $P < 0.0001$; Table 2.2). Biparental inbreeding did not differ between any of the treatment groups. Correlated paternity was lowest in open-pollinated progeny arrays ($r_p = 0.257$) and highest in night-excluded arrays ($r_p = 0.558$), constituting a significant difference and translating to approximately twice as many pollen donors in open-pollinated flowers relative to night-excluded flowers ($P < 0.0001$; Table 2.2). Correlated paternity and pollen donor diversity was intermediate in day-excluded flowers ($r_p = 0.387$) but did not differ from other treatment groups (Table 2.2).

Paternity assignment and pollen dispersal

Of the 359 offspring genotypes analyzed with CERVUS, 292 (81%) paternity tests were resolved with strict (95%) confidence and 24 (7%) were resolved with relaxed (80%) confidence. Paternity was not resolved for the remaining 43 (12%) offspring. High-confidence (95%)

paternity assignments were obtained for 98 open-pollinated seeds, 95 hawkmoth-pollinated seeds, and 99 bee-pollinated seeds, and pollen dispersal analyses were conducted using these high-confidence assignments only. The probability of successful paternity assignment did not vary between exclusion treatments (GLM; $\chi^2 = 2.45$, df = 2, 356, $P = 0.29$).

Effective pollen dispersal distances ranged from < 1 m to > 2.5 km. The average distance of pollen dispersal was lowest in open-pollinated flowers, highest in hawkmoth-pollinated flowers and intermediate in bee-pollinated flowers (Figure 2.6), but average pollination distances did not differ between exclusion treatments (one-way ANOVA; $F_{2,292} = 1.49$, $P = 0.23$). Pollen movement was spatially restricted across all exclusion treatments and followed a strongly leptokurtic distribution, with most pollen movement (48 – 57%) occurring within 10 m of maternal plants (Figure 2.7). Long-distance (> 2 km) pollination events were detected in all three treatments (Figure 2.7), with approximately twice as many of these events in hawkmoth-pollinated flowers (n = 7) than in bee-pollinated flowers (n = 3). However, the probability of long-distance pollen dispersal did not vary between treatment groups (GLM; $\chi^2 = 3.71$, df = 2, 356, $P = 0.16$).

Because some hawkmoth visits were observed during morning hours (Figure 2.4), I also considered a possible scenario in which long-distance (> 2 km) pollination events in the bee pollination treatment (n = 3) were actually the result of hawkmoth visits. In this scenario, pollinator exclusion had significant effects on pollen dispersal (one-way ANOVA; $F_{2,289} = 3.84$, $P = 0.02$), and average pollen dispersal distances were significantly higher under hawkmoth pollination than bee pollination (Tukey HSD; $P_{DE\text{-}NE} = 0.02$; Figure 2.6). Similarly, the probability of long-distance pollen dispersal varied significantly between treatment groups in this scenario (GLM; $\chi^2 = 15.57$, df = 2, 356, $P = 0.0004$).

Discussion

Functionally and temporally distinct pollinators were found to drive associated temporal variation in pollination dynamics in *Oenothera harringtonii*. Consistent with expectations, nocturnal pollinators (hawkmoths) were more common floral visitors and facilitated greater maternal fitness and pollen donor diversity than diurnal pollinators (solitary bees), likely reflecting differences in pollinator morphology and foraging behavior. While I found no differences in the spatial extent of pollen dispersal facilitated by different pollinator guilds, paternity analysis revealed a strongly leptokurtic pattern of pollen movement characterized by abundant dispersal over short spatial scales (~10 m) and infrequent but consistent long-distance (> 2 km) pollination events. Collectively, these results suggest that hawkmoths make the primary contribution to reproduction in *O. harringtonii* and demonstrate the potential for landscape-scale gene flow in this system.

Pollinator identity and plant reproduction

The results of the selective pollinator exclusion experiment suggest that nocturnal pollinators (hawkmoths) are more effective than diurnal pollinators (solitary bees) at facilitating seed set in *O. harringtonii*, consistent with its sphingophilous pollination syndrome. This result is likely explained by morphological and behavioral differences between these pollinator guilds, specifically relating to their physical size, the floral resources to which they are attracted and their behavior when visiting flowers. As hawkmoths are relatively large-bodied animals, they should, on average, have greater contact with the plant's reproductive organs than smaller-bodied pollinators. Further, *Hyles lineata* frequently approach the flowers of *O. harringtonii* by inserting

their heads into the floral tubes while probing for nectar, which maximizes their contact with the flowers' strongly exserted anthers and stigma (Rhodes pers. obs.). Consequently, hawkmoths should accumulate more pollen on their bodies and deposit more pollen on stigmas than smaller-bodied pollinators, and ultimately fertilize more ovules. This is consistent with previous observations of hawkmoth pollination in other *Oenothera* species, in which it was noted that hawkmoths are typically positioned directly in the center of the cradle of stamens when visiting, resulting in extensive pollen accumulation and deposition (Gregory 1963, 1964). In contrast, the bees observed in this system are physically much smaller and forage for pollen as their primary floral reward in addition to nectar. As a result, bees can land on anthers and gather pollen freely without making contact with the stigma and are thereby less likely to facilitate effective pollen transfer (Gregory 1963, 1964). In this sense, the solitary bees in this system may frequently act as pollen thieves, which may be especially prominent in flowers expressing strong herkogamy (Hargreaves et al. 2009). It is thus likely that physical and behavioral differences between pollinators figure prominently in the observed variation in maternal fitness. Taken together, these results contribute to a growing body of literature highlighting the importance of the mechanical fit between flowers and pollinators as a determinant of pollination dynamics (Muchhala 2007).

Mating system analysis also indicates that hawkmoths facilitate greater mate diversity than solitary bees by delivering pollen from approximately twice as many pollen donors. This likely reflects physical differences between these pollinator groups as well as other behavioral distinctions, namely the extent of their foraging ranges and whether or not pollinators groom pollen from their bodies. Previous studies have demonstrated the capacity for long-distance foraging by hawkmoths, with *Hyles lineata* flights having been documented over hundreds of meters to tens of kilometers in a single night (Stockhouse 1973, 1976). Conversely, the solitary

bees we observe in this system are more central-place foragers, with typical foraging ranges for certain *Anthophora* and *Lasioglossum* species having been reported as up to 100 and 500 meters, respectively (Rau 1929, 1931; Batra 1984; Greenleaf et al. 2007). If one assumes a relatively continuous distribution of flowers, pollinators with broader foraging ranges should encounter more flowers and therefore remove and deposit pollen from a greater number of pollen donors (e.g. Campbell 1998). Variation in pollinator foraging range may therefore contribute to observed differences in pollen donor diversity, but it is unlikely that this would completely explain realized mating dynamics in this system. Another important distinction lies in pollinator grooming behavior, which can bear heavily on pollen transfer dynamics (Wilson and Thomson 1991). The solitary bees observed in this system collect pollen and groom (Gregory 1963, 1964; Rhodes pers. obs.) whereas hawkmoths do not (Brunet and Holmquist 2009; Rhodes pers. obs.). Grooming behavior should result in greater pollen removal and lower pollen deposition on a per-visit basis (Castellanos et al. 2003) and also cause more rapid declines in pollen carryover relative to non-grooming floral visitors (Thomson 1986; Harder and Wilson 1998; Castellanos et al. 2003). This should ultimately reduce the number of sires that contribute to offspring arrays (Campbell 1998; Brunet and Holmquist 2009). Accordingly, it is likely that observed differences in pollen donor diversity are strongly influenced by this behavioral distinction.

Few studies to date have established direct links between pollinator activity and realized mating patterns in the flowers they visit, and fewer still have characterized mating dynamics beyond the outcrossing-selfing paradigm (Barrett 2003; Mitchell et al. 2009; Karron et al. 2012). I am aware of only one prior study that has explicitly considered how different pollinators contribute to plant mate diversity, in which it was found that hawkmoths and bumblebees did not differ (Brunet and Holmquist 2009). Pollinator visitation rates may explain this discrepancy.

Multiple sires can result either from single visits from pollinators carrying diverse pollen loads or multiple visits from pollinators carrying less diverse pollen loads (Marshall and Ellstrand 1985; Campbell 1998; Mitchell et al. 2005). Pollinators carrying less diverse pollen could therefore facilitate similar levels of pollen donor diversity by accounting for more floral visits than those carrying pollen from more potential sires (Brunet and Holmquist 2009). Brunet and Holmquist (2009) found no differences in the relative visitation rates of hawkmoths and bumblebees. However, a portion of their night pollination treatment was characterized by low temperatures at which hawkmoth activity is less common (Brunet and Holmquist 2009). If the bumblebees in their system were active throughout the entire daytime pollination treatment (~11 hours), it is possible that flowers were actually subject to more visits from bumblebees than hawkmoths despite the similar visitation rates inferred from pollinator observation. This may account for the similar levels of pollen donor diversity between pollinator groups in their study, especially considering that the flowers of their focal organism stay open and receptive for up to six days (Brunet and Holmquist 2009). In contrast, the daytime pollination treatment in my study was much shorter (~5 hours), as *O. harringtonii* flowers only last for 14 – 16 hours, opening at sunset and senescing before noon the following day. Day-pollinated flowers in my study are therefore likely to have received fewer visits overall than those in Brunet and Holmquist's (2009) study, which may explain the opposing results between these studies. Interestingly, this suggests that floral longevity may have contributed to the observed differences in pollen donor diversity in the present study, prompting the question of whether my results are driven primarily by pollinator behavior or are simply an artifact of limited daytime floral availability. This question warrants further consideration, but would require comparisons of pollen donor diversity facilitated on a single-visit basis.

Pollinator identity and pollen dispersal

Although the exclusion experiment and mating system analyses revealed clear differences between hawkmoths and bees, paternity analysis showed no significant differences in the spatial extent of pollen dispersal facilitated by different pollinator guilds. This contradicts previous work that has demonstrated significantly larger pollen dispersal distances under hawkmoth pollination relative to bees (Young 2002). This discrepancy may be driven by the low-frequency hawkmoth visits observed during morning hours (Figure 2.4). Consequently, all realized pollen dispersal in this treatment cannot be definitively ascribed to bees. If hawkmoths and bees do in fact differ in their foraging ranges and ability to disperse pollen, even a few longer-distance pollination events facilitated by hawkmoths during the morning would greatly inflate the average distance of pollen dispersal attributed to bees in this study. Indeed, if one assumes the pollination events above 2000 m in the bee treatment (n = 3) were actually from hawkmoths, the pollen dispersal data show statistically and biologically significant differences (Figure 2.6). Considering that typical foraging distances for some of the bees observed in this system are between 100 and 500 m (Rau 1929, 1931; Batra 1984; Greenleaf et al. 2007) and that many bees have been active for short periods of time at this point in the day, I suspect that long-distance pollen dispersal during morning hours may well be the result of hawkmoth visits. Overlap in pollinator visitation may therefore prevent the detection of meaningful differences in pollen dispersal between pollinator groups. Alternatively, it is possible that this study did not encompass a large enough area for any underlying differences in pollinator foraging range to be realized. Given that previously reported hawkmoth flight distances span tens of kilometers (Stockhouse 1973, 1976)

and one *Anthophora* species was found to have a maximum foraging distance of 2.3 km (Rau 1929, 1931; Greenleaf et al. 2007), this possibility also warrants consideration.

While I found no effect of pollinator identity on pollen dispersal, paternity analysis still provided useful insights into general patterns of pollen dispersal in *O. harringtonii*. Most pollen movement occurred on restricted spatial scales, typically within ~10 m of maternal plants, regardless of exclusion treatment. This is consistent with a general view of pollen dispersal in animal-pollinated species being strongly leptokurtic, with the bulk of pollen dispersal occurring over short distances (Levin and Kerster 1974). I also found evidence of infrequent but consistent long-distance (> 2 km) pollination events, confirming the presence of landscape-scale gene flow via pollen in this system. While these long-distance events comprised only a small portion (2 – 7%) of realized pollination events, it is important to note that my study spanned a comparably short portion of the flowering season. Considering that *O. harringtonii* flowers for 2 – 3 months, populations likely experience many of these long-distance pollination events during the course of an entire growing season. This view is consistent with weak spatial genetic structure observed in the study population (Chapter 1). Notably, the maximum pollination distance revealed through paternity analysis (2,736 m) approaches the maximum possible distance of pollen movement given the location of sampled individuals at our study site. It is therefore possible that pollen movement occurs over even larger spatial scales than indicated in this study. This would be consistent with low levels of range-wide population genetic differentiation in *O. harringtonii* (Skogen et al. in prep.). Collectively, these results suggest that pollen dispersal in this system is highly variable, but the ecological factors that govern these dynamics remain unclear.

Numerous studies have documented long-distance pollen dispersal in temperate and tropical trees (Ashley 2010), but few have utilized parentage techniques in herbaceous species,

particularly on landscape-level spatial scales. The average pollination distances found in this study (~130 – 260 m) are substantially higher than previous parentage studies of herbaceous species, which typically find average pollen dispersal distances on the order of single to tens of meters (Godt and Hamrick 1993; Tero et al. 2005; Ishihama et al. 2006; Llaurens et al. 2008; Van Rossum et al. 2011; Scheepens et al. 2012; Matter et al. 2013). The maximum observed pollination distances (> 2.7 km) are also considerably larger than those previously reported for insect-pollinated herbs, which rarely exceed 1 km (e.g. Buehler et al. 2012). Indeed, my results are more in line with what has been found in wind-pollinated herbs (e.g. Fénart et al. 2007) and many lower-density insect-pollinated trees (e.g. Chase et al. 1996; Hoebee et al. 2007; Lourmas et al. 2007; Ottewell et al. 2012). These findings underscore the importance of long-distance events in overall pollen dispersal patterns and contribute to a relatively meager literature documenting the potential for landscape-scale gene flow in insect-pollinated herbs.

Implications for O. harringtonii

Overall, my findings emphasize the role of hawkmoths in the reproductive ecology of *O. harringtonii*, demonstrating that these pollinators are crucial to plant fitness and mate diversity. By extension, these results imply that any fluctuations in hawkmoth abundance would have pronounced impacts on this species' reproduction (Raguso and Willis 2003), which may be of particular relevance in light of ongoing pollinator decline (e.g. Kearns et al. 1998). There have been anecdotal reports of local hawkmoth decline in the western United States, tentatively linked to pesticide use in vineyards, having caused subsequent pollen limitation and reduced seed set in another narrow endemic *Oenothera* species (*O. deltoides* ssp. *howellii*) (Buchmann and Nabhan 1996). This case may therefore provide a glimpse into future reproductive dynamics in *O.*

harringtonii if hawkmoths were to decline throughout its range in Colorado. While pesticide use is comparatively much less widespread in these parts of Colorado, other anthropogenic factors may give reasonable cause for concern. Artificial light pollution has long been known to disrupt the behavior of nocturnal insects (Frank 1988), and has been implicated as a driver of recently documented moth declines throughout Europe (Conrad et al. 2006; Groenendijk and Ellis 2010; Fox 2012). Moreover, moth attraction to low-wavelength artificial lighting is correlated with morphology, disproportionately affecting moths with larger body mass, wing dimensions and eyes (van Langevelde et al. 2011). Considering their large size, the hawkmoths we observe at *O. harringtonii* (*Hyles lineata* and *Manduca quinquemaculata*) may therefore be especially prone to the effects of light pollution, suggesting the potential for cascading effects on pollination dynamics in this system (e.g. van Langevelde et al. 2011). This possibility is further magnified by development pressure throughout much of *O. harringtonii*'s range, as human population growth in this region is proceeding at some of the fastest rates in the country (Spackman Panjabi 2004). Although *Hyles lineata* is one of the most widespread and abundant hawkmoth species in North America (Raguso et al. 1996), the possibility for local or regional declines driven by anthropogenic factors warrants attention. In the case of *O. harringtonii*, such a decline would likely have dire consequences for reproductive dynamics, potentially threatening long-term population persistence.

Conclusions

This study demonstrates how manipulative field experiments can be paired with molecular techniques to gain more nuanced insights into plant reproductive dynamics. Temporally and functionally distinct pollinators were found to drive associated temporal

variation in pollination dynamics in *O. harringtonii*, with hawkmoths facilitating greater maternal fitness and pollen donor diversity than solitary bees. While most realized pollen dispersal was spatially restricted and did not vary with pollinator identity, pollen-mediated gene flow still occurred over multiple kilometers, which likely minimizes genetic differentiation both within and between populations of *O. harringtonii*. Collectively, these findings highlight the importance of pollinator morphology and behavior in shaping pollination dynamics, reiterating the need to consider how specific attributes of individual pollinators influence plant reproductive processes. More broadly, these findings imply that any extrinsic drivers of variation in the relative abundance of these pollinator guilds over broader spatial or temporal scales may have predictable and significant consequences for reproductive dynamics in this system.

Table 2.1. Genetic summary statistics for n = 323 adults and n = 359 offspring included in parentage analysis: number of alleles per locus (A), effective number of alleles (A_e), expected heterozygosity (H_e), observed heterozygosity (H_o), and average inbreeding (F_{IS}).

	Adults					Offspring				
Locus	A	A_e	H_e	H_o	F_{IS}	A	A_e	H_e	H_o	F_{IS}
D102	8	3.5	0.716	0.679	0.052	7	2.9	0.650	0.695	-0.069
C106	38	23.2	0.957	0.881	0.080	30	19.2	0.949	0.847	-0.106
OB2	13	6.1	0.837	0.869	-0.038	9	6.1	0.837	0.837	0.000
D111	35	13.3	0.925	0.902	0.025	26	10.9	0.909	0.883	0.028
D2	8	3.9	0.747	0.728	0.026	7	3.6	0.720	0.681	0.055
D118	9	1.8	0.429	0.419	0.022	5	1.6	0.386	0.373	0.033
D5	27	11.8	0.915	0.892	0.026	26	10.4	0.904	0.915	-0.013
Average	**19.7**	**9.1**	**0.789**	**0.767**	**0.028**	**15.7**	**7.8**	**0.765**	**0.747**	**0.020**

Table 2.2. Maximum likelihood estimates of mating system parameters: biparental inbreeding (s_b), correlated paternity (r_p) and the number of effective pollen donors (N_{ep}). Estimates for biparental inbreeding and correlated paternity are reported with their standard deviations.

		Parameter		
Treatment	n	s_b	r_p	N_{ep}
Open-pollinated	126	0.024 (0.019)	0.257 (0.055)	3.9
Hawkmoth (DE)	121	0.036 (0.019)	0.387 (0.086)	2.6
Bee (NE)	123	0.069 (0.020)	0.558 (0.076)	1.8

Figure 2.1. Watercolor image of *O. harringtonii* by Jeremie Fant.

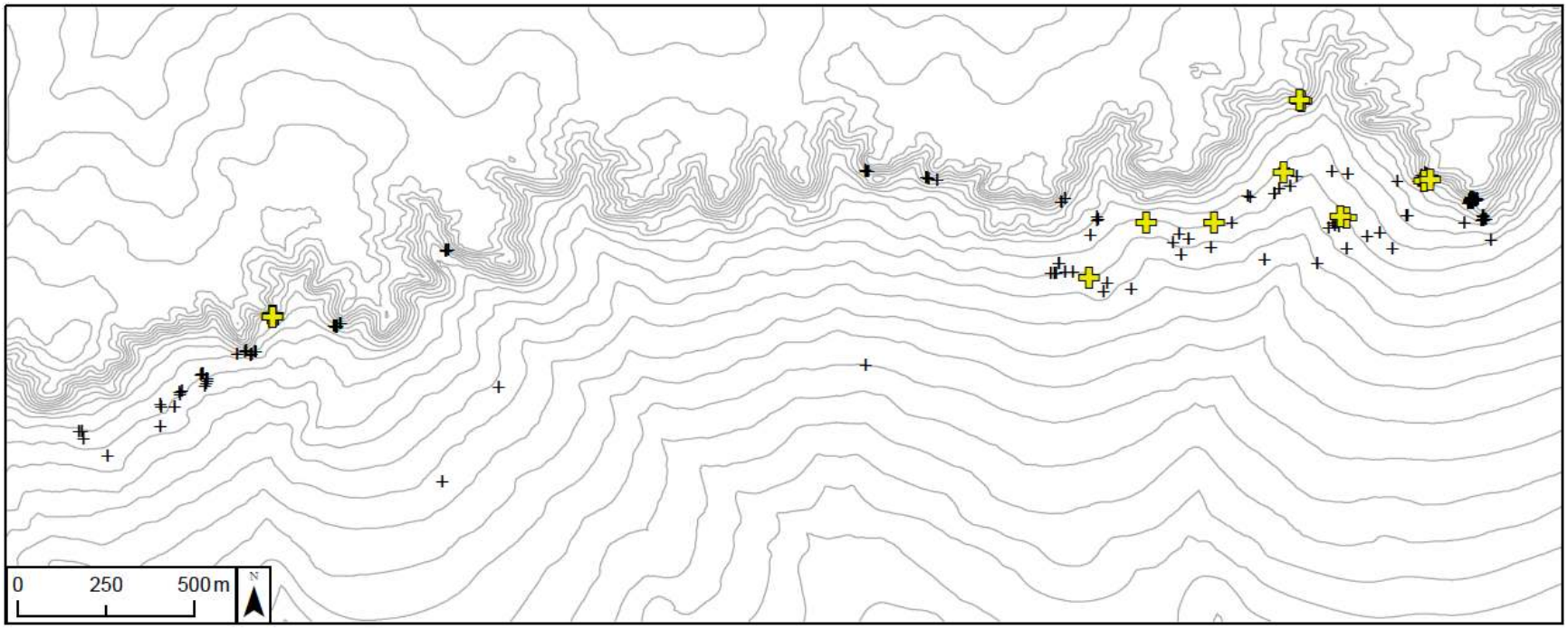

Figure 2.2. Distribution of sampled *O. harringtonii* individuals at Comanche National Grasslands. Yellow crosses indicate maternal plants whereas black crosses indicate possible pollen donors. Contour lines convey elevation changes in 3 m increments.

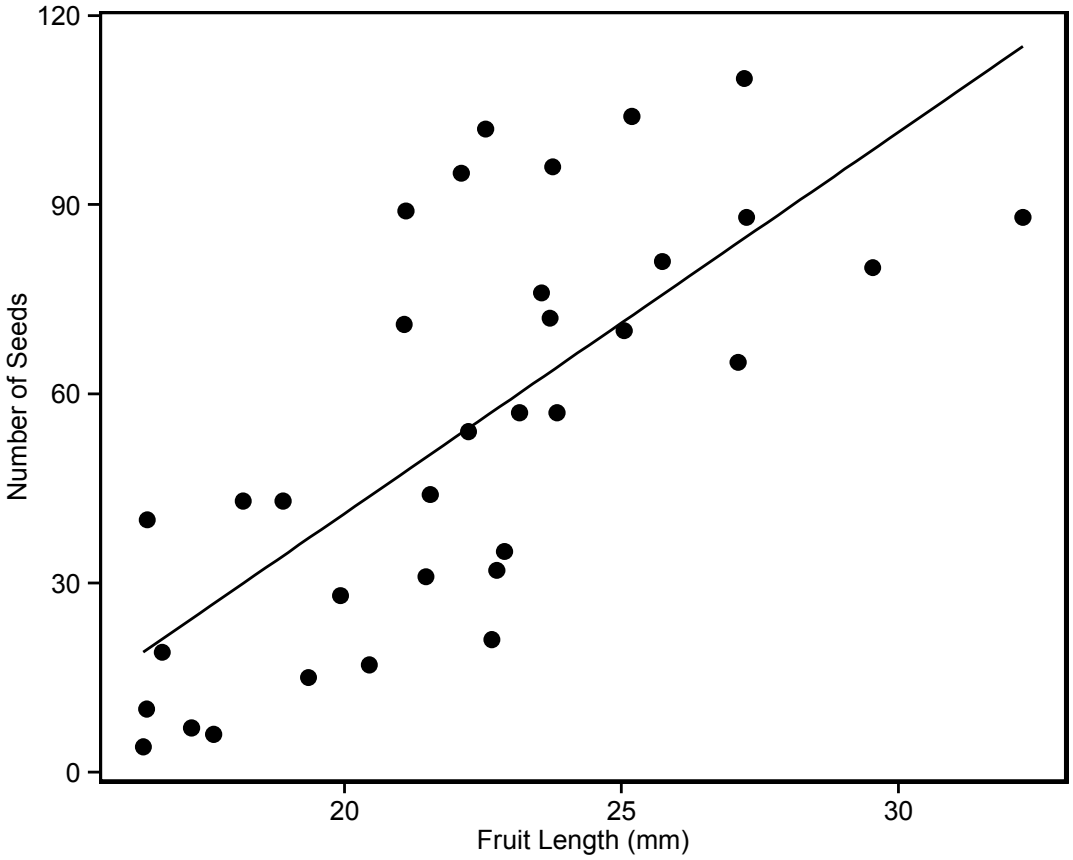

Figure 2.3. Relationship between fruit length and seed set in *O. harringtonii*: number of seeds per fruit is positively correlated with capsule length. Data were collected from open-pollinated fruits that had not released any seeds (n = 34).

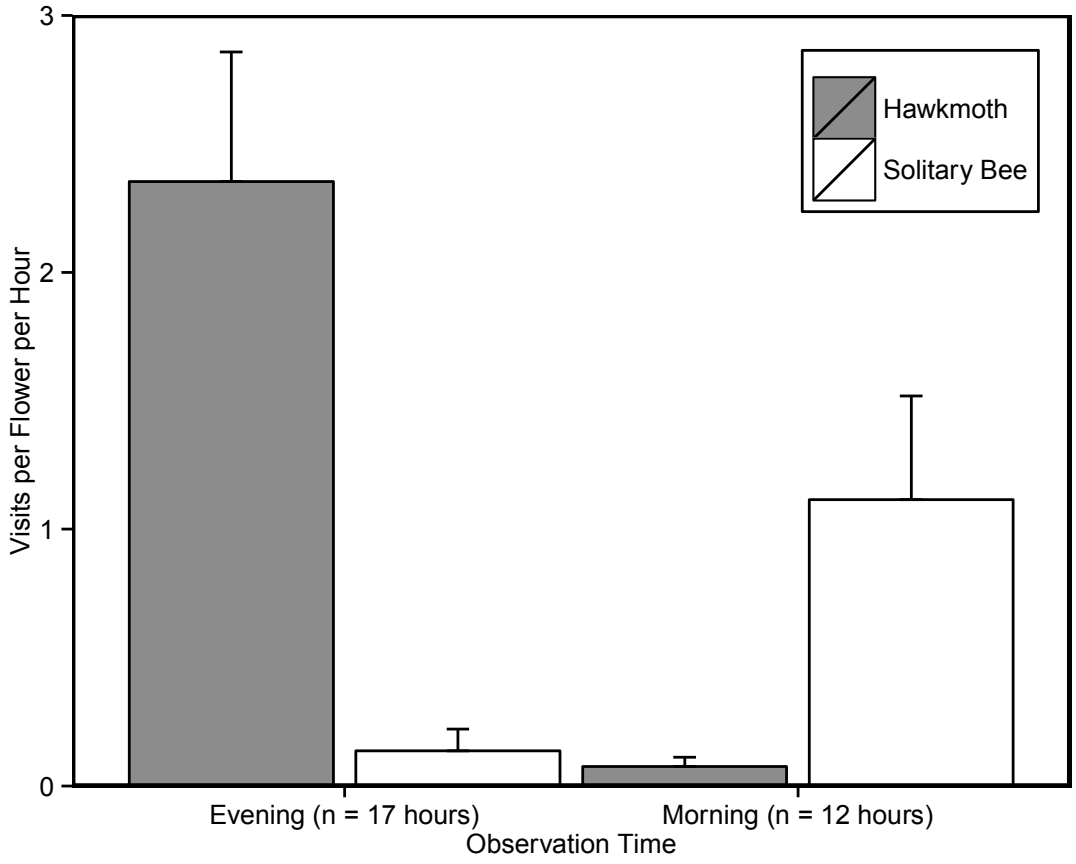

Figure 2.4. Visitation frequency of different pollinator groups during the pollinator exclusion experiment, given as the average number of pollinator visits per flower per hour of observation.

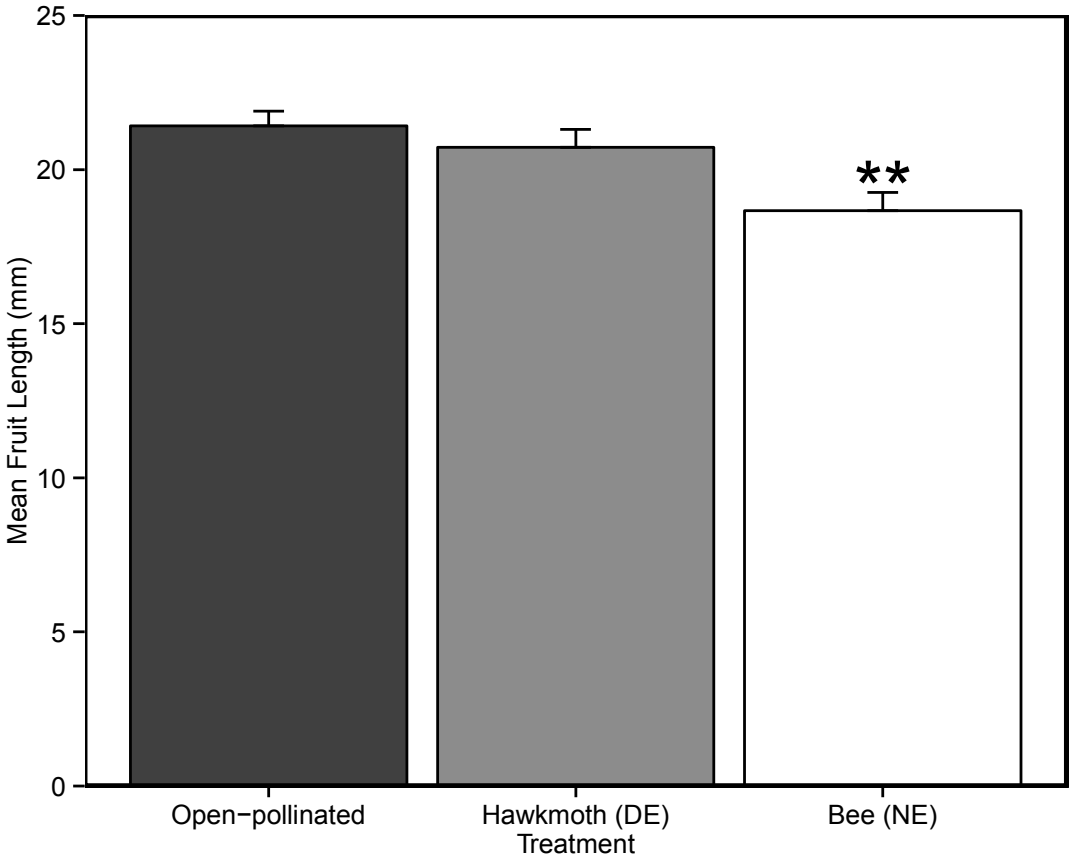

Figure 2.5. Influence of pollinator identity on maternal fitness as measured by average fruit length (mean + SE). Maternal fitness, measured as mean fruit length, is significantly lower in the bee pollination treatment relative to both open-pollinated and hawkmoth-pollinated fruits as determined by a post-hoc Tukey HSD test.

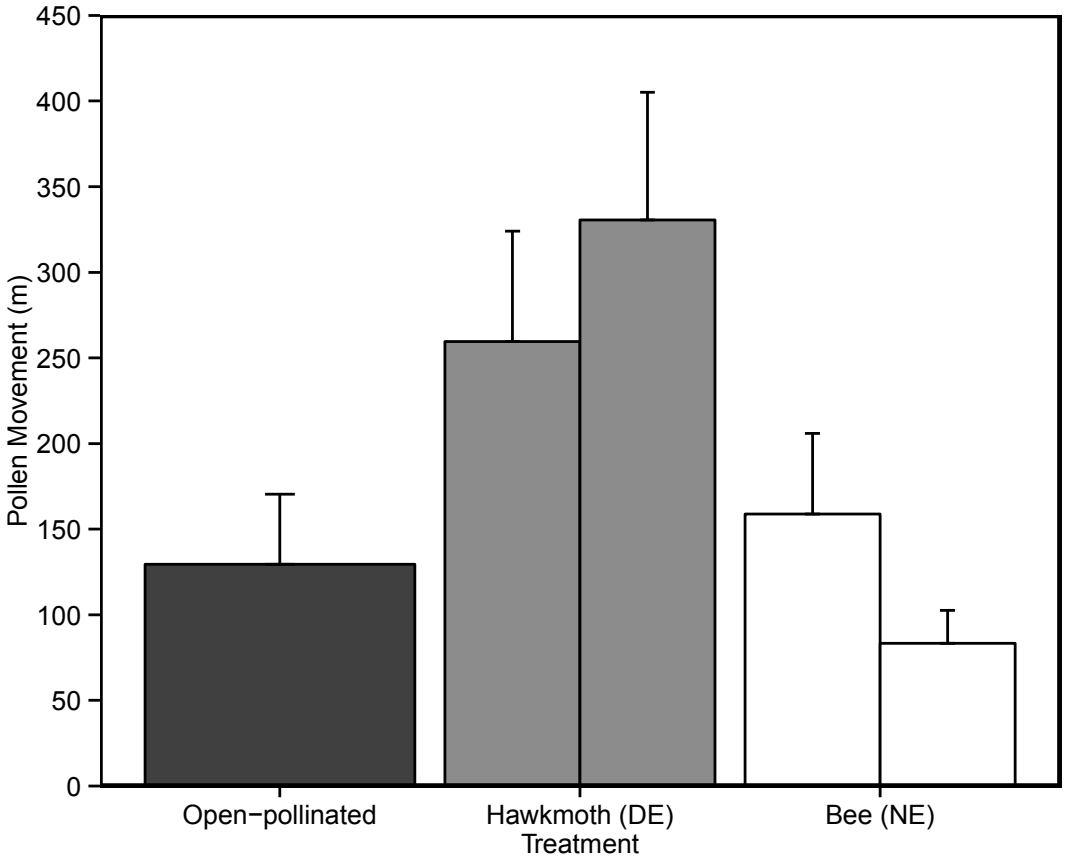

Figure 2.6. Average pollination distances (mean + SE) for each pollinator exclusion treatment as revealed by paternity analysis. Data were log-transformed for analysis, but untransformed data are shown for clarity. Within the hawkmoth and bee treatments, the bars on the left represent the actual data, and the bars on the right represent the average dispersal distances for the scenario in which long-distance pollen dispersal (> 2 km) in the bee treatment is ascribed to hawkmoth visits.

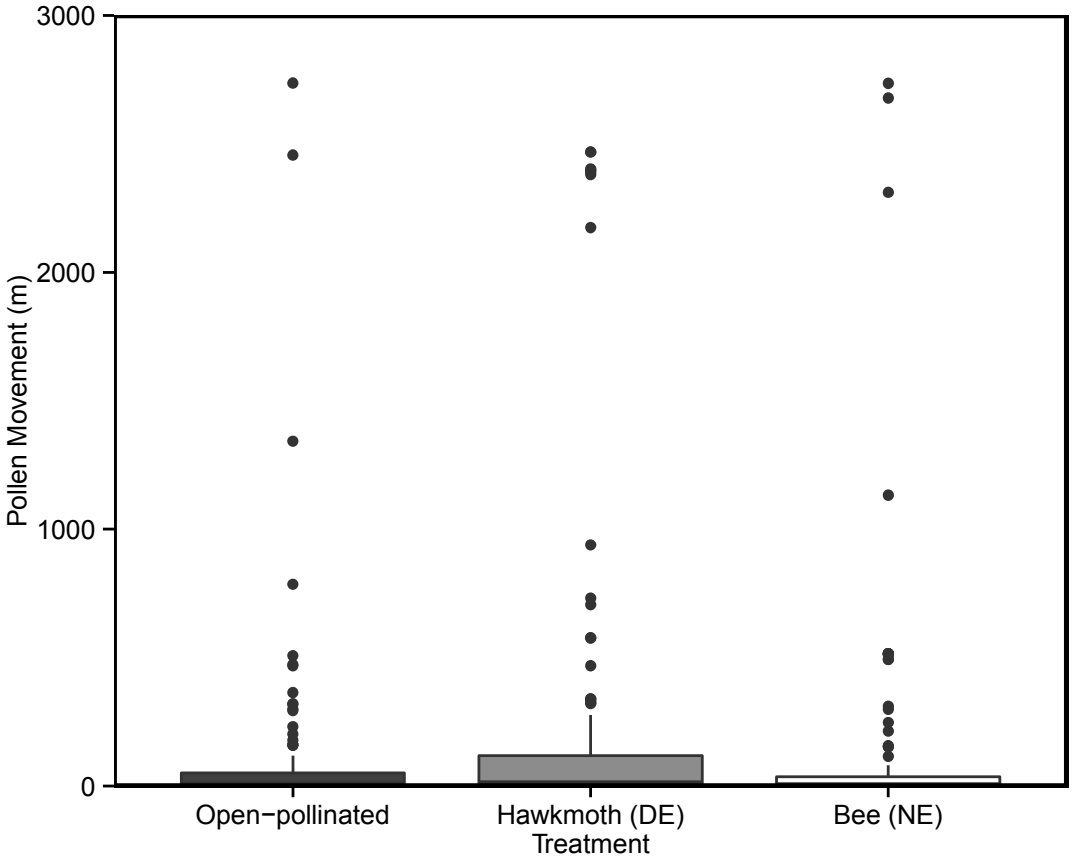

Figure 2.7. Boxplot of realized pollen dispersal distances for each pollinator exclusion treatment.

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APPENDIX

Supplementary Information

Raster images & Simulations of parentage analysis

Below, I have included raster images from which topographic data were extracted for the landscape genetic analysis in Chapter 1. I have also included output from simulations performed in CERVUS 3.03 that guided the selection of microsatellite markers for the parentage study in Chapter 2. The text output details various parameters that estimate the power of these markers for paternity analysis. Below the text output is a histogram of simulated parentage assignments that illustrates the frequency of correct vs. incorrect assignments and their associated delta scores.

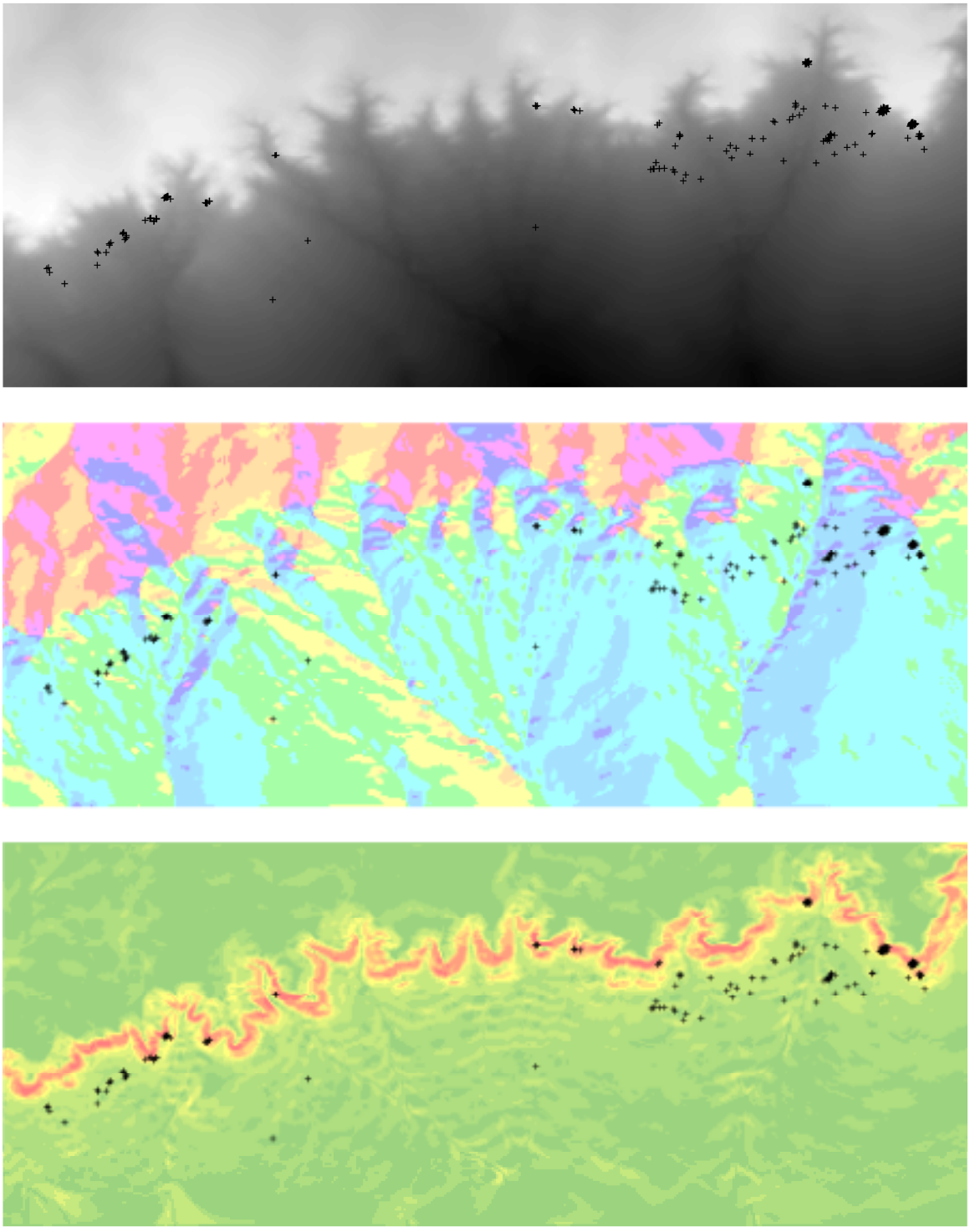

Figure S.1. Raster images for elevation, aspect and slope showing landscape heterogeneity

Table S.1. Results of parentage analysis simulations in CERVUS 3.03 assuming known maternal genotypes. Simulations were based on allele frequencies in the parental dataset and were parameterized as follows: 10000 simulated offspring, 340 candidate fathers, 95% of candidate fathers sampled, genotyping error rate of 1%, and a minimum of four typed loci. Loci included are: OB2, C106, D102, D2, D118, D5, and D111.

Summary Statistics

Level	Confidence (%)	Critical Delta	Assignments	Assignment Rate
Strict	95.00	0.53	9452	95%
Relaxed	80.00	0.00	9791	98%
Unassigned			209	2%
Total			10000	100%

Delta Distributions

Most likely candidate	N	Mean Delta	Std. Dev.
True father	9116	8.13	2.83
Non-father (true father sampled)	371	1.15	1.05
Non-father (true father unsampled)	304	1.86	1.45
None	209		
Total	10000		

Parentage Assignments

Most likely candidate	Confidence Level		None
	Strict	*Relaxed*	
True father	8980 (95%)	9116 (93%)	
Non-father (true father sampled)	231 (2%)	371 (4%)	
Non-father (true father unsampled)	241 (3%)	304 (3%)	
Total	9452	9791	209

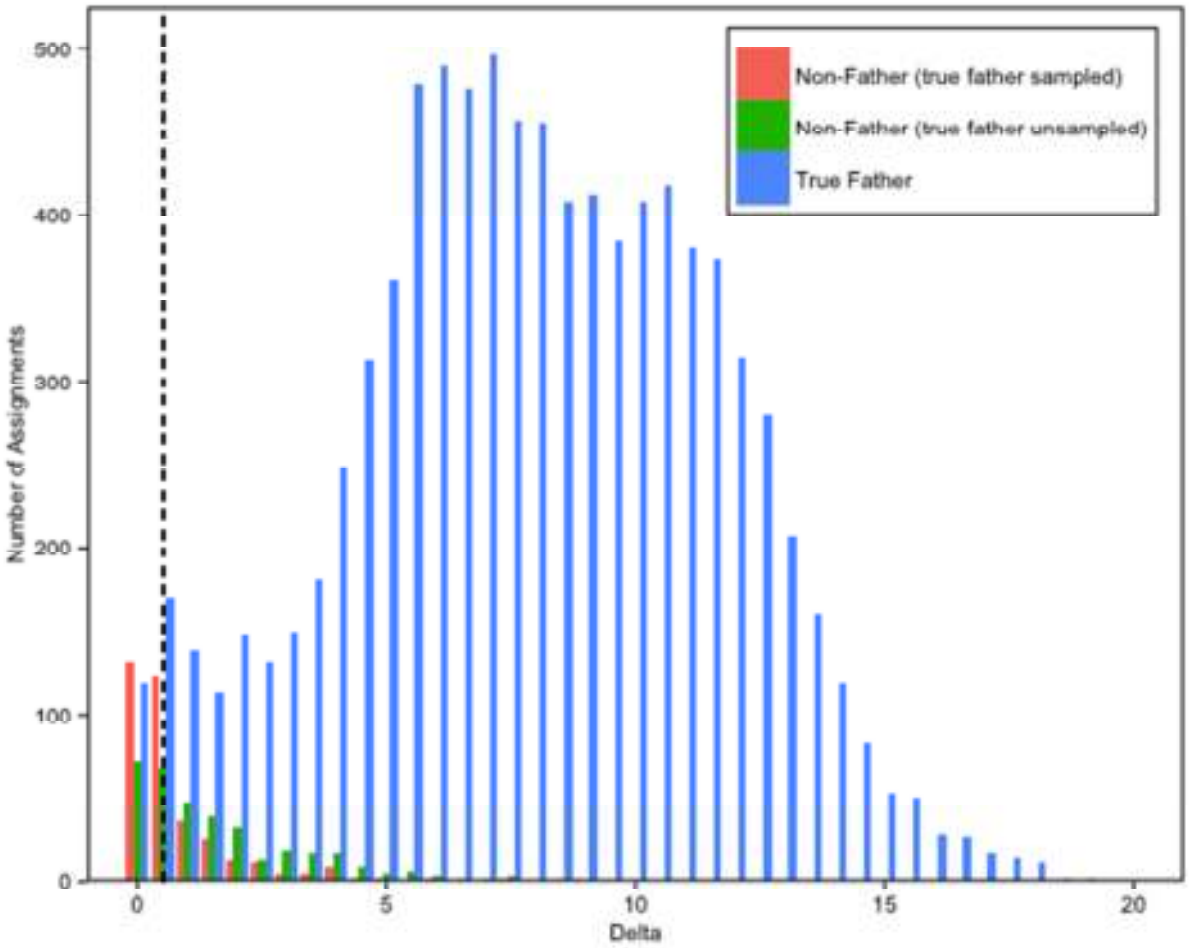

Figure S.2. Frequency histogram of simulated parentage assignments assuming known maternal genotypes. The dashed line is drawn at Delta = 0.53, the point at which 95% of Delta scores exceeding that value come from the distribution of Delta scores for true fathers.