UNIVERSITY OF CALIFORNIA

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# Bat pollination, genetic structure and gene flow in *Crescentia alata* trees in Western México

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy in Biology

by

Pamela Grace Thompson

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ABSTRACT OF THE DISSERTATION

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Professor Victoria L. Sork, Chair

Bats are important pollinators of flowering trees in the tropics. Tropical forests are facing increasing threats from fragmentation, but it is unclear how this affects pollinators, or gene flow in bat-pollinated trees. The goals of this dissertation are to evaluate the impact of forest fragmentation on the abundance of pollinating bats, the reproductive success of trees, and contemporary pollen flow in a bat-pollinated tropical tree species, *Crescentia alata.* We also describe the genetic structure of this plant species, to better understand if the loss of its theorized seed disperser in the Pleistocene has caused restricted seed dispersal. To assess the impact of forest fragmentation, we sampled bat pollinator abundance and seedling genotypes in sites in continuous and fragmented forest, in the area of the Chamela-Cuixmala Biosphere Reserve, in Jalisco, México. We also sampled adult trees at a slightly larger geographic scale. We developed neutral molecular markers for this species and employed spatial genetic structure analyses to

understand gene flow patterns. We hypothesized that adult populations of *Crescentia alata* have high genetic structuring due to the loss of its seed disperser, but that seedlings will show low pollen pool genetic structure, resulting from high gene flow from the long distance foraging movements of bats. We found evidence that bat abundance is a function of floral display, and seems unaffected by forest fragmentation around flowering trees. Fruit-set was higher in fragmented forest sites, as was pollen flow, although the majority of diversity was found at the level of fruit, rather than at the level of site (sub-population).There was little evidence for restricted seed dispersal among adults, which may be due to high pollen flow or seed dispersal by seasonal floods. These findings contradict the idea that pollen flow is reduced by forest fragmentation, and suggest that bats are moving pollen widely across the landscape, and carrying genetically diverse pollen loads to each flower. These results highlight the important role bat pollinators play in maintaining genetic connectivity among plant populations.

The dissertation of Pamela Grace Thompson is approved.

Thomas Bates Smith

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Walter Helmut Metzner

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# DEDICATION

For my sister, Kimberly Thompson. She loved all animals, especially the nocturnal, and unfairly maligned. I am sure she would have been a strong champion for bats.

And for my first mentor, Mary-Joan Dean, who taught me how to be a empathetic member of the Animal Kingdom.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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Thompson PG, Smouse PE, Scofield D, and Sork VL. (2014) What seeds tell us about birds: A multi-year analysis of foraging behavior of acorn woodpeckers on two oak species. *Movement Ecology* **2**:12.

## TALKS

Thompson PG and Sork VL. Bat-mediated genetic connectivity of *Crescentia alata* populations. 16th International Bat Research Conference and 43rd North American Symposium on Bat Research, San Jose, Costa Rica, 8/14/13. (Invited)

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POSTERS

Thompson P, Scofield D, Grivet D, Smouse PE and Sork VL. Foraging Patterns of Acorn Woodpeckers at Sedgwick Reserve, as Seen Through Acorn Movement. UCSB Natural Reserve System Day, Santa Barbara, CA, 2/8/13.

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# Chapter 1

## Introduction: Forest fragmentation, nectar-feeding bats, and bat-pollinated plants

Animals are critical vectors of pollen dispersal for many plant species in tropical forest ecosystems (Bawa, 1990). As tropical forests are transformed by human activities into mosaics of urban areas, agricultural and ranching land, and forest remnants, plant populations become subdivided into fragments (Saunders, Hobbs, and Margules, 1991; Laurance et al., 2002). Fragmentation may change the distribution, abundance and flowering phenology of plants, and their pollinators may likewise be affected, such that their abundance and pollinating behavior and efficiency is altered (Kearns, Inouye, and Waser, 1998; Kremen et al., 2007). These altered interactions may then feed back on one another, causing a loss of pollination function (Knight et al., 2005). Indeed, pollen limitation (where plants receive insufficient pollen to set fruit) appears to be a primary contributor to the reduced reproductive success for plants in forest fragments (Aizen and Feinsinger, 1994; Aguilar et al., 2006). Along with reduced reproductive success, forest fragmentation may also negatively influence the amount of genetic material moving around, which can lead to inbreeding and ultimately jeopardize tree population sustainability (Ellstrand and Elam, 1993; Young, Boyle, and Brown, 1996). Over time, the combination of these two factors can lead to local extinctions of plant and pollinator species, whose interactions form the basis for reproductive health of forest trees. Because humans in the tropics are heavily dependent on forests for food, medicinal products, timber, and ecotourism, understanding the factors that influence forest health is of utmost importance.

Bats are important pollinators in the tropics, with the capacity to move pollen far across the landscape (Bawa, 1990). Due to this ability, there is an untested assumption that pollinating bats are not affected by forest fragmentation (Fleming, Geiselman, and Kress, 2009). Although some bat species have been known to travel long distances to forage (Horner, Fleming, and Sahley, 1998), and cross pastures and forest fragments during foraging trips (Law and Lean

1999), studies of bat-pollinated trees suggest that plant reproductive success may be compromised in disturbed habitats due to less frequent visits by bat pollinators (Quesada et al. 2003). The direct impact on gene flow and genetic connectivity for these trees is unclear, however.

México is an ideal location to study the impacts of forest fragmentation on plant-pollinator interactions. México produces more species of plants cultivated for human consumption than all of the countries in the European Union combined, and over 80% of these species depend on animal pollinators to successfully reproduce (Ashworth et al., 2009). Many different ecosystem types are found within México, but seasonally dry tropical forests (SDTF) are one of the most threatened, due to their long history of human use, conversion of the forest to agricultural areas, and susceptibility to fires (Bullock, Mooney, and Medina, 1995). An analysis of SDTF at the national level found only 27% forest cover remained by 1990, and deforestation continues at a rate of 1.4% annually (Trejo and Dirzo, 2000).

My dissertation research examines the pollination and gene flow of the seasonally dry tropical tree *Crescentia alata,* which is pollinated by nectarivorous bats. At my study site, in and around the Chamela-Cuixmala Biosphere Reserve in Jalisco, México, *C. alata* trees tend to occur in ephemerally dry streambeds called arroyos, which are open landscape elements. Depending on the land-uses around these arroyos, the *C. alata* trees can be surrounding by thick continuous forest, or more permeable fragmented forest (Figure 1-1). The goal of this dissertation is to assess the impact of the landscape around these trees on pollinating bats, and consequences for this on pollen movement and genetic connectivity of the trees. I use an ecological and landscape genetics approach to look at these questions.

In Chapter 2, I examine the abundance of bat pollinators visiting *C. alata* trees within

fragmented and continuous forest sites, by sampling the pollinator community using mist-nets placed next to flowering trees. I also compare the presence of pollen found on different bat species, and evaluate reproductive success of the *C. alata* trees at these different sites, by looking at fruit-set. My results suggest that floral display has a greater effect on numbers of bats visiting trees than the landscape surrounding the tree, and that fruit-set is higher in fragmented sites. Thus, fragmentation may indirectly affect bat movement by influencing the size of the floral display. The results in this chapter point to the importance of the ecosystem services of bats to mitigate the impact of human disturbance.

In Chapter 3, I address the question of whether my study tree species, famous for its now-extinct dispersal agents shows any negative impact of that loss of this dispersal. I describe the development of species-specific neutral genetic markers (SSRs, also known as microsatellites), and use these markers to analyze the genetic diversity and genetic structure of *C. alata* trees across different geographic scales. The findings provide evidence that the extinction of the hypothesized original seed disperser for *C. alata* fruits in the Pleistocene (Janzen and Martin, 1982) has not resulted in high genetic structure of the adult trees. These results suggest that high levels of gene flow may be caused by combined impact of bats as pollinators and seasonal floods as dispersal vectors.

For chapter 4, I assess the genetic consequences of bats as pollinators, by estimating the genetic structure of the *C. alata* pollen pools. This genetic analysis is accomplished by genotyping seedlings from fruit resulting from the same flowering season and open pollinations of flowers described in Chapter 2. I found that pollen flow appears to be higher into forest fragments, indicating that trees in fragments are not isolated from gene flow. However, I observe the paradoxical results that the region is connected, but each tree has few numbers of effective

pollinators, and the number of pollen gametes in each fruit is also low. This result is most understandable if overall allelic diversity in Crescentia alata trees is low, but bats are visiting many flowers in the same night of foraging, and are depositing pollen loads from various (genetically different) trees as they visit flowers.

Bat pollinated-plants and *Crescentia alata*

Bats pollinate over 500 species of flowering plants (Heithaus, Opler, and Baker, 1974; Fleming, Geiselman, and Kress, 2009). These plants tend to have a suite of characteristics that are attractive to bats, termed a "chiropterophilic syndrome" (van der Pijl, 1961; Faegri and van der Pijl, 1966; Dobat and Peikert-Holle, 1985). These characteristics include: a muted color, usually green, brownish-red or white; nocturnal anthesis of flowers; cauliflorous flowers (flowers that grow directly on the trunks of branches of trees); flagelliflorous flowers (flowers which hang down on thin, rope-like branches); "shave-brush" flowers (flowers with many stamens projecting outwards); and bell-shaped flowers (Faegri and van der Pijl, 1966; von Helversen and Winter, 2003; Fleming, Geiselman, and Kress, 2009). They tend to produce lots of nectar, and have an unpleasant odor, which comes from the sulfur compounds frequently found in their nectar (Knudsen and Tollsten, 1995; von Helversen, Winkler, and Bestmann, 2000). Bat-pollinated plants have significantly different sugar concentrations in their nectar compared to passerine-pollinated plants (Baker, Baker, and Hodges, 1998). A recent study on the effects of varying sugar concentrations in nectar on feeding behavior among two captive species (*Leptonycteris yerbabuenae* and *Glossophaga soricina*) indicated that these bats increased their feeding time, and reduced flight time, when sugar concentrations were lowest, indicating high sugar concentration as an adaptive trait in bat-pollinated plants that promotes rapid feeding bouts and

lots of movements between flowers (Ayala-Berdon et al., 2011).

The behavior of bats as pollinators has been shown to have large impacts on the plants they pollinate (Baker and Harris, 1957). For example, one study demonstrated competition for bat pollinators by Bombacaceous tree species served as a strong selective force for the staggered flowering phenology of these trees in dry forests in Costa Rica and México (Lobo et al., 2003). In addition, availability of bat pollinators can affect the mating system of plant species that can switch from an out-crossed to a selfing system; a study of *Ceiba pentandra* showed that populations of this tree with higher visitation rates by bat pollinators was associated with a greater proportion of out-crossed seeds, and less mean relatedness within fruits and between fruits within trees (Lobo, Quesada, and Stoner, 2005).

In our study region, bats pollinate several tree and shrub species. These include columnar cactus species (*Pachycereus pectin-aboriginum*, *Stenocereus standleyi*, *Stenocereus chrysocarpus*, and *Cephalocereus purpusii*), several trees in the Bombacaeae family (*Ceiba pentandra*, *C. grandiflora*, and *C. aesculifolia*), as well as a variety of other plants including *Cleome spinosa*, *Bahuinia paletia*, *B. ungulata*, and *Crescentia alata.*

*Crescentia alata* HBK (family Bignoniaceae, tribe Crescentiae) is a shrubby, cauliflorous tree up to 8 meters tall that is common in seasonally dry tropical forests from México to Costa Rica. There are a variety of common names for the species, including calabash (U.S.), cuastecomate (México), cirian (México) and jícaro (Costa Rica). The tree has soft bark, which permits the ready colonization of epiphytes (Yeaton and Gladstone, 1982). The flowers are hermaphroditic, pale greenish-pink-maroon, and each flower is only open for one night, during which time it produces dilute, copious nectar (Gentry, 1974b; Martínez del Río and Bullock, 1990). At our study site, in Western México, the trees flower from July-August (Lott, 2002), and

this concentrated flowering phenology is in contrast to the "steady-state" pattern Gentry described for the same species (Gentry, 1974a). A study documenting flowering phenology in a Costa Rican tropical dry forest noted that individual trees there flower asynchronously (Gordon, Baker, and Opler, 1974), and for longer time periods than those in dry forest of Chamela, Mexico (Bullock and Solis-Magallanes, 1990). During its flowering period, *C. alata* trees are capable of producing hundreds of flowers (Figure 1-2). The start of the flowering period coincides closely with the start of the rainy season. Bats are the main pollinators, and bees have been reported visiting flowers and robbing nectar; the flowers are large enough that bees do not touch the anthers and are not effective pollinators (Martínez del Río and Bullock, 1990; Tabla and Bullock, 2002).

*Crescentia alata* fruits are large gourds that start developing about one week after pollination, and remain on the tree maturing for several months to one year. Developing fruit have nectarieis on their surface, which may be an adaptation for ant attraction and protection (Elias and Prance, 1978). The fruits have hard outer husks that are difficult to open; an average of 200kg of pressure is required to break a fruit (Janzen, 1982). Each fruit contains hundreds of small winged seeds (hence the species name, *alata*) that are embedded in a sticky black pulp when mature. People in México use the pulp in a medicinal recipe for respiratory ailments (Rojas et al., 2001). The outer husk is also used for ladles, decorative bowls, and maracas (Gentry, 1992). Janzen hypothesized that Pleistocene horses were the original seed dispersers, with modern horses as substitutes, and rodents secondarily dispersing seeds from horse dung (Janzen, 1982; Janzen and Martin, 1982). Fruits are also likely dispersed by seasonal flooding of arroyos (Gentry, 1974b), but often times the fruit will collect underneath the crown of the tree and remained undispersed.

Nectar-feeding bats and pollination behavior

Bats that are highly specialized to feed on nectar can be found in two families: the Phyllostomidae, found in the New World, and the family Pteropodidae (the flying foxes) from the Old World. These bats share adaptations to extract nectar from flowers, such as an elongated rostrum, reduced dentition, and a long tongue tipped with hair-like papillae (Freeman, 1995). Pteropodid bats are larger, and tend to land and hold onto flowers while extracting nectar, while Phyllostomid bats are smaller and tend to hover while drinking (Fleming and Muchhala, 2008). The most specialized nectarivorous bat species are generally those with the longest rostrum and tongue (Winter and von Helversen, 2003), and these bats can reach nectar within flowers with extraordinarily long corollas. For example, the recently discovered nectar-bat *Anoura fistulata* in Ecuador has a tongue length of 84.9mm (150% its body length), and is the sole pollinator of the *Centropogon nigricans*, a plant with a 80-90mm corolla (Muchhala, 2006).

Phyllostomid nectar-bats use a variety of sensory modes to locate flowers, including vision, olfaction, and echolocation; several studies suggest that bell-shaped flowers and dish-shaped leaves surrounding flowers produce characteristic spectral echoes which nectar-bats can identify, and to which they respond (von Helversen and von Helversen, 1999; von Helversen, Holderied, and von Helversen, 2003; Simon, Holderied, and von Helversen, 2006). Beyond visual cues in daylight and normal color ranges, bats (particularly *Glossophaga soricina*) have been shown to have ultraviolet (UV)- sensitive cones, which may be used by nectar bats to detect UV patterning on flowers (Müller et al., 2009). In addition, they have an excellent spatial working memory; an experimental study of *Glossophaga soricina* foraging demonstrated that bats remembered the locations of up to 40 depleted floral resource patches, and avoided these

after a only a few trials (Winter and Stich, 2005).

The floral and nectar resources available to bats have strong effects on bat community structure, and bat behavior. In general, when several nectarivorous bats are in the same location, their degree of specialization on nectar, combined with body size and energy requirements, influence the plants they can and will visit (Gonzalez-Terrazas et al., 2012). However, the variation in available resources constrains the number of bat species an area can support. A study of four nectarivorous bat species in a Costa Rican lowland rainforest determined that two of the species were residents (*Glossohapa commissarisi* and *Hylonycteris underwoodii*), while the other two (*Lichonycteris obscura* and *Lonchophylla robusta*) made seasonal movements into the area when resources were high (Tschapka, 2004). The two resident bat species had different strategies for dealing with periods of low nectar resource times: *G. commissarisi* switched to a more frugivorous diet, while *H. underwoodii* flew farther and fed on low energy, scattered flowers (Tschapka, 2004). Certain feeding behaviors may come into play when resources are high, and there is some evidence that bats will aggressively defend territories around high floral resources such as blooming *Agaves* (Lemke, 1984). Flock-foraging has also been observed in some species (Howell, 1979), and Howell (1979) suggests that the spatially and temporally patchy nature of *Agave palmeri* resources drives the flocking behavior in *Leptonycteris sanborni* (currently *L. yerbabuenae)*, as this behavior increases foraging efficiency.

Bats and bat-pollinated plants vary in their response to fragmentation. In Chamela, México, two of three species of nectarivorous bats visited *Ceiba pentandra* trees significantly fewer times in disturbed vs. continuous forest sites, and trees in these disturbed sites had fewer pollen grains deposited on their stigmas and reduced fruit-set compared to trees in undisturbed areas (Quesada et al., 2003). In a similar study, Quesada et al. (2004) recorded bat visitation rates

for three different tree species (*Ceiba aesculifolia, C. grandiflora* and *C. pentandra)* in three seasonal tropical forests (Chamela, Mexico; Guanacaste, Costa Rica; Osa, Costa Rica). The authors compared bat activity between fragments and continuous forest, and measured the consequences on reproductive success and mating system of these trees. Trees in fragments produced more flowers, but lower fruit-set than continuous forest trees, although only one tree species showed significant differences between landscape types. Mating system seemed unaffected in two of the three tree species. The authors suggest the lack of consistent responses across the tree species is related to their different flowering phenologies, and the other bat-pollinated tree resources available at the time of flowering, which could impact pollinator behavior (Quesada et al., 2004).

Along the Jalisco Coast, thirty-seven species of bats have been recorded, including five species in the family Phyllostomidae that are predominantly nectarivorous: *Leptonycteris yerbabuenae* (formerly *curasoae), Glossophaga soricina, Glossophaga commissarisi, Choeroniscus godmani,* and *Musonycteris harrisoni* (Ceballos and Miranda, 2000). Characteristics for these species are described in Table 1-1. The conservation status of *L. yerbabuenae* and *M. harrisoni* is listed as "Vulnerable" by the International Union for the Conservation of Nature (IUCN), while the remaining three nectarivorous species have a status of "Least Concern" (Arroyo-Cabrales and Álvarez-Castañeda, 2008; Arroyo-Cabrales et al., 2008; Barquez et al., 2008; Miller et al., 2008; Tavares and Molinari, 2008). We consistently caught three of these species visiting *Crescentia alata* flowers during the flowering period (*Leptonycteris yerbabuenae*, *Glossophaga soricina*, and *Choeroniscus godmani*, Figure 1-3).

Lesser long-nosed bats (*L. yerbabuenae*) are large, nectarivorous bats that tend to roost in very large colonies (up to 75,000 individuals in one roost, (Ceballos et al., 1997). This bat is well

known for making annual migrations from South-Central Mexico to Northern Mexico-Southern Arizona in the spring, following the flowering of nectar-rich columnar cacti species, and returning in the early fall following blooming *Agave* species (Fleming, Nuñez, and Sternberg, 1993; Wilkinson and Fleming, 1996). However, some populations do not migrate (Stoner et al., 2003), and there is likely high variation in migratory behavior among populations at our study site. For example, a study of the population dynamics at a sea cave roost in Chamela Bay found bats occupying the cave year-round, evidence for two reproductive bouts per year, and the presence of juveniles in January, which suggests that females give birth at the site or in the nearby area, instead of strictly at maternity roosts in their northern migratory locations (Stoner et al., 2003). In addition, males of this species move secretions from their genitals to their dorsal fur to form a "patch", which has been correlated to reproductive behavior (Rincón-Vargas et al., 2013), and we have observed these patches when capturing *L. yerbabuenae* bats at flowering *Crescentia alata* trees in early July, a time when these bats are theoretically migrating back to Central-Southern Mexico. As our findings indicate that this species was significantly more likely to be captured with pollen, and with higher loads of pollen on its fur than other nectarivorous species, it is likely a highly important pollinator of *Crescentia alata* trees. Further research on the interplay of the movement patterns of this species with their pollination behavior would be helpful to fully understanding plant-pollinator dynamics in this system.

## Conclusions

This dissertation examines how bats shape the genetic structure of a famous tropical tree species. It illustrates the important ecosystem services provided by nectarivorous bats in México for fragmented ecosystems. This work also demonstrates the usefulness of genetic markers for

both the study of pollinator movement, and the analysis of the genetic consequences of forest fragmentation and animal disperser behavior. I hope this work will contribute to a greater understanding of an important plant-animal interaction, especially in tropical and subtropical ecosystems.


Figure 1-1: Examples of fragmented and continuous forest surrounding the focal sampling sites in Chapters 2 and 4. Images are compiled from remotely sensed satellite images, courtesy of Google Earth. Numbers correspond to tree IDs. Bats were mist-netted next to each of the labeled trees.

Figure 1-2: Photographs of *Crescentia alata.* From left: full view of tree and close-up of an open flower.

Figure 1-3: Photographs of the three nectarivorous bat species captured in mist-nets next to flowering *Crescentia alata* trees. From left: *Leptonycteris yerbabuenae*, *Glossophaga soricina*, and *Choeroniscus godmani*.

Table 1-1: Characteristics of the predominately nectarivorous bat species along the Jalisco Coast, México. Information from Ceballos and Miranda 2000 and the IUCN, unless otherwise specified with asterisks (*Horner et al. 1998, **Lemke 1984).

| Species | Common name | Weight (g) | Forearm (mm) | Potential foraging range | Probable roosts | Conservation status |
|---|---|---|---|---|---|---|
| *Leptonycteris yerbabuenae* | Lesser long-nosed bat | 21 | 47-56 | 30km* | Caves, tunnels, mines abandoned buildings | Vulnerable |
| *Glossophaga soricina* | Pallas's long-tongued bat | 8-12 | 30-41 | 1.45km** | Caves, culverts, tree hollows, occasional buildings | Least concern |
| *Glossophaga commissarisi* | Commissarisi's long-tongued bat | 8-14 | 33-38 | unknown | Similar to *G. soricina* | Least concern |
| *Choeroniscus godmani* | Godman's long-tailed bat | 12 | 32 | unknown | Caves, underneath banana leaves and other wide-leaved plants | Least concern |
| *Musonycteris harrisoni* | Trumpet-nosed bat | 12 | 40-43 | unknown | Caves, culverts, tree hollows, underneath banana leaves, rocky overhangs | Vulnerable |

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# CHAPTER 2

## Flowering, bat pollinator abundance, and tree reproductive success in *Crescentia alata* trees in fragments and continuous forest sites

**ABSTRACT**

*Premise of the study:* Forest fragmentation is an ongoing threat to the survival of tropical forest tree species and the animals that depend on them. Highly mobile pollinators, such as bats, that can transport pollen long distances, may mitigate the impacts of fragmentation if foraging by these animals is not inhibited by landscape change. The goal of this study is to evaluate the impact of the landscape type (fragmented or continuous forest) surrounding a bat-pollinated tree species (*Crescentia alata*) on pollinator abundance, and to evaluate whether landscape type and bat pollinator presence enhances *C. alata* reproductive success.

*Methods*: Nine sites (5 sites surrounded by continuous forest, 4 sites surrounded by fragmented forest) in the area of the Chamela-Cuixmala Biosphere Reserve, in Jalisco, México, were chosen as focal points for capturing bat pollinators and recording plant phenology. We placed mist-nets next to flowering *C. alata* trees, and recorded number and species of bats captured. We also recorded the presence and amount of pollen on bats. During the day, five *C. alata* trees per site were surveyed for flowering and fruiting over 6 weeks. We used fruit set (number of fruit produced per number of flowers) and total fruit biomass (number of fruit multiplied by average weight of fruit) as measures of tree reproductive success, and compared pollinator abundance and tree reproductive success between the landscape types.

*Results:* We caught a total of 366 nectarivorous bats, of three species, over 25 nights of mist-netting. The number of nectarivorous bats caught was significantly and positively

correlated with number of open flowers on the *C. alata* tree. The landscape type (fragmented/continuous) surrounding the site did not impact number of nectar-bats caught, although one species (*Choeroniscus godmani*) was only found in sites surrounded by continuous forest. Based on pollen presence data, we also found that lesser long-nosed bats (*Leptonycteris yerbabuenae)* are more likely to be caught with pollen on their fur than Pallas's long-tongued bats (*Glossophaga soricina)*, even though we captured *L. yerbabuenae* much less frequently. For the 45 trees monitored for phenology, we found that fruit biomass per tree did not differ between landscape types. For the 25 focal trees where we monitored bat abundance, we found significantly higher fruit-set in fragmented sites, and significantly lower fruit-set in trees with more *Glossophaga soricina* bats.

*Conclusions*: In this study system, bat pollinator abundance and reproductive success in *C. alata* trees do not differ between landscape types. In fact, the trend is for higher fruit set in fragmented sites, possibly due to high flower production, which strongly attracts pollinators despite the surrounding landscape. These results provide evidence that bat pollinators may mitigate the impact of fragmentation for some tree species, as long as local populations are not eliminated by the deforestation, and points to the important ecosystem services provide by bats.

Keywords: bat pollination; nectarivorous bats; *Crescentia alata*; fruit-set; forest fragmentation, seasonally dry tropical forest

## INTRODUCTION

Forest fragmentation is considered one of several pervasive threats to the survival of tropical forest species and maintenance of ecosystem processes (Laurance et al., 2006; Foley et al., 2007; Brook, Sodhi, and Bradshaw, 2008). Of particular concern are the impacts of forest fragmentation on plant-pollinator interactions because these interactions form the basis of agricultural productivity and wild flowering plants' ability to reproduce and maintain their populations (Buchmann and Nabhan, 1996; Kearns, Inouye, and Waser, 1998; Potts et al., 2010). Not only does the landscape change that creates forest fragments reduce or eliminate plant populations, but the fragmentation itself can influence many aspects of plant-pollinator interactions, by changing the abundance, diversity and foraging behavior of the pollinators, and/or by changing the abundance, diversity, and flowering phenology of the plants (Kremen et al., 2007). Together, these altered interactions can impact the reproductive success of the plant, with consequent effects on offspring genetic diversity and future resources for pollinators (Aizen and Feinsinger, 1994; Cunningham, 2000; Aguilar et al., 2006).

Bats are a small but important group of pollinators in the tropics (Heithaus, Fleming, and Opler, 1975; Bawa, 1990), which are capable of moving pollen far across the landscape (Horner, Fleming, and Sahley, 1998) and delivering large pollen loads to flowers (Law and Lean, 1999; Muchhala and Thomson, 2010). Due to this capacity, pollinating bats have the potential to maintain reproductive connectivity among fragmented plant populations (Fleming, Geiselman, and Kress, 2009). However, research on how forest fragmentation impacts the abundance of bat pollinators has remained equivocal. Based on island biogeographic theory, fragments of habitat are expected to

have fewer species, and certain types of bats (e.g., gleaning insectivores), which are less represented in forest fragments, seem to be follow this prediction and are considered good indicators of habitat disruption (Fenton et al., 1992; Medellin, Equihua, and Amin, 2000). Some studies have shown a decrease in nectarivore bat abundance and species diversity in fragments (Cosson, Pons, and Masson, 1999; Medina et al., 2007), but other studies have shown the opposite trend (Gorresen and Willig, 2004; Willig et al., 2007). Research comparing bat assemblages in different successional stages after land was cleared for farming showed that nectarivorous species were most abundant in early successional stages (Avila-Cabadilla et al., 2012). This trend of nectarivorous bats doing well in fragments seems especially true for the widespread and abundant bat species *Glossophaga soricina* (Medellin, Equihua, and Amin, 2000; Meyer et al., 2008). For bats foraging in the forest understory, it is possible that the opening of the landscape associated with forest fragmentation is beneficial, in terms of creating more flyways for movement; bats are known to use rivers and open spaces to travel (Hein, Castleberry, and Miller, 2009).

Bats in the family Phyllostomidae that primarily feed on nectar are similar in many respects, but different species may respond differently to habitat disruption. For example, Stoner et al. (2002) found that *Musonycteris harrisoni*, an endemic nectarivorous bat from Western Mexico, only visits and pollinates *Ceiba grandiflora* trees in undisturbed forest habitats, and they suggest this bat species is particularly sensitive to habitat fragmentation (Stoner et al., 2002). In addition, Quesada et al. (2004) demonstrated that nectarivorous bat visitation of *Ceiba* trees in fragments depends on both the specific species of tree (*C. aesculifolia*, *C. grandiflora,* and *C. pentandra*), and

the bat species. They found that *Glossophaga soricina* and *Leptonycteris yerbabuenae* visited flowers of *C. aesculifolia* in fragments more frequently than continuous forests, but *L. yerbabuenae* visited flowers of *C. pentandra* significantly more times in continuous forest than fragments, and *G. soricina* showed no difference in visits at all (Quesada et al., 2004). The authors suggest that the flowering phenology and other available chiropterophilic resources are important factors in determining pollinator abundance.

It is also important to examine the impact of forest fragmentation on the plant, especially in measures of reproductive success. Often reproductive success is impacted by fragmentation through influences on pollinator abundance, floral display, and/or their interaction. Some research has shown that trees in fragments can have very high reproductive success (Dick, 2001), and Aldrich and Hamrick (1998) demonstrated that adult *Symphonia globulifera* trees in pastures were reproductively dominant (Aldrich and Hamrick, 1998). The authors inferred that the larger size of the trees in pastures allowed increased floral display, which likely increased hummingbird visitation to the flowers, and bat visitation to the fruit, with subsequent dispersion of seeds (Aldrich and Hamrick, 1998). Research specifically on trees pollinated by bats, however, has found reduced fruit-set in forest fragments (Fuchs, Lobo, and Quesada, 2003; Quesada et al., 2003b; Quesada et al., 2004). The complex interplay of variables affecting how trees and pollinators respond to fragmentation, and to each other, makes further study of these interactions imperative.

The overall goal of this study is to determine whether bats as pollinators have the potential to maintain connectivity of tree populations in fragmented landscapes.

Specifically, we will test the hypothesis that bats are unaffected by landscape structure by determining whether the identity and abundance of nectarivorous bats visiting flowers of the bat-pollinated *Crescentia alata* is influenced by the matrix of continuous versus fragmented forest surrounding these trees. We also test the hypothesis that the reproductive success of *C. alata* trees across sites in different landscapes is shaped by the number of nectarivorous bats visiting them. If the nature of the surrounding landscape does not influence how bats forage around these trees, we expect to find the same number of nectarivorous bats in sites surrounded by continuous and fragmented forest, and equivalent values of reproductive success for *Crescentia alata* trees in these sites.

**METHODS**

*Study site and species*. The study site is located within and around the Chamela-Cuixmala Biosphere Reserve (19º30'N, 105º03'W) in the state of Jalisco, Mexico. The Reserve is operated by the Universidad Autonoma de México (UNAM), and contains approximately 13,200 hectares of tropical dry forest. The Reserve has been the site of numerous studies on seasonally dry tropical forest ecology and encompasses a high diversity of plant species, a large number of which are also endemic to the Jalisco and Colima coast (Bullock, Mooney, and Medina, 1995; Dirzo et al., 2011). The protected area of the Reserve is surrounded by fragments of mature forest, cleared forest areas in various stages of succession, and agricultural and ranching fields that are organized under an a communal property ("ejido") system (Sanchez-Azofeifa et al., 2009).

*Crescentia alata* is a common understory tree in tropical dry forests, ranging from México to Costa Rica. This tree is culturally valued by people in Mexico for its fruits, the

pulp of which is used medicinally to treat respiratory ailments (Rojas et al., 2001), and the husks of which are used to make handicrafts such as maracas and engraved pottery (Gentry, 1992). *Crescentia alata* has been suggested for forest restoration projects in other parts of México due to its rapid growth rates (Foroughbakhch et al., 2006).

*Crescentia alata* flowers are cauliflorous, a life-form adaptation to bat-pollination (van der Pijl, 1961; Gentry, 1974). The flowers are hermaphroditic, pale greenish-pink-maroon, and each flower is only open for one night, during which time it produces large amounts of nectar (Gentry, 1974; Martínez del Río and Bullock, 1990). The trees flower from July-August (Lott, 2002), which coincides with the start of the rainy season in this area. This concentrated flowering phenology is in contrast to the "steady-state" pattern Gentry described for the same species (Gentry, 1974), and flowering of *C. alata* in Chamela, Mexico is over a shorter period of time compared to Camelco, Costa Rica (Gordon, Baker, and Opler, 1974; Bullock and Solis-Magallanes, 1990). During its flowering period, *C. alata* trees are capable of producing hundreds of flowers.

Gentry described bats as the primary pollinators of *Crescentia* species, and noted that many bat species visit *Crescentia alata* and *C. cujete* flowers in Costa Rica (Gentry, 1974). There are several nectar-feeding bat species in this area of Mexico that could be pollinators of *Crescentia alata* trees: *Leptonycteris yerbabuenae, Glossophaga soricina, Glossophaga commissarisi, Choeroniscus godmani, and Musonycteris harrisoni* (Ceballos and Miranda, 2000). Stoner *et al*. (2003) report the presence of *C. alata* pollen in feces from a colony of *Leptonycteris yerbabuenae* bats in the same region, during June, July and September (Stoner et al., 2003), and Sperr *et al*. (2011) found *C. alata* pollen on four nectarivorous bats species (*l. yerbabuenae*, *G. soricina*, *M. harrisoni*, and *Anoura*

*geoffreyi*) from July-September, at a site 167 kilometers southeast of our study area (Sperr et al., 2011). Other species have been observed visiting *Crescentia alata* flowers, including sphyngid moths, hummingbirds and several bee species, but these have been described as nectar and pollen robbers because they do not effectively pollinate flowers (Gentry, 1974; Martínez del Río and Bullock, 1990).

We used nine sites to assess pollinator abundance and phenology of *Crescentia alata* trees. Each site was situated in an arroyo, an ephemerally dry streambed that became rivers during the rainy season, and had a minimum of five reproductive *C. alata* trees growing within or directly alongside the arroyo. Five sites were surrounded by continuous forest, and occurred either in the Biosphere Reserve, or directly adjacent to it, and four sites were surrounded by fragmented forest and other land use types such as ranches and pastures (Figure 2-1). The same or very closely situated sites have been used by other researchers to compare fragmented and continuous forest habitat, and the responses of species (particularly bats and bat-pollinated plants) to fragmentation (Stoner et al., 2002; Quesada et al., 2003a; Quesada et al., 2004; Herrerias-Diego et al., 2006; Rosas et al., 2011; Avila-Cabadilla et al., 2012; Quesada et al., 2013). In addition, a GIS analysis of ASTER satellite images of the region determined that the level of fragmentation increased as a function of distance from the Chamela-Cuixmala Biosphere Reserve; the number of fragments increased substantially beyond a buffer area of 15km from this protected area (Sanchez-Azofeifa et al., 2009). This study also estimated deforested land cover to be approximately 3.7% within the Reserve, and 16-17% in buffer zones up to 30km outside of the Reserve, and the authors attribute the deforestation to local slash-and-burn practices, to clear land for agriculture and cattle

pasture (Sanchez-Azofeifa et al., 2009).

*Pollinator abundance*. We assessed nectarivorous bat abundance at different sites using a mist-net (USA-style, 50/2 denier, 38 mm mesh, 2.6 m wide and 12m long; Avinet Inc.®, Dryden, NY). We placed nets in the arroyos, within 5 meters of a *C. alata* tree, with the bottom shelf of the net slightly above the ground. Pilot data indicated high capture rates of bats near these trees, so a single, 12-meter mist-net was used. The net was open from 9pm to 1am, and patrolled every 10-15 minutes for captured bats. All bats were carefully removed from the net and identified to the species level using field keys and guides (Medellín, Arita, and Sánchez, 1997; Ceballos and Miranda, 2000). We recorded the time of capture, forearm length, weight, sex, age, and reproductive condition of each bat, made note of pollen on bats, collected pollen and feces from nectarivores if available, and then released the bats.

We set up mist-nets once at each site during the dry (non-flowering) season, and 2-3 times for each site during the rainy (peak flowering) season, which occurred in late-June to mid-August in 2011. During the rainy season, bats were sampled 25 times, for a total of 1200 net-hours. For several nights, two teams sampled bats at different locations in a single night, in order to maximize sampling during peak flowering. To demonstrate that nectar-bats are present in the area because of the flowering resource, we tested the number of captures of nectarivorous bats in the dry season against the average number of captures in the rainy season, across the sites, using the Wilcoxon signed rank test. This procedure and the following statistical analyses were preformed in R (R Development Core Team, 2013).

The number of flowers open on the focal *Crescentia alata* tree, and any other

potentially bat-pollinated plant species, within 100m, were recorded during each night of mist-netting. Because bat capture rates are count data, we used a negative binomial regression to analyze the effect of landscape type (continuous vs. fragmented forest), the floral display (number of open flowers on the *C. alata* tree next to the net), the interaction between these, and the tree size (diameter at breast height) on the number of nectar-bats caught.

While removing the bats from the net, we also recorded the presence and subjective amount of pollen on each bat on an index from 1-5 (1= very small amount, 5=covered in pollen). We classified pollen presence/absence on bats, and pollen abundance index on the bats with pollen in contingency tables, and analyzed differences in these groups among landscape type and nectarivorous bat species with separate Fisher's exact tests.

*Reproductive success*. We collected data on 5 trees in each of the 9 sites. We measured the diameter at breast height (dbh) of each tree; if the tree was multi-stemmed, we calculated the square root of the sum of all stems' diameters squared. Trees were surveyed five times across the flowering period (late June- mid August 2011, number of days of surveying ranged from 39-46 across the sites), and the number of mature flowers and fruit on each tree was recorded. Because flowers are only open for one night before they drop, and every site could not be monitored every night, we calculated total flower production for each tree by taking the average number of flowers between the first and second time point, and multiplying the average by the number of days between these points. We did this iteratively for the second and third, third and fourth, and fourth and fifth time points, and summed these values to estimate total flower accumulation (Figure

2-S2).

Fruit count is simpler to estimate because *C. alata* fruits are very hard gourds that stay on the tree for up to a year after they start developing; therefore the highest number of new fruit surveyed was used as the estimate of total fruit production per tree. Several fruit per tree were also collected, measured and weighed. In a previous field season we counted number of seeds and measured the circumference of fruits, and found a significant correlation between these variables ($n= 41$, $r= 0.62$, $P<0.001$, Figure 2-S3). In this field season, we measured circumference and weight of fruits, and also found a significant correlation between these variables ($n= 215$, $r= 0.89$, $P<0.001$, Figure 2-S4). We therefore felt confident using fruit weight as an indicator of seed number, and took the average weight of fruit per tree, multiplied this value by the number of total number of fruits produced per tree, and labeled the result "fruit biomass". Fruit weight was measured when fruits were fully ripe, which is detectable once the pulp and seeds detach from the fruit wall. This weight is preferable to dry weight because fruits and seeds start desiccating once mature, and mature fruit are easily discernible by shaking and feeling the pulp thud against the fruit wall (Janzen, 1982).

We first examine if continuous and fragmented sites have the same relationship between flower production and fruit biomass produced using an ANCOVA for the complete data set of 45 trees. We then test the effect of landscape type, tree dbh, and number of nectar-bats present during mist-netting on fruit count using a negative binomial GLM (offset by the log of the flowers produced) for the reduced data set of 25 trees used to assess pollinator abundance.

## RESULTS

We caught significantly more nectarivorous bats in mist-nets next to flowering trees (in the rainy season, mean= 14.88 bats/tree) than next to trees with no flowers (in the dry season, mean = 0.55, $P < 0.01$, Figure 2-S1). This finding supports our supposition that nectar-bats are drawn to the presence of open bat-pollinated flowers, and not just coincidentally present in the arroyos.

During the rainy season, we captured 490 bats of 16 species. Three species were nectarivores, and together accounted for 366 individuals (75% of total captures). The majority (82%) of these nectar-bats were *Glossophaga soricina.* The second most abundant nectarivore was *Leptonycteris yerbabuenae* (16.7%), and the least frequently captured nectarivore was *Choerniscus godmani* (1.6%) The other 13 species were frugivores, insectivores, and one sanguinivore (Table 2-1). This pattern of high abundance of *G. soricina* and lower abundance of *L. yerbabuenae* was consistent across the sampling localities (Figure 2-2).

We did not find a significant effect of landscape type or tree size on number of nectar bats caught, but floral display (the number of open flowers on the tree next to the net) was significant in the negative binomial regression (GLM, z= 3.567, $P < 0.01$; Table 2-2). The number of nectar bats captured was positively associated with floral display (Figure 2-3).

We captured 66 bats with pollen on their fur (18% of nectarivore captures). There was no effect of landscape type on the likelihood of pollen being found on bats, or on the amount of pollen on bats. However, presence of pollen differed significantly among nectarivore species, and *Leptonycteris yerbabuenae* bats had a greater likelihood of being

captured with pollen ($P < 0.001$, Table 2-3), and a greater likelihood of having more pollen on their fur ($P < 0.05$, Table 2-3).

We compared average values for tree sizes and phenological variables for the 25 trees in the continuous landscape type, and 20 trees in fragmented landscape type. Phenological variables include number of flowers produced, number of fruit produced, weight of fruits, fruit biomass (count times weight), and fruit biomass rate (fruit biomass divided by fruit). None of these variables were significantly different between landscape types (Flower: ANOVA, F=0.185, $P > 0.10$ ; Fruit: ANOVA, F= 2.411, $P > 0.10$; Table 2-4).

We ran an ANCOVA to see the effect of landscape type on fruit biomass, co-varied with the number of flowers produced. Flowers were a significant predictor of fruit biomass, which means that the more flowers produced, the higher the reproductive output of the plant (bigger and more fruit; Flower: ANCOVA, F=23. 188, $P < 0.001$). Landscape type was not a significant factor in the model (Landscape type: ANCOVA, F= 1.253, $P > 0.10$). However, we can see that the positive association between flowers and fruit biomass have slightly different intercepts between landscape types, and the fragmented sites trend towards higher values (Figure 2-4).

For the restricted data set for which we have pollinator abundance data (25 trees), there was a significant effect of landscape type and number of *Glossophaga soricina* bats on fruit number (offset by the log of flowers), when we modeled these variables as well as number of *Leptonycteris yerbabuenae* bats and tree size, using a negative binomial GLM. We found significantly higher fruit set in fragmented forest sites (GLM, z=3.30, $P < 0.001$), and significantly lower fruit set with greater numbers of *Glossophaga soricina*

bats (GLM, z=-2.884, $P < 0.01$, Table 2-5). The other two independent variables were not significant. We also ran models with interaction terms between these independent variables, but each of these models were less supported than the one we present based on their AIC scores.

## DISCUSSION

This study illustrates the vital role of bats as pollinators of *Crescentia alata* trees in this region of Mexico, and the lack of impact of forest fragmentation on their foraging as pollinators. We found that their visitation to these trees is not reduced when trees are surrounded by fragmented landscape. In fact, the number of open *C. alata* flowers was the only significant factor associated with nectar-bat captures, suggesting that these bat species are relatively unimpeded by forest fragmentation in their foraging bouts, at least at the scale that sampling occurred. This finding is in contrast to results from research conducted in the same study area, which showed that *Glossophaga soricina* had significantly fewer visits to bat-pollinated *Ceiba grandiflora* trees in fragmented areas, while there was no difference in *Leptonycteris yerbabuenae* visits in fragmented and continuous areas (Quesada et al., 2003a). One possible reason is that *Crescentia alata* produces flowers in massive quantities over a short time period, unlike *Ceiba grandiflora,* which flowers for 5-6 months. This large, synchronous flower production in *C. alata* may present enough reward to foraging bats to overcome any potential negative influence of moving between fragments. This hypothesis is in line with other research demonstrating higher floral display in forest fragments (Aldrich and Hamrick, 1998; Dick, 2001) and the relative power of floral display over landscape condition in attracting

pollinators (Westphal, Steffan-Dewenter, and Tscharntke, 2003). In addition, another study in the same region demonstrated that the abundance of nectarivorous bats was negatively associated with the area of dry forest patches, which the authors attribute to the high density of chiropterophilic resources in early successional forest fragments, and the resilience of generalist nectarivorous bats to disturbance (Avila-Cabadilla et al., 2012).

Our results also demonstrate the differences in the efficacy among the nectarivorous bat species in this region as pollen dispersers of *Crescentia alata* trees. The higher likelihood of finding *L. yerbabuenae* with pollen, and with more pollen, suggests that it may be the more effective pollinator for *C. alata* trees than *G. soricina*, even though *L. yerbabuenae* were less abundant. This result is somewhat unsurprising, as *L. yerbabuenae* are considered more specialized nectarivores than *G. soricina*, which have a wider diet breadth including fruits and sometimes insects (Heithaus, Fleming, and Opler, 1975; Quiroz et al., 1986; Fleming, Nunez, and Sternberg, 1993). Indeed, recent research suggests that *G. soricina* bats are even more omnivorous than previously suspected, and actively pursue insect prey (Clare et al., 2014). Research on *Ceiba grandiflora* pollination by bats in the same region showed that even though *G. soricina* bats spent more time at flowers than *L. yerbabuenae* or *M. harrisoni*, they were significantly less likely to touch the reproductive parts of the flower (Quesada et al., 2003a). In addition, *L. yerbabuenae* bats were more efficient at removing nectar and sustained longer foraging bouts than *G. soricina* bats, when these two species were compared in experiments of spatial working memory (Henry and Stoner, 2011). The combination of these studies and the pollen presence data suggests that *L. yerbabuenae* is serving a much larger role as pollinator of

*Crescentia alata* plants than *G. soricina*. It is unclear if *Choeroniscus godmani* are better at avoiding capture in the net, or are locally rare; they are categorized as "scarce" in a Mexico-wide survey of nectar-feeding bats (Arita and Santos-del-Prado, 1999). The fact that the few individuals we did capture were only found in continuous forest suggests these bats may be particularly sensitive to habitat fragmentation, and may not move pollen very widely.

Our results for tree reproductive success should be interpreted with some caution, because they come from a restricted data set of 25 trees. In this reduced data set, trees in fragmented sites have higher fruit-set, whereas the significance drops away in the complete data set. However, the trend remains the same in the full data set, where trees in fragmented sites tend to have more flowers and more fruit. Our data above demonstrate the strong correlation between number of flowers and number of nectar-bats, which is suggestive that these pollinators may be playing a role in reproductive success of the trees. However, reproductive success may not be pollinator-limited, and the trend of higher fruit-set in fragments could also be attributed to other factors that we did not measure, such as different levels of herbivory. We observed a high level of herbivory at one site in the continuous forest, and previous research on *Crescentia alata* has shown a negative relationship between leaf damage and fruit-set (Rockwood, 1973).

The abundance of a bat species does not necessarily mean the bat is an effective pollinator. For example, we found that the number of *G. soricina* bats was negatively correlated with fruit-set, while the number of *L. yerbabuenae* bats had no effect. Several things can account for these results. We have shown that the *Leptonycteris* bats are likely the more effective pollinator in this system, and it is possible there is a negative effect

from more *Glossophaga* bats being in the vicinity on the abundance of *Leptonycteris* bats, even though we did not detect a significant interaction between these species in the GLM model. Indirectly, these pollinators could interact to produce lower plant reproductive success. This same outcome was reported in the temperature herbaceous plant, *Campanula americana*, where the combination of a high efficiency pollinator in low abundance with a low-efficiency pollinator in high abundance caused lower fruit-set than when low-efficiency pollinators were excluded (Lau and Galloway, 2004). Directly, there can also be aggressive behavior between pollinators, and competition for floral resources. Aggressive behavior among nectarivorous bats has been documented (Lemke, 1984; Arias-Coyotl, Stoner, and Casas, 2006), and in the case of *Glossophaga soricina*, Lemke (1984) recorded an average of 18.9 aggressions per hour towards con-specifics at blooming *Agave desmettiana* plants in Northern Colombia (Lemke, 1984). In addition, Quesada et al. (2003) report that visits by *G. soricina* and *L. yerbabuenae* to *Ceiba grandiflora* flowers were negatively affected by the visits from other bat species, suggesting some amount of negative interaction between the two (Quesada et al., 2003a). The authors also observed bats breaking off anthers and eating pollen during foraging bouts (Quesada et al., 2003a), and these behaviors, which undoubtedly have negative consequences for plant reproductive success, and may increase when the number of bats in the area reaches a high degree.

In conclusion, our study shows that the abundance of bat pollinators at flowering *Crescentia alata* trees is not affected by landscape type, and *Crescentia alata* reproductive success does not differ between fragmented and continuous forests. Our data show a strong correlation between floral display and number of nectar-bats, suggesting

that this variable is more important in determining pollinator abundance than fragmentation, possibly due to the massive synchronicity of this plant's flowering. We also show a trend for higher flowering and fruiting in fragments, and find some evidence that the bat pollinator species most likely to be found with pollen may be negatively affected by interactions with other bat species. Future studies of pollen-mediated gene movement among *C. alata* trees will provide insight about the effectiveness of bats as pollinators in fragmented landscapes.

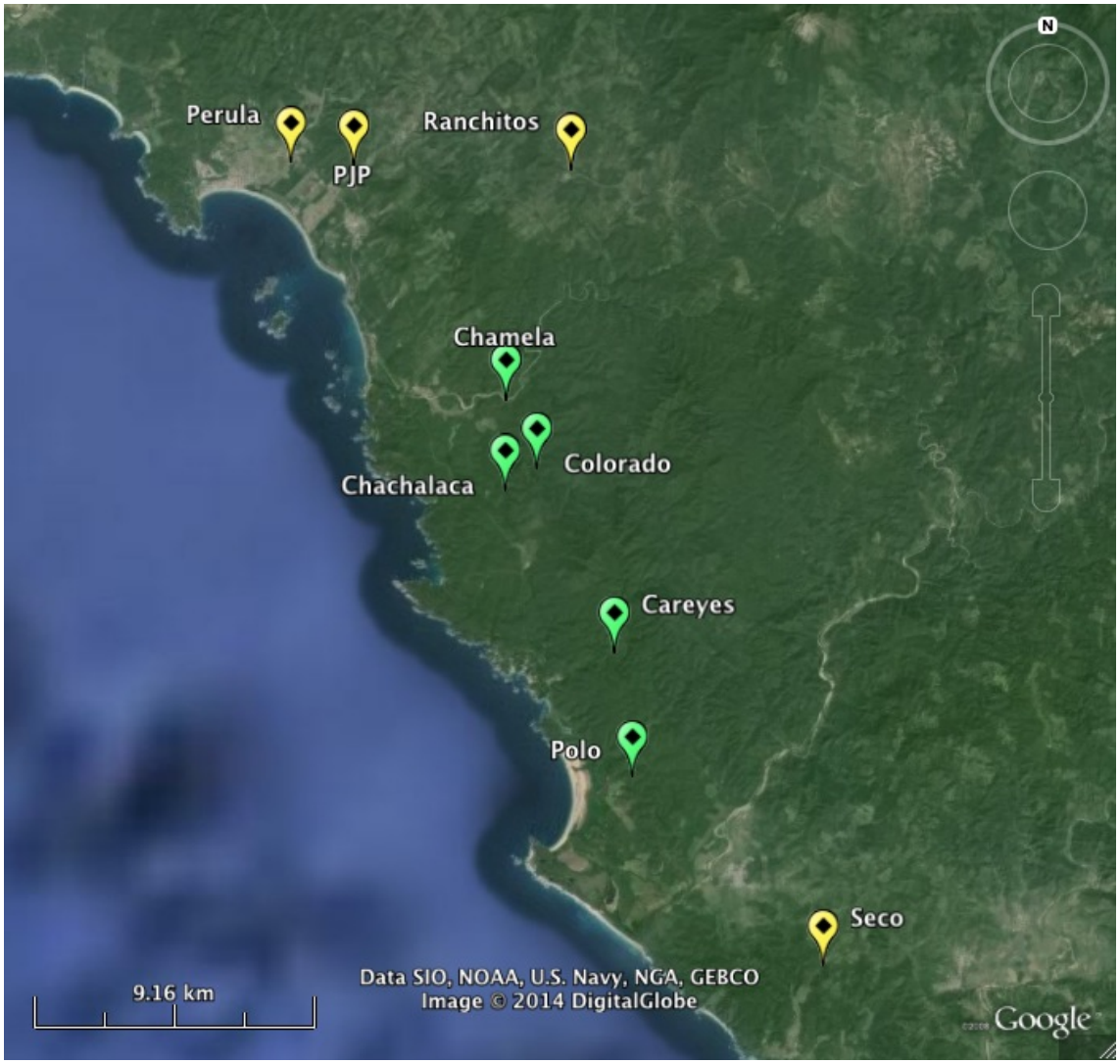


Figure 2-1: Map of study region and sampling sites, from Google Earth satellite images. Fragmented forest sites are labeled in yellow, continuous forest sites are in green.

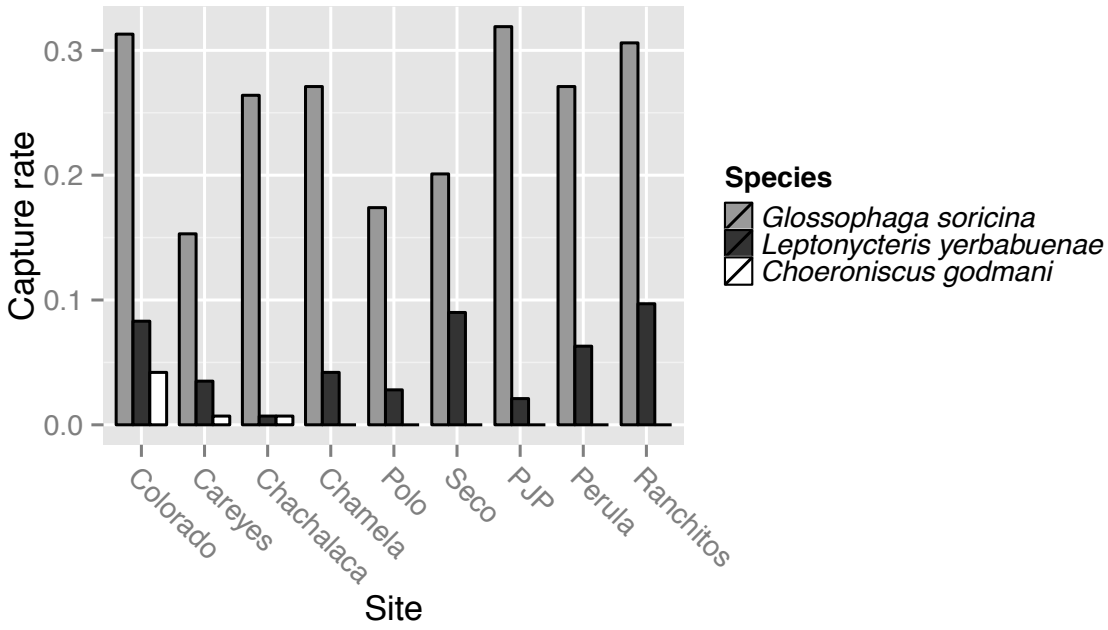


Figure 2-2: Site averages for bat capture rates (bats per meter-net-hours), separated by nectar-bat species.

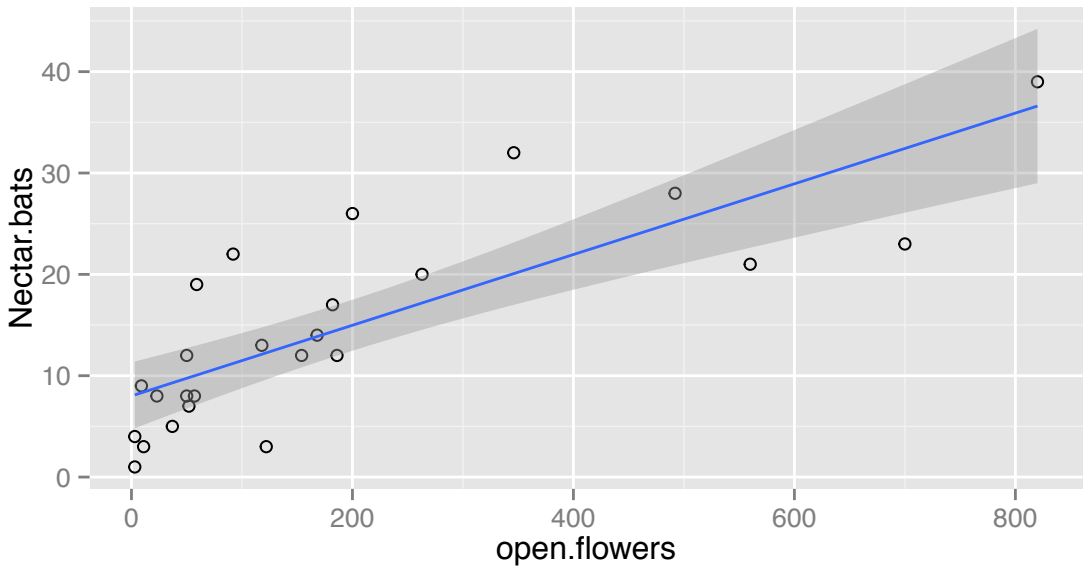


Figure 2-3: Linear regression of the number of open *Crescentia alata* flowers on the number of nectarivorous bats caught next to the flowering tree. Each point represents a different sampling event, the fit of the linear regression is the blue line, and the shaded area is the 95% confidence region (adjusted $R^2$= 0.619).

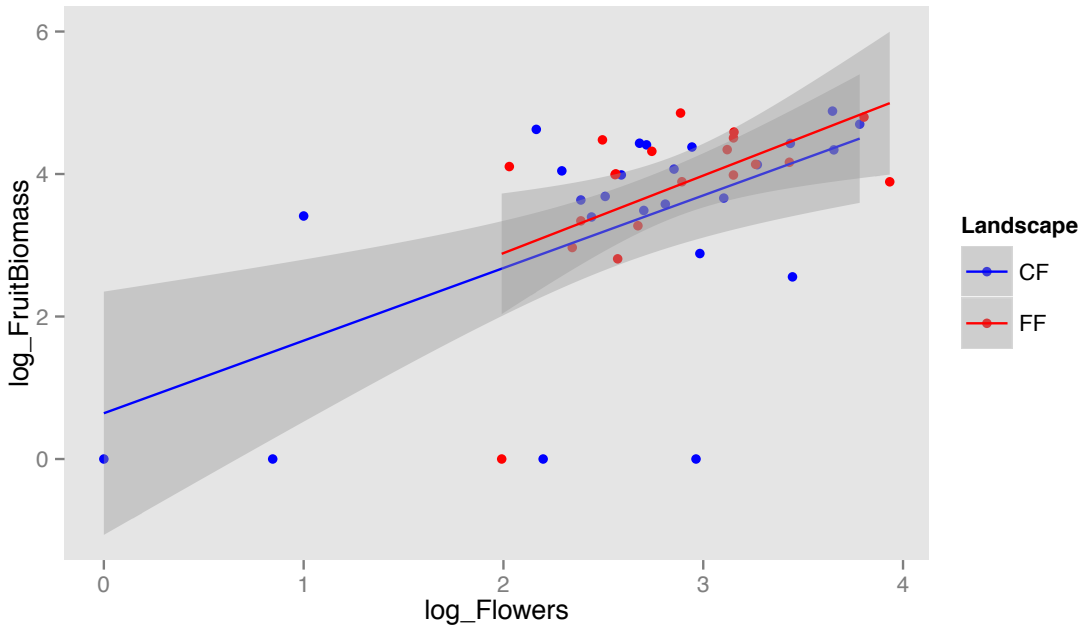


Figure 2-4: Linear regression between flowers and fruit biomass (both log-transformed) for the complete tree data set, separated by landscape type (CF=25 trees, blue line; FF=20 trees, red line). The shaded area are the 95% confidence region. For CF, adjusted $R^2$ = 0.307, p = 0.002; for FF, adjusted $R^2$ = 0.252, p =0 .014.

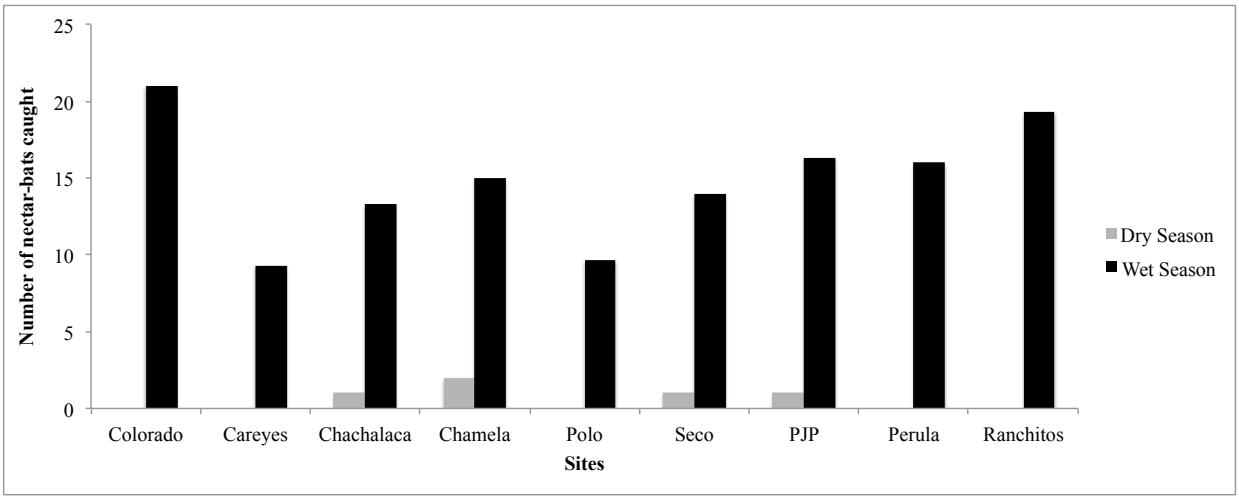


Figure 2-S1: Nectarivorous bat captures during the dry (one sampling night) versus the wet season (average of 2-3 sampling nights).

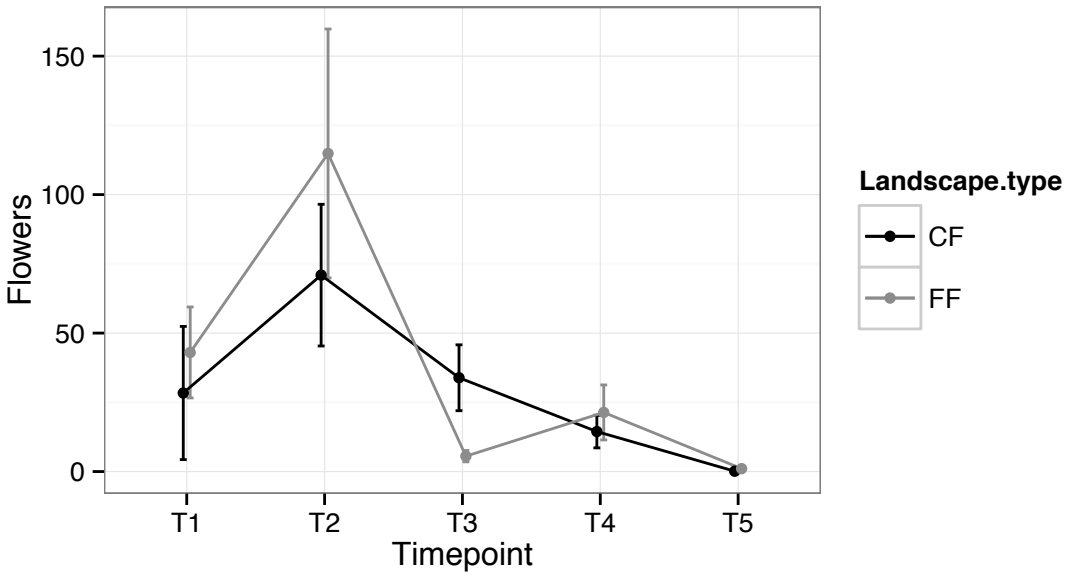


Figure 2-S2: Average number of mature flowers across 25 trees in continuous forest sites (CF), and 20 trees in fragmented forest sites (FF), at five time-points across the flowering period from late June-mid August 2011. Bars around the mean are standard error.


Figure 2-S3: Relationship between fruit size (circumference) and seed number, for 41 fruit sampled in 2008 and 2009. The shaded area is the 95% confidence region around the correlation (Pearson's correlation, r= 0.62, t = 4.88, df = 39, P <0.001).

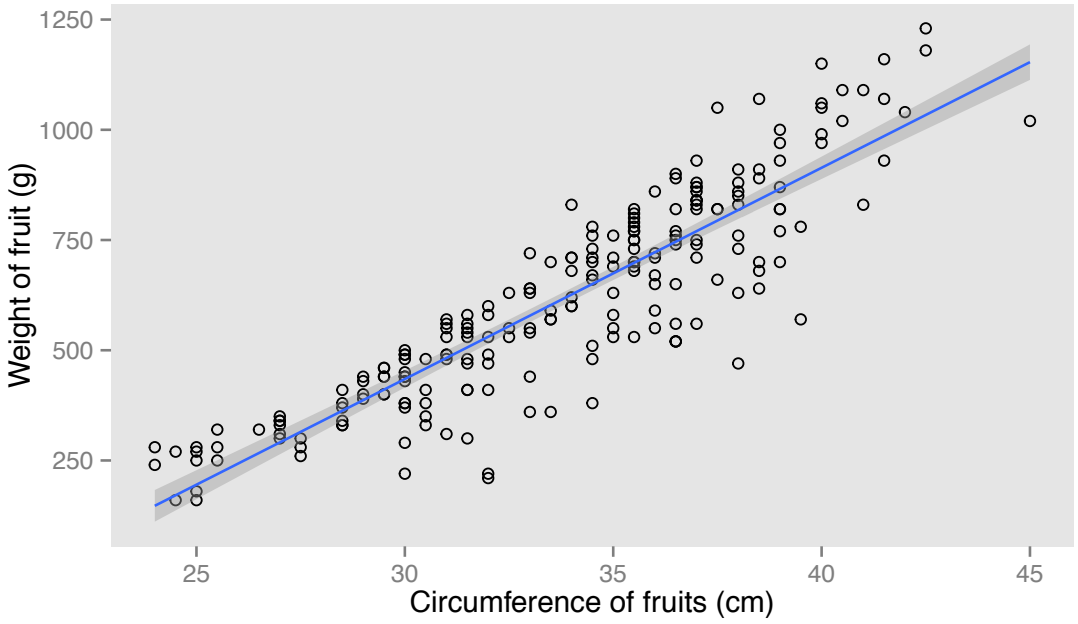


Figure 2-S4: Relationship between fruit size (circumference) and weight for 215 fruit sampled in 2011. The shaded area is the 95% confidence region around the correlation (Pearson's correlation, $r = 0.89$, $t = 28.19$, $df = 213$, $P < 0.001$).

Table 2-1: Total number of bat captures by site, for all species, categorized by trophic guilds. Sampling is from 25 nights (1200 net-hours) over the rainy season; the number of nights of sampling per site is in parentheses below the site name; CF= Continuous Forest sites, FF= Fragmented Forest sites.

| | CF | | | | | FF | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Colorado (2) | Careyes (3) | Chacha-laca (3) | Chamela (2) | Polo (3) | Seco (3) | PJP (3) | Perula (3) | Ranchitos (3) |
| **Nectarivores** | | | | | | | | | |
| *Glossophaga soricina* | 30 | 22 | 38 | 26 | 25 | 29 | 46 | 39 | 44 |
| *Leptonycteris yerbabuenae* | 8 | 5 | 1 | 4 | 4 | 13 | 3 | 9 | 14 |
| *Choeroniscus godmani* | 4 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Frugivores** | | | | | | | | | |
| *Artibeus phaeotis* | 0 | 0 | 0 | 4 | 0 | 1 | 0 | 0 | 0 |
| *Artibeus toltecus* | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| *Artibeus jamaicensis* | 4 | 2 | 1 | 0 | 1 | 0 | 0 | 2 | 0 |
| *Artibeus intermedius* | 2 | 0 | 1 | 2 | 0 | 1 | 1 | 4 | 0 |
| *Artibeus lituratus* | 0 | 0 | 0 | 2 | 0 | 0 | 1 | 0 | 0 |
| *Artibeus sp.* | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| **Insectivores** | | | | | | | | | |
| *Pteronotus parnelli* | 17 | 4 | 19 | 8 | 0 | 0 | 23 | 1 | 1 |
| *Pteronotus personatus* | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Lasiurus blossevillii* | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Lasiurus xanthius* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Rhogeesa parvula* | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Myotis fortidens* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| **Sanguinivore** | | | | | | | | | |
| *Desmodus rotundus* | 1 | 0 | 0 | 0 | 6 | 5 | 1 | 0 | 0 |

Table 2-2: Summary of generalized linear model (GLM) results for change in number of nectar bats due to the landscape type, the number of flowers open on the tree (floral display), the tree size (dbh), and the interaction between landscape type and floral display.

| | Estimate | SE | z value | *P*-value |
|---|---|---|---|---|
| Landscape type | -0.164 | 0.285 | -0.577 | 0.564 |
| Floral display | 0.002 | 0.001 | 2.637 | 0.008 |
| Tree size | -0.006 | 0.009 | -0.624 | 0.532 |
| Landscape type * Floral display | 0.001 | 0.001 | 1.102 | 0.270 |

Table 2-3: Contingency tables for pollen presence or absence on nectar bats and an index of pollen abundance (higher numbers indicating more pollen) on bats captured with pollen, separated by landscape type and bat species.

| | | Pollen Presence | | Index of Pollen Abundance (1-5) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Yes | No | 1 | 2 | 3 | 4 | 5 |
| Landscape type | CF | 36 | 133 | 18 | 7 | 6 | 3 | 2 |
| | FF | 30 | 167 | 15 | 3 | 6 | 3 | 3 |
| Bat Species | *G. soricina* | 41 | 258 | 24 | 7 | 8 | 2 | 0 |
| | *L. yerbabuenae* | 24 | 37 | 8 | 3 | 4 | 4 | 5 |
| | *C. godmani* | 1 | 5 | 1 | 0 | 0 | 0 | 0 |

Table 2-4: The means ± standard errors for tree size (dbh), flower accumulation, number of fruit, fruit weight, fruit biomass (number of fruit * fruit weight), and fruit biomass/ flower accumulation, for 25 trees in continuous forest sites, and 20 trees in fragmented forest sites.

| | Continuous Forest | Fragmented Forest |
|---|---|---|
| Number of trees | 25 | 20 |
| Tree size (dbh) | 43.5 ± 2.5 | 40.6 ± 3.8 |
| Flowers | 1350.2 ± 279.7 | 1485.9 ± 489.0 |
| Fruit count | 28.8 ± 4.6 | 36.6 ± 7.9 |
| Fruit weight (g) | 467.6 ± 36.5 | 454.4 ± 45.2 |
| Fruit biomass (g) | 16,383.2 ± 2869.6 | 18,519.8 ± 4472.5 |
| Fruit biomass / Flowers | 31.3 ± 8.8 | 26.3 ± 7.8 |

Table 2.5: Summary of generalized linear model (GLM) results for change in number of fruit produced (offset by the log-transformed number of flowers), due to the landscape type, the number of *L. yerbabuenae* bats captured near the tree, the number of *G. soricina* bats captured near the tree, and the tree size.

| | Estimate | SE | z value | *P-value* |
|---|---|---|---|---|
| Landscape type | 1.259 | 0.382 | 3.300 | 0.001 |
| *L. yerbabuenae* | -0.007 | 0.054 | -0.135 | 0.892 |
| *G. soricina* | -0.084 | 0.029 | -2.884 | 0.004 |
| Tree size (dbh) | -0.005 | 0.014 | -0.364 | 0.716 |

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# CHAPTER 3

## Patterns of genetic structure in a bat-pollinated, extinct megafauna-dispersed, tropical tree species (*Crescentia alata*) from Western Mexico

**ABSTRACT**

*Premise of study*: The genetic structure of plant populations is shaped by pollen and seed dispersal, and by landscape features that influence those processes. We examined the fine-scale genetic structure of populations of *Crescentia alata*, a tropical dry forest tree species in Jalisco, México that is pollinated by bats, and which were purportedly dispersed by megafaunal seed dispersers that went extinct in the Pleistocene. If no other seed dispersal vector is present to move these hard-to-open fruit, we might expect the loss of the original dispersal agent to create highly localized genetic structure.

*Methods*: To assess genetic structure, we developed 8 neutral microsatellite markers *de novo* for *Crescentia alata*, and genotyped 161 trees sampled from 16 localities across a geographic region of 92,000 hectares. We used a Bayesian clustering program to detect the number of possible subpopulations. We then estimated the genetic structure among the sites and looked for evidence of isolation by distance. Finally, we tested the central hypothesis that the loss of historical seed dispersal agent results in restricted dispersal by quantifying the patterns of spatial autocorrelation at broad and fine geographic scales.

*Results*: Our STRUCTURE analysis identified two North-South genetic clusters, and we found low but significant genetic structure between these two regions ($F_{RT}$ = 0.019), and high structure between sampling localities ($F_{ST}$ = 0.049). We found evidence for isolation by distance between sites, at a large geographic scale. We observed significant spatial autocorrelation at a broad scale (0-12km) with significant and low kinship coefficients at these distance classes (0.08 > r >

0.027). Most importantly, the fine scale spatial autocorrelation was significant (0-800m, 0.030 > r > 0.106), but it did not reveal the degree of spatial clustering that would be apparent for a species without a dispersal agent.

*Conclusions*: We conclude that the overall low level of genetic structure of *Crescentia alata* trees across our study region suggests that gene flow is not restricted, as might be expected with the loss of the original dispersal agent in the Pleistocene. Instead, high pollen-mediated gene flow via bat pollinators is a plausible mechanism causing the homogenization of genetic diversity of *Crescentia alata* trees in this region. Moreover, the lack of strong fine scale genetic structure indicates a dispersal vector other than gravity (which is assumed if the seed disperser is no longer there). Given the fact that *C. alata* trees are found along arroyos, it is possible that flooding of arroyos during the rainy season may promote seed-mediated gene flow.

Keywords: genetic structure; seed dispersal; megafauna dispersal syndrome; bat pollination; hydrochory; isolation by distance; spatial autocorrelation

## INTRODUCTION

The genetic diversity within plant populations is often non-randomly distributed in space, leading to genetic structure (Linhart et al., 1981; Loveless and Hamrick, 1984; Epperson et al., 1990; Heywood, 1991; Vekemans and Hardy, 2004). For plant species, genetic structure is influenced by pollen and seed flow, which in turn is influenced by the behavior of pollinators and seed dispersers (Levin and Kerster, 1974; Levin, 1981; Loveless and Hamrick, 1984; Hamrick, Murawski, and Nason, 1993; Hamrick and Nason, 1996). Plant population genetic structure is also broadly shaped by landscape features, which set the ecological stage for pollinators and seed dispersers, as well as plant adaptive responses to the environment (Sork et al., 1999; Sork and Smouse, 2006; Holderegger et al., 2010).

Plants that are pollinated by highly mobile vertebrate pollinators, such as birds and bats, might be expected to have high gene flow and low genetic structure (Loveless and Hamrick, 1984). Many studies have demonstrated that gene flow is higher among plants that are pollinated by birds and plants than plants pollinated by other vectors. For example, within three species of *Penstemon* in the Great Basin of the U.S., the hummingbird-pollinated *P. rostriflorus* had much lower genetic structure than the bee-pollinated *P. deustus* and *P. pachyphyllus* (Kramer, Fant, and Ashley, 2011). Similarly, a study on two species of *Streptocarpus* found lower genetic structure in the sunbird-pollinated *S. dunnii* than the fly-pollinated *S. primulifolius*, which the authors attribute to the larger range of the bird pollinators (Hughes et al., 2007). Even when not strictly compared to an insect-pollinated relative, bird and bat-pollinated plants often show relatively high levels of pollen flow; one study found pollen moving over 5km (Byrne et al., 2007) and another showed extremely low spatial genetic structure coupled with multiple - paternity within fruit, indicating high pollen carry-over (Krauss et al., 2009). In addition, two

studies of the bat-pollinated tropical tree *Hymenaea courbaril*, which were conducted in different countries (Puerto Rico; Brazil) with different molecular markers (allozymes; microsatellites), reported similarly low estimates of genetic structure ($G_{ST}$=0.053 and pairwise population $F_{ST}$= 0-0.015), indicating high pollen flow (Dunphy, Hamrick, and Schwagerl, 2004; de Lacerda, Kanashiro, and Sebbenn, 2008).

Seed dispersal is perhaps even more influential than pollination as a process that shapes plant genetic structure, as it is the first step in moving plant genetic material to new areas, (Nathan and Muller-Landau, 2000; Wang and Smith, 2002; Simon et al., 2003; Grivet, Smouse, and Sork, 2005; Sork and Smouse, 2006). In addition, seed movement tends to be more restricted than pollen flow; a model developed to estimate the ratio of pollen to seed flow suggested a range of 4:1 for barley, and 200:1 for oaks (Ennos, 1994). Several techniques are traditionally deployed to detect population genetic structure using neutral molecular markers, and make inferences about the level of gene flow among populations (Ouborg, Piquot, and Van Groenendael, 1999; Wang and Smith, 2002). Studies frequently report genetic structure using *F*-statistics (Wright, 1951; Weir and Cockerham, 1984) , and infer restricted seed dispersal from high $F_{ST}$ values (Dutech et al., 2002; Latouche-Halle et al., 2003). Other studies have used a Bayesian clustering approach such as STRUCTURE (Pritchard, Stephens, and Donnelly, 2000) to infer discrete genetic clusters, which can be the result of historically restricted gene flow (Kramer, Fant, and Ashley, 2011; Fant et al., 2014). This approach can be advantageous because one does not assume the number of populations that individuals belong to *a priori;* in addition, if clusters are identified, assignments of individuals to groups can be overlaid on a geographic map to develop hypotheses about possible landscape features that affect gene flow and genetic structure (Anderson et al., 2010).

Restricted dispersal can also be inferred by observing significant patterns of isolation by distance (e.g. Tero et al., 2005), due to genetic drift acting on populations to make them more divergent as the distance between them increases (Wright, 1943). However, distinguishing between isolation by distance patterns and concrete genetic clustering can be difficult in plant populations, especially when individuals are not evenly sampled across the landscape (Anderson et al., 2010). Additional analyses that examine the genetic relationship between individuals at different geographic scales, such as spatial autocorrelation analysis, can be advantageous to resolve inferences about gene flow and the spatial scale of dispersal (Heywood, 1991; Gonzales et al., 2010). For some plants, this analysis has revealed dispersal may be very limited, at the scale of 5 meters (Sork, Huang, and Wiener, 1993; Loiselle et al., 1995), while others species completely lack fine scale genetic structure (Dewey and Heywood, 1988; Berg and Hamrick, 1995; Gugger et al., 2008).

As predicted with highly mobile vertebrate pollinators, large, mobile frugivorous animals should promote long-distance seed dispersal resulting in low genetic structure (Hamrick, Murawski, and Nason, 1993; Jordano et al., 2007; Karubian et al., 2010). For example, many plant species whose seeds are dispersed by bats show high levels of gene flow and low population genetic structure (Nassar, Hamrick, and Fleming, 2003; Parra et al., 2008; Bizoux et al., 2009). Conversely, there is evidence that when population densities of flying foxes (large bats in the Family Pteropodidae) fall below a certain threshold, seed dispersal of plants with large seeds functionally ceases to occur (McConkey and Drake, 2006). For plants that have lost their co-evolved seed disperser to extinction events, seed dispersal is likely highly restricted, resulting in high genetic structure of adult plants (Guimarães, Galetti, and Jordano, 2008; Pires et al., 2014).

Here we examine the genetic structure of *Crescentia alata*, a common shrubby tree in seasonally dry tropical forest, which is pollinated by bats and whose fruit often accumulate below the maternal tree, undispersed. Janzen and Martin proposed that this plant species originally co-evolved with megafaunal species that ate the fruits and dispersed the seeds, but went extinct in the Pleistocene (Janzen and Martin, 1982). They suggest that the extinction of this megafauna circa 10,000 years ago caused a constriction in the range of *C. alata*, until the re-introduction of horses to Mexico and Central America during Spanish colonization, at which point the introduced horses became substitute seed dispersers. Guimarães Jr. et al. (2008) recently analyzed traits of potentially extinct-megafauna-dispersed fruit, and identified two distinct types: large fruits with extremely large individual seeds, and extremely large fruits with many small seeds (Guimarães, Galetti, and Jordano, 2008). Fruits from *Crescentia alata* trees are consistent with this latter category.

Our overall goal is to determine if the extinction of *Crescentia alata*'s putative dispersal agent (a Pleistocene horse species) has impacted the plant's population genetic structure. To investigate the distribution of genetic diversity in *Crescentia alata* populations, we developed 8 novel microsatellite primers for the species, and genotyped 161 individuals from our study area in Jalisco, Mexico. If seed dispersal now occurs mainly through gravity, we expect there to be locally restricted gene flow. Our objectives are to analyze patterns of genetic diversity, to look for 1) genetic sub-structuring across the region; 2) high genetic structure among putative populations; 3) evidence for isolation by distance; 4) evidence for spatial autocorrelation among individuals at a small spatial scales.

## METHODS

*Study species. Crescentia alata* HBK (Bignoniaceae) is a shrubby tree distributed from México to Costa Rica. The flowers are bat-pollinated and have many traits consistent with a bat pollination syndrome (van der Pijl, 1961): they are large, greenish-pink, hermaphroditic, produce copious nectar, have a sulfur-fetid smell, and anthesis occurs shortly after dusk. In our study area, the bat species *Leptonycteris yerbabuenae* and *Glossophaga soricina* are the main pollinators (see Chapter 2). Trees flower shortly after the start of the rainy season, in late June-July, and flowering lasts for 2-4 weeks. Each flower is open for a single night.

*Crescentia alata* fruits start developing about one week after pollination, and remain on the tree maturing for several months to one year. They are large, with 200-1000 seeds (see Chapter 2 for details on fruit size and seed number). The fruits have hard outer husks that are difficult to open; an average of 200kg of pressure is required to break a fruit (Janzen, 1982b). The pulp of the fruit turns black and sticky when it is mature, and is used by people in México in a medicinal recipe for respiratory ailments (Rojas et al., 2001). The outer husk is also used for ladles, decorative bowls, and maracas (Gentry, 1992).

The seed dispersal mechanism remains unclear, and may be variable at different locations. Human activity and cultivation may influence the distribution of this tree, although a congener, *Crescentia cujete,* has a more established history of domestication (Aguirre-Dugua et al., 2012). Janzen hypothesized that Pleistocene horses were the original seed dispersers, with modern horses as substitutes, and rodents (particularly *Liomys salvini*) secondarily dispersing seeds from horse dung (Janzen, 1982b; Janzen and Martin, 1982). We have personally observed a substantial number of fruit remaining uneaten and unbroken within cattle pens around our study sites, confirming Janzen's statement that cattle do not eat the fruit or consequently disperse

the seeds (Janzen, 1982a). Fruits are also able to float in water (Gentry, 1974), and have been observed floating for at least 20 days and remaining intact (personal observation). Seedlings have also been observed germinating in the remains of broken fruit. The distributions of these trees along arroyos, which seasonally flood with rain, make the potential for water dispersal a possibility, and there are reports of *C. alata* seedlings growing along sandbars in Guanacaste, Costa Rica (Gentry, 1974).

*Study site and sampling*. Our study site was in and around the Chamela-Cuixmala Biosphere Reserve in Jalisco, Mexico (19º30'N, 105º02'W, Figure 3-1). This area is dominated by a seasonal tropical dry forest ecosystem. The *Crescentia alata* trees in this region tend to occur along the highways, in arroyos, and as remnant trees on farms and ranches. We searched for trees along all accessible arroyos within 10km of Federal Highway 200, along a span of 92 km, which resulted in extensive surveying of approximately 81.4 hectares. The sampling localities are described in Table 3-1. We collected leaf samples and marked each sampled tree using a handheld GPS unit.

*Molecular marker development*. Limited genetic data is available for *Crescentia alata*, so we developed microsatellite markers *de novo* for the species following the general procedure outlined in Abdelkrim et al. (2009) and Gaddis et al. (2014). This involves the use of high-throughput sequencing technology to generate thousands of short DNA fragments, which can then be screened for microsatellites (short tandem repeats of base pairs). We extracted ~ 3 µg of genomic DNA from dried *C. alata* leaves using a modified Cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987), and prepared the DNA library for $1/16^{th}$ of a run on the Roche 454 Genome Sequencer FLX system at the UCLA Genotyping and Sequencing Core. The run yielded 30,000 sequences, which we screened for microsatellites using the software

program MSATCOMMANDER (Faircloth, 2008). We also used this program to design primers, and tested them for polymorphism and reliable amplification on 8 *C. alata* individuals that were sampled across the range of the study area. Once we had a set of appropriate candidate markers, we ordered fluorescent dye-labeled primers, and used these in multi-plex PCR to genotype the samples. We assessed the loci for null alleles, allele dropout, and evidence of stuttering using MICRO-CHECKER (Van Oosterhout et al., 2004).

*Genotyping.* Leaves were collected from each tree and dried using silica gel in the field. We extracted DNA from each sample using the previously-mentioned CTAB protocol, and amplified the DNA with the fluorescently-tagged microsatellite primers, using a Qiagen multiplex mix and the following recipe: 2.5 µl DNA, 5 µl multiplex mix, 1.1 µl water, 0.4µl bovine serum albumin (BSA), and 1µl forward and reverse primer mix.

The reaction cycle consisted of an initial 15 minute denaturing at 95 °C, followed by 26 cycles at 94 °C for 30 seconds, then annealing at 53 °C for 90 second, and extension at 72 °C for 60 seconds. We ran the following 20 cycles at 94 °C for 30 seconds, 53 °C for 90 seconds, and 72 °C for 60 seconds (Schweizer *et al.*, 2009). We ran the final extension step at 60°C for 30 minutes. We did fragment analysis at the UCLA Genotyping and Sequencing Core and we made genotype calls in the lab using GeneMapper® (Applied Biosystems, Waltham, Massachusetts).

*Genetic Analyses.* We analyzed the genotypes from 161 individuals, across 8 microsatellite loci, using GenAlEx version 6.501 (Peakall and Smouse, 2006, 2012). Assuming the sampling localities as putative sub-populations, we averaged values across loci and report the following genetic diversity measures: $N_a$ (number of different alleles), $P_a$ (number of private alleles), $H_o$ (observed heterozygosity), $H_e$ (expected heterozygosity), and $F$ (fixation index).

To determine whether our sampled sites represent one or more than one subpopulations, we used the Bayesian assignment program STRUCTURE 2.3 (Pritchard, Stephens, and Donnelly, 2000) to look for clustering in the genetic data. We set the following parameters for the STRUCTURE analysis: length of burn-in period: 50,000; number of MCMC repetitions: 500,000; use of an admixture model; use of sampling site information for LOCPRIOR; set allele frequencies as correlated; and set the range of clusters to the highest possible number of populations (K=1-16). Following advice from Gilbert et al. (2012), we ran 20 iterations of this parameter set for better reproducibility of our results. Results from the run were analyzed with StructureHarvester (Earl and Vonholdt, 2012), and the most likely K (number of clusters) was chosen from the ΔK graph, following the Evanno method (Evanno, Regnaut, and Goudet, 2005). The iterations of the chosen K were organized using the program CLUMPP (Jakobsson and Rosenberg, 2007), and visualized using Excel. The coefficients of membership in each cluster were also used to create pie charts of proportional membership in the groups, and overlaid on a base map of the region in ArcGIS® (Esri, Redlands, California).

We tested for the effect of regional substructure by performing a hierarchical AMOVA among the number of clusters suggested by the STRUCTURE analysis, and among sampling localities as sub-populations. The significance of the F-statistics is calculated in GenAlEx based on a permutation test; the *P*-value indicates if there is significant population differentiation away from the null of zero. We report the results of this AMOVA using $F_{RT}$ (estimated variance among regions / total variance), $F_{SR}$ (estimated variance among populations / (estimated variance within and among individuals + estimated variance among populations)), and $F_{ST}$ (estimated variance among regions and populations / total variance).

We used GenAlEx to create a codominant genotypic distance matrix between individual trees, as well as a geographic distance matrix (Peakall and Smouse, 2006). To determine the codominant genotypic distance between pairs of individuals, the distance between the four possible allele combinations is squared; the resulting values can range between 0-4 for each locus (Smouse and Peakall, 1999). Because each locus is considered independent, the values for distance are added together to get total genetic distance between individuals. The geographic distance between two individuals is determined by calculating Euclidean distances between sampling coordinates (in the UTM system). We then tested for significant isolation by distance using a permutation-based Mantel test, which iteratively shuffles values in rows and columns in one matrix, and assesses the correlation with the other matrix. The correlation values from the permutated samples are compared with the actual correlation between the genetic and geographic matrices.

We also used GenAlEx to assess for patterns of spatial autocorrelation among individuals. This procedure utilizes the genetic and geographic distance matrices mentioned previously, and gives correlation coefficients of genetic similarity between individuals that fall within specified distance classes (Smouse and Peakall, 1999). Similarly to the Mantel test described above, GenalEx implements a permutation test to give a distribution of random correlations between individuals, and tests for significant departures from the random distribution in the real data. We looked at two scales for spatial autocorrelation: regional (including the whole study region, 92 kilometers by 10 kilometers), and fine-scale (focused on a subset of samples that occur within 800 meters of each other). For the regional scale, we modified the geographic distance matrix by dividing each value by 1,000, so that the resulting correlelogram can be read in kilometers, rather than meters. For both spatial scales, we chose 5

distance classes, with an endpoint that increased logarithmically (regional endpoints are 6km, 12km, 24km, 48km, and 96km; fine-scale endpoints are 50m, 100m, 200m, 400m, and 800m). This created semi-even sampling in each distance class.

## RESULTS

*Molecular Marker development.* We tested 41 potential simple sequence repeat (SSR or microsatellite) markers, and selected 8 dinucleotide microsatellites that were polymorphic and amplified reliably across the tested individuals of *Crescentia alata* (Table 3-2). None of the loci showed evidence of null alleles, stutter, or large allele dropout, and the total population of adults exhibits frequencies consistent with Hardy Weinberg equilibrium. The number of alleles ranged from 4-13 across loci. The loci were 100% polymorphic in each putative population (sampling locality).

*Genetic structure***.** Using STRUCTURE, we detected significant clustering of individuals into two groups (K=2), when we added the sampling locality as prior information before running the simulation. The least negative average values for ln(prob(K)) were found for K=2, and the ΔK peaked at K=2. We sorted the individuals based on spatial arrangement (latitude multiplied by longitude), and then visualized their membership in the two groups (Figure 3-2). We then visualized their proportional STRUCTURE assignments on a map of the region (Figure 3-3), and inspected these relationships at a fine-scale to determine where clusters separated (Figure 3-4).

We performed a hierarchical AMOVA, using the two regions suggested by the two clusters from the STRUCTURE analysis, and populations based on the sampling localities. We found significant genetic structure at the defined regional and population levels ($F_{RT}$= 0.019, $F_{SR}$= 0.030, $F_{ST}$= 0.049; P < 0.001 for all values).

We did not find a significant correlation between genetic and geographic distance, when we looked at these distances among individuals ($r_{xy}$=0.051, p= 0.150). However, when we separated the samples into groups by their sampling locality, and performed a correlation between population-level mean genotypic distance and geographic distance, there was a significant signal ($r_{xy}$=0.468, p= 0.010, Figure 3-5). The correlations within each sampling site were mostly non-significant, probably due to the small sampling size for these comparisons. Two sites, Colorado (13 individuals, sampled across 1.9km) and Careyes_a (12 individuals, sampled across 1.8km) had significant correlations between genetic and geographic distance, but in opposite directions. Colorado showed an expected correlation between increasing genetic distance with increasing geographic distance ($r_{xy}$=0.378, p= 0.019) while Careyes_a showed a correlation of increasing genetic distance with decreasing geographic distance ($r_{xy}$= -0.237, p= 0.048). This latter result might be spurious, but these correlation results at the within-population level help explain the lack of significance found at the individual distance level.

At the regional scale, we found significant positive spatial autocorrelation at the first two distance classes (0-6km, 6-12km, Figure 3-6), but the correlation coefficients as these distance classes were very low (r =0.08 for 0-6km, r= 0.027 for 6-12km; Table 3-S1). There was significant positive spatial autocorrelation in all distance classes we examined at the finer spatial scale (0-800m, Figure 3-7), and kinship coefficients were higher among the individuals (Table 3-S2). Samples at this fine-scale mostly represent individuals within the same site. These findings do not provide support for restricted gene flow.

## DISCUSSION

Our findings demonstrate considerable gene flow among *Crescentia alata* trees in this region of México, and no evidence that the extinction of the plant's putative dispersal agent has created high genetic structure. Instead, we found relatively low genetic structure, and evidence for low genetic relatedness among the trees when we examined spatial autocorrelation at a regional scale. We did find a correlation between genetic and geographic distance, but only at the inter-population level, and most of the within-population correlations were not significant, indicating isolation by distance is only occurring at a large geographic scale.

One likely explanation for the low genetic structure is pollen movement by bats. Bats are known to use natural corridors such as trails (Hein, Castleberry, and Miller, 2009), and streams for movement through the landscape, and these landscape elements may influence nectarivorous bat foraging patterns, and subsequent pollen movement of bat-pollinated trees. Thus, it is likely that they are contributing to both high and non-random gene flow. This interpretation is supported by our findings on contemporary gene flow from an analysis of genetic structure among pollen pools (Chapter 3). We found that that pollen movement among *C. alata* trees is more extensive in fragmented sites, further supporting the idea that open landscape elements promote pollen flow. We also found that the number of effective pollinators per tree was low in both continuous and fragmented sites, but that bats maintained connectivity via pollen flow across large geographic areas.

In a study of another species with an extinct megafaunal seed disperser, Gonzalez et al. (2010) found that gene flow in *Enterolobium cyclocarpum* remained high, likely due to wind-pollination and high adult densities (Gonzales et al., 2010). They also found that adult densities helped explain spatial autocorrelation patterns, where the average kinship coefficient across 4

sites was high at the first distance class (r = 0.321, 1-64m), but then grew smaller and insignificant in higher distance classes, conforming to trends in other studies (Hardy et al., 2006). Although our regional spatial autocorrelation results follow this pattern, our findings indicate significant relatedness between individuals over much larger distances (0-12km), but with a weak kinship coefficient ($0.008 < r < 0.027$). At the fine scale, our data indicates significant positive autocorrelation between all distance classes (0-800m), but again, the kinship coefficient is lower ($0.038 < r < 0.106$) than for the study of *Enterolobium cyclocarpum*. Most of the pairwise comparisons in the fine-scale distance classes are between individuals at the same sampling locality (an arroyo or patch), since the average length of a sampling site is 1.77km; therefore their significant autocorrelation might be expected, but also indicates some geographic sub-structuring that is not apparent with our results from the analysis of $F_{ST}$.

This pattern of fine scale genetic structure does not suggest seed dispersal by gravity, which would cause high correlations between individuals at the scale of 50 meters, which would then drop off at higher distance classes. We therefore propose an alternative explanation. The distribution of the *C. alata* trees in arroyos can also influence seed dispersal, by affecting the foraging patterns of secondary dispersers such as rodents, and/or the movement of seeds by flowing water. As suggested by Gentry (1974) for *C. alata* in Costa Rica, fruit may move extensively during seasonal rains that cause flooding in low elevation areas. There is evidence that some plant species in low-lying floodplains have traits such as indehiscent fruits and fruiting phenology coinciding with rainfall, to take advantage of seasonal rains (Kubitzki and Ziburski, 1994). In addition, there is evidence that suggests the genetic structure of plants can be influenced by seed dispersal along riverbanks, which promotes long distance seed movement (Jacquemyn et al., 2006).To investigate this hypothesis, we created a map of hydrologic

connections in the region, and overlaid the STRUCTURE pie charts on the map (Figure 3-8). We found that two sampling sites located very near each other, Chamela and Colorado, seemed particularly different genetically. Closer examination of the watersheds revealed that these sites are connected, but that each side of the stream seems to come from separate headwaters, with potentially separate source populations (Figure 3-9). The hierarchical AMOVA results also support the idea that this region is sub-structured based on watersheds. Future research should focus on explicitly testing the role of hydrologic connectivity and divergence in shaping genetic diversity patterns in this species.

In conclusion, our findings indicate widespread gene flow in *Crescentia alata* populations in this region of Mexico, in contrast to our predictions of restricted seed dispersal due to the loss of the original seed dispersal agent. Some genetic structuring was detectable, and may be related to watershed and water dispersal of the fruits and seeds, but the genetic homogenizing effect of bat-mediated pollen flow is also likely influencing genetic diversity patterns.


Figure 3-1: Map of the sampling sites on the Central Western coast of México, created using the imagery base-map in ArcGIS 10.0 (Esri, Redlands, California).

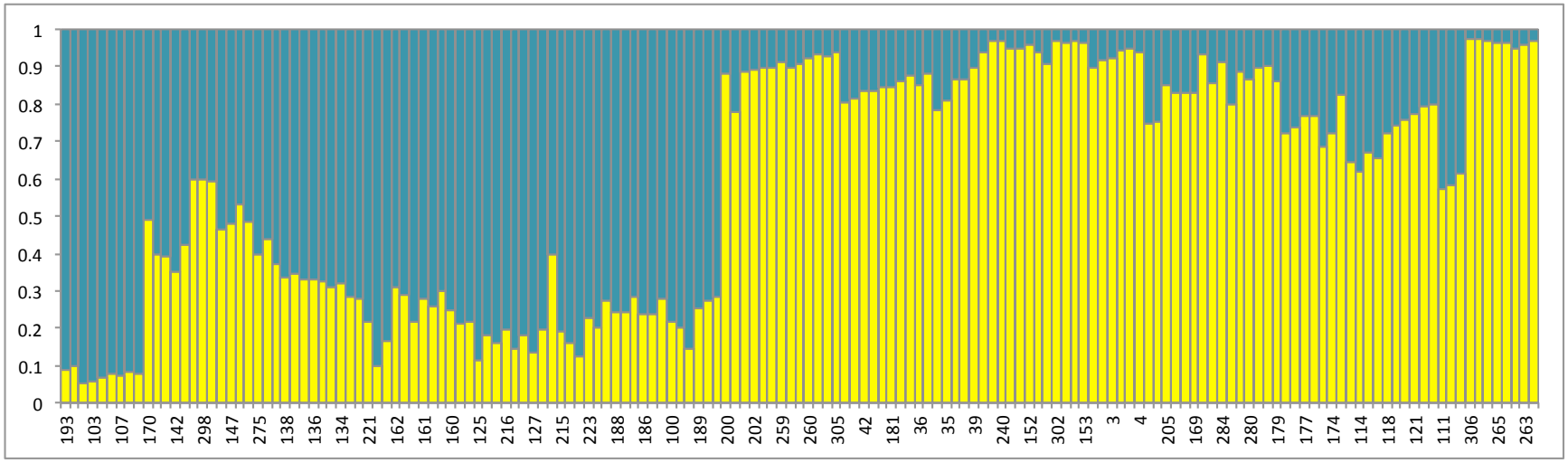


Figure 3-2: Results of the STRUCTURE analysis, with K=2, and individuals sorted by latitude multiplied by longitude. The numbers on the bottom of the graph are the IDs of individual trees.

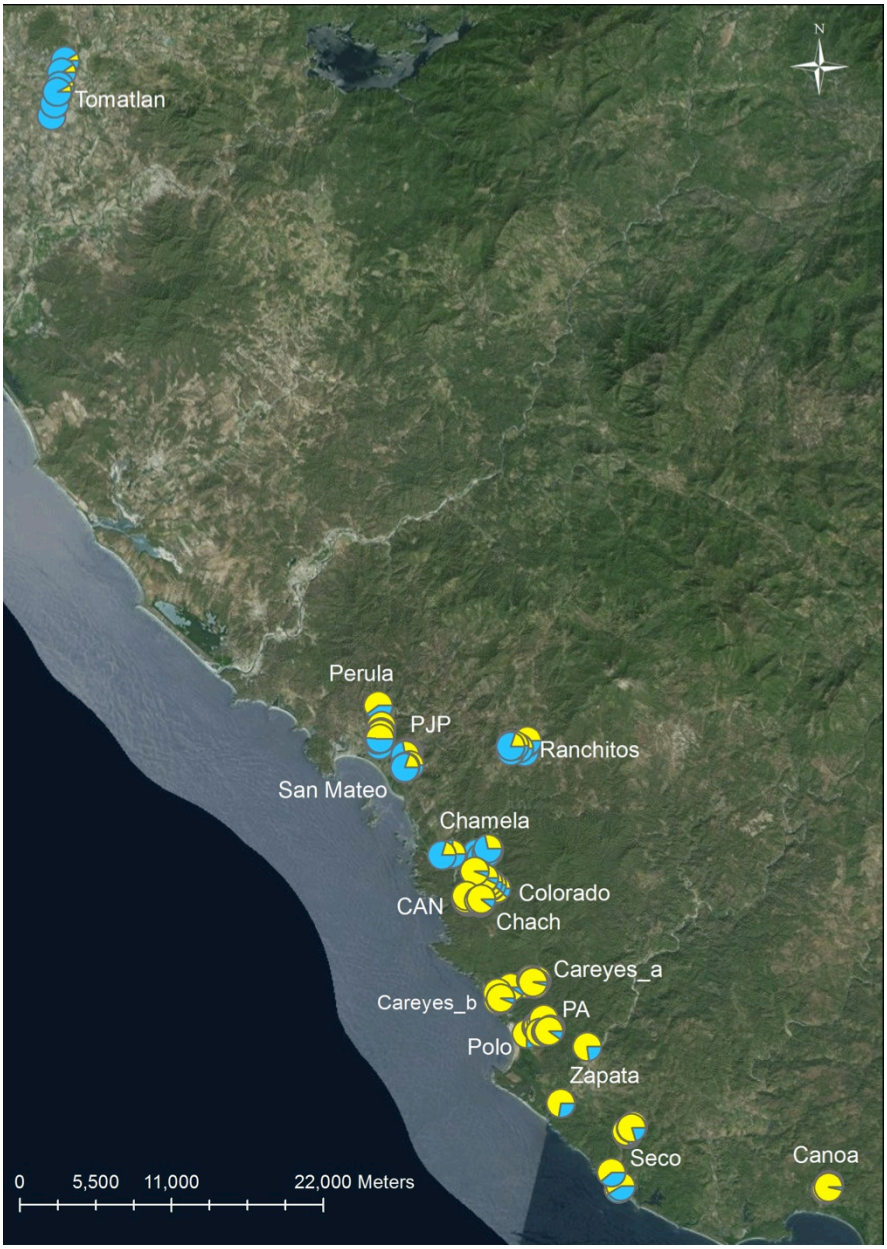


Figure 3-3: Structure assignments of individual trees represented as pie charts showing proportion of membership in one of two groups; landscape at broadest view, which includes all sites sampled. Map created using the National Geographic imagery base-map in ArcGIS 10.0 (Esri, Redlands, CA).

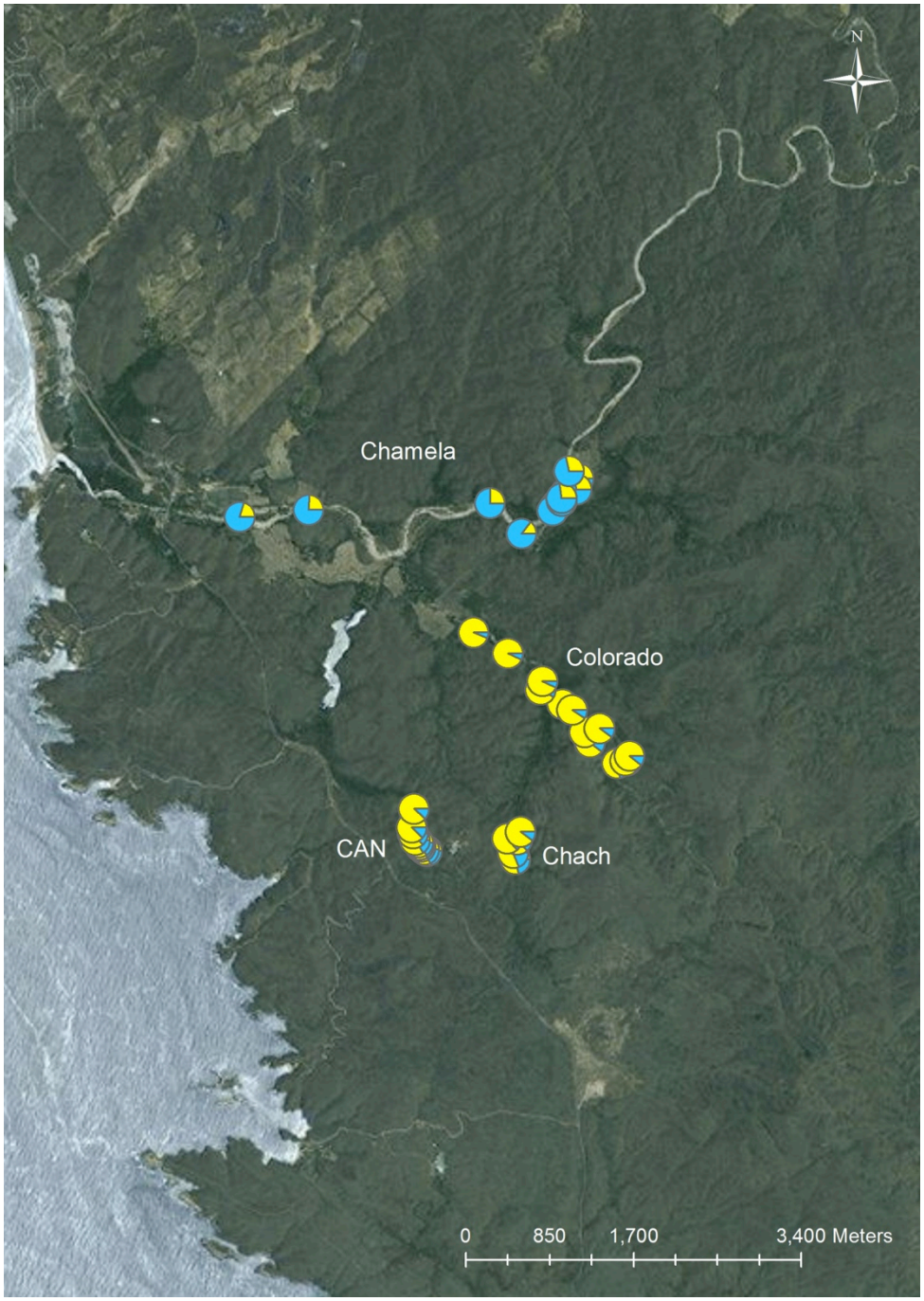


Figure 3-4: Structure assignments represented as pie charts showing proportion of membership in one of two groups; close-up image of the area where the two clusters separate most clearly. Map created using the National Geographic imagery base-map in ArcGIS 10.0 (Esri, Redlands, CA).

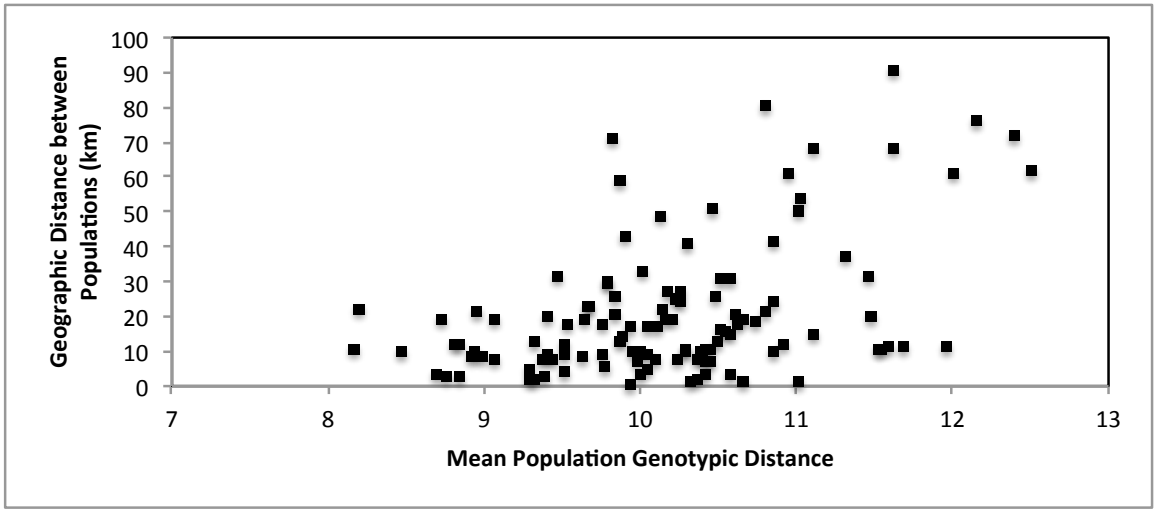


Figure 3-5: Results from a permutation test of the correlations between the population (sampling locality) genotypic distance matrix and matrix of geographic distances between sampling localities, in km ($r_{xy}$=0.468, $P < 0.010$).

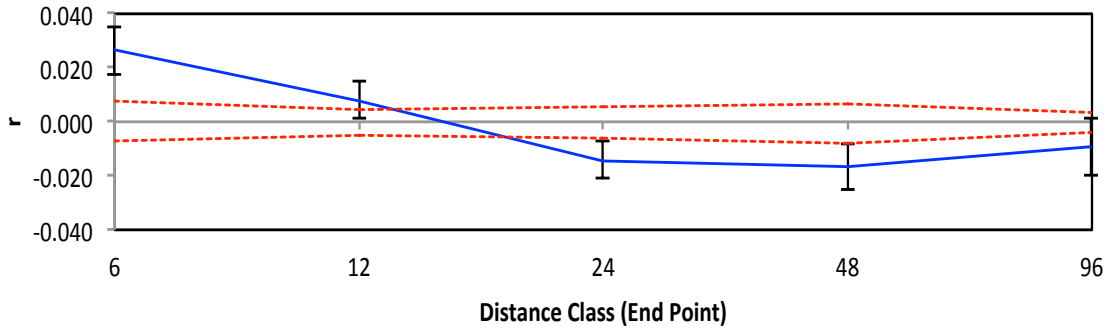


Figure 3-6: Spatial autocorrelation analysis across five distance classes, for regional scale distances (in kilometers). The blue line is the average correlation coefficient (r) across the permuted samples; the red dotted lines represent the jackknife estimates of what the null hypothesis ($r = 0$) would allow, while the error bars at each distance class are the bootstrap estimates for the alternative hypothesis ($r \neq 0$).

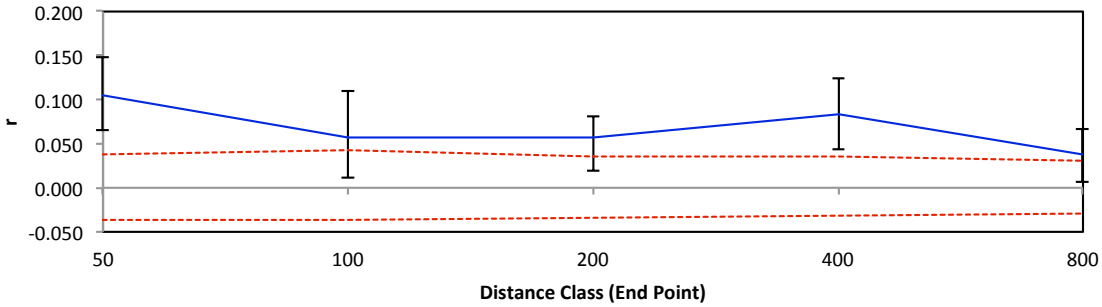


Figure 3-7: Spatial autocorrelation analysis across five distance classes, for fine scale distances (in meters). The blue line is the average correlation coefficient (r) across the permuted samples; the red dotted lines represent the jackknife estimates of what the null hypothesis ($r = 0$) would allow, while the error bars at each distance class are the bootstrap estimates for the alternative hypothesis ($r \neq 0$).

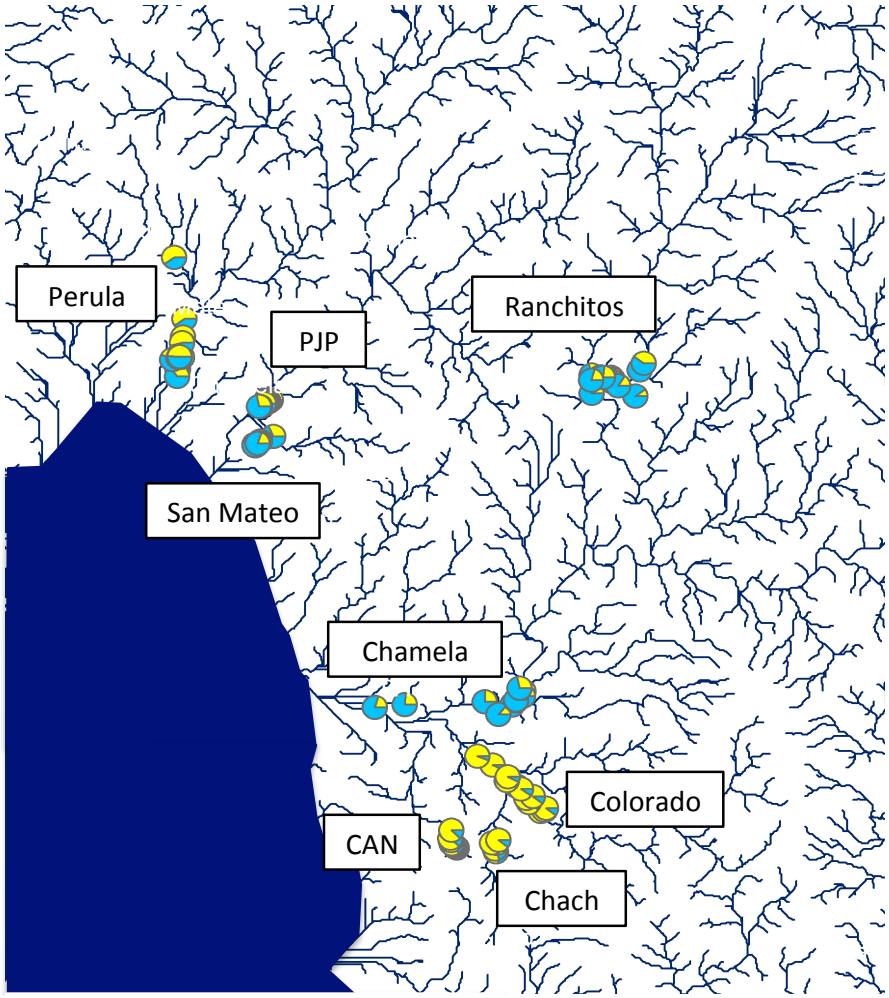


Figure 3-8: Maps of STRUCTURE assignments overlaid on a map of hydrologic connectivity; focus is on several study sites at the center of the study area with the site names labeled. Map created with a digital elevation model provided by the USGS, and hydrology functions in ArcGIS 10.0 (Esri, Redlands, CA).

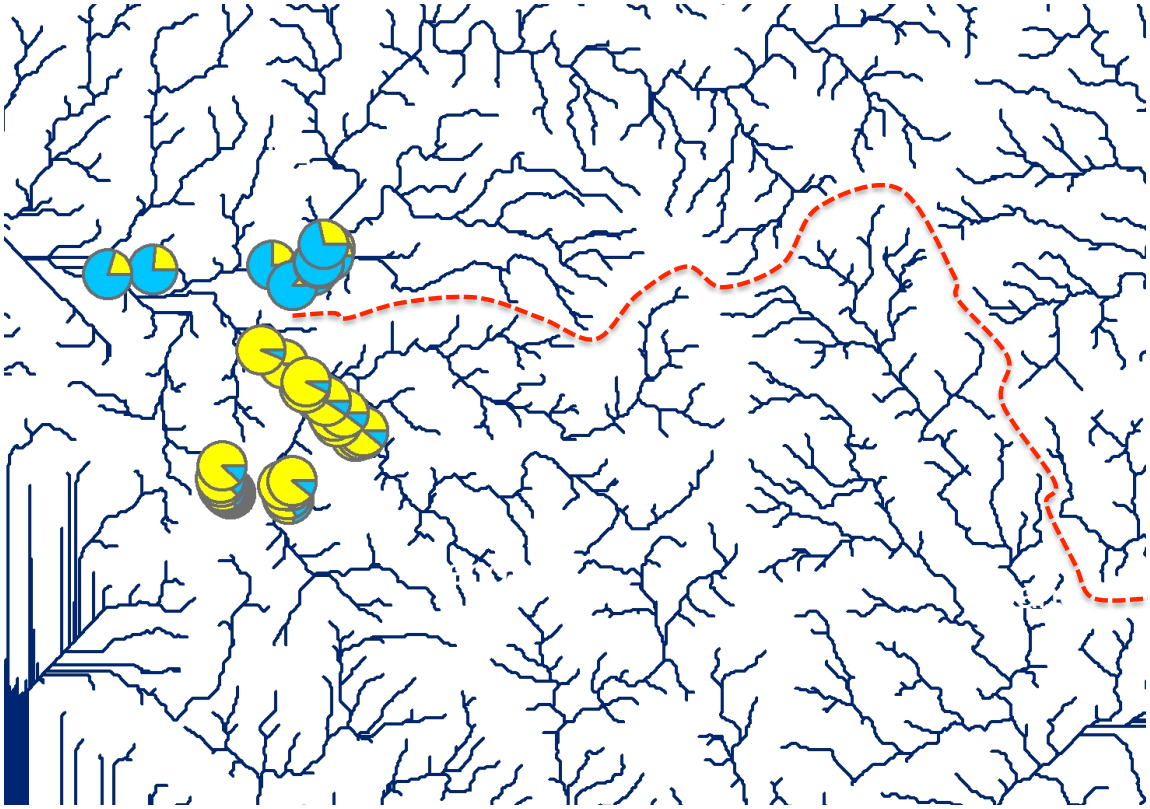

Figure 3-9: Maps of STRUCTURE assignments overlaid on a GIS map of hydrologic connectivity; focus is on the sites where genetic structure was most divergent, with a dashed line placed at a possible divergence in headwaters (area where hydrologic flows do not connect). Map created with a digital elevation model provided by the USGS, and hydrology functions in ArcGIS 10.0 (Esri, Redlands, CA).

Table 3-1: Locations and description of sites, arranged from North to South. Coordinates are in decimal degrees and elevation is in meters. The area of the site was estimated using straight-line distances between sampling points for length and width in meters, and converted to hectares. Density was estimated based on the number of trees sampled at the site divided by the estimated area of the site.

| Site ID | Site Name | Number of trees | Latitude | Longitude | Elevation (m) | Area (ha) | Density (trees/ha) |
|---|---|---|---|---|---|---|---|
| 8 | Tomatlan | 9 | 19.98925 | -105.31662 | 42.0 | 7.71 | 1.17 |
| 9 | Perula | 12 | 19.59583 | -105.10483 | 20.8 | 8.09 | 1.48 |
| 10 | Ranchitos | 13 | 19.59470 | -105.01910 | 127.5 | 3.93 | 3.31 |
| 11 | PJP | 12 | 19.58923 | -105.08608 | 16.2 | 0.45 | 26.94 |
| 12 | San Mateo | 13 | 19.58038 | -105.08848 | 14.2 | 0.44 | 29.58 |
| 7 | Chamela | 13 | 19.52838 | -105.03477 | 22.0 | 19.53 | 0.67 |
| 6 | Colorado | 13 | 19.50862 | -105.03130 | 34.2 | 3.81 | 3.41 |
| 4 | Chach | 6 | 19.49972 | -105.03848 | 50.2 | 0.27 | 22.48 |
| 5 | CAN | 9 | 19.49880 | -105.04783 | 51.8 | 0.42 | 21.46 |
| 2 | Careyes_a | 12 | 19.44900 | -105.00513 | 39.3 | 2.79 | 4.30 |
| 1 | Careyes_b | 6 | 19.43807 | -105.02583 | -2.5 | 2.93 | 2.05 |
| 3 | PA | 7 | 19.42108 | -105.00243 | 17.7 | 1.55 | 4.51 |
| 19 | Polo | 7 | 19.41820 | -104.99628 | 29.6 | 1.60 | 4.38 |
| 13 | Zapata | 7 | 19.37407 | -104.98648 | 10.0 | 8.53 | 0.82 |
| 14 | Seco | 14 | 19.35622 | -104.94350 | 32.6 | 17.63 | 0.79 |
| 18 | Canoa | 8 | 19.32186 | -104.81252 | 10.0 | 1.71 | 4.69 |

Table 3-2: Primer sequences for the 8 microsatellite loci, their dinucleotide motifs, size ranges and number of alleles.

| Locus | Primer sequence | Motif | Size range (bp) | Number of Alleles |
|---|---|---|---|---|
| CRE1 | F: TGCTATTGATGGGTTGCGG | (CA)9 | 254-264 | 5 |
| | R: GCTCAGATTGACTGTATGCTCC | | | |
| CRE4 | F: GCAGTGGCTACCTGAGACC | (AT)10 | 211-233 | 10 |
| | R: ACATTACACACATCCTCTTGTATCG | | | |
| CRE9 | F: TCCAAGTTACGTGATTGTTCCAG | (TG)11 | 209-235 | 13 |
| | R: GACATATAGTTCCACTGCTCCG | | | |
| CRE25 | F: TGAAACCCAATTACTTTCCGATG | (TG)10 | 167-179 | 7 |
| | R: AACTACCCTTCCGTGTGCC | | | |
| CRE27 | F: TGCCTCCAGGTTTCCTTCG | (CA)10 | 253-271 | 5 |
| | R: GGAGTCCAATAAGCGTCCC | | | |
| CRE28 | F: TCACTGTTAACTGGCCACC | (TA)10 | 163-173 | 5 |
| | R: AAACCTCCCTCAGGTTCGG | | | |
| CRE29 | F: CAGATGATCGACACTTGTTGAATTG | (TA)9 | 151-161 | 4 |
| | R: CGTTCCTGCCTGAAGTTTCC | | | |
| CRE30 | F: ACTCCGTTGAGTAACTTCAGTTTG | (AG)10 | 222-274 | 10 |
| | R: TGAGAAGGCTGTACGCTGG | | | |

Table 3-3: Summary statistics, with the following parameters: $N_a$= average number of alleles, $P_a$= average number of private alleles, $H_o$= observed heterozygosity, $H_e$=expected heterozygosity, $F$=fixation index.

| Site ID | Site Name | n trees | $N_a$ | $P_a$ | $H_o$ | $H_e$ | F |
|---|---|---|---|---|---|---|---|
| 8 | Tomatlan | 9 | 3.625 | 0.125 | 0.556 | 0.563 | 0.001 |
| 9 | Perula | 12 | 4.750 | 0.000 | 0.667 | 0.568 | -0.170 |
| 10 | Ranchitos | 13 | 4.750 | 0.500 | 0.558 | 0.573 | 0.028 |
| 11 | PJP | 12 | 4.500 | 0.000 | 0.656 | 0.625 | -0.053 |
| 12 | SM | 13 | 4.500 | 0.125 | 0.692 | 0.611 | -0.148 |
| 7 | Chamela | 13 | 4.625 | 0.000 | 0.731 | 0.600 | -0.210 |
| 6 | Colorado | 13 | 4.375 | 0.000 | 0.644 | 0.574 | -0.096 |
| 5 | CAN | 9 | 4.625 | 0.000 | 0.625 | 0.596 | -0.060 |
| 4 | Chachalaca | 6 | 3.375 | 0.000 | 0.604 | 0.589 | -0.028 |
| 2 | Careyes--Arroyo | 12 | 4.000 | 0.000 | 0.552 | 0.516 | -0.022 |
| 1 | Careyes--Beach | 6 | 3.625 | 0.000 | 0.667 | 0.566 | -0.173 |
| 3 | PA | 7 | 3.625 | 0.125 | 0.714 | 0.577 | -0.251 |
| 19 | Polo | 7 | 4.000 | 0.000 | 0.571 | 0.568 | -0.023 |
| 13 | Zapata | 7 | 3.875 | 0.000 | 0.661 | 0.551 | -0.219 |
| 14 | Seco | 14 | 4.375 | 0.000 | 0.696 | 0.603 | -0.169 |
| 18 | Canoa | 8 | 4.375 | 0.000 | 0.688 | 0.626 | -0.062 |

Table 3-S1: Results from the regional-scale spatial autocorrelation analysis across 5 distances classes (distances in kilometers), including samples sizes of permutations in each distance class, the estimates of kinship coefficients (r), and upper and lower confidence intervals around the estimate.

| **n** | **2417** | **3721** | **3448** | **1944** | **1350** |
|---|---|---|---|---|---|
| **Distance Class (end point)** | **6** | **12** | **24** | **48** | **96** |
| P(r-rand >= r-data) | 0.001 | 0.001 | 1.000 | 1.000 | 1.000 |
| U | 0.008 | 0.005 | 0.005 | 0.006 | 0.003 |
| r | 0.027 | 0.008 | -0.014 | -0.017 | -0.009 |
| L | -0.007 | -0.005 | -0.006 | -0.008 | -0.004 |
| P(r-rand <= r-data) | 1.000 | 1.000 | 0.001 | 0.001 | 0.001 |

Table 3-S2: Results from the fine-scale spatial autocorrelation analysis across 5 distances classes (distance in meters), including samples sizes of permutations in each distance class, the estimates of kinship coefficients (r), and upper and lower confidence intervals around the estimate.

| **n** | **103** | **95** | **128** | **141** | **185** |
|---|---|---|---|---|---|
| **Distance Class (end point)** | **50** | **100** | **200** | **400** | **800** |
| P(r-rand >= r-data) | 0.001 | 0.009 | 0.001 | 0.001 | 0.008 |
| U | 0.038 | 0.042 | 0.036 | 0.035 | 0.030 |
| r | 0.106 | 0.058 | 0.057 | 0.083 | 0.038 |
| L | -0.037 | -0.037 | -0.035 | -0.033 | -0.030 |
| P(r-rand <= r-data) | 1.000 | 0.992 | 1.000 | 1.000 | 0.993 |

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# CHAPTER 4

## Bat-mediated pollen flow among *Crescentia alata* trees in fragmented and continuous forest

**ABSTRACT**

*Premise of the study*: Forest fragmentation is considered a pervasive threat to the maintenance of plant-pollinator interactions in tropical forests because of its potential to disrupt the foraging of pollinators, and the subsequent impact on their pollen delivery. This study examines gene flow in the bat-pollinated tree, *Crescentia alata*, located in continuous and fragmented sites in seasonally dry tropical forest near the Chamela-Cuixmala Biosphere Reserve in Jalisco, Mexico. Due to the ability of bats to travel long distances during foraging bouts, we hypothesize that populations of *Crescentia alata* will have high levels of gene flow among populations, and high genetic diversity within populations. Further, if foraging bats visit many trees, then they will carry pollen from multiple sources resulting in high genetic diversity within fruit.

*Methods*: We sampled leaves from adult trees and germinated seedlings from nine sites across an area of approximately 30km by 5km. Four of these sites were located in continuous forest populations (CF) and five in fragmented forest populations (FF). We genotyped 273 seedlings from 17 maternal trees in CF, and 249 seedlings from 17 maternal trees in FF, using seven microsatellite markers previously developed for this species. We analyzed the consequences of gene flow by examining pollen pool genetic structure, connectivity among populations using a network analysis, and genetic diversity of seedlings at multiple sampling scales.

*Results*: We found unexpectedly high genetic structure of the pollen pools, and higher structure from CF maternal trees ($\Phi_{ST\ cont} = 0.164$ vs. $\Phi_{ST\ frag} = 0.140$) suggesting more restricted pollen movement in continuous forest landscapes. However, we also found high connectivity among all trees and sites, and the diversity in pollen gametes was similar among seedlings from continuous

and fragmented forest sites. Interestingly, the highest diversity in pollen gametes was at the smallest stratum of sampling, among seeds within fruit.

*Conclusion*: At smaller spatial scales, pollen transport by bats appears to result in high genetic structure and low alpha diversity among seedlings, while resulting in high genetic connectivity across *C. alata* trees at larger spatial scales, without apparent impact from forest fragmentation.

Key words: bat pollination; forest fragmentation; gene flow; genetic connectivity; pollen pool genetic structure; genetic diversity

## INTRODUCTION

Forest fragmentation is a major driver of landscape changes and biodiversity loss in tropical forests (Laurance and Bierregaard, 1997; Foley et al., 2007; Asner et al., 2009). The tropics are also home to many diverse plant-animal interactions, raising the question of how forest fragmentation might impact these interactions and other critical ecosystem processes (Dirzo and Raven, 2003). Fragmentation can alter interactions between seed dispersers and the plants they disperse (Wright and Duber, 2001; Cordeiro and Howe, 2003), and also between animal pollinators and the plants they pollinate (Aizen and Feinsinger, 1994; Kearns, Inouye, and Waser, 1998; Harrison and Bruna, 1999; Cunningham, 2000; Aguilar et al., 2006). The expected negative effects of plant population fragmentation include reduction in population size, and isolation of remaining individuals by reduction in gene flow, which can then lead to reduced fruit-set, inbreeding depression, and genetic erosion (Ellstrand and Elam, 1993; Young, Boyle, and Brown, 1996; Sork and Smouse, 2006).

One potential impact of landscape change is its disruption of the movement of pollinators, which in turn influences pollen movement. By focusing on pollen-mediated gene flow into fragmented and continuous forest, it is possible to test whether this type of landscape change has influenced pollen movement. One method of assessing pollen flow that is feasible on a landscape-scale (Smouse and Sork, 2004) is to estimate the genetic structure of pollen pools resulting from pollen-mediated gene flow (Smouse et al., 2001), and then compare these structure estimates ($\Phi_{FT}$) among sites that differ in the amount of forest fragmentation. Sork et al. (2005) used this analysis to compare pollen pool structure among three forest management types, and found that pollen-mediated gene flow in *Cornus florida* increased in more open landscapes, where clear-cutting of trees had occurred (Sork et al., 2005). Estimates of $\Phi_{FT}$ can

also be compared with geographic distance between trees or between sites, to assess the extent to which landscape features are influencing pollinator movement and subsequent gene flow, over and above the effects of sheer distance between parent trees.

How much does fragmentation contribute to the isolation of plant populations, by reducing connectivity by pollen flow? Trees can remain technically connected, even when the number of pollen donors is very low. This seeming paradox can be explained by the fact that pollen movement is often very leptokurtic, such that most pollination events occur locally but with a long tail (e.g. Pluess et al., 2009). For example, single trees and small forest patches contributed pollen and maintained genetic connectivity among *Gomortega keule* trees in a fragmented landscape, even though the majority of pollination events occurred within the same patch, according to parentage analysis (Lander, Boshier, and Harris, 2010). A useful method to study genetic connectivity is a graph-theoretic approach (Dyer and Nason, 2004), which measures how significant the co-variance is among pollen pools contributing to each tree. By putting the pollination graph on a spatially explicit map, Dyer et al. (2012) found that pathways of pollinator movement seem to be most strongly influenced by spatial distribution of *Cornus florida* canopies. These genetic connectivity analyses are helpful in clarifying other analyses of gene flow, and for visualizing how pollinators respond to landscape features, such as level of fragmentation.

Ultimately, the effects of forest fragmentation may impact the genetic diversity of the seedlings that result from pollen-mediated gene flow. Do these progeny have reduced genetic diversity? Some studies have found lower genetic diversity of seedlings in fragments vs. the continuous forest (Hall, Walker, and Bawa, 1996; Fernandez and Sork, 2007), while others have shown no difference in genetic diversity between landscape types (Dick, 2001; Cascante et al.,

2002). Traditionally, these genetic diversity measures varied between numbers of alleles to measures of heterozygosity, but it is also possible to summarize genetic diversity using traditional species diversity measures familiar to ecologists (e.g. as modified Shannon diversity indices applied to seed dispersal, (Scofield et al., 2012). Diversity measures can be used to compare the genetic consequences of plant progeny at sites with varying levels of fragmentation, and can be utilized at multiple scales (e.g., within a fruit, within a tree, within a site, within a landscape type), scaling up to an overall regional diversity measure.

Both the type of pollinator and its interaction with landscape features will influence pollen flow. Bats are an important group of pollinators in the tropics, especially because they have the capacity to fly great distances to forage (Bawa, 1990). For instance, Horner et al, (1998) observed the nectarivorous bat *Leptonycteris curasaoe* (= *L. yerbabuenae*) commute 100 km round-trip each night from their roost on an off-shore island to foraging areas on mainland Mexico, where there were dense stands of columnar cacti. Additionally, Law and Lean (1999) reported high mobility across a fragmented landscape mosaic for the common blossom bat (*Syconycteris australis*) as they foraged on the flowers of the rainforest tree *Syzygium cormiflorum*, and one bat was observed flying 5.8 km over cleared land. Therefore, bats can potentially move pollen great distances, potentially connecting plants in fragmented landscapes (Law and Lean, 1999; Fleming, Geiselman, and Kress, 2009). However, connecting the observations of bat foraging movements and the explicit evidence of pollen delivery by bats (as seen in realized gene flow) is still quite difficult. A recent study of gene flow in the bat-pollinated tree *Ceiba aesculifolia* found that isolated trees in forest fragments had higher relatedness of progeny between fruits within a tree, and higher genetic structure of pollen pools,

indicating lower gene flow into fragments (Quesada et al., 2013), but the ability to generalize this finding to all bat-pollinated species is not possible.

The central goal is to test whether the pollen-mediated gene flow in the bat-pollinated tree *Crescentia alata* differs across continuous versus fragmented forest. We predict that bats will fly great distances to forage, carrying pollen in the process, and we therefore expect high pollen flow into trees surrounded by both fragmented and continuous forest. We also expect high connectivity between neighboring trees, regardless of the surrounding landscape, and equivalent genetic diversity in seeds from trees in continuous versus fragmented sites. To provide a robust test of the extent to which highly mobile bat pollinators can mitigate the impact of forest fragmentation on pollen flow, we used microsatellite markers to genotype the progeny of adults trees surrounded by continuous and fragmented forest, and ask the following questions: (i) Is there lower structure of the pollen pools and higher effective number of pollen donors in continuous compared to fragmented forest sites? (ii) What is the influence of landscape on patterns of pollen movement and is there isolation by distance? (iii) Do trees in continuous sites show greater connectivity than those in fragmented ones? (iv) Does the genetic diversity of pollen pools differ between forest types, and at what spatial scale is genetic diversity greatest?

## METHODS

*Study species*. *Crescentia alata* HBK (Bignoniaceae) is a shrubby, cauliflorous tree that is distributed from México to Costa Rica. The flowers are hermaphroditic and pollinated by bats. In our study area, *Leptonycteris yerbabuenae* and *Glossophaga soricina* are the most common pollinators of the flowers (see Chapter 2). Trees flower shortly after the start of the rainy season, in late June-July, and flowering lasts for 2-4 weeks. Each flower is only open for one night, but

there are usually several to hundreds of flowers open on a tree the same night, and the trees can produce hundreds to thousands of flowers over their short flowering season.

*Crescentia alata* trees produce large, hard gourds, which may have originally been dispersed by megafauna that went extinct in the Pleistocene (Janzen and Martin, 1982). The fruit take several months to mature on the tree, have very durable outer husks, and often fall directly beneath the crown, undispersed. Introduced horses may now disperse the seeds (Janzen, 1982b, a), but fruit may also be dispersed by water when the arroyos flood during the rainy season (see Chapter 3).

*Study site and sampling*. Our study site was in and around the Chamela-Cuixmala Biosphere Reserve in Jalisco, México. The *Crescentia alata* trees in this region tend to occur in arroyos, which are ephemerally dry streambeds. During the rainy season, the arroyos fill with water and become streams and rivers. Because each sampling site is an arroyo, we characterized the site as fragmented forest (FF) or continuous forest (CF) based on the landscape around the site. The same or very closely situated sites have been used by other researchers to compare fragmented and continuous forest habitat, and the extent to which pollen flow within a species is impacted by forest fragmentation (e.g. Rosas et al., 2011; Quesada et al., 2013). In addition, the degree of deforestation within and around the Reserve has been analyzed using ASTER satellite images of the region (Sanchez-Azofeifa et al., 2009). This study found deforested land cover was approximately 3.7% within the Reserve, and 16-17% in buffer zones up to 30km outside of the Reserve (Sanchez-Azofeifa et al., 2009). Deforestation in the area is attributed to local slash-and-burn practices, to clear land for agriculture and cattle pastures.

In 2011, we collected fruit from four sites surrounded by CF, and five sites surrounded by FF (Figure 4-1). Within each site, we sampled leaves from 3-5 maternal trees, and collected 3-4

fruit from each of these trees. We germinated 4-7 seedlings from each fruit (14-20 seedlings from each tree), and dried leaves from the maternal trees and seedlings using silica gel. The sampling design included a total of 522 progeny from 34 maternal trees (Table 4-1).

*Genetic Analysis*. Genomic DNA was extracted from the dried leaves from *C. alata* maternal trees and seedlings, using a modified CTAB method (Doyle and Doyle, 1987). We used the following seven fluorescent-tagged microsatellite primers (Cre1, Cre4, Cre9, Cre25, Cre28, Cre29, and Cre30; see chapter 2 for details), developed for this species, to genotype the samples. Microsatellite regions were amplified in 10µl PCR reactions. Samples were genotyped at the UCLA Sequencing and Genotyping Core, using the ABI 3730 DNA Analyzer® (Applied Biosystems, Waltham, Massachusetts), and allele peaks were manually called using GeneMapper® (Applied Biosystems, Waltham, Massachusetts).

*Data Analysis.* Due to the paucity of genetic data from this species, we analyzed mating system patterns as a first step, to determine whether the species showed any evidence of self-fertilization. This knowledge is critical for subsequent analyses and interpretations of gene flow (Burczyk and Koralewski, 2005). We examined the multi-locus outcrossing rate ($t_m$) in CF and FF separately, using MLTR version 3.4 (Ritland, 2002), and used the following parameter estimates to analyze the mating system in MLTR: Newton-Raphson (NR) numerical method; 500 bootstraps for standard error; and re-sampling using families.

To test the effect of the surrounding landscape type on gene flow patterns, we used several statistical methods. First we estimated the genetic structure of the pollen pools, using a TwoGener approach (Smouse et al., 2001), implemented in GenalEx 6.501 (Peakall and Smouse, 2012). TwoGener uses the known maternal genotype and a set of offspring genotypes, to infer the male gametic contribution (pollen) and estimate the genetic structure statistic ($\Phi_{FT}$), based on

how different the pollen profiles are among the maternal trees. We determined $\Phi_{FT}$ at the landscape level, separately for (CF and FF) and calculated the number of effective pollen donors ($N_{ep}$) using the equation $N_{ep} = 1/(2 \cdot \Phi_{FT})$, to see how this varies between the five CF and four FF sites. We also checked for the degree to which distance between trees is influencing pollen pool genetic structure, by calculating $\Phi_{FT}$ separately for each site, and comparing it to the average geographic distance (measured as distance along the arroyo, between maternal trees within a site).

To examine how bat pollinators are visiting trees in more detail, we looked at the hierarchical genetic structure of the pollen pools among fruit and among trees. It is important to look at these two different levels of structure, because they let us estimate the effective number of bat pollinators at each of these levels, and also help us determine how much differentiation between fruit in the same tree is contributing to the overall structure. We did this by doubling the inferred male gametes for seedlings into "genotypes" and performing an AMOVA using fruit as populations, and trees as regions, calculating $N_{ep}$ for each of these strata using the equation above.

To determine whether fragmented sites were isolated via pollen flow and to assess connectivity among *Crescentia alata* adults across the landscape, we used a graph theory approach (Dyer and Nason, 2004), modified for pollen-flow networks and dubbed "Pollination Graphs" (Dyer et al., 2012). Each node in the network represents a maternal tree, and the edges between them represent significant connections, based on conditional genetic covariance between the pollen pools. We constructed the pollination graph and tested for isolation by distance between connected trees and their conditional genetic covariance using the gstudio package in R (Dyer, 2013). We tested the hypothesis that landscape matrix did not affect

connectivity by conducting a two-sample t-test on the number of edges in CF and FF sites, using the base stats package in R (R Development Core Team, 2013).

To determine the genetic consequences for progeny in each landscape type, we analyzed the doubled-male gametes (described above) using a modification of the diversity approach introduced by Scofield *et al*. (2012). We estimated allelic diversity for seeds using the Shannon statistic in GenalEx 6.501 for each locus separately, then averaged the estimate across the seven loci. We compared the diversity of effective alleles in the different landscape types, at different levels of strata: within fruit, within maternal trees, within arroyos, and within a landscape type (Shannon, 1948; Jost, 2007; Scofield et al., 2012). We then standardized these different components of diversity by comparing them to their maxima, which were determined by the sampling scheme. This method focuses on the genetic consequences of pollen delivery using an allelic diversity measures, which differs from the method described previously, which estimates the number of pollen donors ($N_{ep}$).

**RESULTS**

The mating system analysis confirmed that *Crescentia alata* is largely out-crossing ($t_{m\text{-}CF}$ = 0.966) > ($t_{m\text{-}FF}$ = 0.933), as is common among tropical tree species. The genetic structure of the pollen pools was higher in the continuous forest than in the fragmented forest ($\Phi_{FT\ cont}$ = 0.164) > ($\Phi_{FT\ frag}$ = 0.140), translating into a slightly smaller number of effective pollen donors in the continuous forest ($N_{ep\text{-}CF}$ = 3.05) < ($N_{ep\text{-}FF}$ = 3.60) (Table 4-2). When we examined patterns of genetic structure of the pollen pools at each site separately, we found variation among the within-site level $\Phi_{FT}$ values, and these structure estimates were significantly correlated with the average distances between maternal trees within a site ($r$ = 0.88, df = 7, $p$-value < 0.002, Figure 4-2).

When we examined how the molecular variance was partitioned among fruit (within a tree) and among trees, we found that the fruit contributed more to overall structure of the male gametes. Trees in fragmented forest sites also had consistently lower structure, and thus higher numbers of effective pollen donors, at both the inter-fruit ($N_{ep\text{-}CF} = 1.79$) < ($N_{ep\text{-}FF} = 1.946$) and inter-tree ($N_{ep\text{-}CF} = 4.464$) < ($N_{ep\text{-}FF} = 6.410$) levels of analysis (Table 4-3).

All of the trees had significant connections (edges) to other trees in the network analysis. Edges ranged from 3 to 8, and there was not a significant difference between the number of edges in CF and FF trees ($t = 0.27$, df = 29.3, $p$-value = 0.79; Figure 4-3). We also found a significant correlation between conditional genetic distance and physical distance between the maternal trees, using a Spearman's rank correlation (rho = 0.16, $p$-value = 0.0001, Figure 4-4).

The Shannon diversity analysis revealed that most of the allelic diversity is found at the level of seeds within fruit (CF = 56%) ≈ (FF = 59%), suggesting that there is multiple paternity within each fruit (Table 4-4). The effective number of alleles increases only marginally from the level of within fruit, to that among arroyos, and at that level, the differences between the landscapes is minimal (CF = 74%) ≈ (FF =75%). This indicates that the delivery of genetically diverse pollen grains to a flower via bat pollinators is a critical component of gene flow in this species, and that pollen is moving freely over the scale of at least 30 km (Table 4-4).

**DISCUSSION**

We found strong evidence that forest fragmentation is not reducing bat-mediated pollen flow among populations of *Crescentia alata* trees in this region, and that gene flow may actually be higher in fragmented sites. This conclusion is supported by the lower genetic structure of pollen pools for trees in fragmented sites than in continuous forest sites. In addition, the network

analysis of pollen flow indicates trees in both landscape types are equivalently connected. These results add to our findings that bat pollinator abundance is not significantly different between landscape types (see Chapter 2), and provide evidence that there is consistently negative effect of fragmentation on the pollinators and pollen flow in this plant species. In addition, we found support for isolation by distance among the pollen pools, suggesting that bats are not strongly responding to landscape features such as forest cover. All of these results suggest that the opening of the landscape from forest fragmentation may increase pollen flow, and provide increased opportunities for foraging movements by nectarivorous bats.

These effective pollen flow results are in stark contrast to those of Quesada et al. (2013), who found consistently higher structure of pollen pools in *Ceiba aesculifolia* trees in disturbed sites across 4 years of sampling. Because the data from Quesada et al. (2013) on *C. aesculifolia* was collected in the same region of México as our study, and both *C. aesculifolia* and *C. alata* are pollinated by the same bat species, these data represent an intriguing point of comparison. It is possible that the differences in flowering phenology between the tree species affects the foraging decisions by bats, with consequent effects on pollen movement. In Chamela, México, *Crescentia alata* trees have a short, massive flowering period of about one month (late June-July, see Chapter 1), while *Ceiba aesculifolia* flowers over a longer period of time (~ 3-4 months, April-July, (Lobo et al., 2003). The short time period during which *C. alata* nectar is available to bats, and reduced number of chiropterophilic resources available during its flowering period compared to *C. aesculifolia*'s flowering period (Stoner et al., 2003), may make *C. alata* so attractive that bats will visit trees regardless of the habitat conditions surrounding them.

Our previous work showed non-significant differences between the numbers of flowers produced by *C. alata* trees in continuous versus fragmented forest areas (Chapter 2), but others

report that bat-pollinated *C. aesculifolia* trees in disturbed sites in this region have lower conspecific density and produce more flowers (Herrerias-Diego et al., 2006). Quesada *et al*. (2013) point to this higher floral display as a factor driving the higher level of relatedness among seeds within fruit for *C. aesculifolia* trees in disturbed sites. The hypothesis is that the forest fragmentation lowers the density of flowering trees and increases the number of flowers in bloom for *C. aesculifolia*, concentrating bat pollinators on local trees in isolated patches, thereby reducing the pollen flow to trees in disturbed sites. Our study shows that these effects of forest fragmentation on flowering number and pollen flow seemingly do not occur for *C. alata* in the same region, and point to a very species-specific plant response to landscape disturbance.

Using a hierarchical approach, we found different patterns of pollen pool genetic structure, at the level of seeds sampled within a fruit, versus seeds sampled within a tree. It is interesting to note the level at which the sub-sampling of the pollen pool that is captured through the creation of different fruit within a tree results in high genetic structure. The $N_{ep}$ at the fruit level was similar for CF and FF ( ~2), but at the tree level was higher in FF (~6.5) than CF (~4.5), again demonstrating that trees in fragmented sites receive higher numbers of pollen donors, counter to the initial expectations about forest fragmentation reducing gene flow. Interestingly, these $N_{ep}$ values are similar to those found by de Lacerda et al.(2008) for the bat-pollinated tree *Hymenaea courbaril* in the Brazilian Amazon. Although those authors found the $N_{ep}$ was around 4, they detected a large percent of the pollen was coming from outside of their study plot, presumably a considerable distance away. This pattern seems to correspond with our findings, where seemingly low $N_{ep}$ does not contradict the probably few long-distance pollination events that keep sites connected.

The diversity of alleles reaching trees in fragmented and continuous sites were fairly similar, corresponding to our other results of equivalent gene flow among trees in these landscape types. The most interesting result was that the majority of allelic diversity that did get sampled by the trees happened at the level of seeds within fruit, and sampling at higher strata did not add much diversity. Our interpretation is that in this low diversity system, bat pollinators are playing an important role in bringing genetically diverse pollen loads to flowers, which results in single fruits with multiple paternity.

In conclusion, we find that despite the high local structure of the pollen pools, bat pollinators are maintaining genetic connectivity between trees in fragmented and continuous forests, and are delivering pollen from multiple sources to flowers of *Crescentia alata* trees, and allelic diversity within fruit represents the majority of the diversity within this system. These findings highlight the important role nectarivorous bats play in keeping plant populations genetically connected.


Figure 4-1: Map of study region and the nine sampling sites for pollen flow. Fragmented forest sites are in yellow, continuous forest sites are in green. Map courtesy of Google Earth.

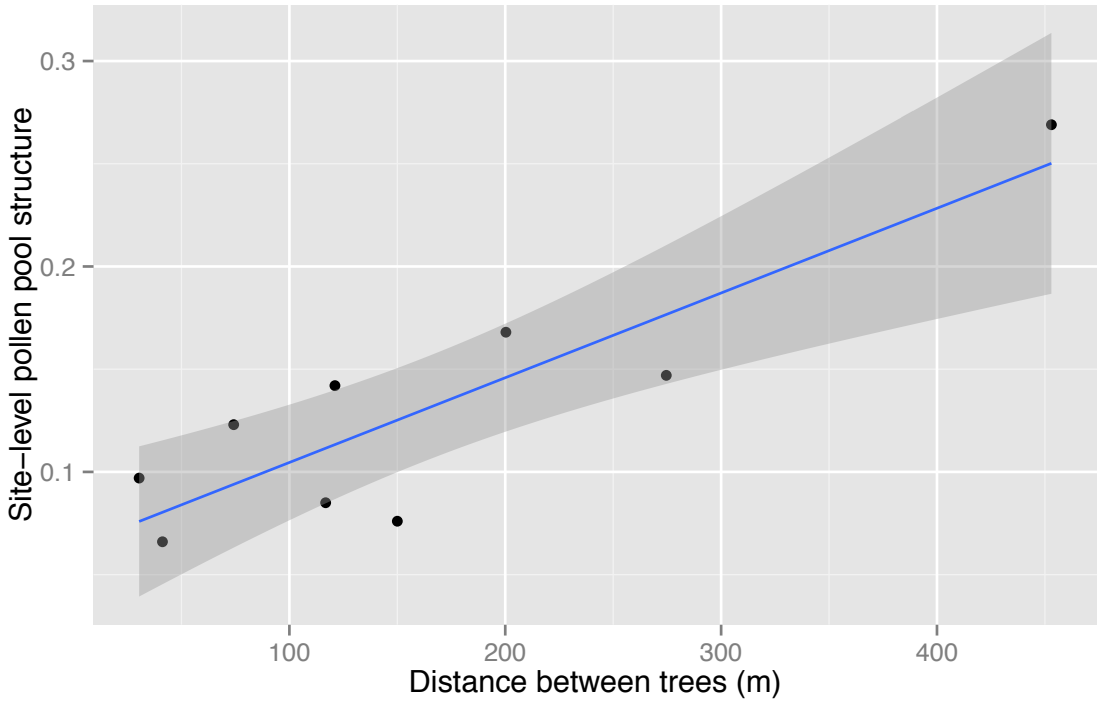


Figure 4-2: Correlation of average distance between maternal trees within a site, and the genetic structure of the pollen polls ($\Phi_{FT}$) estimates for the site ($r = 0.88$, $P < 0.002$). Distance between trees is measured in meters, and was calculated along an arroyo, using ArcGIS10.0 (Esri, Redlands, California).


Figure 4-3: Network analysis of conditional genetic connectivity between maternal trees, on a map of the study region (map courtesy of Google Earth). Green nodes are maternal trees in continuous forest; yellow nodes are maternal trees in fragmented forest; white lines between the nodes are significant connections.

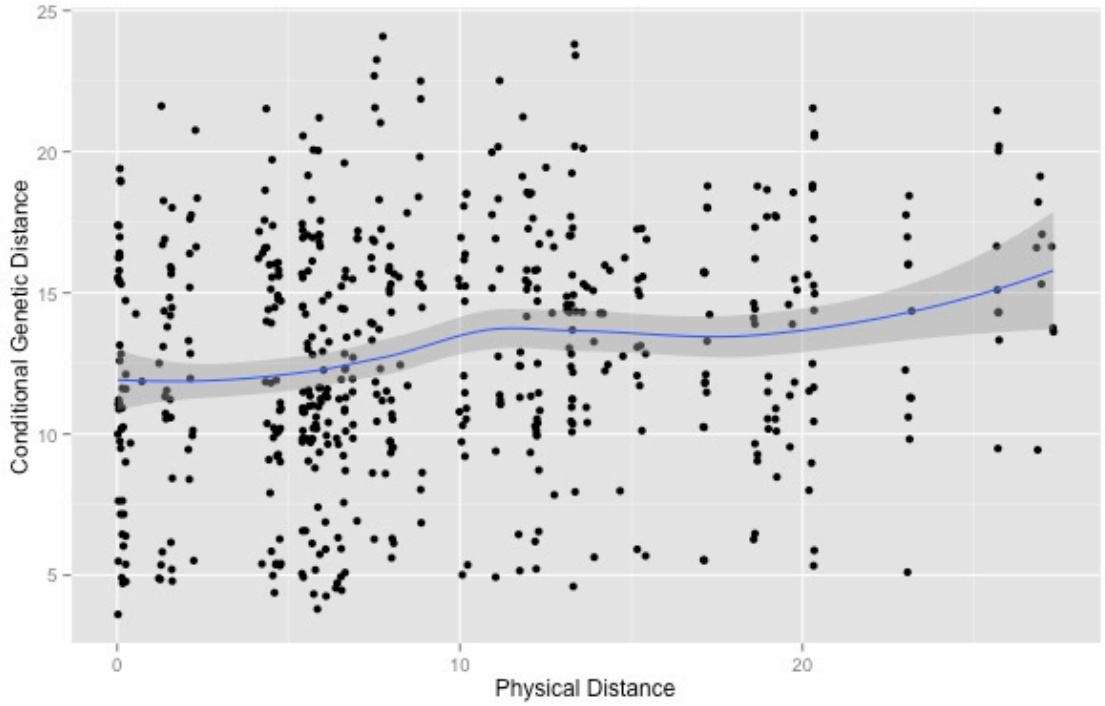


Figure 4-4: Loess regression between physical distance of maternal trees and conditional genetic distance between trees (via covariance in pollen pools, estimated in gstudio). Spearman's rank correlation, $P < 0.0001$.

Table 4-1: Sampling design for genetic analysis of seedlings, fruit, trees and sites, within continuous forest (CF) and fragmented forest (FF).

| CF | | | | | FF | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Site** | **Tree ID** | **fruit /tree** | **seeds /tree** | **seeds/site** | **Site** | **Tree ID** | **fruit /tree** | **seeds /tree** | **seeds/site** |
| Careyes | 149 | 3 | 16 | 81 | Perula | 144 | 3 | 15 | 42 |
| | 150 | 4 | 20 | | | 239 | 3 | 13 | |
| | 153 | 3 | 15 | | | 299 | 3 | 14 | |
| | 154 | 3 | 15 | | Ranchitos | 24 | 3 | 14 | 59 |
| | 302 | 3 | 15 | | | 289 | 3 | 15 | |
| Colorado | 258 | 3 | 14 | 64 | | 290 | 3 | 15 | |
| | 259 | 3 | 15 | | | 291 | 3 | 15 | |
| | 260 | 3 | 15 | | PJP | 131 | 3 | 14 | 44 |
| | 287 | 4 | 20 | | | 132 | 3 | 15 | |
| Chamela | 98 | 3 | 15 | 80 | | 133 | 3 | 15 | |
| | 99 | 3 | 14 | | Seco | 116 | 3 | 14 | 59 |
| | 100 | 3 | 16 | | | 117 | 3 | 15 | |
| | 189 | 3 | 15 | | | 118 | 3 | 15 | |
| | 190 | 4 | 20 | | | 123 | 3 | 15 | |
| Polo | 279 | 4 | 19 | 48 | Canoa | 262 | 3 | 15 | 45 |
| | 280 | 3 | 15 | | | 265 | 3 | 15 | |
| | 283 | 3 | 14 | | | 306 | 3 | 15 | |
| Total | | | | 273 | Total | | | | 249 |

Table 4-2: Genetic structure of pollen pools ($\Phi_{FT}$) by landscape type (continuous forest =CF; fragmented forest = FF) and by sampling site. The number of effective pollinators ($N_{ep}$) is calculated from the equation $N_{ep} = 1/(2\ \Phi_{FT})$.

| **Landscape*** | **Maternal Trees** | **Seedlings** | $\Phi_{FT}$ | $N_{ep}$ |
|---|---|---|---|---|
| CF | 17 | 273 | 0.164 | 3.049 |
| FF | 17 | 249 | 0.140 | 3.571 |

| **Landscape** | **Site** | **Maternal Trees** | **Seedlings** | $\Phi_{FT}$ |
|---|---|---|---|---|
| CF | Careyes | 5 | 81 | 0.142 |
| CF | Colorado | 4 | 64 | 0.168 |
| CF | Chamela | 5 | 80 | 0.097 |
| CF | Polo | 3 | 48 | 0.147 |
| FF | Perula | 3 | 42 | 0.269 |
| FF | Ranchitos | 4 | 59 | 0.08 |
| FF | PJP | 3 | 44 | 0.066 |
| FF | Seco | 4 | 59 | 0.123 |
| FF | Canoa | 3 | 45 | 0.085 |

Table 4-3: Hierarchical genetic structure of pollen, partitioned among seeds within fruit, and fruit within trees.

| | **CF** | **FF** |
|---|---|---|
| $\Phi_{RT}$ (Among Maternal Trees) | 0.112 | 0.078 |
| $N_{ep}$ to a tree | 4.464 | 6.410 |
| $\Phi_{PR}$ (Among Fruit) | 0.279 | 0.257 |
| $N_{ep}$ to a fruit | 1.792 | 1.946 |

Table 4-4: Diversity of seeds (in terms of effective number of alleles), nested among successively higher levels, using a modified Shannon diversity analysis.

| | Components | CF | | | FF | | |
|---|---|---|---|---|---|---|---|
| | | Cumulative diversity | Max diversity | Compared to maxima | Cumulative diversity | Max diversity | Compared to maxima |
| Among seeds within a fruit | α | 1.81 | 4.99 | 0.56 | 1.90 | 4.89 | 0.59 |
| Among fruit within a tree | β | 1.39 | 9.67 | 0.31 | 1.38 | 6.86 | 0.32 |
| Among seeds within a tree | μ (=α*β) | 2.51 | 48.21 | 0.61 | 2.61 | 33.56 | 0.64 |
| Among moms within an arroyo | χ | 1.29 | 4.30 | 0.29 | 1.29 | 3.44 | 0.32 |
| Among seeds within an arroyo | ρ (=α*β*χ) | 3.23 | 207.37 | 0.69 | 3.37 | 115.30 | 0.71 |
| Among arroyos within CF/FF | δ | 1.16 | 3.92 | 0.19 | 1.19 | 4.94 | 0.20 |
| Among seeds within CF/FF | ϒ (=α*β*χ*δ) | 3.75 | 273.00 | 0.74 | 3.99 | 249.00 | 0.75 |

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