Population genetics of the endangered tropical tree *Guaiacum sanctum* (Zygohphyllaceae)

by

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(Under the direction of James L. Hamrick)

Abstract

This dissertation describes the reproductive biology and patterns of genetic diversity of the endangered tropical tree *Guaiacum sanctum* (Zygophyllaceae). First, I analyzed the abundance and spatial distribution of different size classes of *G. sanctum* within Palo Verde National Park (PVNP). Results showed a large proportion of individuals in the smaller size categories, suggesting population growth. All size classes were spatially aggregated and associated with canopy openings; seedlings occurred in shaded areas and saplings and juveniles were found in open areas.

Allozyme analyses were used to examine patterns of fine-scale genetic structure (FSGS) in three plots within PVNP. A lack of fine-scale genetic structure was observed for all size classes, suggesting the mixing of seeds from several different adults. A parent-pair analysis indicated that populations of *G. sanctum* populations were probably established by bird mediated long-distance seed dispersal.

To broaden the spatial scale, I quantified genetic diversity within and among seven *G. sanctum* populations in Costa Rica. I compared genetic diversity differs between populations in continuous habitats and trees in fragmented sites. Results indicated that *G. sanctum* maintains high levels of genetic diversity. Trees in fragmented habitats did not have less genetic diversity than trees in protected habitats. We concluded that the observed patterns of genetic structure in this species are probably caused by the historic separation of populations in different geographic regions, and extensive historical rates of gene flow among populations within regions.

To determine if fragmentation has affected gene-movement, I estimated the mating system and pollen flow patterns of *G. sanctum*. A fractional paternity analysis was used to estimate average gene flow distances. Our results showed that *G. sanctum* is a mixed-mating species with the ability to transport pollen over large distances. Isolated trees function as stepping-stones between clusters of individuals, assisting long-distance pollen movement. These individuals also sired a disproportionate number of seeds, and are thus important components of the reproductive success of the population.The high levels of genetic diversity maintained as a consequence of long-distance gene-flow suggest that this endangered species may have the potential for future adaptation and population expansion.

INDEX WORDS: allozymes, fragmentation, demography, fine-scale genetic structure, genetic structure, mating systems, paternity analysis, pollen flow, endangered, tropical dry forest, Costa Rica

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Dedication

I would like to dedicate this dissertation to my wife Tatiana Robles, for her help, encouragement, patience and dedication. I could have never done this without you, mi amor.

I also dedicate this work to my parents, for their continuous help, to the Robles-Cordero family, for having me in their hearts and to La Virgen de los Angeles, for always watching over me.

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Chapter 1

Introduction and Literature Review

Tropical forests are disappearing at alarming rates, with deforestation and urban expansion being identified as the proximate causes for habitat reduction (Geist and Lambin, 2002). It's been estimated that between 1990 and 1997, yearly deforestation rates for Latin America averaged 2.5×10^6 ha$\times yr^{-1}$ (Achard *et al.*, 2002). Within the tropics, some forest types are more threatened than others. Sader and Joyce (1988) estimated that by 1961 tropical dry forests in Costa Rica were completely deforested, while more than 70% of tropical rain forest remained intact. Deforestation leads to a reduction in habitat area, which in turn has two direct impacts on species' populations: fragmentation of continuous habitat and reduction in population sizes (Bissonette and Storch, 2002). Fragmentation transforms continuous forest into a collection of isolated forest patches, surrounded by disturbed habitats. Secondly, a reduction in forest cover generally reduces natural population sizes, mainly due to a lack of suitable habitat for reproduction and recruitment, or as a consequence of increased human extraction. In plants, separation into discontinuous forest patches may affect reproductive success by separating conspecifics over distances larger than those supported by their pollen vectors, thus modifying their mating systems and their abillity to reproduce adequately (Murawski and Hamrick, 1991). Additionally, a reduction in population size may increase the probability of population extinction by the interaction of ecological and genetic factors (Young *et al.*, 1996).

Fragmentation and small population size can contribute to reductions in genetic diversity by altering gene flow (Hamrick *et al.*, 1993a) and increasing the combined effects of genetic drift and inbreeding (Hedrick and Miller, 1992). Small finite populations lose genetic diversity through genetic drift, as a consequence of random changes in allele frequencies. The loss of genetic diversity is directly related to population size, with smaller populations maintaining less genetic diversity (Frankham, 1996). A reduction in genetic diversity may curtail the ability of the population to

adapt to a changing environment. Lower levels of genetic diversity in small populations have been associated with a decrease in fitness (Reed *et al.*, 2003), probably due to an increase in inbreeding and the resulting inbreeding depression (Husband and Schemske, 1996). In small populations with relatively little gene-flow, the average relatedness of individuals increases gradually, which leads to consanguineous matings (Wang *et al.*, 1999). Such matings increase the frequency of recessive deleterious homozygotes in the population (Ellstrand and Elam, 1993; Charlesworth and Charlesworth, 1999). Almost all organisms have been observed to display some form of inbreeding depression in small populations (Falconer and Mackay, 1996; Lynch and Walsh, 1998; Hedrick and Kalinowski, 2000) and inbreeding has been shown to increase the probability of extinction (Brook *et al.*, 2002).

Tropical trees are more vulnerable to the effects of fragmentation caused by anthropogenic intervention, due to their sessile life forms, low densities, and high economic value (Cascante *et al.*, 2001). Additionally, many tropical tree species require a critical number of conspecifics for adequate reproduction, due to their predominantly outcrossing mating system (Bawa, 1974; Bawa *et al.*, 1985a). Fragmentation may reduce the reproductive success of trees, as well as impact the viability of the progeny produced. Current research in the conservation genetics of tropical trees is primarily interested in determining current levels of genetic diversity within and among populations, as well as patterns of gene movement. Since trees are so long-lived, it is difficult to determine the direct effects of fragmentation, as they may become obvious over much longer periods of time. Therefore, indirect estimates such as changes in genetic diversity, or patterns of gene movement, may allow predictions of the sustainability of populations of these tropical tree species (Hamilton, 1999).

Genetic and ecological information is even more important for endangered species. Species are generally categorized as threatened or endangered, based on their abundances, spatial distribution and economic value (CITES, 2000). *Guaiacum sanctum* (Zygophyllaceae) is an endangered, slow growing, dry forest tree, distributed from southern Central America to Northern Mexico and Florida, and throughout the Greater Antilles (Holdrige and Poveda, 1975). Due to severe reductions in population sizes caused by over exploitation, this species has recently been included in Appendix I of the CITES convention (CITES, 2000) and is also recognized as "Endangered" in the World List of Threatened Trees (Oldfield *et al.*, 1998). *G. sanctum* has been predominantly harvested for its hard wood and medicinal value. Its extremely dense wood was popularly used for the construction

of propeller shafts on steamships. Additionally, for over five centuries, the decoction of *Guaiacum sanctum* was believed to cure syphilis (Voeks, 2004). Therefore, most populations of *G. sanctum* were decimated by the end of the 19th century with a few populations remaining in Mexico, Central America, and the Florida Keys (Quirico, 1993). In Costa Rica, *G. sanctum* is only found in the dry forests of the northwest Pacific coast, an area with the highest levels of deforestation within the country (Janzen, 1988).

The main objectives of this dissertation are to characterize ecological and genetic parameters of extant populations of *Guaiacum sanctum* in Costa Rica. Presently, very little information exists on the population ecology and genetics of this species, and most published contributions have focused on its medicinal value (Gariepy, 1981). Nonetheless, this species continues to be threatened by commercial and artesanal use of its wood. To develop proper conservation and management strategies, information on a species' life history and habitat requirements are needed (Soul and Orians, 2001). For most endangered species, knowledge of their abundance and distribution are lacking, which makes conservation strategies harder to implement, since habitat requirements are not known.

Spatial distribution and abundance of individuals are fundamental components of the reproductive biology of a species. These estimates provide information about a species habitat requirements and interspecific interactions. Research conducted on tropical forest trees have determined that most tropical trees are generally spatially aggregated (Condit *et al.*, 2000; Hubbell, 1979). Spatial aggregation may arise in two ways, localized (i.e. clumped) seed dispersal, or heterogeneity in the distribution of microenvironmental requirements for recruitment or growth (Hamill and Wright, 1986). For tropical dry forest species, such as *G. sanctum*, the presence of a marked dry season may influence the spatial distribution of different size classes. Increased mortality during the dry period has been associated with differential recruitment under specific canopy environments which reduce the chance of desiccation. Research conducted in tropical dry forests in Ghana (Lieberman and Li, 1992) and Costa Rica (Gerhardt, 1996) showed that a large proportion of seedlings die due to moisture stress. Seedling mortality was higher in areas with high irradiance or within canopy gaps. Similar results were reported by McLaren and McDonald (2003) for tropical dry forest trees in Jamaica. They showed that seedling establishment and survival was higher in heavily shaded

environments, which conserved moisture during the dry season. The effects were more marked for evergreen shade-tolerant species, such as *G. sanctum*. These habitat requirements may promote spatial aggregation in areas that promote survival and recruitment. Finally, abundance of different size classes or life stages has been identified as an important initial assessment of population resilience. The current stage structure of a population may allow the identification of populations in danger of extinction, distinguished by a lack of individuals in the early size classes. Conversely, a large proportion of seedlings may suggest ample reproduction and population expansion (Odum, 1983a).

In the second chapter, I determined the abundance and spatial distribution of different size classes of *G. sanctum* located within Palo Verde National Park in northwestern Costa Rica. We also determined whether canopy cover had a direct impact on the abundance and distribution of this species. In this chapter we looked at the abundance of different size categories to infer indirectly whether the proportion of individuals in the younger size categories suggests an expanding population. The results demonstrated that *G. sanctum* populations within PVNP are expanding, due to a large proportion of individuals in the smaller size classes. Additionally, we showed that all size classes are highly spatially aggregated. Seedlings, saplings and juveniles of *G. sanctum* prefer different canopy environments with the smaller size categories being more likely to recruit in areas with closed canopy. Conversely, juveniles were more common in open canopy areas. These results suggest that the spatial distribution in this species is mediated by the patchy distribution of suitable habitats for recruitment. Nonetheless, clumped distributions may arise by localized seed dispersal, directly impacting patterns of genetic diversity within populations (Epperson, 2000)

The distribution of genetic diversity within populations is often patchily distributed, and is greatly influenced by gene dispersal patterns both within and among populations (Hamrick *et al.*, 1993a). Localized seed dispersal, low levels of pollen flow, high adult densities, vegetative growth and the non-homogeneous distribution of suitable habitats for germination can all influence the non-random distribution of genetic diversity within populations, and may cause fine-scale genetic structure (FSGS) to arise (Epperson, 1993; Tonsor *et al.*, 1993; Epperson, 2000; Chung *et al.*, 2000; Kalisz *et al.*, 2001). Most studies of FSGS in tropical trees have found a strong signature of family structure in seedlings (Loveless *et al.*, 1998), and have attributed those patterns to localized seed dispersal patterns (Hamrick *et al.*, 1993b). In trees with clumped seed dispersal patterns and little

overlap among seed shadows, progeny from a single maternal tree will germinate in close proximity to each other, creating patches of related individuals. In the absence of density-dependent mortality, family groups will be formed within populations, and if near-neighbor mating occurs, inbreeding may increase in the population (Turner *et al.*, 1982). This in turn may lead to a reduction in the population's fitness (Reed *et al.*, 2003). Conversely, species with long distance or scatter dispersed seeds (sensu Howe, 1989), will have a mixture of progeny from different maternal trees germinating close to each other. In these cases, family structure will not develop. It has been shown for tropical trees that differ in dispersal syndromes that animal dispersed species are generally less likely to produce family structure because propagules are dispersed away from maternal trees, reducing the probability of sib-ships being dispersed simultaneously. Most studies of FSGS have focused on reproductive individuals and only a handful have included different age or size classes (Hamrick *et al.*, 1993a; Tonsor *et al.*, 1993; Hamrick and Nason, 1996; Epperson and Alvarez-Buylla, 1997; Kalisz *et al.*, 2001). Studies that fail to analyze the spatial genetic structure across size classes may not detect structure due to combining cohorts with different levels of FSGS. Additionally, changes in the magnitude of structure in different age categories may provide information on how ecological processes such as demographic thinning impact FSGS. If FSGS disappears in the older size categories, this may be caused by only one or a few trees representing the previous seed shadow. Most endangered species lack a proper description of the spatial distribution of genetic diversity within populations or FSGS. This is of vital importance for conservation genetics, because FSGS may reduce effective population size, increase inbreeding and the loss of genetic diversity through drift (Epperson, 1993). Low genetic diversity and effective number of individuals may increase the extinction probability of populations and may reduce long-term species survival (Frankham, 1995; Newman and Pilson, 1997; Brook *et al.*, 2002; Henle *et al.*, 2004).

In the third chapter, I looked at changes in spatial patterns of genetic diversity among size classes within a continuous population of *Guaiacum sanctum*. The study was conducted in PVNP, on the plots used to estimate spatial distribution patterns. The main objectives were to determine and compare patterns of genetic diversity and FSGS across different size classes. Additionally, to determine if the presence or absence of FSGS was attributable to patterns of seed dispersal, I conducted a parent-pair analysis on the younger size categories within each plot. This analysis

allowed us to assess what proportion of these individuals could be attributed to matings and seed dispersal events occurring within plots. The results of this project indicated a lack of FSGS across all size classes within PVNP populations. Parent-pair analysis revealed that extensive levels of seed flow by birds, may produce a mixture of progeny from several individuals, effectively eliminating family structure. Our results also showed that *G. sanctum* within PVNP had high levels of genetic diversity, which was unexpected for an endangered species.

It has been suggested that populations of rare or endangered Neotropical tree species should have low levels of genetic diversity (Hamrick and Murawski, 1991). Lower genetic diversity may increase extinction probability, by reducing the population's ability to track evolutionary changes (Ellstrand and Elam, 1993). Additionally, forest fragmentation may alter patterns of gene movement within and among fragments, reducing genetic diversity (Murawski and Hamrick, 1991; Dayanandan *et al.*, 1999). If gene-flow is low, fragmented populations will gradually accumulate differences in allele frequencies through the effects of genetic drift. High levels of gene flow, in contrast, will transfer genetic diversity across populations, homogenizing allele frequencies among populations (Bohonak, 1999). Therefore, genetic structure estimates allow indirect measures of historical patterns of gene-flow among populations (Wright, 1969). Secondarily, a description of the magnitude of genetic diversity within and among populations of endangered species has direct implications for the development of conservation or management practices. High levels of genetic structure suggest that *in situ* conservation strategies should be implemented to preserve genetic variation at all sites (Lemes *et al.*, 2003). Additionally, populations with low-levels of genetic diversity, may warrant the introduction of novel genetic diversity by transplantation or artificial pollinations. Conversely, populations with high levels of genetic diversity may serve as sources of seedlings or vegetative clones which may be grown in nurseries or forestry plantations to preserve highly diverse germoplasm. Studies conducted on tropical trees have generally found that most of the genetic diversity is partitioned within rather than among populations (e.g. Hamrick *et al.* 1992; Hall *et al.* 1996; Dayanandan *et al.* 1999; Collevatti *et al.* 2001; Pither *et al.* 2003; Rossetto *et al.* 2004). These studies have shown, that generally gene flow among extant populations is frequent enough to sustain high levels of genetic diversity and to elude the deleterious effects of fragmentation (Rossetto *et al.*, 2004 but see Pither *et al.*, 2003).

The fourth chapter of my dissertation is aimed at studying patterns of genetic diversity within and among extant populations of *G. sanctum* in Costa Rica. In this study, I located all populations of *G. sanctum* and conducted allozyme analyses to determine patterns of genetic structure. Many *G. sanctum* trees are located in disturbed habitats such as roadsides, or as ornamental plants in gardens, pastures or as part of living-fences. Therefore, we also analyzed differences in genetic diversity among populations located within continuous habitats, such as National Parks or Forest Reserves; and trees in fragmented populations. The results of this study showed that populations of *G. sanctum* in Costa Rica have unexpectedly high levels of genetic diversity for an endangered species. Our findings indicate that trees located in fragmented or disturbed habitats do not show evidence of a reduction in genetic diversity relative to trees in continuous forests. Our results suggest that observed patterns of genetic structure in this species are probably caused by historic separation of populations in different geographic regions, and extensive historical rates of gene flow among populations within a region.

Conservation strategies for tropical trees rely on accurate estimates of the mating system and pollen flow distances as functions of population density, to accurately estimate minimum population sizes needed to maintain the reproductive success of the population (Sork *et al.*, 2002; Degen and Roubik, 2004). Pollen flow distances directly impact the magnitude and structure of genetic diversity, but will also affect the reproductive success of outcrossing species by reducing inbreeding. Recent studies have shown that pollen flow in tropical trees is higher than previously expected (Eguiarte *et al.*, 1993; Nason *et al.*, 1996), with many examples showing pollen flow over several hundred of meters (reviewed in Nason and Hamrick, 1997). Forest fragmentation reduces population densities and increases the distance between reproductive conspecifics. These factors may lower pollinator densities (Bawa, 1990) and increase selfing rates (Murawski and Hamrick, 1991; Fuchs *et al.*, 2003), consequences which have generally been attributed to lower levels of pollen flow. Nonetheless, recent work has shown that in fragmented habitats, pollen may travel larger distances due to changes in pollinator abundances or behaviour (Sork and Smouse, 2006). For example, Dick *et al.* (2003) showed that in fragmented populations of *Dinizia excelsa* pollen moved larger distances than in continuous forests, primarily because of long-distance movement by Africanized bees. Similarly, in discontinuous populations of *Swietenia humilis*, White *et al.* (2002) determined

that pollen flow commonly occurred between individuals separated by more than 2 km. Our genetic structure analyses, suggest that *G. sanctum* has the ability for long-distance gene flow, at least for distances within a few kilometers. Nonetheless, direct estimates of pollen flow distances will enhance our understanding of gene movement for this species.

In my fifth chapter I estimated the mating system and pollen flow patterns in continuous and fragmented populations of *G. sanctum* (Zygophyllaceae). I collected seeds from reproductive adults in two populations that differ in habitat quality. Fractional paternity analysis was used to estimate pollen flow distances, and the reproductive success of different pollen donors. I also analyzed the role of isolated trees in patterns of pollen movement across groups of adults. My results show that *G. sanctum* is a predominantly outcrossing species with which can transport pollen over large distances. Isolated trees function as stepping-stones between clusters of individuals in fragmented landscapes, assisting pollen movement over distances of at least 3.5 km. Isolated individuals also sire a large proportion of the progeny and are thus important components of the reproductive success of populations.

Even though *Guaiacum sanctum* is an endangered species, currently very little knowledge exists on the reproductive biology of this species. The results of this dissertation, may be used to develop effective management and conservation strategies for this species, since they provide information on habitat requirements and patterns of gene movement both within and among populations. Our results will enable future researchers to identify populations with high levels of genetic diversity, as targets for conservation efforts. Additionally, our pollen flow data shows that isolated individuals are an integral part of gene movement and hence should receive a higher priority in conservation strategies. Finally, within continuous populations seed dispersers promote the admixture of genetic diversity preventing the formation of family structure. Therefore, forested areas should be preserved to maintain large populations of birds, which mostly live in continuous forests (Boswell *et al.*, 1998; Bleher and Bohning-Gaese, 2001; Graham *et al.*, 2002).

Chapter 2

Spatial distribution of *Guaiacum sanctum* (Zygophyllaceae) seedlings and saplings relative to canopy cover[1]

[1]Fuchs, E.J. and Hamrick, J.L. To be submitted to *Journal of Plant Sciences*.

2.1 Introduction

Spatial distribution and patterning of plants is an important characteristic of communities and is a fundamental property of most species. Hutchinson (1953) determined that at least five causal factors shape the spatial pattern of species: 1) environmental factors such as nutrients or light availability, 2) reproductive factors including propagule dispersal and seasonality, 3) interspecific or social factors such as territoriality, predation and competition, 4) intraspecific components such as competition and density dependent factors and 5) stochastic variation in any of these causal factors. Evidence for micro-environmental heterogeneity (Forget *et al.*, 1999; Palmiotto *et al.*, 2004), localized seed dispersal (Russo and Augspurger, 2004) and density-dependent factors affecting spatial distribution have been previously determined for many tree species (Schupp, 1992; Gilbert *et al.*, 1994; Grau, 2000; John *et al.*, 2002; Lambers *et al.*, 2002), with some authors supporting a combination of multiple factors (Itoh *et al.*, 1997). Many authors have shown that most tropical tree species have aggregated spatial distributions (Hubbell, 1979; Armesto *et al.*, 1986; He *et al.*, 1997; Okuda *et al.*, 1997; Condit *et al.*, 2000), generally attributed to a combination of dispersal limitation and micro-site variation. Light availability and gap-phase dynamics have also been described as important factors shaping the spatial distribution and regeneration patterns of tropical trees (Denslow, 1987). In tropical dry forests, light availability is directly related to seedling mortality and recruitment patterns. Areas with low canopy cover generally have few seedlings due to increased desiccation (Gerhardt, 1996). Therefore, light availability should be taken into account when studying the spatial distribution and regeneration of tropical dry forest tree species. Condit *et al.* (2000) showed that rare species are generally more aggregated than common species, and Hubbell and Foster (1986) proposed that light availability was the main proximal factor shaping aggregated distributions, with a significant proportion of rare or endangered species in the heliophyle category. For many endangered species information on their spatial distribution and the effect of abiotic factors on spatial patterns is lacking. This will be the focus of our investigation.

Guaiacum sanctum (Zygophyllaceae) is a slow growing tropical and subtropical dry forest tree, distributed from southern Central America to Northern Mexico and Florida, and throughout the Greater Antilles (Holdrige and Poveda, 1975). This species, also known as Lignumvitae or the tree of life, has been heavily exploited for its hard wood and medicinal value. Its extremely dense

wood was popularly used for the construction of propeller shafts on steamships, gears, and for mallets. Additionally, for over five centuries, the decoction of *Guaiacum sanctum* was believed to cure syphilis (Voeks, 2004). Therefore, most populations of *G. sanctum* were decimated by the end of the 19th century with a few remnant populations remaining in Mexico, Central America, and the Florida Keys. In Costa Rica, *G. sanctum* is distributed throughout the dry forests of the northwest Pacific coast. Tropical dry forests are the most endangered biome in the Neotropics with less than 0.1 % of its original cover remaining (Janzen, 1988), and currently many sites suitable for *G. sanctum* populations have been transformed to agricultural fields or pastures. Therefore, extant populations of *G. sanctum* in Costa Rica are rare, and generally restricted to National Parks or reserves which continue to be menaced by habitat loss, fire or exploitation (Oldfield *et al.*, 1998). Because of its restricted distribution and reduced population sizes, *Guaiacum sanctum* is now included in Appendix I of the CITES convention (CITES, 2000) and is also termed "Endangered" in the World List of Threatened Trees (Oldfield *et al.*, 1998).

The ratio of different age classes in a population (*i.e.* demographic structure) can be used to infer its current reproductive status (Odum, 1983b but see Condit *et al.*, 1998). Species with declining numbers or local demographic instability generally display a large proportion of adults and few seedlings (Sagar and Singh, 2004). Conversely, species with large seedling to adult ratios generally indicate growing populations. It has been argued that demographic stochasticity is an important extinction factor associated with small, fragmented populations (Soul and Orians, 2001). Dry forests are continuously threatened by fire, deforestation and forest fragmentation (Murphy and Lugo, 1986; Janzen, 1988). The demographic status of *Guaiacum sanctum* populations is currently unknown, and a description of their demographic structure may allow us to determine the susceptibility of this species to future fragmentation or habitat reduction.

Presently, little information exists on the biology of *G. sanctum* and most published contributions have focused on its medicinal value (Gariepy, 1981, and citations therein). Nonetheless, most available information agrees that this is a slow-growing species with restricted distributions, limited reproduction and declining populations (Quirico, 1993; CITES, 2000). Soul and Orians (2001) emphasize that restoration and management of endangered species are only possible when detailed descriptions of a species' life history and habitat requirements are available. Currently, life history

traits such as age or size structure, patterns of spatial distribution and micro-habitat selection are not available for *G. sanctum*.

In this study we test whether *G. sanctum* is spatially aggregated and examine the role of canopy cover on recruitment. Additionally, differences in spatial patterns between size classes may be used to obtain important information on the regeneration requirements of this species (Okuda *et al.*, 1997; Yamada and Suzuki, 1997; Nichols *et al.*, 1999). Differences in spatial distribution across size classes may allow us to infer if factors such as density or distance dependent mortality (Janzen, 1970; Connell, 1971) or different micro-environmental requirements (Hamill and Wright, 1986) are shaping the spatial distribution of *G. sanctum*. Therefore, detailed descriptions of the spatial distribution of different size classes of *G. sanctum*, as well as a description of light availability may provide insights into the regeneration of this endangered tropical species.

2.2 Methods

2.2.1 Study site

This study was conducted within Palo Verde National Park (PVNP) in the lower Tempisque River basin on the Pacific lowlands of northwestern Costa Rica (10 21' N, 85 21' W). Upland portions of the 19,000-hectare park are mainly composed of dry tropical forest on limestone outcrops, with a mean annual rainfall below 1500 mm and a mean annual temperature of 30C. The area is characterized by an extended dry season from December through April and a rainy season from May to November. Palo Verde National Park has areas of continuous forest, which have been preserved from forest fires or logging for the last 25–30 years. On its dry limestone slopes, there occurs one of the last remaining populations of *Guaiacum sanctum* in Costa Rica.

2.2.2 Spatial distribution

In 2003, a 50 ha plot (i.e. 1 km x 500 m) was created on the southwestern slope of "Guayacan" hill in Palo Verde National Park (Figure 3.1). All adult *G. sanctum* within this 50 ha plot were marked and mapped using GPS technology and densities and spatial distribution of *G. sanctum* adults were determined. To determine the density and spatial distribution of seedlings and juvenile *G. sanctum*, three permanent 50 x 50 m subplots were established in areas of high *G. sanctum* density

within the 50 ha plot (Figgure 3.1). The three sub-plots have recent tree-falls (i.e. within 5-10 years) and hence vary in canopy structure. Within these subplots all *G. sanctum* were mapped to the nearest centimeter using tape meassures and were marked with permanent aluminum or plastic tags. Height (h) and diameter at ground level (DGL) were determined for all individuals within the subplots; DBH was also measured for individuals with DBH¿ 5 cm. All data were collected during the wet season, between June and December 2004.

Individuals within all three subplots were grouped into five height classes or life stages: seedlings ($h < 15$ cm), saplings (15 ¡ h ¡ 30 cm), juveniles (30 cm ¡ h ¡ 2 m), sub-adults (2 ¡ h ¡ 5 m) and adults (h ¿ 5 m). Height is used as a proxy for age, since no prior information exists on growth rates for this species. Size and height will be used interchangably. Spatial patterns for each size class were tested using Ripley's $K(t)$ function (Ripley, 1977). This function describes two-dimensional spatial distribution patterns. The $K(t)$ function tallies the expected number of points that fall within a circle of radius t at any point in two-dimensional space based on the Poisson distribution. That is, $K(t)$ shows the proportion of points that fall within each t distance class. The $L(t)$ square-root transformation of $K(t)$:

$$L(t) = \sqrt{\frac{K(t)}{\pi}} - t$$

is generally preferred since it linearizes the function and homogenizes the width of the confidence intervals, which allows easier interpretation (Besag 1977). Graphical representation of the $L(t)$ function may be interpreted as follows: complete random distribution of points if $L(t)$ does not deviate from zero; aggregated spatial distribution $L(t) > 0$; and $L(t) < 0$ suggests a regular distribution. For each subplot, we analyzed the spatial distribution of the three youngest size classes (juveniles and sub-adults were lumped together due to a low number of individuals in the latter category) at 1 meter intervals, for t distances ranging from 1 to 25 meters (i.e. half the length of the subplot). Due to their low densities, the spatial pattern of adult trees was analyzed by means of Ripley's K function using data for the entire 50 ha plot.

To determine the spatial distribution between different life stages, we performed Ripley's second order analysis (Ripley, 1976). This function describes the spatial relationship between two size classes (i.e. aggregated, random or repulsion). Different categories are aggregated when individuals

from different groups are found at closer distances than expected by random dispersion. Repulsion is the contrary effect, when individuals from different size classes are rarely found in close proximity. If no pattern is observed, the distribution of the two size classes relative to one another is considered random. We examined the spatial relationship between all pairwise comparisons of different size classes by means of the $L(t)$ function. A 95% confidence envelope was created for all $L(t)$ functions by means of 1000 Monte Carlo simulations, where the position of individuals is randomly shifted in a toroidal plane. All calculations were performed using the SpatStat library in R (R Development Core Team, 2005). To determine if the average distance to an adult varies with size, we measured the Euclidean distance of every individual within a sub-plot to every adult within the subplot. Average distances to the nearest adult were calculated for each size class and averaged across plots. We also estimated the average distance to the second and third nearest adult.

2.2.3 Canopy cover

To determine the effect of canopy cover on seedling and sapling abundance, we created a two-dimensional map of canopy cover for each subplot. A 5×5m grid was created for each subplot and canopy opening estimates were taken at each grid node. Canopy cover was measured using a spherical convex densiometer. We took four readings in orthogonal directions for each node and three measurements were conducted during the day: 07:00, 12:00 and 17:00. Densiometer readings were transformed to percent canopy opening. The average of all node measurements was used for further analysis. To estimate the canopy opening of each marked individual, a canopy cover map was created using an Ordinary Kriging spatial interpolation technique implemented by the geoR library (Ribeiro and Diggle, 2001) in R (R Development Core Team, 2005). A canopy opening index (COI: % canopy opening) was estimated for each marked *G. sanctum* individual in each subplot using the canopy cover map.

To determine if the average canopy opening index of different size classes varied from random, a null distribution was created by re-sampling random points from each sub-plot. Random points (i.e. random locations) from each subplot where drawn based on a uniform probability distribution and their canopy opening index were determined using the Kriging estimates. For each size category the number of random points drawn was equivalent to the number of observed individuals. An average

canopy opening index was estimated for the simulated points and compared to the average canopy opening index of observed individuals. This process was repeated 10000 times for each size category and 95% confidence intervals were built around the mean of simulated values using the PopTools add-in for Excel. All central statistics are presented ± their standard error, unless otherwise stated.

2.3 Results

Thirty-five adults were marked within the 50 ha plot (i.e. 0.7 adults per hectare, Figure 3.1). Adults had an average DBH of 35 cm (±8.2) with a skewed distribution towards lower diameters. Adults were spatially aggregated forming clumps of 2 to 4 individuals within ≈ 20 meters of one another.

A total of 2155 *G. sanctum* individuals were marked in the three sub-plots. Over 65% of these individuals belonged to the seedling category: saplings and juveniles represent 16.7% and 17.6% of the entire sample, respectively. Adults (11 individuals) and sub-adults (7 individuals) comprised less than 1% of the total sample. The percentage of individuals in different size classes varied among subplots ($G^2_{[8]} = 445.606$; $p < 0.001$; Figure 2.2), with juveniles being the most common size class in subplot 3. Overall, the average seedling to adult ratio was 106.33 (± 49.38). Sapling and juvenile to adult ratios were 35.5 (± 6.22) and 43.9 (± 20.56), respectively. We observed a two-fold decline in the number of juveniles relative to seedlings.

Seedlings, saplings and juveniles have distinct clumped distribution (i.e. $L(t) > 0$, Figure 2.3). Seedlings have the highest aggregation across all distances, and juveniles are more aggregated than saplings. The spatial distribution of saplings becomes random at higher spatial distances for all three subplots, a trend not generally observed for the other size categories (Figure 2.3). Across all subplots, seedlings, saplings and juveniles are always aggregated with each other (data not shown). Seedlings, saplings and juveniles are generally randomly distributed relative to adults (Figure 2.4), with seedlings showing sporadic departures from random (Figure 2.4). Nonetheless, deviations are usually only found at a single distance point, and the $L(t)$ function quickly returns within the confidence intervals (Figure 2.4), therefore the spatial distribution of seedlings relative to adults is effectively random. Average distance to the nearest adult increases significantly with size (Figure 2.5; $F = 159.73$, $p < 0.001$; box-cox transformation $\lambda = 0.2$). The average distance of seedlings to

the nearest adult is 7.75 m (± 0.144), while for saplings and juveniles, it is 13.28 m (± 0.484) and 14.50 (± 0.470) meters, respectively.

Seedlings tend to be found in areas with dense canopy cover with an average canopy opening index (COI) of 39.7% (±0.25). Conversely, juveniles occur in canopy openings (Figure 2.6). On average, the COI for juveniles is 58.2% (± 0.63). The distribution of saplings in reference to light availability does not differ from random expectations (Figure 2.6). Overall, a positive relationship between size class and light availability is observed (Spearmans r = 0.76, $p < 0.001$), with larger size classes more common in areas with higher light availability.

2.4 Discussion

Populations of *G. sanctum* in PVNP are characterized by a highly skewed size distribtions, with disproportionate numbers of small individuals and few representatives in the sub-adult and adult categories (Figure 2.2). This distribution is typical of expanding populations of species with long life spans and slow growth rates (Korning and Balslev, 1994; Clark and Clark, 1996; Zuidema and Boot, 2002; Sagar and Singh, 2004). A preliminary study with a similar plot system (0.7 ha vs. 0.75 ha) conducted by Ribbens (1990 Tropical Biology course, OTS 90-1), showed a size class distribution skewed towards seedlings and saplings, but lacked individuals in the juvenile category. Although it was previously suggested that the regeneration of *G. sanctum* in PVNP is almost negligible (Quirico, 1993), both studies (OTS course and ours) are consistent with the possibility of population expansion. PVNP forests have only in the last several decades (post 1975) been protected from harvesting and fires. During the first half of the 20th century, timber extraction occurred in most of PVNP and fires were relatively common. It is likely that during this time, many adult and sub-adult *G. sanctum* were removed by logging. This could have impacted the reproductive success of the population, by reducing the number of individuals recruiting into the younger size categories. Seedlings, saplings and juveniles are also sensitive to fire, while adults and subadults are more resistant (Otterstrom *et al.*, 2006). Seasonal fires have tend to reduce the density of individuals in the younger cohorts of climax tropical tree species, and repeated fires (i.e. within 15 years) increase the probability of local extinction of many climax trees species (Slik and Eichhorn, 2003). Fires in Palo Verde are generally anthropogenic in origin, although lightning strikes

occur infrequently. Most adults in our plots have large fire-scars (E.J.F. personal observation), suggesting that this site experienced fires in the relatively recent past. After PVNP was established (1978), reproduction from the remaining adults began to generate a pool of seedlings and saplings. Otterstrom *et al.* (2006) has shown that regeneration of *G. sanctum* is greatly increased in post-fire treatments. Additionally, sub-adults spared during logging may have become reproductive, increasing the number of progeny produced. Insufficient time had passed for the new recruits to contribute significantly to the juvenile size class by the time of Ribbens' (1990) study. However, nearly two decades later, we do not see a noticeble gap of individuals between the sapling and juvenile size classes, but still see a significant lack of sub-adults and adults. Judging from the changes in age distribtuion seen in this population since PVNP was created, we believe that these populations are actively growing, and that over the course of the next several decades, subadults and adults will increase as individuals in the population grow.

Individuals of *G. sanctum* are spatially aggregated at all size classes (Figure 2.3), with more aggregation in the seedling category. Spatial aggregation may occur due to two non-mutually exclusive processes: localized seed dispersal (Howe and Smallwood, 1982; Barot *et al.*, 1999; Bleher *et al.*, 2002; Russo and Augspurger, 2004) and heterogeneity in the distribution of microhabitats suitable for germination, establishment and growth (Forget *et al.*, 1999; Silla *et al.*, 2002; Souza and Martins, 2004). Localized seed dispersal has been proposed as a major factor contributing to aggregated distributions, with evidence in animal and wind dispersed species (Nathan and Muller-Landau, 2000; Greene *et al.*, 2004). Howe (1989) defined bird dispersed species as 'scatter-dispersed' (sensu Howe, 1989) meaning they recruit as isolated individuals with random or over-dispersed distributions. Although *G. sanctum* has ornithochorous fruits (Wendelken and Martin, 1987), random distribution is clearly not the case for this species, where spatial aggregation is the norm. *G. sanctum* seeds are dispersed by a large array of frugivorous birds, with: *Trogon melanocephalus*, *Trogon elegans*, *Tityra semifasciata*, *Eumomotus momota, Pitangus sulphuratus* and *Calocitta formosa* having been observed actively foraging on seeds. These birds generally swallow the entire seed, either during flight (i.e. Trogons) or while foraging at the tree (i.e. Tityras and Magpie-jay's), suggesting that most consumed seeds are dispersed away from maternal trees. A clumped or aggregated distribution of *G. sanctum* seedlings may be produced by spatially contagious seed dispersal. Birds may

become 'clumped dispersers' (sensu Howe, 1989) if they defecate or regurgitate seeds in specific areas such as seed processing sites, display roosts, along foraging routes or in sites where predation is minimized (Howe and Smallwood, 1982). Our results show that seedlings are more commonly found in areas with ample canopy cover (Figure 2.6). If contagious seed dispersal is the predominant factor associated with the spatial distribution of younger *G. sanctum* cohorts, this would suggest that seeds are more likely to be defecated in specific areas under dense canopy cover. Wheelwright (1991) has shown that birds minimize their time in foraging trees and digest in other trees to reduce predation. Most species feeding on *G. sanctum* have conspicuous coloration and may move to areas with dense canopy cover to reduce predation risk, thus depositing seeds in closed canopy environments.

It is also possible that birds disperse seeds randomly and spatial microhabitat heterogeneity may cause an aggregated distribution of seedlings by increasing the frequency of recruitment in areas with suitable abiotic conditions. It has been suggested that seed germination and seedling establishment in tropical dry forests is regulated by moisture and light (Ray and Brown, 1995; Gerhardt, 1996). Research conducted in tropical dry forests in Ghana (Lieberman and Li, 1992) and Costa Rica (Gerhardt, 1996) showed that a large proportion of seedlings die during the extended dry season, due to moisture stress, with mortality increasing in areas with high irradiance or within canopy gaps. Palo Verde has a marked dry season between November and May. Establishment in open canopy areas may increase the desiccation of seedlings during the dry season, therefore there is probably an advantage for *G. sanctum* seedlings to germinate or recruit in shaded environments to overcome dessication and mortality. Our results agree with this argument. Seedlings of *G. sanctum* are more often found in areas with significantly higher canopy cover. Similar results were reported by McLaren and McDonald (2003) for tropical dry forest trees in Jamaica. They showed that seedling establishment and survival was higher in heavily shaded environments, which conserved moisture. The effects were more marked for evergreen shade-tolerant species, such as *G. sanctum*. Although their results show that plants in high light environments produced more above ground biomass, this advantage could not overcome the increase in mortality caused by dehydration in these environments. Therefore we conclude that the high proportion of *G. sanctum* seedlings in shaded environments, which results in an aggregated distribution, is the result of a combination of

the deposition of seeds in heavy canopy sites coupled with greater recruitment in areas with less desiccation. Clumped distributions arise through the combined effects of non-random seed dispersal and site specific mortality during the dry season. Plants located in gaps or high radiance sites, are less likely to survive the dry months, and therefore would not occur in our censuses, which were conducted in the rainy season.

Juvenile and adult *Guaiacum sanctum* are randomly distributed relative to each other (Figure 2.4); nonetheless, the average distance between adults and juveniles is significantly higher than between adults and other size categories. This trend has been shown for other tropical tree species (Clark and Clark, 1984; Sterner *et al.*, 1986; Condit *et al.*, 1992; Itoh *et al.*, 1997; Okuda *et al.*, 1997). Density dependent mortality (Grau, 2000) and selection for specific environments (Itoh *et al.*, 2003) have been proposed as the predominant explanations for repulsion between these two size categories. A large proportion of young individuals in close proximity to adults may increase the affluence of pathogens and herbivores, thus increasing mortality near adults (Janzen, 1970; Connell, 1971). Thus, the large proportion of saplings and juveniles found at greater distances from adults may reflect the effects of density dependent mortality i.e. the Janzen-Connell effect. However, we have not observed significant signs of herbivory or pathogen infections on *G. sanctum* seedlings or juveniles. Also, our spatial analysis shows that seedlings, saplings and juveniles are randomly distributed relative to adults with no signs of repulsion (Figure 2.4), suggesting that areas suitable for germination, establishment and growth are randomly dispersed relative to adults. Increase in average distance between juveniles and adults, relative to that of seedlings and adults, could be caused by population thinning. Therefore, biotic factors are probably not the predominant factor shaping the spatial distribution of this species.

The distribution of juveniles indicates that light availability may be a predominant factor shaping the spatial distribution of seedlings and juveniles. Juvenile plants were predominantly found in areas with higher light availability (Figure 2.6), while seedlings were commonly found in closed canopy environments. The environmental requirements of a species may change during development, and conditions advantageous for one life stage may be disadvantageous for another. While seedlings may require low levels of light or dense canopy cover for recruitment, juveniles may require increased light availability to grow into larger size classes (Denslow, 1987; Schupp, 1995).

Sites located near adults lack enough light for juveniles to grow, due to the dense evergreen canopy of the adults. Therefore, suitable sites for growth may only become available during gap formation, away from adults. Delayed gap-phase dynamics, where saplings and juveniles experience increased growth following gap formation, has been shown for many tropical rainforest trees (Denslow, 1987). Gerhardt (1996) showed for the tropical dry forest evergreen species, *Hymenaea courbaril* and *Swietenia macrophylla*, that increased light availability was negatively correlated with survival during the dry season. Nonetheless, higher irradiance associated with canopy openings were positively correlated with high growth rates. She also showed that seedling vulnerability decreased with plant height. Therefore it is likely that once *G. sanctum* seedlings are established in shaded areas, they develop root systems that access moisture in deeper soil strata, making them more desiccation resistant. Canopy openings caused by tree falls provide juveniles with increased light regimes which translate into higher growth rates. These gaps not only allow increased photosynthetic activity, but also signal the availability of space, which should promote recruitment of younger size classes into the sub-adult and adult categories. As mentioned previously, all subplots have had minor to large gaps formed by tree falls within the last five to ten years, creating the necessary environments for juvenile and sapling growth. Therefore, the larger proportion of *G. sanctum* juveniles found in canopy openings is likely caused by micro-environmental selection for areas which allow greater growth rates.

In conclusion, populations of *G. sanctum* in PVNP, are probably expanding due to the large number of seedlings, saplings and juveniles observed. The spatial patterns observed demonstrate that light availability due to canopy openings is an important factor determining the spatial distribution of small plants. We have shown that the demographic structure of *G. sanctum* is dependent on forest gap dynamics which has direct implications for the conservation of tropical dry forests. Endangered species require detailed descriptions of life history traits in order to develop well suited management and conservation strategies, which may insure the future survival of these species.

Figure 2.1: Diagram of adult *G. sanctum* individuals within the 50 ha plot. Adults are depicted as black dots, subplots are shown as squares. A rectangle within Costa Rica's map, depicts the approximate location of the 50 ha plot.

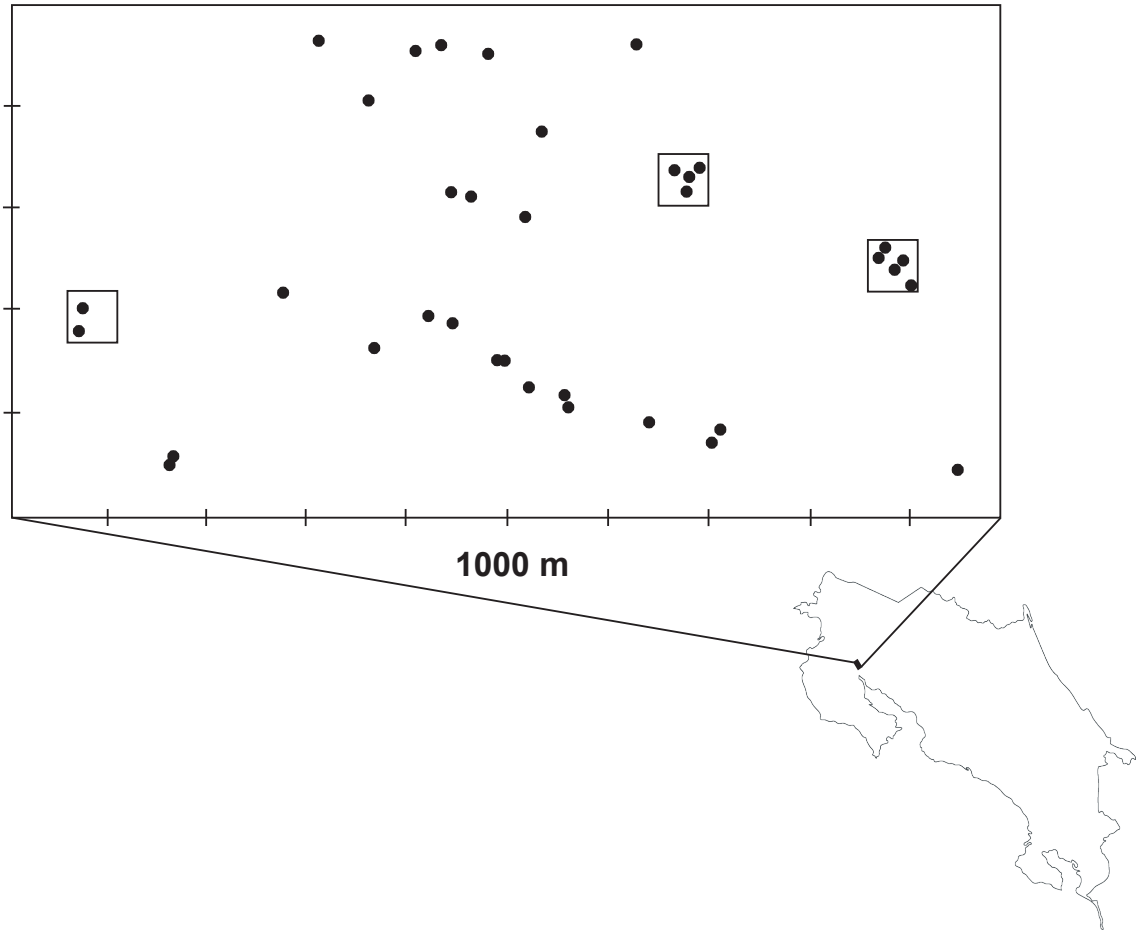


Figure 2.2: Number of *Guaiacum sanctum* individuals found in each of the 50 x 50 m sub-plots in Palo Verde National Park, Costa Rica.

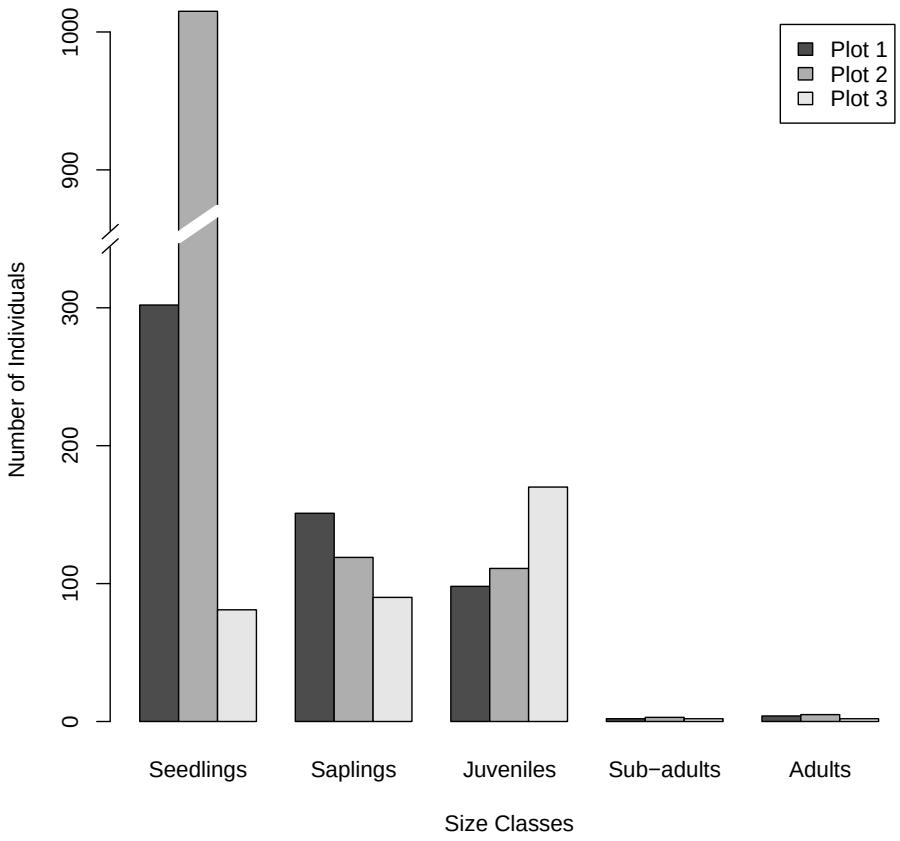


Figure 2.3: Ripley's $L(t)$ function for seedlings (S), saplings (A) and juveniles (J). Solid line depicts the $L(t)$ function, 95% confidence intervals are shown as dotted lines. Results for all three plots are shown in consecutive lines.

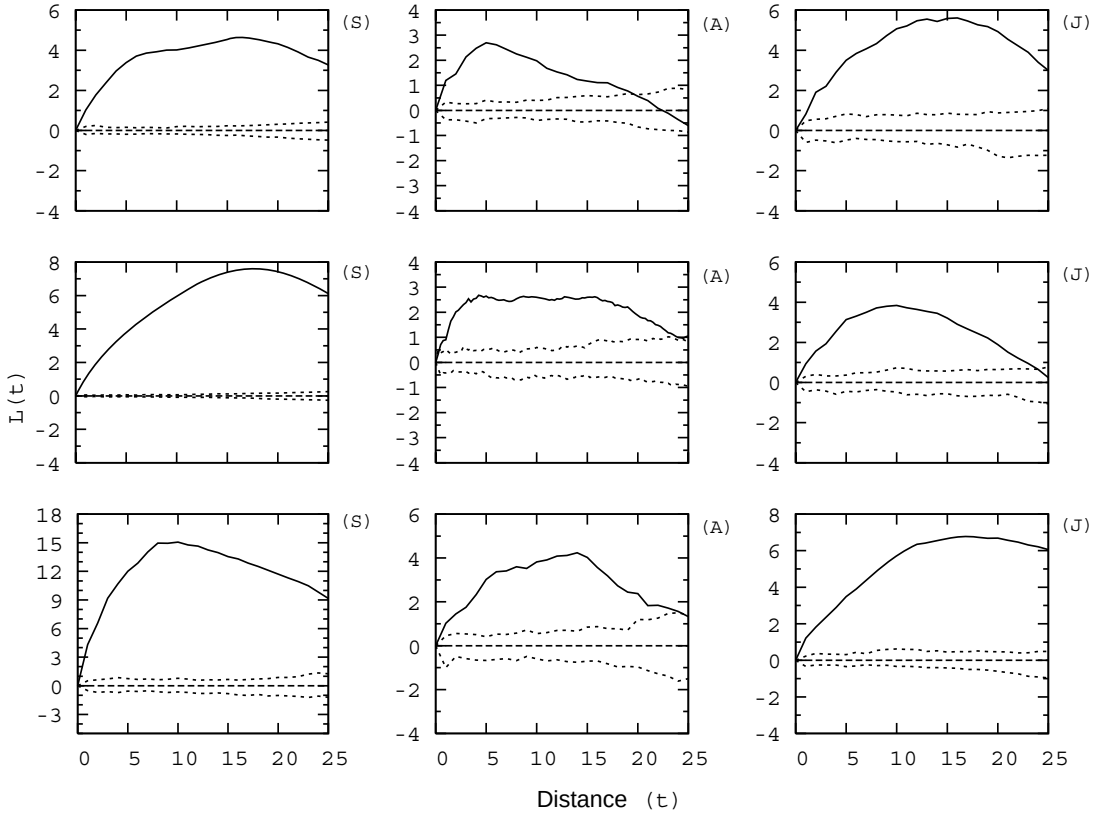


Figure 2.4: Ripley's second order function for seedlings (S), saplings (A) and juveniles (J) relative to adults. Solid line depicts the $L_{12}(t)$ function, 95% confidence intervals are shown as dotted lines. Results for all three plots are shown in consecutive lines.

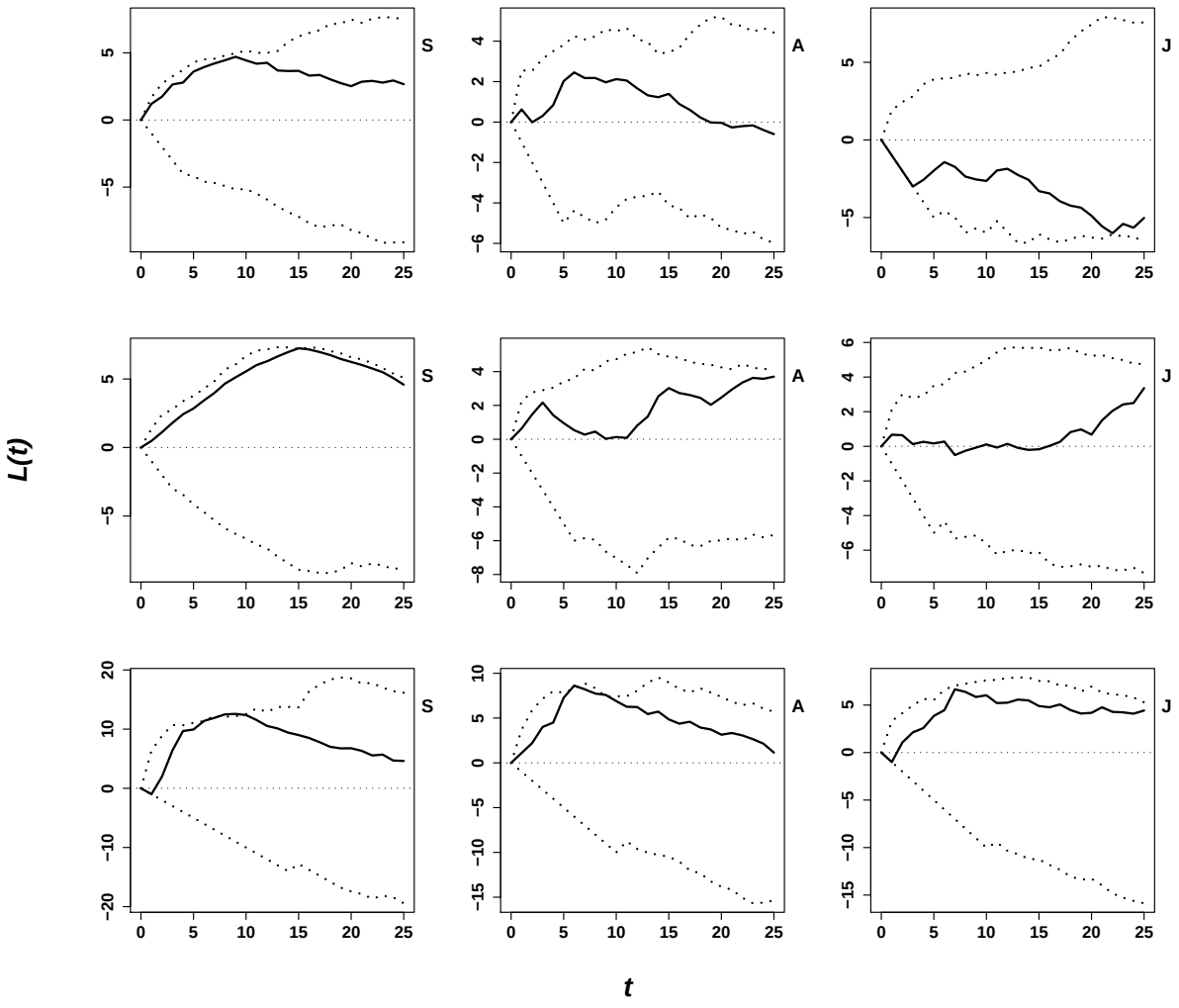


Figure 2.5: Histograms of the distance between seedlings (S), saplings (A) and juveniles (J) and the nearest adult *Guaiacum sanctum* tree. Results for all three plots are shown in consecutive lines.

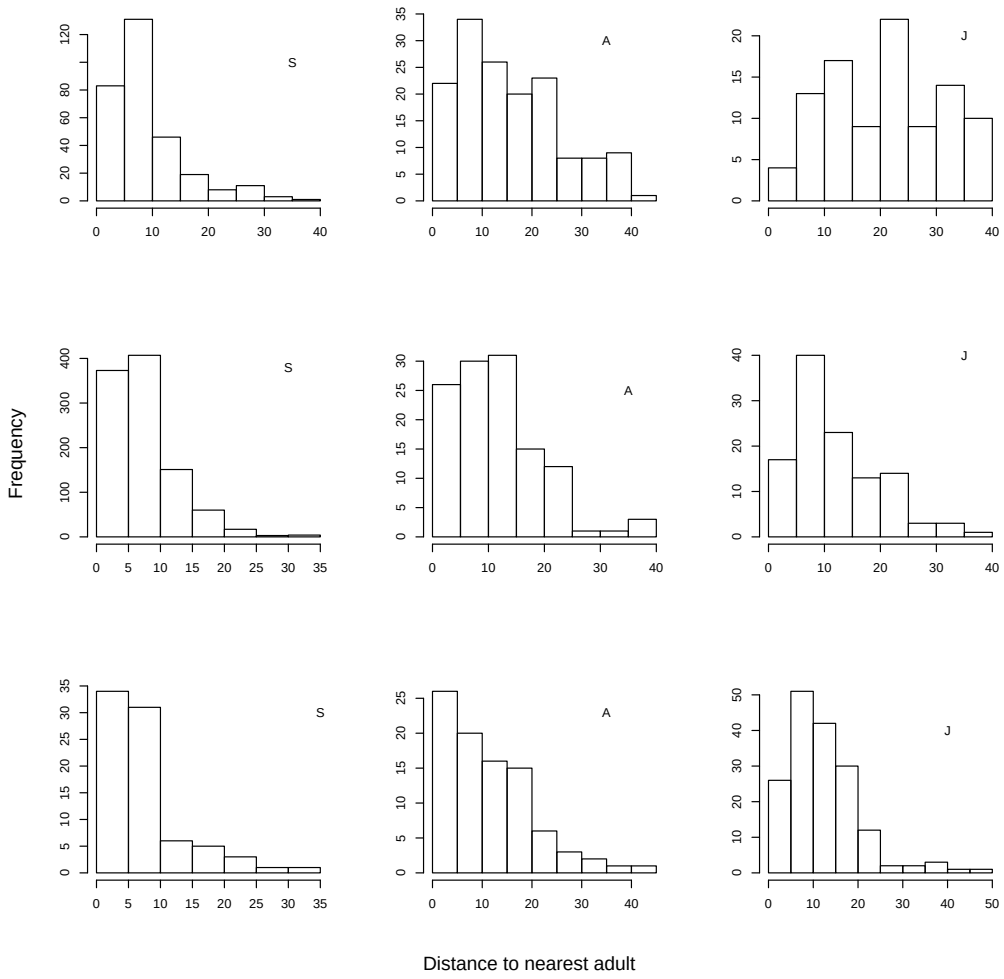
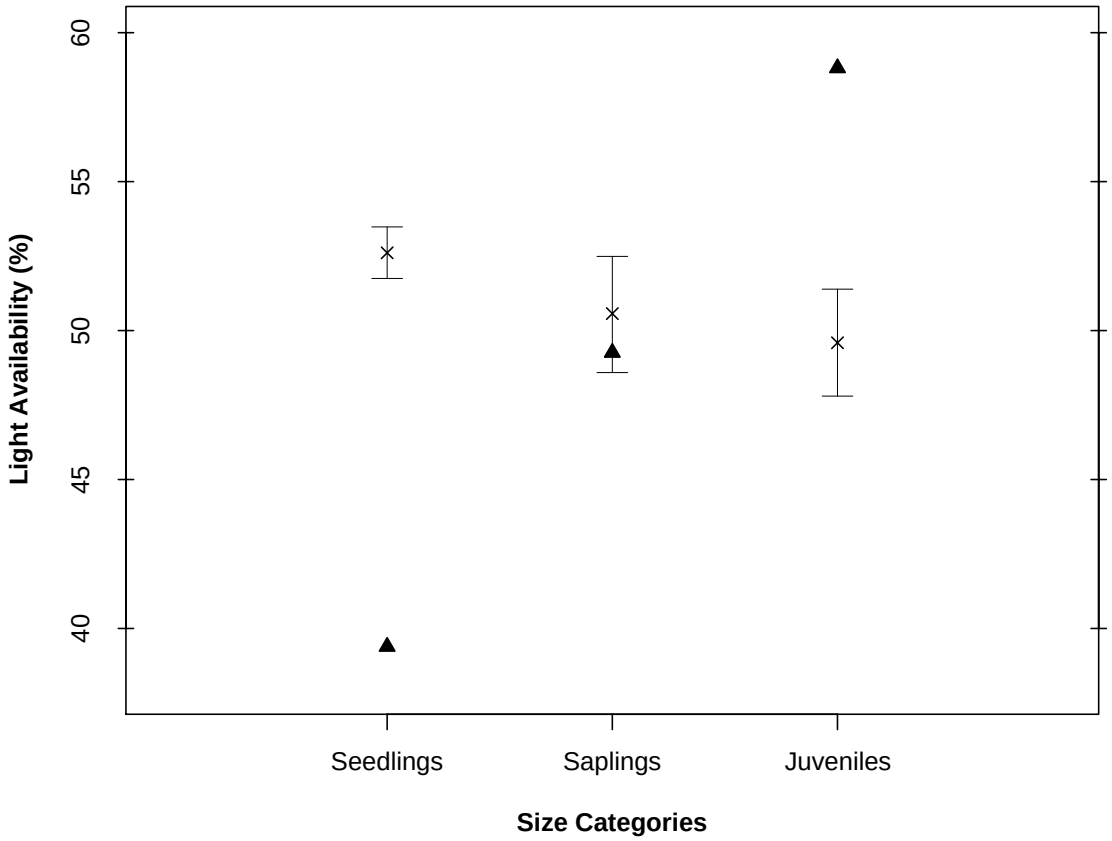


Figure 2.6: Results of the Monte Carlo simulation for light availability (Canopy Opening Index, COI) and presence of different size classes of *G. sanctum*. Average canopy opening (%) is depicted by a solid triangle. The average of Monte Carlo simulations are shown with an "×" with surrounding 95% confidence intervals.



Chapter 3

Fine-scale genetic structure in different size categories of the endangered tropical tree *Guaiacum sanctum* (Zygophyllaceae)[1]

[1]Fuchs, E.J. and Hamrick, J.L. To be submitted to *Molecular Ecology*.

3.1 Introduction

Successful conservation strategies depend, in part, on detailed knowledge of the levels and distribution of genetic diversity within and among populations (Holsinger and Gottlieb, 1991). Highly structured plant populations are more vulnerable to the loss of genetic variability due to stochastic processes and small effective population sizes (N_e) (Wright, 1946). In plant populations, gene flow via seed and pollen increases N_e and maintains genetic diversity within populations. In contrast, limited seed dispersal may create patches of related individuals or fine-scale genetic structure (FSGS), may reduce effective population size and promote the loss of genetic diversity through genetic drift (Schnabel *et al.*, 1998; Epperson, 2000). Thus, the magnitude and patterns of gene movement play a central role in the maintenance and structure of genetic diversity within plant populations.

The spatial distribution of genetic diversity within populations is influenced by seed dispersal patterns (Berg and Hamrick, 1995), mating system (Vekemans and Hardy, 2004), adult density (Hamrick and Nason, 1996), colonization events (Parker *et al.*, 2001; Premoli and Kitzberger, 2005), competition, demographic structure (Chung *et al.*, 2003b) and micro-habitat heterogeneity (Epperson, 1993). FSGS within populations occurs primarily through localized seed dispersal, either to specific locations (*i.e.* multiseeded fruits dispersed simultaneously) or around maternal plants (*i.e.* seed shadows). Pollen movement only affects the level of genetic relatedness between individuals, and thus cannot eliminate family structure created by limited seed dispersal. Therefore, patterns of FSGS structure provide an indirect look into gene dispersal processes and their possible influence on effective population size.

The study of spatial genetic structure is greatly enhanced by incorporating comparisons among different age classes (Tonsor *et al.*, 1993; Kalisz *et al.*, 2001; Chung *et al.*, 2003a), allowing one to detect changes in genetic composition across various life stages. This information may be used to elucidate potential ecological and evolutionary processes shaping the genetic composition of the population. A reduction in intrapopulation genetic structure in adult cohorts is expected if juvenile populations are thinned by demographic processes coupled with a reduction in adult densities to the point that one or a few adults represent a previous seed shadow (Hamrick *et al.*, 1993a; Epperson and Alvarez-Buylla, 1997; Parker *et al.*, 2001). In contrast, when several individuals within a seed shadow survive to larger size classes the FSGS found in seedlings should remain (Chung *et al.*,

2003a), or increase due to factors such as founder effect and local or post dispersal selection (Tonsor *et al.*, 1993; Kalisz *et al.*, 2001). If FSGS occurs in adults, this may affect mating patterns and increase inbreeding in progeny produced by these adults. Therefore, FSGS studies across different life stages provide indirect information on the demographic structure of the population, as well as predictions on the inbreeding coefficients of future generations.

Most FSGS studies are conducted on a single population or plot (but see Gehring and Delph, 1999). These studies implicitly assume that proximate causes of within population structure are homogeneous across populations. Even if different plots are located within the same habitat type, differences in ecological conditions, adult densities, reproductive success of adults, community assemblages of dispersers and micro habitat availability may lead to different patterns of family structure (Gehring and Delph, 1999). For example, variation in abundance or diversity of seed dispersers may lead to changes in mean seed dispersal distances and deposition patterns, leading to variation in the strength of family structure (Pacheco and Simonetti, 2000). For animal dispersed plants, disperser abundances may fluctuate among habitats due to the availability of roosting or nesting sites, and the repertoire of food resources. This, in turn, may cause fine-scale genetic structure patterns to vary across different habitat conditions. Therefore, FSGS analyses should include replications that confirm the effect of ecological processes shaping patterns of intrapopulation genetic structure.

Guaiacum sanctum (Zygophyllaceae) is an endangered tree species, found mainly on limestone outcrops of dry tropical or sub-tropical forests. Populations of this species have been heavily decimated due to its hard wood and medicinal value. Lignumvitae, as it is commonly known, has one of the densest known woods, and the resins produced by the bark possess antibiotic properties. Currently, most populations of *G. sanctum* are restricted to National Parks or Forest Reserves due to past exploitation (Holdrige and Poveda, 1975; Oldfield *et al.*, 1998). Previous work conducted in Palo Verde National Park (PVNP), Costa Rica (Fuchs and Hamrick in prep), showed that *G. sanctum* populations are characterized by a large number of young individuals (i.e. seedlings, saplings and juveniles), with few sub-adults and adults. This previous study also showed that size classes are highly spatially aggregated, probably due to increased survival in microhabitats based on light availability and gap-phase dynamics (Denslow and Spies, 1990). Species with spatially

clumped distributions are predicted to display FSGS in younger size classes due to localized seed dispersal (Doligez *et al.*, 1998; Hamrick *et al.*, 1993a). Therefore, given the spatial distribution of *G. sanctum*, we expect high levels of spatial genetic structure in younger cohorts. The lower densities of older individuals may have been caused by mortality due to competition or by the harvesting of adults. In Palo Verde, adults were harvested during the first half of the twentieth century, but since the creation of the National Park in 1978, this population has experienced an increase in reproduction and recruitment (Fuchs and Hamrick in prep). We predict that if genetic structure is present in the smaller size classes (seedlings), the magnitude of FSGS will decline in subsequent stages, mainly due to reduced tree density. Here we examine the spatio-temporal distribution of genetic diversity within populations of *Guaiacum sanctum* (Zygophyllaceae). We used allozyme analysis to address the following questions: 1) Are there differences in genetic diversity among size-classes? 2) Does the clumped distribution of individuals produce significant FSGS in younger cohorts? 3) Are patterns of FSGS stable through different size classes? 4) Are there differences in genetic diversity and FSGS between different plots located within Palo Verde National Park?

3.2 Materials and Methods

3.2.1 Study species

Guaiacum sanctum L. (Sapindales: Zygophyllaceae) is a slow growing tropical and subtropical dry forest tree, distributed from southern Central America to Northern Mexico and Florida, and throughout the Greater Antilles (Holdrige and Poveda, 1975). Trees grow up to 10–15 meters in height, and are usually found as part of the canopy and sub-canopy of certain tropical dry forests (González-Rivas *et al.*, 2006). Trees have opposite, compound leaves about 7–12 cm long, with oblong to lanceolate leaflets. *G. sanctum* is a non-deciduous tree, with perfect, conspicuous blue-purple flowers, that open between March and July. Only a few individuals flower each year, with most adults displaying a supra-annual phenology (Fuchs and Hamrick, in prep). In Costa Rica the current main pollinator is Africanized bees, but wasps and solitary bees have also been observed visiting flowers (Quirico, 1993) and were probably the main pollinators before the introduction of Africanized bees. Fruiting occurs from May to November, fruits are 5-lobed ovoid capsules, with one arilloid seed per locule. Seeds are dark brown to black, oblong and measure 1-2 cm in length.

During fruit maturation seeds are covered by a bright red arill, which attracts birds, the main dispersers.

3.2.2 Study Site:

The study was conducted in Palo Verde National Park (PVNP), in northwestern Costa Rica (10 21' N, 85 21' W). The 19,000 hectare park is mainly composed of dry tropical forest on limestone outcrops, with a mean annual rainfall of 1500 mm. This area is characterized by an extended dry season from December through April and a rainy season from May to November. PVNP contains one of the largest populations of *Guaiacum sanctum* in Costa Rica.

Forests in PVNP have been preserved from human intervention and forest fires for the last 30 years. Before that, this area was subject to regular burning and wood extraction. Populations of commercially important trees such as *G. sanctum*; were heavily decimated by logging. After the creation of PVNP in 1978, natural regeneration of seedlings and juveniles has increased, as sub-adult individuals spared during harvesting have become adults and are reproducing. This practice led to an increase in the proportion of individuals in the smaller size classes, and not enough time has passed since anthropogenic intervention for the younger cohorts to reach adult sizes, therefore, adults are not common in Palo Verde (0.7 individuals per hectare). Populations of *G. sanctum* outside the protection of National Parks or Forest reserves do not show evidence of recruitment.

3.2.3 Sampling

In the summer of 2003, three 50 x 50 m plots were placed in mature undisturbed forests on a southwestern slope of the limestone hills of PVNP. We placed the plots in areas with high densities of *G. sanctum* and care was taken that at least one adult was located within each plot. Plots are separated by an average distance of 315 meters (Figure 3.1). All *G. sanctum* within each plot were permanently marked and mapped to the nearest centimeter. The height of each individual was recorded and diameter at soil-height was measured using calipers.

All individuals were classified into four size categories, depending on height (h): seedlings (h ¡ 15 cm), saplings (15 cm ¡ h ¡ 30 cm), juveniles (30cm ¡ h ¡ 200 cm), sub-adults (200 cm ¡ h ¡ 5 m) and adults (h ¿ 5 m). Individuals were classified as adults if flowering or fruiting was observed,

regardless of height. Due to a very low number of individuals in the sub-adult category ($n < 10$), these trees were lumped into the juvenile size class. Flower and fruit production of adults was recorded yearly between 2003 and 2006.

3.2.4 Sample collection and allozyme analysis

Due to the large number of seedlings per plot, 10% of the seedlings within a plot were randomly selected for genetic analysis, while all saplings, juveniles and adults were collected from each plot, for a total of 884 samples. Two mature leaves were collected in the summer of 2004 from each sampled individual, placed in liquid nitrogen and transported to the University of Georgia in an ultra-cold shipper for allozyme analysis. Leaves were crushed in chilled mortars and pestles with liquid nitrogen to release cell content. Enzymes were extracted with a phosphate buffer according to Soltis *et al.* (1983). Protein extracts were absorbed onto 4×6 mm paper wicks (Whatmann 3 chromatography paper). Electrophoresis was conducted on 10% starch gels, with five buffer systems.

Thirteen polymorphic loci were resolved from 10 enzyme systems. Buffer and stain recipes followed Soltis *et al.* (1983) except UTP-glucose-1-phosphate and diaphorase, which were adapted from Manchenko (1994). We resolved triosephosphate isomerase (TPI1, TPI2) on system 8. Malate dehydrogenase (MDH1, MDH2), phosphoglucomutase (PGM), and shikimic acid dehydrogenase (SKDH) were scored on buffer system 4. Aspartate aminotransferase (AAT1, AAT2) and diaphorase (DIA1, DIA2) were stained in buffer system 7, while UTP-glucose-1-phosphate (UGPP) and isocitric acid dehydrogenase (IDH) were scored in a morpholine-citrate buffer. System 6 was used to score phosphoglucoisomerase (PGI). Banding patterns were consistent with expectations based on Mendelian inheritance (Wendell and Weeden, 1989).

3.2.5 Genetic Diversity

Genetic diversity parameters were calculated between size classes within plots. Genetic diversity was characterized by four parameters: mean number of alleles per locus (A), effective number of alleles (A_e), mean observed heterozygosity (H_o) and expected heterozygosity (H_e). Standard errors were estimated based on 1000 permutations across loci. All calculations were performed using GDA and Arlequin (Excoffier *et al.*, 2005) software packages.

Wright's fixation index (f_w) was estimated for each locus in each size category, as the deviation between observed and expected heterozygosities (Wright, 1969). Significance of f_w was assessed by means of 10000 permutations among loci, and p-values were adjusted according to the Bonferroni correction. A fixation index for the entire population (F) was calculated by pooling individuals from all size classes. Genetic structure among size classes and among plots was determined in terms of hierarchical F-statistics (Weir and Cockerham, 1984) using Arlequin (Excoffier *et al.*, 2005). Differences in gene frequencies were assessed among plots, and among age classes within plots. A null distribution for all F-statistics was obtained by 30,000 permutations of individuals among size classes. Pairwise F_{ST} values were used to compare differences in gene frequencies among different size classes, a Bonferroni correction was used to adjust observed p-values for multiple comparisons. Unless otherwise stated, all statistics are presented ± their standard error.

3.2.6 Spatial autocorrelation

Fine-scale genetic structure was assessed with autocorrelation analyses. A multilocus genetic correlation coefficient (r_j) was calculated for all pairs of individuals within a size class, separated by a specific j-th distance. A positive r_j value indicates that pairs of individuals separated by a $j-$distance, have more alleles in common than expected by chance. The r_j statistic is closely related to Moran's I coefficient (Peakall and Smouse, 2001), where an $r_j = 0.5$ is expected for full-sibs. Statistical significance of genetic correlation was assessed by comparing observed r_j values with 95% confidence envelopes generated under the null hypothesis of no spatial genetic structure. Confidence intervals are created by randomly permuting individuals among geographic locations (10,000 permutations). All calculations were performed with the GenAlEx V6 program created by Peakall and Smouse (2001).

Correlograms were created for all size classes on each of the three plots (Adults were excluded from the analysis due to insufficient sample size). Five meter distance classes were chosen for all analyses, to insure that the first category would include seedlings that fall under the crown of adult *G. sanctum* trees. Genetic correlation was computed for each size class in separate plots and 95% confidence intervals generated by 10,000 permutations of individuals across different distance classes. Correlograms were compared among plots using a modification of a t-test suggested by

P. E. Smouse (pers. comm). One thousand genetic correlation coefficients (r'_{ij}) were generated by bootstrap within each j-th distance class in each of the i plots. Correlation coefficients were standardized with the standard deviation of the bootstrap values ($U'_{ij} = \frac{r_{ij}}{\sigma_r}$). A t-test was performed to compare the mean of the standardized bootstrapped values between plots ($H_0 : \bar{U}'_{ij} = \bar{U}'_{ik)}$). The calculated student t was compared to the null distribution of t-statistics, estimated by performing a t-test on each of the 1000 bootstrap values. Significant differences for a specific distance class was inferred, if the calculated t-value was higher than the 99% confidence interval for the null distribution (i.e. after Bonferroni correction).

Correlogram analyses were also performed over the combined set of plots for each size class, using the "Multiple populations" option in GenAlEx. This analysis assumes similar biological processes are responsible for the observed patterns of spatial genetic structure. The calculation of autocorrelation over multiple populations allows an increase in sample size and statistical power, enabling the analysis to clearly detect signatures of genetic spatial structuring within populations.

3.2.7 Parentage analysis

To determine what proportion of seedlings, saplings and juveniles resulted from seed dispersal events originating outside the plots, we conducted a parentage analysis on these size-classes. For each individual the most likely parents were chosen based on LOD ratios (Likelihood Odds ratio) as implemented on the FaMoZ software (Gerber *et al.*, 2000, 2003). LOD ratios determine the likelihood that two adults are the parents of any progeny, based on their multilocus genotypes and Mendelian segregation ratios. For each individual within a plot, four parentage scenarios are possible: i) both parents are within the plot; ii) pollen donor is within the plot, but seed parent is not; iii) maternal plant is within the plot, but pollen donor is outside; iv) both parents are outside the plot. Given our data, we are not able to determine if a likely parent is the pollen donor or the maternal tree. Therefore to obtain a minimum estimate of seed dispersal we assume that if only one parent is within the plot, it is the maternal plant. We estimated the proportion of individuals with no-likely parent in each plot, and considered those seed dispersal events. Analyses were performed in each plot, and all adults within a surrounding 50ha (500m x 1000m Figure 3.1) plot were assumed to be candidate parents.

Parentage was assigned to candidate trees if they had the highest LOD-score above a threshold value estimated using simulations. We simulated 30,000 progeny by random mating of all candidate parents. Separately, 30,000 offspring were created with parents whose transition probabilities are based on population-wide allele frequencies. LOD values were calculated for all parent-progeny combinations. The intersection between the distribution curves of LOD values for both simulations, was used as the threshold value as suggested by Gerber *et al.* (2000). The proportion of seed dispersal events was determined as the proportion of plants with no likely parent within each plot. In some cases, multiple parents (i.e. likely parents) could be assigned to a single individual. In these cases, if one of the possible parents was within the plot, even if it did not have the highest LOD-score, we scored it as a non-immigration event. Using this procedure, we obtain a minimum estimate of seed dispersal from individuals outside the plots. Cryptic gene-flow, gene flow events falsely assigned to an individual within the plot, was estimated using the simulation procedure implemented in FaMoZ (for details see Gerber *et al.*, 2000, 2003).

3.3 Results

A total of 884 individuals were analyzed across four size classes and three 0.25 ha plots. All 13 analyzed loci were polymorphic and displayed similar levels of polymorphism in each plot. For the pooled population, the average number of alleles for each locus was $A = 2.71$ (± 0.166), and the effective number of alleles was $A_E = 1.543$ (± 0.02). The pooled genetic diversity of *G. sanctum* depicted by observed and expected heterozygosities are $H_O = 0.233$ (± 0.01) and $H_E = 0.302$ (± 0.02); respectively (Table 3.1).

Mean inbreeding coefficients calculated for each size class within each plot, in general, suggest a deficit of heterozygotes for all individuals (Table 3.1). A regression analysis on inbreeding coefficients versus size class –pooled for all plots– suggests a decrease in the inbreeding coefficient with an increase in size class ($\beta_1 = -0.06$; $F = 7.235$; $p = 0.007$). Similar regression analyses performed within each plot were not significant. Seedlings generally have lower levels of genetic diversity and higher inbreeding coefficients than the other size classes (Table 3.1). Inbreeding coefficients averaged over loci, plots and size classes; suggest a significant deficit of heterozygotes ($F = 0.185 \pm$

0.05). Expected heterozygosity varies less across size classes and different plots than observed heterozygosities.

A hierarchical analysis of molecular variance (AMOVA) revealed that the majority of the genetic diversity is partitioned within size classes, nested within plots (96.4%, $p < 0.001$). Only three percent (3.3%, $p < 0.001$) is partitioned among plots, with the remaining 0.3% ($p = 0.088$) of the variation due to differences among size classes within plots. Pairwise F_{ST} values revealed, after Bonferroni corrections ($\alpha = 0.0042$), that allele frequencies in adult populations are not statistically significant from other size classes regardless of plot locality. Pairwise F_{ST}'s show that allele frequencies are also homogeneous among all age classes within every plot. There are significant differences between similar size categories across the three plots.

3.3.1 Spatial genetic structure

Pairwise genetic correlations between individuals were used to assess the spatial genetic structure of different size classes of *G. sanctum*. Our results indicate a significant, but low correlation coefficient for seedlings in the first distance class (5 m) in all plots (Appendix). Correlograms for different plots were compared with a modified t-test, based on 1000 bootstrap r'_{ij} values. No significant differences were observed in autocorrelograms of any size class among plots, across all distances. Therefore, pooled samples were used for subsequent analyses.

Our results indicate significant fine-scale genetic structure for seedlings separated by less than 5 meters ($r_j = 0.02$, Figure 3.2). Saplings have significant family structure for the 10 meter distance class ($r_j = 0.02$), while juveniles show substructure of genetic diversity at 30 meter intervals (Figure 3.2). Although significant, the magnitude of genetic correlations is very low ($r_j < 0.02$), suggesting that there is little fine-scale genetic structure in this *G. sanctum* population. The significant correlations observed may be caused by large sample sizes obtained when individuals are combined across plots.

3.3.2 Parentage analysis

We estimated parent-pair exclusion probabilities for each plot, since the progeny (i.e. seedlings, saplings and juveniles) under scrutiny are different in each case. Nonetheless, values were very

Table 3.1: Summary of genetic diversity estimates for each size class within three plots of *Guaiacum sanctum* in Palo Verde National Park, Costa Rica. N: number of individuals, A: average number of alleles per locus, A_e: effective number of alleles, H_o: observed heterozygosity, H_e: HWE expected heterozygosity, f_w: inbreeding coefficient. Values in parenthesis are standard errors.

Plot	Age Class	N	A	A_e	H_O	H_E	f_w
Plot 1							
	Seedlings	64	3.083	1.528	0.184	0.283	0.253
			(0.358)	(0.147)	(0.037)	(0.061)	(0.065)
	Saplings	85	2.667	1.575	0.244	0.309	0.194
			(0.256)	(0.145)	(0.047)	(0.058)	(0.062)
	Juveniles	96	3.000	1.619	0.258	0.336	0.227
			(0.213)	(0.134)	(0.046)	(0.053)	(0.058)
	Adults	4	1.750	1.439	0.167	0.245	0.219
			(0.179)	(0.133)	(0.047)	(0.061)	(0.136)
Plot 2							
	Seedlings	104	3.000	1.593	0.196	0.315	0.281
			(0.174)	(0.142)	(0.032)	(0.059)	(0.063)
	Saplings	85	2.750	1.591	0.232	0.321	0.191
			(0.279)	(0.135)	(0.035)	(0.056)	(0.058)
	Juveniles	114	3.167	1.582	0.243	0.325	0.217
			(0.386)	(0.127)	(0.037)	(0.049)	(0.051)
	Adults	5	2.000	1.504	0.250	0.283	0.089
			(0.174)	(0.13)	(0.07)	(0.056)	(0.132)
Plot 3							
	Seedlings	75	2.833	1.470	0.218	0.285	0.173
			(0.271)	(0.109)	(0.029)	(0.044)	(0.059)
	Saplings	88	3.250	1.584	0.250	0.337	0.232
			(0.305)	(0.11)	(0.034)	(0.043)	(0.044)
	Juveniles	162	3.250	1.597	0.266	0.343	0.210
			(0.25)	(0.109)	(0.038)	(0.043)	(0.06)
	Adults	2	1.667	1.439	0.292	0.240	-0.257
			(0.188)	(0.141)	(0.074)	(0.064)	(0.058)

similar with $P_E = 0.89$ for plot 1, $P_E = 0.94$ for plot 2, and $P_E = 0.92$ for plot 3. We could assign at least one parent to 45.3%, 46.8%, 46.7% of the progeny, respectively for each plot. A parent-pair was resolved for only 20.4%, 8.8%, and 2.1% off the progeny in each plot. Apparent gene-flow into the plots was high, with an average across plots of 43.3 percent. The proportions of individuals without a likely parent within the plots are: 34.3%, 44.4% and 51.2%, respectively for each plot. Cryptic gene-flow is estimated to be 5.8%, 4.3% and 2.5%, with an average across plots of $\bar{x} = 4.2\%$ ± 0.95. Therefore, at a minimum, we can say that at least 48% of the progeny within a plot is the product of seed dispersal events from outside the plot. The nearest adult to a plot is $\approx$150 meters away, suggesting that the minimum distance for seed dispersal is 150 meters.

3.4 Discussion

3.4.1 Genetic diversity

Allozyme analyses of different size categories of *G. sanctum* in PVNP revealed high levels of genetic diversity (Table 3.1). At the population level, observed and expected heterozygosities are $H_O = 0.222$ and $H_E = 0.353$; respectively. All analyzed loci were polymorphic and allele numbers ranged from three to six, per locus. Life history characteristics are probably responsible for the relatively high levels of genetic diversity observed in this species. *G. sanctum* is a long lived, woody species, with mixed-mating system and animal ingested seeds. Even though Lignumvitae is an endangered species with reduced local populations, its geographical distribution encompasses Central America, Mexico, and southern Florida; as well as the Caribbean. Hamrick *et al.* (1992) in their review show that long-lived woody species with large geographic ranges and animal dispersed seeds generally maintain higher levels of genetic diversity within their populations than species with other life history traits. Other long-lived, widespread species from the Zygophyllaceae have been reported to posses high levels of genetic diversity. A study on diploid creosote bush (*Larrea divaricata*: Zygophyllaceae) (Cortes and Hunziker, 1997) reported heterozygosity values of $H_E = 0.290$, and a similar study on *Kallostremia grandiflora* reported H_E values of 0.267. In both cases, their wide distribution is considered the proximate cause of high levels of genetic diversity.

Populations of *G. sanctum* in Costa Rica have likely suffered substantial reductions in number and size due to over exploitation, deforestation and the advancement of the agricultural frontier

during the 1960's and 1970's. *Guaiacum sanctum* in PVNP were actively harvested during the first half of the 20th century, and regular burning of these areas until the mid 1970s probably reduced recruitment on this site. It is likely that during this time, many adult *G. sanctum* individuals were logged, reducing population sizes, which could have caused a bottleneck effect on genetic diversity. Nonetheless, many sub-adults and small adult trees were probably spared, mainly due to their low economic value relative to large adults. The current high levels of genetic diversity at PVNP sites suggest that enough individuals escaped logging to retain a high degree of genetic diversity, suggesting that only rare, low frequency alleles were lost due to logging. Additionally, since the creation of the National Park in 1978, many sub-adults have grown into large reproductive adults, leading to an increase in the reproductive success of the population (Fuchs and Hamrick in prep. Chapter 2). A reduction of genetic diversity caused by a decline in population number can recover fairly quickly if populations quickly reattain their original size (Nei, 1975).

Our results indicate a general deficit of heterozygotes relative to Hardy-Weinberg expectations, with more pronounced differences in the seedling category (Table 3.1). An excess of homozygotes in plant populations has generally been explained as being caused by two related factors: bi-parental inbreeding and selfing (Hedrick and Miller, 1992). *Guaiacum sanctum* has a mixed-mating breeding system. Estimates of outcrossing rates in PVNP average $t_m = 0.74$ (Fuchs and Hamrick in prep, Chapter 5). These results agree with breeding system estimates for a related species. Through hand pollinations, Bullock (1985) estimated a 30% selfing rate in *G. officinalis* –a congener from Mexico. Additionally, our observations of flowering indicate that *G. sanctum* in PVNP do not flower every year. In 2003, only two out of five adults were reproductive in plot 2. And during 2004, only a single individual reproduced within this plot. The low number of reproductive adults will usually lead to an increase in the distance between reproductive conspecifics, causing phenological isolation which has been shown to increase the proportion of geitenogamous progeny (Fuchs *et al.*, 2003). Lower levels of observed heterozygosity may also be caused by bi-parental inbreeding. Using Queller and Goodnight (1989) multilocus relatedness estimator (r_{xy}), we determined that some adults have relatedness values over $r_{xy} > 0.25$, suggesting half-sib lineages. Thus some of the progeny produced may be the result of consanguineous matings. In fact, during the 2003 reproductive season, the only two reproducing adults in sub-plot 2, had a relatedness value of $r_{xy} = 0.205$, suggesting that a

proportion of the progeny produced during that year could have been the result of consanguineous matings. Finally, a temporal Wahlund effect may increase estimated inbreeding coefficients. Given the slow-growth rates of *G. sanctum* individuals, our size-classes may group individuals from different reproductive events into the same size category. This indiscernible sub-structuring of the population may contribute to the observed reduction in heterozygosity. In conclusion, a deficiency of heterozygotes is likely to be caused by moderate amounts of selfing, with correlated matings and Wahlund effects as contributing factors shaping the genetic structure of this population. Our results show a low structuring of genetic diversity among plots separated by relatively short distances ($\approx$ 300 meters). Although significant, genetic differences are small suggesting ample gene movement among plots. The significance of these differences could be attributed to an artifact of large sample sizes (N=884) and we conclude that plots are effectively panmictic.

Seedlings generally had higher levels of inbreeding than older size classes (Table 3.1). This reduction in homozygosity may be caused by selection acting against inbred individuals in early life stages favouring higher heterozygosity in older size classes. Husband and Schemske (1996), determined that predominantly outcrossing species exhibited stronger inbreeding depression in early and late life stages. Purging of deleterious homozygotes during the seedling stage, may account for the observed reduction in inbreeding coefficients in larger *Guaiacum sanctum*. Similar results were found by Mandk *et al.* (2006) in *Atriplex tartarica*, where heterozygote deficits were greater in early stages than in older categories, due to strong selection and a reduction in seedling density through mortality and competition. Hufford and Hamrick (2003), working on the tropical tree *Platypodium elegans* (Fabaceae), demonstrated dramatic changes in outcrossing rates between germinated seeds and established seedlings, suggesting that strong inbreeding depression acted in these early stages, rapidly changing the frequency of homozygous individuals. These results indicate that selection against inbred progeny may act in early developmental stages of *G. sanctum*, to reduce the frequency of homozygosity.

3.4.2 Fine-scale genetic structure

This study demonstrates an overall lack of spatial genetic structure in different size classes of the endangered tropical tree *G. sanctum*. Significant family structure was observed for seedlings at the

five meter distance class (Figure 3.2.a); however, the magnitude of the genetic correlation is too low ($r_{ij} = 0.02$) to establish any family relationship between individuals. This effectively means that seedling genotypes are randomly distributed in space. These conclusions contradict our original prediction of clusters of related individuals caused by localized seed dispersal. Previous empirical and theoretical work has demonstrated that consanguineous matings and low levels of gene flow should increase genetic relatedness between progeny, and coupled with short distance seed dispersal, should produce spatially aggregated individuals with strong family structure (Turner *et al.*, 1982). This does not seem to be the case in Lignumvitae. Two non-mutually exclusive processes may explain this observation: long-distance seed dispersal and spatial-temporal overlap in seed shadows.

At least five species of birds were observed feeding on *G. sanctum* seeds in PVNP: *Trogon melanocephalus*, *Trogon elegans*, *Tityra semifasciata*, *Pitangus sulphuratus* and *Calocitta formosa*. These birds normally have long feeding bouts or territories, and generally swallow the entire seed either during flight (i.e. Trogons) or perching at the tree (i.e. Tityras and Magpie-jays). For most species, evidence suggests that most ingested seeds are dispersed away from maternal trees (Howe, 1977; Jordano, 1983). A study conducted on *G. sanctum* trees in Guatemala (Wendelken and Martin, 1987) listed 19 species of birds as regular foragers of Lignumvitae, which typically ingested seeds and flew from the tree. No regurgitation was observed in any of the cases, suggesting that these birds are probably dispersing seeds over relatively long distances. Twelve of the nineteen species reported by Wendelken and Martin are present in PVNP and may be potential long-distance seed dispersers. Given variation in the number of *G. sanctum* trees fruiting within a year, birds may travel significant distances to feed on different reproductive adults. This effectively creates multiple seed migration events, which should decrease any local family structure within populations. The absence of FSGS (even with strong clustering of individuals) may be the consequence of these repeated dispersal events (Chung *et al.*, 2003a). Our paternity data confirms this interpretation. As we have shown, on any given plot, approximately 48 percent of the progeny cannot be assigned to adult trees within the plot, suggesting seeds are being dispersed over distances larger than 150 meters. Additionally, birds may mix the progeny of different maternal trees by feeding on multiple *G. sanctum* adults before regurgitating or defecating. A mixtures of seeds from multiple maternal trees will eliminate any signature of FSGS in the progeny. These results are consistent with those

of other bird-dispersed tropical trees. Hamrick *et al.* (1993a) showed that *Swartzia simplex,* a bird dispersed treelet, had less spatial genetic structure than a co-occurring wind dispersed species. Degen *et al.* (2001) in an analysis of the FSGS of eight tropical trees, also determined that animal dispersed trees had less family structure than wind dispersed species. Although long-distance seed dispersal seems the most likely explanation for the lack of spatial structure in different cohorts of *Guaiacum sanctum*, specific data on seed dispersal are needed to complement and support this hypothesis. Nonetheless, by integrating paternity assignment strategies with FSGS estimations, we can more accurately interpret patterns of seed dispersal and the resulting levels of intra-population structure.

Alternatively, family structure may be eliminated by local seed shadow overlap (Chung *et al.*, 2004; Sato *et al.*, 2006; Ueno *et al.*, 2006). Seeds dispersed around adults, as a seed shadow, will create family structure when sibs germinate in close proximity to each other (Epperson, 1993). If adults are separated by distances smaller than the diameter of a seed shadow, overlap will occur, and family structure will be attenuated. Seed shadow overlap may also occur on a temporal basis. When seed dispersal patterns vary among years, or there is variation in the number of reproductive individuals across years, the magnitude of seed shadow overlap may vary across years. Additionally, seed dormancy or slow seedling growth rates may mix multiple reproductive cohorts, effectively eliminating FSGS. *G. sanctum* has always been categorized as a slow growing species (Schaffer and Mason, 1990; Quirico, 1993; CITES, 2000). If adults contribute differently to the seedling pool in different years, this would indicate that multiple annual cohorts may be lumped together into our size categories, creating temporal overlap which may reduce any signature of fine-scale structure. These processes may explain the low significant relatedness values observed in the 5 meter category (Figure 3.2). Nonetheless, given the large number of pairwise comparisons, one would expect some values to land outside the 95% confidence intervals by chance alone.

We conclude that the absence of family structure in different size categories of *G. sanctum* in PVNP, is probably caused by long-distance seed dispersal and the mixing of progeny from multiple maternal trees in space or time.

3.4.3 Conclusion

In conclusion, *Guaiacum sanctum* has high levels of genetic diversity at the intrapopulation level, even in the presence of apparent inbreeding due to moderate amounts of selfing. Long-distance seed dispersal likely inhibits the formation of fine-scale genetic structure within PVNP. Long distance seed dispersal coupled with estimates of high genetic diversity, suggest a potential for regeneration and restoration of this species through colonization and expansion of extant populations into suitable habitats. Nonetheless, seed dispersal is dependent on bird species which generally are more abundant in undisturbed habitats (Boswell *et al.*, 1998; Laurance *et al.*, 1998). The continuing threat of deforestation and habitat fragmentation, may have detrimental consequences for *G. sanctum*, by reducing disperser abundances and seed dispersal into suitable microhabitats for germination and establishment. Continuous inbreeding with localized seed dispersal, will rapidly create family patches with reduced genetic diversity and effective population sizes, which increase the probability of extinction (Brook *et al.*, 2002). Therefore, maintaining natural populations and supporting habitat conservation, may result in larger population sizes of this endangered tropical tree.

Figure 3.1: Diagram of adult *G. sanctum* individuals within a 50 ha plot. Adults are depicted as black dots, study plots are shown as squares. A rectangle within Costa Rica's map, depicts the approximate location of the 50 ha plot.

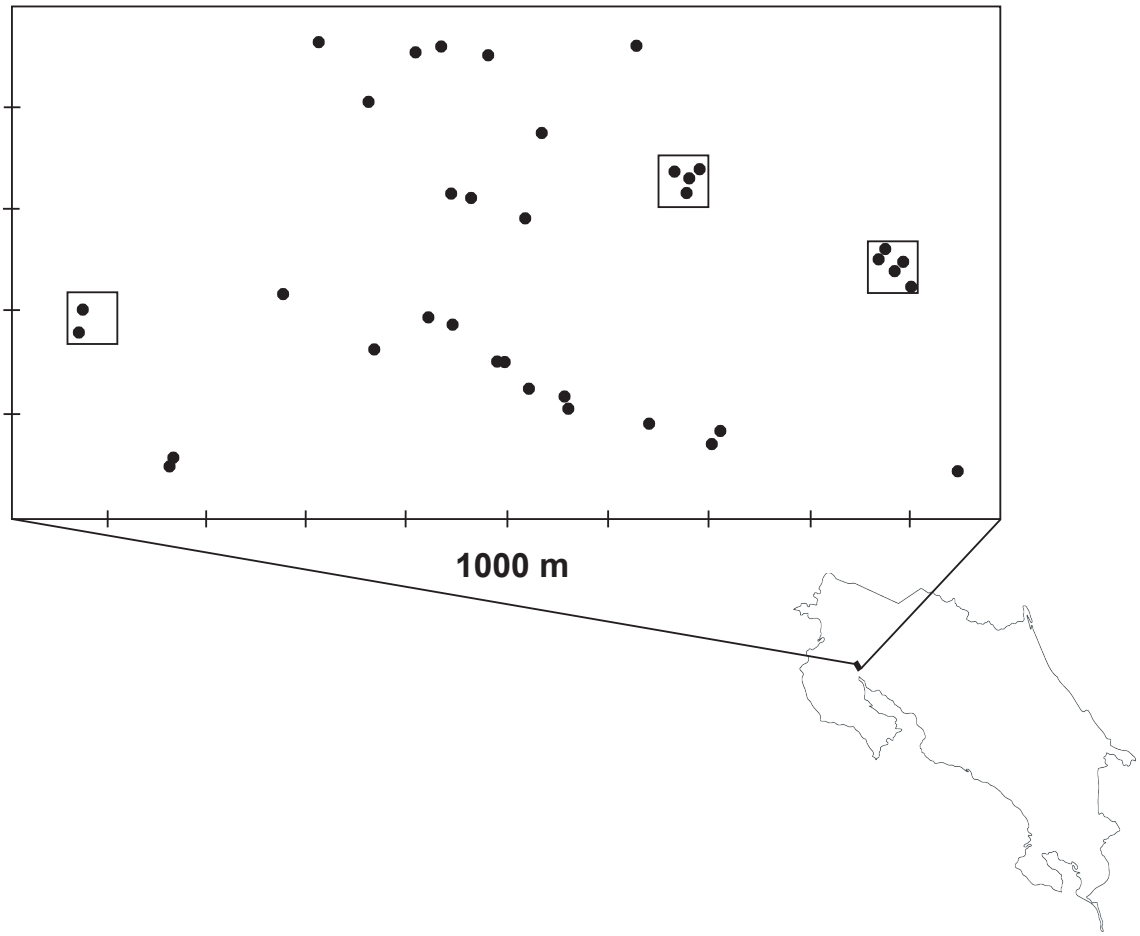
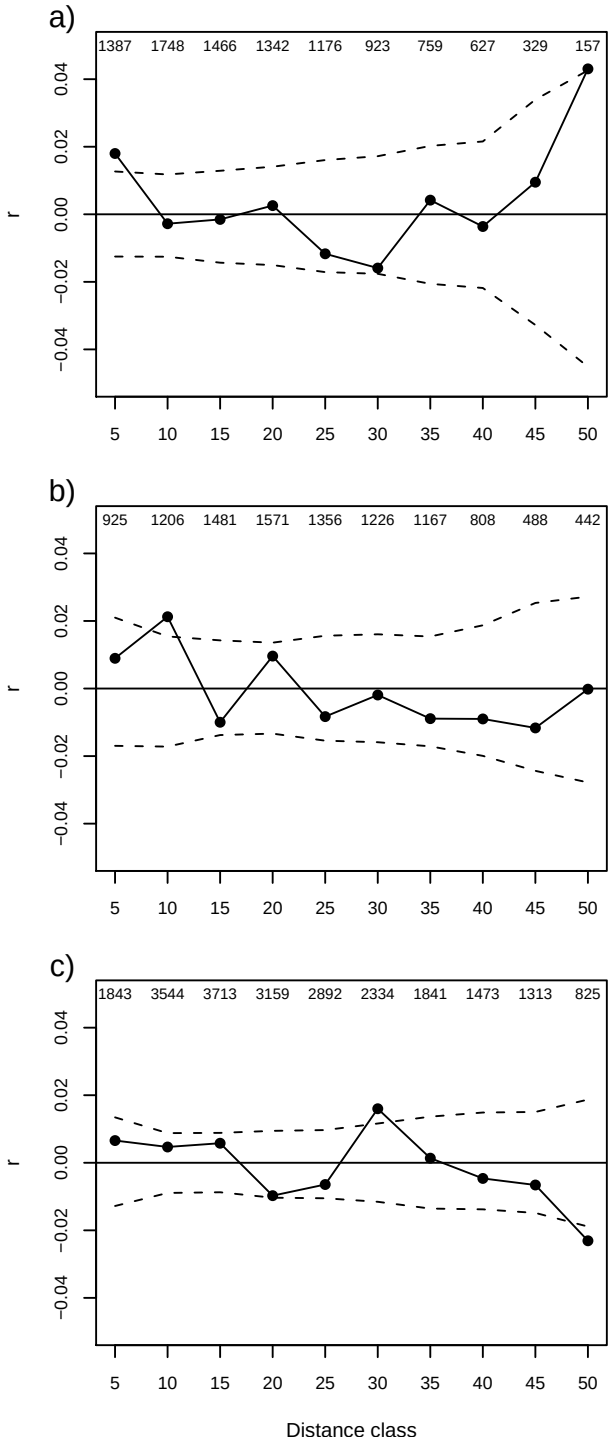


Figure 3.2: Correlograms for estimated genetic correlation r_j for three size classes: a) Seedlings, b) Saplings, c) Juveniles. The solid line represents calculated r_j values for each distance class. Dashed lines represent upper and lower 95% confidence envelopes for the null hypothesis of $r_j = 0$. Sample size for each distance class is represented on the top margin of each graph.



Chapter 4

Genetic Diversity in the endangered tropical tree *Guaiacum sanctum* (Zygophyllaceae). The effects of forest fragmentation.[1]

[1]Fuchs, E.J. and Hamrick, J.L. To be submitted to *Journal of Heredity*.

4.1 Introduction

Endangered species are often characterized by small population sizes, limited geographic distributions or specific habitat requirements (Soulé, 1986). Many such species have suffered reductions in their population sizes due to exploitation and/or habitat reduction and fragmentation. One of the main objectives of conservation genetics is to quantify and describe the spatial distribution of genetic diversity in extant populations of threatened or endangered species (Hedrick and Miller, 1992). A species' evolutionary potential depends primarily on the amount of genetic diversity present within its populations, allowing adaptation to changing environmental conditions (Huenneke, 1991). For endangered species, a detailed analysis of the levels and spatial distribution of genetic diversity is important for the development of effective conservation strategies and management practices. Populations with high levels of genetic diversity could serve as potential sources of propagules and reservoirs of genetic variation and, thus, are important targets for conservation. Conversely, populations with little genetic variability may be candidates for the introduction of novel genetic variation (Lande and Barrowclough, 1987; Holsinger and Gottlieb, 1991).

Fragmentation of tropical forests due to deforestation and human expansion has changed once continuous tropical landscapes into a network of poorly connected fragments surrounded by agricultural fields, urban settlements or abandoned sites. The immediate effects of fragmentation are a reduction of population sizes, extinction of local populations, and increased genetic isolation (Ellstrand and Elam, 1993; Young *et al.*, 1996). Fragmentation of continuous populations may also lead to a reduction of genetic diversity and the eventual loss of alleles through genetic drift. Additionally, individuals in populations located in small forest patches may become increasingly homozygous for deleterious alleles due to increased inbreeding (Alvarez-Buylla *et al.*, 1996; Hedrick and Kalinowski, 2000). In combination, these effects may increase the risk of extinction from stochastic and catastrophic events.

Tropical dry forests are specially threatened by deforestation and fragmentation, and are considered among the most endangered ecosystems (Janzen, 1988). Tropical tree species are often more susceptible to forest fragmentation due to their long generation times, low densities and commercial importance (Cascante *et al.*, 2001). Despite their significance, only a few studies have quantified the effects of forest fragmentation on the genetic diversity of these species (Lowe *et al.*, 2005, and

references therein). Studies conducted on fragmented populations of ecologically important tree species such as *Pachira quinata*, *Samanea saman* and *Symphonia globulifera* (Aldrich *et al.*, 1998; Cascante *et al.*, 2001; Fuchs *et al.*, 2003) have shown that fragmentation alters pollinator visitation rates, and the genetic composition of progeny.

Guaiacum sanctum L. (Zygophyllaceae) has a wide geographic distribution, but is often locally scarce and is considered endangered due to the overexplotiation of its valuable wood. *G. sanctum*, also known as Lignumvitae, has one of the densest woods known and was commonly harvested to manufacture propeller shafts for steam ships. Currently, even under the protection of the CITES convention (CITES, 2000), *G. sanctum* is still used as an ornamental wood and for handicrafts. Additionally, the bark of *G. sanctum* produces a resin called *guaiacin*, that possesses antibiotic properties, and has been used since the 1500's to cure sexually transmitted diseases, such as gonorrhea and syphilis (Voeks, 2004). This extensive use has led to a significant reduction in its population sizes, and large continuous populations are presently almost only found within protected areas. Currently, there is little knowledge of the status of *G. sanctum* populations throughout its distribution. The continuous exploitation of this species requires a proper description of genetic parameters to classify populations into conservation and restoration priorities.

In Costa Rica, *G. sanctum* is locally rare and is only found in the tropical dry forests of Guanacaste province. To our knowledge, there are only a few remnant populations, some of which are protected within national parks; others are located within fragmented or disturbed habitats under private ownership. These populations are of special interest, since they comprise the southernmost distribution of the species (Quirico, 1993). It is generally accepted that these peripheral populations are at greater risk due to smaller population sizes and reduced gene flow, making them more prone to extinction through stochastic processes (Lesica and Allendorf, 1995).

This study quantifies genetic diversity within *G. sanctum* populations in Costa Rica, and describes how this diversity is structured among populations. We also describe how genetic diversity differs between populations located within continuous habitats in national parks and populations located in fragmented sites or within agricultural/urban settings. Given that populations of *G. sanctum* in Costa Rica are at the southern extreme of the species' range and deforestation has fragmented its populations, we expected to observe relatively low levels of genetic diversity within

its remaining populations. We also hypothesized that due to restricted gene flow, genetic differentiation among populations will be correlated with geographic distance as suggested by the isolation-by-distance model (Rousset, 1997). Finally, we expected that trees within fragmented habitats have less genetic diversity and higher inbreeding values than populations in the more continuous protected habitats, as a direct consequence of smaller population sizes and reduced gene flow.

4.2 Materials and Methods

4.2.1 Study species

Guaiacum sanctum L. (Sapindales: Zygophyllaceae) is a slow growing tropical and subtropical dry forest tree, distributed from southern Central America to Northern Mexico and Florida, and throughout the Greater Antilles (Holdrige and Poveda, 1975). Trees grow to 10–15 meters in height, and are usually found as part of the sub-canopy of tropical dry forests (González-Rivas *et al.*, 2006). Trees have opposite, compound leaves about 7–12 cm long, with oblong to lanceolate leaflets. Guayacan, as it is known throughout the tropics, is an evergreen tree, with conspicuous perfect blue-purple flowers that open between March and July. In Costa Rica the main pollinators are africanized bees, but wasps and solitary bees have also been observed visiting flowers (Quirico, 1993). Fruiting occurs from May to November; fruits are 5-lobed ovoid capsules, with one arilloid seed per locule. Seeds are dark brown to black, oblong and measure 1-2 cm in length. Mature seeds are covered by a bright red aril which attracts birds, the main dispersers.

4.2.2 Study sites

We located and mapped all extant populations of *Guaiacum sanctum* in northwestern Costa Rica (Figure 4.1 and Table 4.1). Populations were separated into two regional groups: Northern and Southern populations. Sta. Rosa, Cuajiniquil and RaboMico are located in the northern part of Guanacaste province, and are separated by at least 70 km from the four southern populations (Figure 4.1). Populations may also be categorized into continuous and disturbed (fragmented) habitats. Palo Verde, Catalina, Sta. Rosa and Rosario, are all located within national parks or forest reserves and have been protected from anthropogenic disturbance since the late 1960's. The remaining populations are located within pastures, fields, or are part of living fences or gardens.

Table 4.1: Geographic location, sample size and habitat type for seven populations of *G. sanctum* in Costa Rica

Site	Code	N	Latitude (N)	Longitude (W)	Region	Habitat type
Cuajiniquil	CU	32	11°00′	85°41′	North	Fragmented
RaboMico	RM	30	10°55′	85°38′	North	Fragmented
Sta. Rosa	SR	40	10°16′	85°39′	North	Continuous
Rosario	RO	35	10°16′	85°23′	South	Continuous
PozoAgua	PA	27	10°17′	85°20′	South	Fragmented
Palo Verde	PV	30	10°21′	85°20′	South	Continuous
Catalina	CA	47	10°20′	85°15′	South	Continuous

Within each population we located, marked and mapped all adults using GPS technology. During Spring, 2006, two mature leaves were collected for each adult, placed in liquid nitrogen and transported to the University of Georgia for allozyme analysis. Between 27 and 47 individuals were sampled from each population (Table 4.1).

4.2.3 Allozyme electrophoresis

Leaves were crushed in chilled mortars and pestles with liquid nitrogen in a phosphate buffer according to Weeden and Wendell (1989). Protein extracts were absorbed onto 4×6 mm paper wicks (Whatmann 3 chromatography paper), and stored at -70°C until needed for electrophoretic analyses. We performed starch gel electrophoresis (10%) on five buffer systems. We scored a total of fifteen polymorphic loci from 11 enzyme systems. Buffer and stain recipes followed Soltis *et al.* (1983) except UTP-glucose-1-phosphate and diaphorase, which were adapted from Manchenko (1994). We resolved triosephosphate isomerase (TPI1, TPI2) in system 8; malate dehydrogenase (MDH1, MDH2), phosphoglucomutase (PGM) and shikimik acid dehydrogenase (SKDH) on buffer system 4; aspartate aminotransferase (AAT1, AAT2), diaphorase (DIA1, DIA2) and superoxid dismutase (SOD) were stained in buffer system 7; and UTP-glucose-1-phosphate (UGPP) and isocitric acid dehydrogenase (IDH) were scored in a morpholine-citrate buffer. System 6 was used to score phosphoglucoisomerase (PGI) and colorimetric esterase (CE). Banding patterns were consistent with

expectations based on Wendell and Weeden (1989), and we considered a locus polymorphic if at least two alleles were observed.

4.2.4 Genetic Analysis

Genetic diversity parameters and allele frequencies were calculated within populations (subscript p), at the regional level (subscript 'r') and pooled over all populations (subscript 's'). Genetic diversity was also analyzed by averaging across populations belonging to either the "Continuous" or "Fragmented" habitats (subscript 'h'). Standard measures of genetic diversity were characterized: proportion of polymorphic loci ($\%P$), mean number of alleles per locus (A), number of alleles for polymorphic loci (AP), effective number of alleles (A_e), mean observed heterozygosity (H_o) and expected heterozygosity (H_e). These statistics were calculated separately for each locus and averaged over loci. To estimate diversity parameters for the different regions (i.e. North and South) and for the different habitat types (i.e. Continuous and Fragmented), we used two methods: pooled estimates and averages across populations. For pooled estimates, we grouped individuals within a category regardless of population relationship. For mean estimates (referred to as 'Mean') we averaged the diversity estimates from populations belonging to similar categories. All calculations were performed using the Lynsprog program created by Loveless and Schnabel (University of Georgia, unpublished program), and GenAlEx (Peakall and Smouse, 2001).

We used Wright's fixation index (f) to measure the deviation of observed heterozygosities from expectations under Hardy-Weinberg equilibrium (Wright, 1969). Significance of inbreeding coefficients were assessed by 10,000 permutations among loci, using the F-stat program. A mean fixation index for the entire species (f_s) was calculated by averaging inbreeding coefficients across loci and populations. We estimated Nei's G_{ST} statistic to determine genetic structure among populations.

To assess differences in the pooled estimates of genetic diversity (H_e) between regions and between habitat types, we performed a permutation test conducted in R (R Development Core Team, 2005). Differences in genetic diversity between regions or habitats, was calculated for 10,000 permutations of individuals among categories. The proportion of values smaller than the calculated difference was used as an estimate of α. Average estimates were compared with a Mann-Whitney test due to small sample sizes.

Genetic structure was also analyzed by means of hierarchical F-statistics (Weir and Cockerham, 1984) using an Analysis of Molecular Variance (AMOVA) to test for differences in allelic frequencies among regions, among populations within regions, and within populations. These analyses were performed using Arlequin version 3.1 software (Excoffier *et al.*, 2005). Differences in gene frequencies were also assessed by means of AMOVA between fragmented and continuous populations. Statistical significance of F-statistics was attained by 30000 permutations of individuals or populations depending on the level of the analysis.

4.2.5 Spatial analysis of genetic diversity

To test whether population differentiation is caused by dispersal limitation (i.e. isolation by distance IBD), we estimated the correlation between geographic distance and genetic differentiation between populations as suggested by Rousset (1997). Pairwise F_{st} values were calculated for all population pairs, using Arlequin software. Geographic distances were log transformed, while genetic differentiation was transformed to $F_{st}/(1 - F_{st})$ as suggested by Rousset (1997). A Mantel correlation was performed between the geographic and genetic distance matrices and its significance was assessed by 10,000 permutations in R (R Development Core Team, 2005).

Given the geographic distances between northern and southern populations, we analyzed the effect of region on IBD. A partial Mantel test was performed by creating a third binary distance matrix, where populations belonging to the same region were given the value 1.0, and pairs of populations in different regions received a zero value. A partial Mantel test determines the correlation between two distance matrices (i.e. geographic distance, and genetic differentiation) removing the effect of a third distance matrix (i.e. region). Significance was attained by 10000 permutations using the Vegan package in the R statistical language.

To determine if genetic differences between populations correspond to geographic distribution patterns, Nei's (1972) genetic distance (D) and genetic identity (I) were calculated between all pairs of populations and a clustering algorithm was used to graphically represent genetic dissimilarities. Genetic distances (D) were used to construct an unrooted phenogram with the neighbour-joining algorithm. Calculations and graphics were produced using the Ape (Analysis in Phylogenetics and Evolution) library in R.

4.3 Results

We resolved a total of 50 alleles at fifteen loci, seven of which were private alleles to specific populations. Three loci (TPI2, CE and IDH) were monomorphic in at least one population. Two loci were highly polymorphic (CE, UGPP) with a total of five alleles each, and AAT1 and PGI had four alleles. On average, most loci in the sample had at least three alleles ($\bar{x}$= 3.33; SD = 0.211). Genetic diversity statistics for each population are given in Table 4.2.

Pooling across populations resulted in all loci being polymorphic ($\%P = 100$). Across populations the percent of polymorphic loci ranged between 87% and 100%, with an average of 94.3%. Two populations (RO and PA) located in the southern region had 100% polymorphism; while the other two southern populations (CA and PV) had the lowest $\%P$ values. At the regional level, the average percentage of polymorphic loci was slightly higher in the northern populations than in southern populations. Pooled estimates show no difference among regions, with both having 100 % polymorphism. On average, individuals located in fragmented populations had a slightly higher proportion of polymorphic loci compared to trees in relatively undisturbed populations.

Across populations, allele number per locus ranged between 2.47 and 2.85. On average the number of alleles did not differ significantly (Mann-Whitney's U=7, $p = 0.685$) between regions, nor did the pooled estimates. A similar pattern was observed for the number of alleles at polymorphic loci and the effective number of alleles. All pooled estimates of allelic diversity were slightly higher in fragmented than in continuous populations (permutation test, $p = 0.057$). Trees in fragmented habitats have on average two private alleles, contrasted to three in continuous habitats. The average across populations in fragmented and continuous habitats echo the results for the pooled samples.

Observed heterozygosity values (H_{op}) across loci ranged between 0.033 (polymorphic loci) and 0.760. The highest average for a population was found in PozoAgua ($H_{op} = 0.299$) and the lowest in Catalina ($H_{op} = 0.204$), both populations located in the southern region. Average observed heterozygosity values for populations within regions, were not significantly different ($U = 5$; $p = 0.85$). Although populations in fragmented habitats had higher observed H_{oh} values, no statistical differences were found for mean observed heterozygosity across habitat types ($U = 2$; $p = 0.23$).

Genetic diversity (H_e) estimates for each locus ranged between 0.033 (polymorphic loci) and 0.716, the pooled value was $H_{es} = 0.329$ and the mean genetic diversity across populations was

Table 4.2: *Guaiacum sanctum* genetic diversity estimates at the species, region and population levels. Diversity statistics were also computed for populations in fragmented and continuous habitats. $\%P$: percent polymorphic loci. A: average number of alleles. AP: alleles per polymorphic loci. A_e:effective number of alleles. H_o: observed heterozygosity. H_e: HWE expected heterozygosity. f: inbreeding coefficient. (Standard errors are shown in parenthesis).

	%P	A	AP	A_e	H_o†	H_e	f†
Overall	100	3.40	3.40	1.63	—	0.329 (0.057)	—
Regions							
North	100	2.93	2.93	1.58	—	0.296 (0.053)	—
Mean	95.6	2.70	2.62	1.55	0.253	0.287 (0.024)	0.094 (0.028)
South	100	3.00	3.00	1.61	—	0.327 (0.049)	—
Mean	93.3	2.64	2.74	1.59	0.254	0.311 (0.020)	0.174 (0.051)
Populations							
Cuajiniquil	93.3	2.67	2.85	1.69	0.284	0.332 (0.056)	0.137 (0.147)
RaboMico	100	2.73	2.73	1.49	0.247	0.280 (0.048)	0.084 (0.107)
Sta. Rosa	93.3	2.47	2.57	1.47	0.227	0.249 (0.054)	0.060 (0.185)
Rosario	100	2.67	2.67	1.65	0.264	0.335 (0.053)	0.180 (0.382)
PozoAgua	100	2.80	2.80	1.69	0.299	0.356 (0.047)	0.230 (0.386)
Palo Verde	86.7	2.47	2.69	1.49	0.250	0.286 (0.045)	0.085 (0.162)
Catalina	86.7	2.60	2.85	1.52	0.204	0.268 (0.060)	0.201 (0.242)
Mean	94.3	2.63	2.74	1.57	0.254 (0.012)	0.301 (0.015)	0.139 (0.025)
Fragmentation							
Continuous	100	2.87	2.87	1.57	—	0.302 (0.051)	—
Mean	91.7	2.55	2.70	1.53	0.236 (0.013)	0.285 (0.018)	0.132 (0.084)
Fragmented	100	3.27	3.27	1.68	—	0.348 (0.051)	—
Mean	97.8	2.73	2.77	1.62	0.277 (0.015)	0.322 (0.022)	0.156 (0.132)

†: Pooled estimates include Wahlund effect and are thus omitted

$H_{ep} = 0.301$. The population with the highest average was PozoAgua, and Sta. Rosa had the lowest estimate of genetic diversity (Table 4.2). Across regions, southern populations exhibited higher expected heterozygosity values than populations in the northern sites ($U = 1$; $p = 0.042$). No differences were found in expected heterozygosity values between fragmented and continuous populations nor for pooled estimates (permutation test; $p = 0.42$), or averages across populations ($U = 3$; $p = 0.4$).

The mean inbreeding coefficient, estimated as an average across loci and populations, showed a significant deficit of heterozygotes ($f_s = 0.142$; $p < 0.05$). Across loci, inbreeding coefficients ranged between -0.114 and 0.650, with most loci displaying an excess of homozygotes. In both fragmented and continuous habitats, heterozygote deficiency was not significantly different from zero.

Significant heterogeneity in allele frequencies was found across populations ($G_{ST} = 0.101$). G_{ST} values across loci ranged between 0.016 (CE) and 0.252 (AAT1), and loci with low values were characterized by very skewed allele frequencies (frequency of most common allele ¿ 0.90). A hierarchical analysis of molecular variance revealed significant structure among regions ($\theta_r = 0.070$; $p = 0.022$) and among populations within regions ($\theta_p = 0.064$; $p < 0.001$). Nonetheless, the largest proportion of genetic variation (87%) was found within populations, and the remaining variation is partitioned nearly equally between regions and, among populations within regions. The effect of fragmentation on allele frequencies was also tested with an AMOVA. Our results showed no significant effect of habitat type ($\theta_h = -0.014$; $p = 0.7725$).

Populations within regions were separated by an average distance of 11.5 km, but separation in northern populations (16.7 km) was significantly higher than inter-population distances among southern populations (7.2 km, $U = 2$; $p = 0.033$). A significant pattern of isolation-by-distance was observed for all populations when genetic differentiation (i.e. $F_{st}/(1 - F_{st})$) was correlated with the natural logarithm of pairwise spatial distances among populations ($r = 0.653$; $p = 0.0061$). The correlation ceased to be significant however, when region was taken into account ($r = 0.035$; $p = 0.437$); within regions there is no correlation between genetic differentiation and geographic separation (i.e. no IBD).

The unrooted neighbour joining phenogram created with Nei's distances clearly depicts two clades separated by a large distance, corresponding to the northern and southern regions (Figure

4.2). Within the southern region, two smaller clusters correspond to the PaloVerde-Catalina populations, both located within 10 km of each other in Palo Verde National Park. The second group consists of the Rosario and PozoAgua populations, located on the Nicoya Peninsula west of the Tempisque River (Figure 4.1). A comparison of the phenogram (Figure 4.2) and the geographic position of populations (Figure 4.1) shows a marked correspondence (with the exception of the RaboMico population) between geographic location and genetic differences. Average genetic identity for populations within a region ($\bar{I} = 0.963$) was significantly higher than the average identity among populations in different regions ($\bar{I} = 0.929$; $t = 5.94$, $p < 0.001$).

4.4 Discussion

4.4.1 Genetic diversity

We observed high levels of genetic diversity for *G. sanctum*, even though these populations were located at the southern margin of its range. All loci were polymorphic in the pooled sample, with only two loci monomorphic in some populations. Heterozygosity for the pooled populations ($H_{es} = 0.329$) were higher than the mean for species with similar life history characteristics. Even though our populations represent a restricted geographic area, *G. sanctum* exceeds the mean values for long-lived tropical woody species ($H_{es} = 0.191$; Hamrick *et al.* 1992), and for long-lived species with animal-dispersed seeds ($H_{es} = 0.225$; Hamrick and Godt, 1996). Although no genetic studies have been conducted on other *Guaiacum* species, genetic diversity estimates are consistent with values observed for other Zygophyllaceae; values range from 0.322 for *Larrea tridentata* (2x)(Duran *et al.*, 2005), to 0.098 for *Larrea nitidia* (Lia *et al.*, 1999), with an average of 0.234 ($\pm$0.032) for six Zygophyllaceae species (Cortes and Hunziker, 1997; Lia *et al.*, 1999; Ge *et al.*, 2003; Duran *et al.*, 2005; Cuevas *et al.*, 2006). These results are not consistent with our initial expectation, that this endangered, uncommon tree should have low levels of genetic diversity. It has been suggested that trees with wide geographic distributions with predominantly outcrossing mating systems and capable of long-distance seed dispersal are generally characterized by high levels of genetic diversity (Hamrick *et al.*, 1992, 1993a; Hamrick and Godt, 1996; Duran *et al.*, 2005). Although *G. sanctum* is locally scarce, its geographic distribution encompasses North and Central America, as well as the Caribbean Islands (Holdrige and Poveda, 1975). Seed dispersal is by birds, animals with large

potential dispersal abilities (Wendelken and Martin, 1987). These characteristics are associated with higher levels of genetic diversity; therefore, we conclude that these life-history traits may explain the high levels of genetic diversity in *G. sanctum*.

There is a consistent deficiency of heterozygotes across populations and habitat types, which is usually indicative of some form of inbreeding. Allozyme analyses conducted on the progeny of *G. sanctum* from two of the populations (PV and CU), show that outcrossing rates vary between $t_m = 0.72$ to 0.94, suggesting that *G. sanctum* is a mixed-mating species. Some adult trees in our populations may be inbred, which would increase the deviation from random mating. Additionally, a temporal Wahlund effect may contribute to the observed excess of homozygotes. Previous results show that *G. sanctum* has a supra-annual flowering phenology, with few individuals reproducing each year (Fuchs and Hamrick in prep. Chapter 3). By using all adults within a population, different reproductive cohorts may be grouped together. Ignoring this temporal subdivision may result in a slight increase in homozygosity.

Higher estimates of genetic diversity in the more southern populations could be due to higher levels of gene flow among these sites. On average, populations located within the southern region are separated by smaller distances than populations located in northern Guanacaste province (Figure 4.1). The shorter inter-population distances in the southern region probably allow pollinators and seed dispersers to move freely across the landscape. This opportunity for gene exchange may reduce the loss of genetic variation through drift, allowing higher levels of genetic diversity to be maintained. Proximity across different landscapes might even ameliorate the effects of fragmentation. PozoAgua (Table 4.1) is a fragmented population located between continuous sites, and may serve as a stepping stone for gene transfer among basins of the Tempisque river. Similarly, Hall *et al.* (1996) found that genetic diversity measures in populations of *Pithecellobium elegans*, a tropical rainforest tree, were negatively correlated with distance from continuous forest. This decrease in genetic diversity was probably caused by a reduction in gene flow and effective population size. These results suggest that gene dispersal distances may influence genetic diversity parameters for this tropical endangerd species.

4.4.2 Genetic structure

Calculated G_{ST} values suggest a low but significant structuring of genetic diversity among populations ($G_{ST} = 0.101$; $p < 0.001$). This value is slightly lower than that observed for 26 long-lived tropical woody species ($G_{ST} = 0.119$; Hamrick *et al.* 1992) and somewhat higher than the mean value for 28 long-lived perennial species with animal dispersed seeds ($G_{ST} = 0.099$; Hamrick and Godt 1996). Several studies of other tropical tree species have also observed high levels of genetic diversity within populations, and moderate to low amounts of genetic structure among populations (Hamrick and Murawski, 1990; Hamrick *et al.*, 1993a). Since statistics such as G_{ST} and AMOVA's θ measure the results of historical levels of gene flow, these results suggest that prior to fragmentation *G. sanctum* experienced high to moderate levels of gene flow.

However, although tropical dry forests in Costa Rica have been recently fragmented (see below), it is not likely that *G. sanctum* was continuously distributed throughout its range in Costa Rica. Currently, populations of this species occur in areas characterized by thin, xeric soils located on lime-stone hills. Additionally, *G. sanctum* populations are found in geologically similar areas, arising in the early Pleistocene later than the majority of Guanacaste province (Burnham and Graham, 1999). Based on data from Costa Rica's digital Atlas (Provided by Costa Rica's National Geographic Institute, `www.mopt.go.cr/ign`) only two areas in the country have these conditions: the Tempisque River basin, where Palo Verde National Park is located; and northwestern Guanacaste province (populations in the northern region). We recognize that these analyses are conducted *a posteriori*, and extinct *G. sanctum* populations which were located in other habitats could clearly modify these conclusions. Nonetheless, herbaria collections suggest that these seven populations are the only known sites for *G. sanctum* in Costa Rica.

High levels of historical gene flow between populations within regions seems to be a better explanation for the current patterns of genetic structure in *G. sanctum*. We found evidence for isolation-by-distance only when populations belonging to different regions were included in the analysis. No correlation was found when populations within a region were analyzed, suggesting that gene flow is not restricted between adjacent populations. Pollination of *G. sanctum* is performed mostly by bees and wasps; all of which can transport pollen over large distances (Janzen, 1971; Frankie *et al.*, 1976). Seed dispersal is mostly performed by birds belonging to the Momotidae,

Trogonidae and Tyranidae (Wendelken and Martin, 1987 and Fuchs and Hamrick in prep). Populations within regions are typically separated by less than 20 km, well within the flying capabilities of pollen and seed dispersers. Previous work conducted on a population of *G. sanctum* within Palo Verde National Park, has shown that seed dispersal over distances of 150 m is commonplace (Fuchs and Hamrick in prep). Pairwise F_{st} values among populations within a region ($\bar{x}$=0.04) suggest that inter-population migrations may frequently occur. Other studies conducted in tropical dry forest tree species have shown similar results, with high rates of gene movement between populations located in fragmented habitats (Apsit *et al.*, 2001; White *et al.*, 2002).

More than 50% of the variation among populations is due to regional differences in allele frequencies, indicating that gene flow between regions is more limited than gene flow within regions. Even if continuous suitable habitat between northern and southern populations had once existed, these regions are separated by 70 kilometers, probably beyond the range of pollinators and seed dispersers. Alternatively, differences between regions may be explained by historical vicariance events. Populations in the northern and southern regions may have been separated since the late Pliocene (Burnham and Graham, 1999). This separation, coupled with limited contemporary gene dispersal among regions, may have produced the genetic differences observed. The neighbour joining phenogram (Figure 4.2) corroborates these findings, by clustering populations in almost perfect association with their geographic locations. The most evident separation is between northern and southern clades, indicating that although gene flow occurs frequently enough to minimize genetic drift ($Nm > 1$), gene exchange occurs predominantly among populations located within a few kilometers ($\sim$10 km) of each other.

4.4.3 Effects of fragmentation

Theory predicts that small isolated populations in fragmented habitats should have less genetic diversity due to the combined effects of genetic drift and inbreeding (Young *et al.*, 1996; Hedrick and Kalinowski, 2000). These effects are most evident when populations undergo rapid declines caused by anthropogenic disturbances such as habitat degradation or extraction. Tropical trees may be more vulnerable to fragmentation and population declines due to their low densities, outcrossed breeding systems and dependence on animals for pollen and/or seed dispersal (Cascante *et al.*,

2001). Nonetheless, a recent review by Lowe *et al.* (2005) contradicts this conclusion. Most tropical tree populations located in fragmented habitats do not have reduced genetic diversity compared to trees in continuous habitats. Our results are consistent with these observations. *Guaiacum sanctum* trees located in disturbed or fragmented habitats had very similar levels of genetic diversity, when compared to trees in national parks or forest reserves (Table 4.2).

The lack of differences in genetic diversity between populations in altered or pristine habitats should not be surprising since fragmentation has usually occurred within the lifetime of the remaining trees (Lowe *et al.*, 2005, and references therein). Although *G. sanctum* has been actively used and extracted due to its precious wood and medicinal values since the 1500's (Voeks, 2004), fragmentation of tropical dry forests in Costa Rica has primarily occurred in the last 100 years with a drastic increase in the second half of the twentieth century (Quesada and Stoner, 2003). Since *G. sanctum* is a long-lived species with individuals surviving up to a 1000 years (Wilson and Eisner, 1968), is possible that trees analyzed in this study may have been alive before habitat fragmentation occurred. Therefore, factors such as genetic drift and inbreeding would not have had enough time since European intervention to exert changes in genetic diversity. Habitat fragmentation influences genetic diversity by reducing effective population sizes within populations and reducing gene flow into populations (Frankham, 1996). Our results have shown that historically gene flow was probably not limited between populations separated by less than 20 km. The elimination of continuous habitat between populations may alter the behaviour of pollen or seed vectors and, thus, modify gene movement between fragments. Thus, the impacts of forest discontinuity may become more obvious in the progeny produced by trees found in low densities in disturbed habitats (Fuchs *et al.*, 2003; but see Cespedes *et al.*, 2003) . Finally, many national parks and forest reserves in Costa Rica previously existed as ranches, or were logged prior to protection. PVNP populations, for example, were subject to selective logging until the 1970s when the park was created. Although harvesting may have occured, forest cover was not eliminated. Nevertheless, selective logging in these sites may have reduced allelic diversity somewhat, perharps explaining the lack of observed difference between continuous and fragmented populations.

4.4.4 Implications for conservation

The results of the present study can be used directly to infer conservation strategies for *Guaiacum sanctum*. Two populations located in the southern region display somewhat higher levels of genetic diversity than northern populations, making them appropriate candidates for *in situ* conservation. Additionally, trees in fragmented habitats within this region are commonly found next to gravel roads, in gardens, agricultural fields or as part of living-fences (pers.obs), areas susceptible to further perturbation. Guanacaste province has the only remaining areas of tropical dry forest in Costa Rica, currently estimated to represent less than 0.01% of its original cover (Janzen, 1988). This area continues to be threatened due to tourism related developments and agriculture. Guanacaste has the second largest rural population growth rate (Estado de la Nacion, 2005) and one of the highest rates of urbanization of rural areas relative to the rest of Costa Rica. Therefore extant populations of *G. sanctum* in Guanacaste province are threatened and should receive a high priority for conservation.

We determined that *G. sanctum* populations have low levels of genetic differentiation. This has direct implications for *ex situ* conservation strategies. A large number of individuals may be collected from fewer populations, properly representing the species' genetic diversity. Since populations such as as CU and PA have high levels of genetic diversity and are threatened by human intervention, seedlings and vegetative clones should be collected from these sites and grown in nurseries or plantations. This may insure the preservation of important allelic diversity found in these populations.

Finally, our results indicate that gene exchange may occur between populations within a region. Most fragmented populations are located within 10 km of continuous habitats, allowing seed and pollen dispersal (Dick, 2001; Dick *et al.*, 2003). Ample gene exchange may occur between trees in continuous and fragmented habitats, allowing the maintenance of genetic diversity (Chapter 5). Trees in fragments and isolated trees may also function as stepping stones between larger populations and, thus, are important components of the population dynamics of these endangered tropical trees. Conservation strategies should provide sufficient protection for these trees to insure their continued presence in disturbed landscapes.

Figure 4.1: Location of sampled *Guaiacum sanctum* populations in Costa Rica. Population codes are shown in Table 4.1.

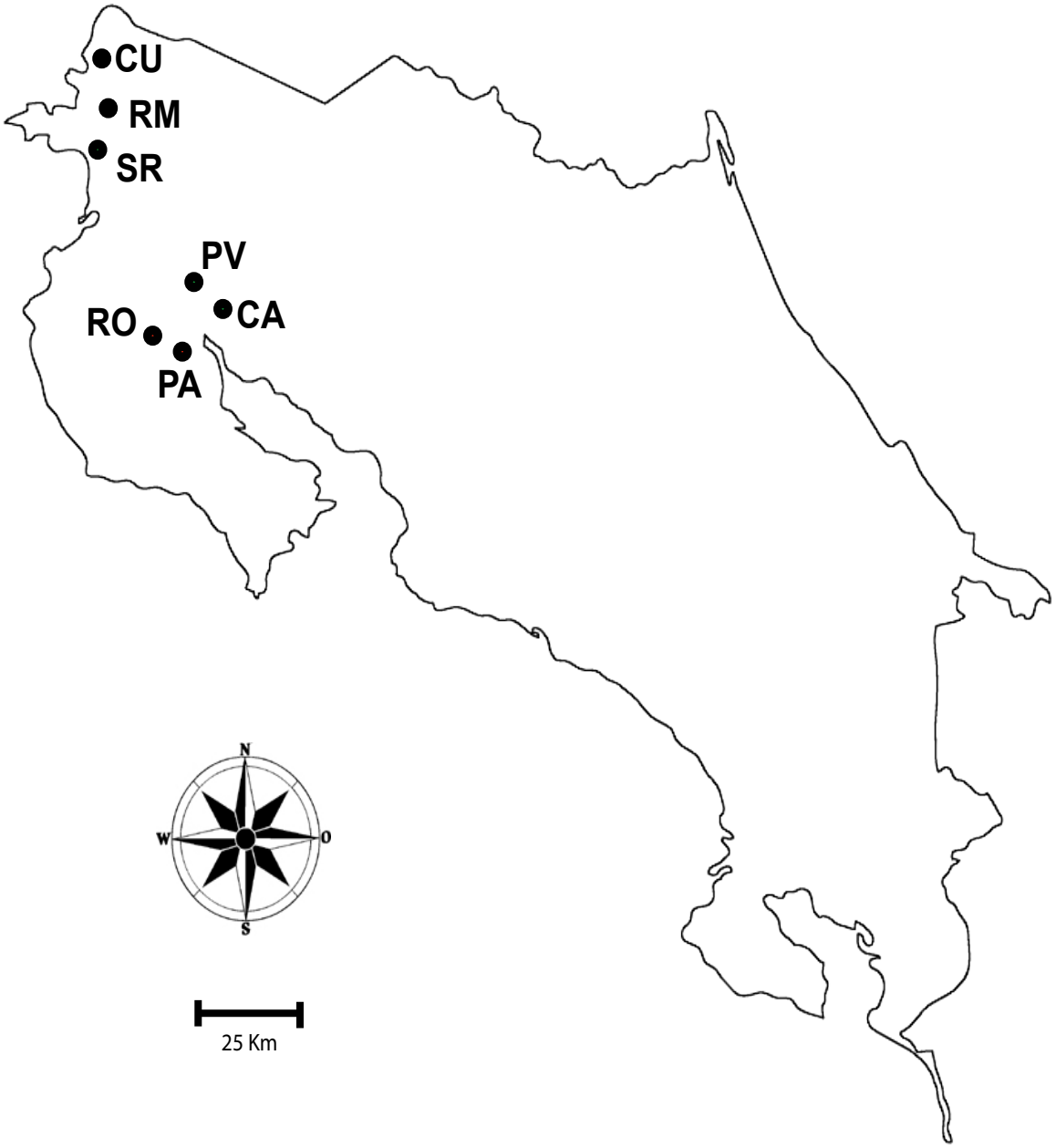
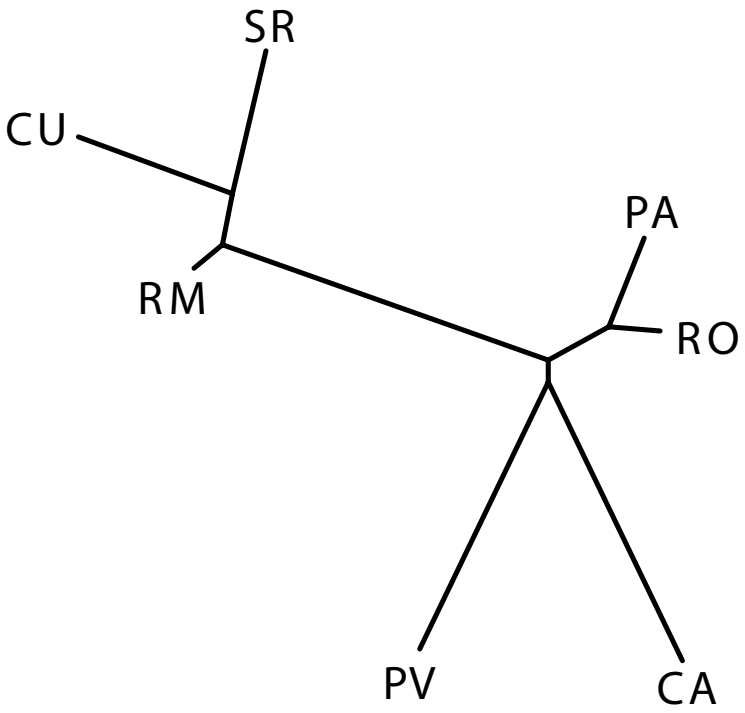


Figure 4.2: Neighbour joining phenogram based on Nei's genetic distance, depicting populations of *Guaiacum sanctum* in Costa Rica. Population codes are shown in Table 4.1.



Chapter 5

Mating System and pollen flow among populations of the endangered tropical tree, *Guaiacum sanctum* (Zygophyllaceae). The importance of isolated trees.[1]

[1]Fuchs, E.J. and Hamrick, J.L. To be submitted to *Molecular Ecology*.

5.1 Introduction

Tropical forests have suffered drastic reductions in extent and continuity due to deforestation and urbanization (Geist and Lambin, 2002). Fragmentation of once continuous populations has created a mosaic landscape of cleared areas and forest fragments. Populations of tropical trees are in greater peril of fragmentation due to their generally lower densities relative to temperate tree species. Fragmentation and overexploitation usually reduce population sizes and increase the distance between conspecifics. This reduction in effective population size (N_e) may lead to the random loss of rare alleles, and if isolation persists, alleles may become fixed by genetic drift. Inbreeding within fragments should increase and leads to the loss of heterozygosity and a possible reduction in overall fitness. Gene flow, in contrast, ameliorates the effects of drift by increasing effective population sizes and introducing novel variation from adjacent fragments or isolated trees (Young *et al.*, 1996).

Tropical trees are generally long-lived, which makes it difficult to assess the long-term effects of fragmentation on levels of genetic diversity. However, gene flow estimates may provide indirect information on the probability of conserving genetic diversity in extant forest fragments (Hamilton, 1999). In plants, gene flow occurs via seed and pollen dispersal but recent work has determined that the major contribution to gene movement is usually via pollen (Loveless and Hamrick, 1984; Trapnell and Hamrick, 2004; Sork and Smouse, 2006). Thus, predicting the impact of fragmentation on the maintenance of genetic diversity is largely dependent on the availability of direct estimates of pollen flow. To this end, marker assisted paternity analyses can provide accurate measures of pollen flow patterns and distances (Streiff *et al.*, 1999; Konuma *et al.*, 2000; Dick *et al.*, 2003).

Although originally it was believed that pollen flow did not have a significant impact on the genetic makeup of populations (Ehrlich and Raven, 1969), recent work using molecular markers has shown that pollen flow into tropical tree populations can occur over large distances and is frequent enough to directly impact the genetic structure of populations (Hamrick and Nason, 1996; Apsit *et al.*, 2001). Nason *et al.* (1996) determined that wasp pollinators of tropical figs could routinely disperse pollen over distances greater than 10 km, and pollen movement over distances larger than 500 meters has been documented for a number of tropical trees (Chase *et al.*, 1996; Hamrick and Nason, 1996; Loveless *et al.*, 1998). Additionally, recent work has shown that pollinator communities, abundance and behaviour may change in fragmented habitats promoting long-distance

pollen flow (Aldrich and Hamrick, 1998; Dick, 2001; White *et al.*, 2002). In fragmented habitats, pollinators may increase their foraging ranges in search of food and may be able to move pollen over several kilometers.

At the extreme of fragmentation, tropical trees may be left as isolated individuals surrounded by a matrix of disturbed habitats. These individuals may be separated by large distances from any conspecifics, or continuous populations. The role of these trees in respect to gene flow has led to contradictory hypotheses. Janzen (1986) proposed that isolated individuals were unlikely to receive or donate pollen to trees in adjacent populations, and hence were excluded from the gene-pool. This reasoning led Janzen to call them the "living dead". In contrast, Hamrick (1994) proposed that isolated trees could serve as "stepping stones" between groups of individuals and, as a result, could play a critical role in determining gene movement within fragmented tropical landscapes. In this latter view, isolated individuals would facilitate the transfer of genetic diversity between populations and would be, therefore, an integral part of the mechanisms that allow the maintenance of genetic diversity in fragmented landscapes. The two arguments have direct and opposite consequences for the management of isolated trees. The former implies that isolated individuals could be safely removed without significant effects on the genetic composition of the landscape. Conversely, the latter hypothesis suggests that these individuals should be preserved to maintain higher effective levels of gene movement and to retard the loss of genetic diversity in remnant populations.

Plant mating systems directly influence patterns of pollen movement between individuals, which in turn, affect the genetic composition of the progeny produced. Although tropical trees exist at low densities (less than one individual per hectare), most studies conducted with molecular markers indicate high rates of outcrossing (Lowe *et al.*, 2005, and citations therein), suggesting the movement of pollen between individuals. Outcrossing rates are often susceptible to reductions in the density of reproductive individuals as a result of habitat alteration or fragmentation. Generally, isolation has been associated with an increase in the proportion of selfed progeny (Murawski and Hamrick, 1991, 1992; Fuchs *et al.*, 2003) and the reduced performance of outcrossed progeny (Rocha and Aguilar, 2001). Pollinators spend more time foraging in isolated trees, and are more likely to cross barriers among fragments (Quesada *et al.* 2003 but see Apsit *et al.*, 2001; White *et al.*, 2002). Hence, pollen movement and outcrossing rates may have correlated responses to fragmentation.

Pollen dispersal distances and mating system estimates are vital for the development of effective conservation and management strategies of endangered species. These parameters may be directly translated into information about the minimum density of individuals or the minimum area required to avoid reproductive isolation (Nason *et al.*, 1998). Pollen flow studies involving isolated individuals are also important to assess the role of these individuals in pollen movement dynamics within fragmented landscapes. In this study, we estimate the mating system and pollen flow patterns in continuous and fragmented populations of the endangered tropical tree *Guaiacum sanctum* (Zygophyllaceae). Specifically, we address the following questions: 1. What is the mating system of *G. sanctum*? 2. Is the mating system affected by habitat type and the number of reproductive individuals? 3. What are the patterns of pollen movement within and between populations? 4. Do isolated individuals play a significant role determining mating patterns within fragmented landscapes?

5.2 Materials and Methods

5.2.1 Study species

Guaiacum sanctum L. (Sapindales: Zygophyllaceae) is a slow growing tropical and subtropical tree distributed from southern Central America to Northern Mexico and Florida, and throughout the Greater Antilles (Holdrige and Poveda, 1975). Trees grow up to 10–15 meters in height, and are usually found on limestone outcrops in tropical dry forests (González-Rivas *et al.*, 2006). Trees have opposite, compound leaves about 7–12 cm long, with oblong to lanceolate leaflets. *G. sanctum* is evergreen, with perfect conspicuous blue-purple flowers. Flowering occurs between March and July, and although some trees flower every year, the species' phenology may be characterized as supra-annual. In Costa Rica the main pollinators are africanized bees, but wasps and solitary bees have also been observed visiting flowers (Quirico, 1993). Fruiting occurs from May to November, fruits are 5-lobed ovoid capsules, with one arilloid seed per locule. Seeds are dark brown to black, oblong and measure 1-2 cm in length. During fruit maturation seeds are covered by a bright red arill, which attracts frugivorous birds, the main dispersers.

Guaiacum sanctum has been heavily exploited because of its extremely dense wood and medicinal value. The decoction of *Guaiacum sanctum* is believed to cure syphilis, and reports of its use

date back to the 16th century (Voeks, 2004). Currently, most populations of *G. sanctum* have suffered drastic reductions due to logging and habitat loss. In Costa Rica, *G. sanctum* is distributed throughout the dry forests of the northwest Pacific coast, but many sites suitable for *G. sanctum* populations have been converted to agricultural fields or pastures (Janzen, 1988). Continuous populations of *G. sanctum* in Costa Rica are rare, and generally restricted to national parks or reserves which continue to be threatened by habitat loss, fire or exploitation (Oldfield *et al.*, 1998). Because of its restricted distribution and reduced population sizes, *Guaiacum sanctum* is now included in Appendix I of the CITES convention (CITES, 2000) and is also termed "Endangered" in the World List of Threatened Trees (Oldfield *et al.*, 1998).

5.2.2 Study sites and Sampling Scheme

Guaiacum sanctum trees were located in Palo Verde National Park (PVNP, 10°21′ N, 85°20′ W) and Cuajiniquil (11°00′ N, 85°41′ W), both populations located in Northwest Costa Rica. Both sites are tropical dry forests, and differ mostly in habitat use. PVNP is a 19,000 ha continuous habitat, which has been preserved from forest fires or logging for the last ∼30 years. Upland portions of the park are mainly composed of dry tropical forest on limestone outcrops, with a mean annual rainfall below 1500 mm and a mean annual temperature of 30 C. The area is characterized by an extended dry season from December through April and a rainy season from May to November. On its dry limestone slopes, there occurs one of the densest remaining populations of *Guaiacum sanctum* in Costa Rica. Within PVNP a permanent 50 ha plot was established and all *G. sanctum* within the plot (see Chapter 2) were located and mapped using corrected GPS technology and permanently marked with aluminum tags. A total of 35 adults occurred within the 50 ha plot, but only six individuals reproduced during the 2005 reproductive season.

Cuajiniquil is a disturbed habitat mainly comprised of small patches of forest embedded in an agricultural matrix. Forest cover in this area has been reduced by the creation of extensive pastures and sugar cane plantations. Forest patches remain in the vicinity of rivers and private reserves. As in PVNP, vegetation type corresponds to tropical dry forest with a marked dry season in the Spring. During 2005, 21 reproductive *G. sanctum* adults were located within pastures, plantations or as part of living fences, in an 8 km^2 area (Figure 5.1). Trees in Cuajiniquil have a disjunct

distribution with 9 adults grouped in a northern cluster, 11 trees found in a southern cluster and an intermediate tree between the two clusters (#18, Figure 5.1). Great effort was taken to locate and map all adults within Cuajiniquil; nonetheless a few inaccessible areas could not be surveyed. Although *G. sanctum* may be located in these sites, they are generally separated by at least 6 km from the sampled individuals.

We monitored flowering and fruiting phenology on a bi-weekly basis for all adults between March 2005 and July 2006. Trees were classified as either non-reproductive, flowering or fruiting, and there was no overlap between phenological categories within a tree. To estimate pollen flow distances and mating system parameters for *G. sanctum*, we collected a random sample of 18 seeds from each reproductive individual in both populations. Seeds were cleaned, air dried and placed in individual paper envelopes. During Spring, 2006, two mature leaves were collected from each adult and snap-frozen in liquid nitrogen. Leaves were stored at -70° until used for electrophoresis. Seeds and leaf samples were transported to the University of Georgia (Athens, GA. USA) for allozyme analysis.

5.2.3 Allozyme Electrophoresis

Guaiacum sanctum seeds have very poor germination under greenhouse conditions. Therefore, the embryo was manually removed from the seed coat by cutting seeds longitudinally with a razor blade. To soften the tissue we soaked seeds in warm water for 24–48 hours before embryo extraction. Embryos were crushed in chilled mortars and pestles in a phosphate buffer according to Wendell and Weeden (1989). Liquid nitrogen was used to crush leaves from adult individuals. Protein extracts from embryos or leaves were absorbed onto 4×6 mm paper wicks (Whatmann 3 chromatography paper), and stored at -70°C until needed for electrophoretic analyses. We used 10% starch gel electrophoresis on five buffer systems to score a total of thirteen polymorphic loci from eight enzyme systems. Buffer and stain recipes are based on Soltis *et al.* (1983), except UTP-glucose-1-phosphate and diaphorase which were adapted from Manchenko (1994). We resolved triosephosphate isomerase (TPI1, TPI2) in system 8; malate dehydrogenase (MDH1, MDH2) and phosphoglucomutase (PGM) on buffer system 4; aspartate aminotransferase (AAT1, AAT2) and diaphorase (DIA1, DIA2) were stained in buffer system 7; and UTP-glucose-1-phosphate (UGPP) and isocitric acid dehydrogenase

(IDH) were scored in a morpholine-citrate buffer. System 6 was used to score phosphoglucoisomerase (PGI1, PGI2). Banding patterns were consistent with expectations based on Wendell and Weeden (1989), and we considered a locus polymorphic if two alleles were observed.

5.2.4 Statistical analysis

Mating system

The mating system of *G. sanctum* was estimated from progeny arrays using the procedure developed by Ritland (1989b, 2002). Maternal genotypes were available for all loci with the exception of the PGI2 locus, which does not express in adult tissue. We estimated single (t_s) and multilocus outcrossing rates (t_m) by means of maximum likelihood using the Newton-Rapson maximization algorithm implemented in the Mltr program (Ritland, 2002). We also estimated the paternity correlation index (r_p), which determines the proportion of full siblings among outcrossed progeny. This index may be interpreted as the probability that any two randomly chosen outcrossed seeds are sired by the same pollen donor (Ritland, 1989a). Pollen and ovule allele frequencies were estimated separately, using the same maximization algorithm as above. Standard errors for all estimated parameters were determined by 10,000 bootstrap replicates, using families as the sampling unit.

Paternity assignment and gene flow

A fractional paternity analysis was used to determine mating patterns and average gene flow distances for the progeny of *G. sanctum* (Devlin *et al.*, 1988). We determined potential pollen donors for each seed based on the calculation of LOD ratios (i.e. Likelihood Odds ratio). LOD ratios determine the likelihood that an individual is the father of a seed, relative to the likelihood that the two individuals are unrelated. The LOD ratios are calculated based on transition probabilities for different genotypes and allele frequencies in the population (Meagher and Thompson, 1986). Fractional paternity analysis performs better than single paternity assignment, especially in cases where multiple adults cannot be unambiguously assigned to a progeny. Additionally, single paternity assignment based on LOD ratios tends to assign higher likelihoods to homozygous genotypes given that their transition probabilities for a given allele are higher than those for heterozygotes. Fractional paternity assignment (Devlin *et al.*, 1988), in contrast, does not always assign a single

father to each offspring, but instead divides paternity proportionally among all possible fathers. Each potential father receives a fraction of a seed's paternity, proportional to its likelihood of being a father (i.e. the LOD score). We will refer to this proportion as the fractional score. Fractional paternity reduces bias toward homozygotes. Even though homozygous individuals will always have a higher LOD score, and hence be assigned a larger proportion of the progeny, heterozygote individuals produce a larger array of gametes and thus are more likely to be potential fathers for more seeds than homozygous individuals. Therefore the contribution of heterozygote individuals to the overall progeny array is increased.

Fractional paternity assignments were conducted using the FAMOZ computer program (Gerber *et al.*, 2003). Allele frequencies were estimated using all adult individuals and the progeny of reproductive adults. We assumed scoring errors were less than 1% (a confident estimate for allozymes). All possible pollen donors were chosen for each progeny array and for each potential father, the LOD scores were used to assign fractional paternity (i.e. fractional score). Average pollen movement distances were calculated for each seed using the average distance between all possible fathers and the maternal tree, weighted by the pollen donor's fractional score. Additionally, we estimated the proportion of progeny sired by each adult by averaging fractional scores for each pollen donor across all progeny. To determine the proportion of pollen flow events between the northern and southern clusters, we estimated the proportion of seeds sired by each father, depending on the location of the maternal tree (i.e. northern cluster, southern cluster or isolated individual). Given that the isolated tree does not belong to any cluster, we also estimated the proportion of progeny sired by this tree for each cluster. Seeds with no possible fathers were assumed to represent gene flow from unsampled trees. Cryptic gene flow (i.e. gene flow event falsely assigned to a father within the stand) was calculated using the FAMOZ test option, by simulating 30,000 offspring from trees within the sampled individuals, and 30,000 offspring originating from a father outside the stand with transition probabilities equal to the allele frequencies of the population. Unless otherwise stated, all statistics are presented ± their standard errors.

5.3 Results

There were 35 adults within the 50 ha plot in PVNP and 32 in Cuajiniquil. For both populations flowering occurred during June and July, and fruits bore mature seeds between July and September, with peak fruit production in August. Only six individuals in PVNP flowered and produced fruits during the 2005 reproductive season, and a large proportion of seeds aborted before fruit maturation. Twenty-one individuals reproduced in Cuajiniquil, with all trees producing an ample amount of seed.

We scored 13 polymorphic loci from the embryos. Average heterozygosity in these loci is $H_e = 0.446 \pm 0.04$, and the effective number of alleles is $A_e = 1.932 \pm 0.15$. The cumulative paternity exclusion probability was $P_E = 0.81$ for Cuajiniquil, suggesting that for each seed we can correctly exclude 81% of the potential fathers (Chakraborty *et al.*, 1988). For PVNP, the exclusion probability was lower ($P_E = 0.64$).

Mating system estimates (Table 5.1) suggest that the breeding system of *G. sanctum* ranges from mixed-mating to predominantly outcrossing. Multilocus outcrossing rates were lower in PVNP ($t_m = 0.719 \pm 0.145$) than in the progeny from Cuajiniquil ($t_m = 0.948 \pm 0.019$), nonetheless estimates were not significantly different from each other (t-test, $p = 0.082$). Single and multilocus outcrossing estimates differed significantly in Cuajiniquil ($t_m - t_s = 0.101$; $p < 0.001$), suggesting that bi-parental inbreeding was occurring. No significant differences were found between multilocus and single locus estimates in PVNP ($t_m - t_s = 0.099$; $p = 0.132$).

Correlation of paternity estimates indicate that the probability of fullsibship ranges from 51% to 78%. This may be interpreted as the probability of randomly drawing two full-sibs from the outcrossed progeny of a single mother. The effective number of outcross pollen donors for Cuajiniquil is $N_e \approx \frac{1}{r_p} = 1.96$, suggesting that on average each mother samples pollen from approximately two effective pollen donors. In Palo Verde $N_e \approx \frac{1}{r_p} = 1.27$, implying that reproductive trees on average sample pollen from about one individual.

Given the low number of seeds and reproductive individuals in PVNP, exclusion probabilities were too low to perform an accurate paternity analysis. Therefore, pollen flow estimates were only performed on progeny from Cuajiniquil. A single pollen donor was assigned to 153 of the 378 (40.5%) seeds analyzed in Cuajiniquil. Sixty-one seeds (16.1%) had no potential pollen donor within the

Table 5.1: Mating system parameters estimated from the progeny of 21 trees in Cuajiniquil, and six adults in PVNP, Guanacaste, Costa Rica. t_m: multilocus outcrossing rate; t_s: average single locus outcrossing rate; r_p: paternity correlation index; f: paternal inbreeding coefficient; SE: standard error.

	Cuajiniquil		Palo Verde	
Parameter	Estimate	SE	Estimate	SE
t_m	0.948	0.019	0.719	0.145
t_s	0.847	0.034	0.619	0.211
$t_m - t_s$	0.101	0.026	0.099	0.088
r_p	0.510	0.073	0.785	0.246
f	−0.070	0.052	0.101	0.075

sampled population, and hence where considered as pollen-flow events from outside the sampled areas. Cryptic gene flow was determined as 3.1%, based on 30,000 simulations, giving a total gene flow rate of 19.2%. Fractional paternity was assigned to the remaining (44.4%) seeds. Each seed had between 2 and 9 possible fathers with an average of 2.54 (SE=0.16). Adult fecundity was not homogeneously distributed. Three adults were responsible for over 40% of progeny assignments: 18 (13.9%), 14 (10.2%) and 2201 (11.1%); while tree 19 did not sire any progeny (Figures 5.1, 5.2). In Cuajiniquil, the average distance between reproductive individuals is 1864 m (±101.4). The distribution of inter-adult distances is bimodal (Figure 5.3), with a large proportion of trees separated by less than 500 meters (i.e. intra cluster), few individuals occurred at the intermediate categories (between 1000 and 2500 m), and a large proportion of individuals were separated by 3000–4000 meters (i.e. inter cluster). Average pollen movement distance between each maternal and paternal individual, weighted by the fractional score, is 1276 ± 91.8 meters, 32% less than the average among randomly selected adults. The distribution shows that pollen flow distances occur almost uniformly across all possible distances (Figure 5.4), with a slightly higher proportion of pollen flow events from the 3000 meter category. Twenty-three percent of pollen flow events

originate with distances below 1000 m, with the majority of pollinations occurring at distances over 2000 m (Figure 5.4). A weighted average of pollen flow distances by the fractional paternity may underestimate pollen movement by increasing the proportion of intermediate distances. This averaging effect provides an inaccurate estimation of the pollen movement distribution. Therefore, a more accurate description of pollen movement is attained by analyzing the 153 seeds with a single pollen donor (shaded bars in Figure 5.4). This analysis revealed that over half of the pollen flow events occur at distances over 2000 meters, with the largest proportion of matings originating at 3500 meters. The average pollen flow distance estimated from single-sire seeds is 2085 ± 148.4 meters, 63% higher than estimated from fractional paternity. These results show that long-distance pollen flow in *G. sanctum* is commonplace, and also indicate that pollinations commonly occur among clusters. However, given that the distribution of inter-adult distances is bimodal (Figure 5.3), we estimated the difference between the observed pollen flow events (fractional and single-sire) and the proportion expected based on inter-adult distances as suggested by Kenta *et al.* (2004). By performing this calculation we can determine how pollen flow distances deviate from values expected if adults mate at random. The resulting distribution shows that observed values deviate significantly from expectations under random amting ($\chi^2 = 295.7$, df=8, $p < 0.001$), with significant deficits of pollen flow events at the tails of the distribution and an excess of pollinations from distances between 1000–3000 meters (Figure 5.5). Deviations calculated using seeds with a single pollen donor, show a large excess of pollinations coming from distances betwee 1500–2000 meters (shaded bars in Figure 5.5). Only tree #18 (See Figure 5.1), is separated by a distance between 1.5 km and 2.0 km from other reproductive individuals, suggesting that this tree fathered a disproportionally large proportion of seeds, relative to the number expected based on the spatial distribution of adults.

Analyzing the proportion of sired seeds, taking into account the cluster location of the pollen donor and the maternal tree, shows that pollen flow among clusters occurs asymmetrically (Table 5.2). Pollen donors located in the north sired a larger proportion of seeds in southern trees (21.7%), relative to seed sired within the same cluster (8.2%). Conversely, within-cluster pollinations were slightly higher in the south (20.5%), relative to among-cluster pollinations (16.7%). Likewise, tree 18 (Isolated individual) sired a slightly larger proportion (7.8%) of seed in the southern mothers,

Table 5.2: Percentage of seeds sired by fathers in different clusters or as isolated individuals, depending on the position of the maternal tree. Unknown: proportion of seeds with no likely father (i.e. gene-flow).

Location of:		Maternal Tree			
	Pollen Donor	North	South	Isolated	Total
	North	8.2	21.7	1.6	31.5
	South	16.7	20.5	1.3	38.5
	Isolated	5.4	7.8	0.7	13.9
	Unknown	9.7	4.6	1.8	16.1
	Total	40	54.6	5.4	100.0

Table 5.3: Proportion of pollen flow into the clusters and isolated individual, partitioned among different sources. Unknown: proportion of seeds with no likely father.

	Pollen source			
Cluster	North	South	Isolated	Unknown
North	20.5	41.8	13.5	24.2
South	39.7	37.5	14.3	8.4
Isolated	29.6	24.1	13.0	33.3

than in the northern trees (5.4%). The directionality of pollen flow is geared towards the southern cluster with a significantly higher number of seeds sired in this area ($\chi^2 = 8.45$, d.f.=2, $p = 0.015$). Similarly, the proportion of unexplained pollen flow events is larger for northern trees, suggesting that pollen from unsampled trees is probably moving south.

The amount of gene flow each cluster received indicates that most pollinations are the result of inter-cluster pollen movement (Table 5.3). Forty-two percent of successful pollinations in the northern trees originated from pollen donors in the southern cluster. Similarly, in the southern cluster, the proportion of seeds sired by northern pollen donors is slightly higher than the proportion of seeds produced by intra-cluster pollinations. Both clusters received a similar amount of pollinations from the isolated individual (i.e. tree 18); however, the northern cluster received

a significantly higher proportion of pollen flow events from unknown pollinators ($\chi^2 = 7.4$, df=1, $p = 0.001$; Table 5.3). The origin of pollen flow is not homogeneous across clusters ($\chi^2 = 25.1$, df=3, $p < 0.001$), with inter-cluster pollinations representing a higher proportion in each cluster.

5.4 Discussion

5.4.1 Mating system

The mating system of *G. sanctum* varies from mixed-mating (70%) to predominantly outcrossing (95%), depending on location. Bawa *et al.* (1985b) suggested that self fertilization is uncommon in tropical tree species, due mainly to widespread self incompatibility systems. Nonetheless, selfing in *G. sanctum* is probably not avoided altogether, since in PVNP 30% of the progeny are the products of self fertilization. A higher proportion of selfing in PVNP is consistent with previous results, which show that there is a significant deficit of heterozygosity at all life stages in this location ($F = 0.185$; SE=0.05, Fuchs and Hamrick in prep, Chapter 3). If PVNP were at equilibrium, the observed outcrossing rate would produce an inbreeding coefficient (F_e) of $F_e = (1 - t_m)/(1 + t_m) = 0.163$; which is very similar to the observed value. This suggests that the observed inbreeding values in PVNP are caused primarily by self-fertilization.

When the density of reproductive individuals decreases, selfing may become more prominent due to a reduction in the availability of outcross pollen (Murawski and Hamrick, 1991; Fuchs *et al.*, 2003). A lower density of reproductive adults reduces outcrossing rates by increasing the distance between reproductive individuals, and the proportion of geitonogamous pollinations. Additionally, tropical trees tend to favor outcross pollen tube growth under microgametophytic competition (Quesada *et al.*, 2001), but may allow self pollen to reach the ovaries when outcrossed pollen loads are low. Murawski and Hamrick (1991) showed for nine tropical tree species that a reduction in the density of flowering individuals among years negatively impacted outcrossing rates. Our results are consistent with these conclusions by showing lower outcrossing rates in PVNP, which had only six reproductive individuals in 2005 (i.e. 0.12 flowering individuals/ha). Although most studies suggest that fragmentation is an important factor shaping the breeding dynamics of tropical trees (Lowe *et al.*, 2005), the proximate cause is generally identified as a reduction of adults and an increase in inter-mate distances. Only 20% of the adults flowered in PVNP in 2005, while 65% flowered

at Cuajiniquil. It is likely that this reduction promoted geitonogamous matings, which decreased outcrossing in PVNP. As this study shows, habitat type (i.e. continuous versus fragmented forest) may not be the most important factor shaping the mating patterns of tropical trees, but rather, it is the number and/or density of pollen producing adults.

5.4.2 Paternity Analysis and Gene flow

Inter-adult distances at Cuajiniquil (Figure 5.3) are bimodally distributed with around 50% of the distances below 1000 meters (within cluster distances), few intermediate distances (1500–2500 meters) and the other half at distances greater than 3000 meters (among cluster distances). The average distance between trees within a cluster is 560 meters, among clusters is 3274 meters, and the isolated individual is separated by an average of 1704 meters from each cluster. Our estimates of pollen flow distance based on fractional paternity indicate in contrast, that successful pollinations occur roughly uniformly over all distances (Figure 5.4), with 49% of the pollinations occurring between individuals separated by distances over 2 km and only 23% of the pollen flow distances occurring within 1000 meters. However, a more accurate description of pollen flow based on single-sired seeds shows that the distribution of pollen flow distances has three peaks at: 500 (9%), 1500 (7%) and 3500 (10%) meters, respectively (shaded bars in Figure 5.4). Additionally, 56% of the single sire pollinations occur at distances higher than 2000 meters; therefore, we conclude that pollinations occur predominantly between clusters and that pollinators are capable of long-distance pollen flow in this disturbed habitat. *G. sanctum* is pollinated by a large array of solitary, stingless and Africanized bees (pers. obs.); most of which are capable of long distance pollen flow (Frankie *et al.*, 1976). Our results support previous studies that show that insect pollinators travel long distances across disturbed landscapes (Apsit *et al.*, 2001; White *et al.*, 2002; Dick *et al.*, 2003).

The long-distance pollen movement observed in Cuajiniquil maybe an artifact of fragmentation. Although some studies have shown that fragmentation may have detrimental effects on the mating system, pollen flow distances and pollinator abundances (Quesada *et al.*, 2003; Lowe *et al.*, 2005), recent work on entomophyllous tropical tree species indicate that fragmentation may actually increase pollen flow distances (e.g. Apsit *et al.* 2001). Dick *et al.* (2003) showed that pollen moved larger distances in fragmented populations than in continuous forests, primarily because

of the long-distance movement of Africanized bees. Similarly, White *et al.* (2002) determined in a tropical dry forest tree, *Swietenia humilis*, that in fragmented landscapes pollen flow is common between individuals separated by more than 2 km. Insects, and especially bees, can move large distances (Janzen, 1971; Frankie *et al.*, 1976), and their foraging behaviour –trap-lining and pollen caching– may promote pollen carryover and, thus, long distance pollen flow. In our case, though, pollen movement over distances larger than 2 km may be mediated, at least in part, by the presence of individuals at intermediate distances that serve as stepping stones between larger groups of trees.

We found that the largest proportion of seeds are sired by three adults: 18 (13.9%), 14 (10.2%) and 2201 (11.1%), with 18 being the most prolific pollen donor (Figure 5.2). The disproportionate number of seeds sired by tree 18 may be due to tree 18 producing a large amount of pollen, or because it receives more pollinator visitations. There is evidence that mating success may be associated with adult size (Latouche-Hall *et al.*, 2004)). However, although tree 18 is a large individual, it is not the largest tree in the study area and its DBH (32.7 cm) is not significantly different from the population mean (28.11 ± 3.15 cm, $t = 1.46$, $p > 0.05$). Given the location of this tree (i.e. isolated by 1500m and 2000m from the northern and southern clusters, respectively), it is conceivable that the high proportion of paternity assignments are caused by a large array of pollinators visiting this individual as they move between the two clusters. Hamrick (1994) suggested that in fragmented landscapes, isolated trees may serve as stepping stones between populations allowing the dispersal of genetic diversity between fragmented populations. Our results confirm this prediction, with tree 18 appearing to serve as a bridge between the northern and southern clusters. Bees and other insects have been shown to use trap-lining pollination routes, and visit flowering individuals sequentially (Janzen, 1971; Levin and Kerster, 1974). Since our trees are somewhat linearly distributed, pollinators may travel along roads or pastures and visit all trees until they reach the end of a cluster where flowering individuals become scarce. Insects reaching the end of a cluster would appear to visit intermediate tree 18, before reaching the next cluster of trees. Because tree 18 is conspicuous, isolated and surrounded by pastures, insects may detect it from a far. Bees will collect pollen from this tree and disperse it to trees in adjacent clusters. A detailed analysis of the pollinations conducted by tree 18 shows that aside from being the most prolific pollen donor (Figure 5.2), progeny sired by this individual occur in both clusters with somewhat

more pollinations occurring in the southern cluster (Table 5.2). Additionally, as shown in figure 5.5, tree 18 sires a disproportionate number of seeds, compared to expectations based on random mating among the adults. These results demonstrate that tree 18 is an important component of the pollen flow dynamics of *G. sanctum* in this fragmented landscape. It is clear that our results are not consistent with Janzen's "living dead" hypothesis, since tree 18, although isolated by at least 1500 meters, plays a major rule in the pollination dynamics of this landscape by acting as a stepping-stone between the two clusters of trees.

The discontinuous distribution of adults in Cuajiniquil allows us to treat clusters as distinct populations separated by more than 3 km. Our paternity analysis demonstrated that pollen flow into these clusters ranges between 80% for the northern cluster and 62% for the southern cluster, respectively (Table 5.3). These results show that pollen flow among populations of *G. sanctum* occur frequently and over large distances. The high levels of pollen immigration observed in Cuajiniquil are consistent with estimates from other insect pollinated tropical dry forest species (Hamrick and Apsit, 2004). Nason and Hamrick (1997) showed that pollen flow into island populations of *Spondias mombin* (Anacardiaceae) ranged between 45% and 100%. Similarly, White *et al.* (2002) showed that in a fragmented landscape, pollen flow into isolated fragments ranged between 64% and 72%, and over distances between 1 and 4.5 km. Previous work conducted on this species (Fuchs and Hamrick in prep. Chapter 4) has shown that gene flow is probably homogenizing allele frequencies across populations separated by less than 10 km, which should reduce the short and medium term effects of fragmentation. In Cuajiniquil, the ability of pollinators to travel long distances and effectively disperse pollen between individuals separated by as much as 4 km, should encourage the retention of genetic diversity in this fragmented landscape. The heterozygosity of adult trees in Cuajiniquil ($H_e = 0.332 \pm 0.052$) and of the progeny produced ($H_e = 0.446 \pm 0.04$) is high when compared to other long-lived outcrossing perennials (Hamrick *et al.*, 1992). These results clearly show that the severity of fragmentation is directly dependent on patterns of gene movement between populations and the actual spatial arrangement of fragments and isolated individuals. Insect pollinated species with ornithochorous seed dispersal, such as *G. sanctum*, are probably less sensitive to reductions in density and fragmentation than species with different pollination and seed dispersal syndromes (Quesada *et al.*, 2003).

Our results (Tables 5.2 and 5.3, Figure 5.2) demonstrate that pollen flow occurs predominantly in a north-to-south direction, and that the largest proportion of intra-cluster pollinations occurs for trees in the southern cluster. The north-to-south gradient in pollen transfer may be caused by two factors: a larger number of reproductive individuals in the southern cluster, or a larger availability of foraging and roosting sites for bees in the southern region of Cuajiniquil. Bees have been shown to follow trap-lining routes and visit trees sequentially in search of food (Janzen, 1971; Frankie *et al.*, 1976). A larger proportion of reproductive adults in the southern cluster probably provides larger floral cues for pollinators, which will be attracted to these trees preferentially. Bees in the northern cluster travel south in a trap-lining fashion and visit the isolated tree (18) on their way to the southern cluster. Once they arrive at the southern cluster, they will forage longer on these individuals, given the greater resource availability. The greater seed set seen in the southern cluster (Table 5.2) supports this scenario. As shown by Franklin (1983) bees move between groups of flowering individuals due to shifts in nectar production, which may account for individuals returning towards the northern cluster. When nectar or pollen resources are temporarily depleted in the southern trees, bees will move north to forage on those trees, which explains the observed 42% of pollinations conducted by southern pollen donors on northern maternal trees. Again, many pollinators may visit tree 18 on their way north. Another explanation for the southern movement involves the availability of foraging and nesting sites. The Junquillal National Wildlife Refuge is located about 10 km south of the Cuajiniquil site. This area contains remnants of pristine tropical dry forest, which may contain a higher diversity of flowering plants and food resources, attracting pollinators towards the southern end of Cuajiniquil. Similarly, the largest proportion of unknown pollinations occurs in trees in the northern cluster (Table 5.3), suggesting that unsampled trees may be located north of Cuajiniquil, and insect movement towards the south increases the proportion of gene flow events in these trees. These pollen movement trends may vary among years, and hence further studies are warranted to support these observations.

Although some areas could not be sampled, we determined based on satellite imagery (Google Earth) that the average distance of a tree to unsampled forest patches is more than the distance to known conspecifics. However, the high pollen dispersal distances observed suggests that if *G. sanctum* are located within these neighboring sites, successful pollinations may occur. We obtained

a low estimates of gene flow from outside the sampled areas (19%), relative to gene-flow estimates for other tropical trees (Smouse and Sork, 2004). Therefore, we conclude that we have probably sampled a large proportion of possible pollen donors in this area. Nevertheless, our demonstration of pollen movement over 4 km raises the possibility of long-distance pollen flow from unsampled sites beyond 4 km. As a result, our estimates of pollen dispersal distances should be considered minimum estimates. Additionally, we only sampled seeds from a single reproductive event. Schnabel and Hamrick (1995) have shown that pollen flow distances may vary between years, due to changes in pollinator abundances and variation in the distribution of flowering trees. Therefore, we suggest that further studies should provide additional insights into the annual variation in pollen flow distances.

5.4.3 Conservation issues

Guaiacum sanctum is an endangered tropical tree, and currently very little knowledge exists on the reproductive biology of this species. Conservation genetics theory proposes that populations of significant size should be preserved to ensure the preservation of ample genetic diversity, thus reducing the detrimental effects of drift and inbreeding. Mating system and gene-flow distance estimates allow better predictions of minimum viable population sizes, which ensure reproductive success. Our results have shown that *G. sanctum* is predominantly outcrossing. We have also shown that supra-annual flowering may reduce the number of reproducing individuals, affecting the proportion of outcross progeny. Populations of this species should preserve enough adults to ensure seed production even in years when few individuals reproduce. We demonstrated that pollen flow distances are high, and that pollen may even travel through fragmented or altered habitats. Nonetheless, the ability of pollen to travel long distances is at least partially dependent on the presence of mature individuals functioning as stepping stones between fragments or groups of reproductive adults.

Trees in isolated pastures may act as stepping stones between populations, and therefore great effort should be devoted to identify and preserve such isolated individuals (Aldrich *et al.*, 1998). Detailed studies of fragmented populations should identify the relative importance of certain trees as key factors shaping the genetic diversity of populations. In our study, tree 18 is extremely important

for conservation because it mediates pollen transfer over large distances, and hence the maintenance of genetic diversity. Adult 18 contributes ∼14% of the matings in the Cuajiniquil population (Table 5.2). Removal of this individual may reduce the movement of pollen between clusters, increasing the effects of genetic drift. We also show that the number of pollen producing parents affects outcrossing rates. Tree 18 increases the number of pollen donors by encouraging long distance dispersal, and perhaps the higher outcrossing rates observed in the Cuajiniquil population. Although conservation genetics strives to preserve populations as the conservation unit, results such as these, illustrate the relative importance of certain individuals in a population. Conservation efforts should be directed to preserve such individuals, whose removal may have negative impacts on the genetic structure of remnant populations.

Figure 5.1: Map of *G. sanctum* individuals in the Cuajiniquil population. The inset shows the position of the population in Costa Rica. Numbers refer to tree ID's. Tree # 2202 is not shown, since it overlaps with tree 22.

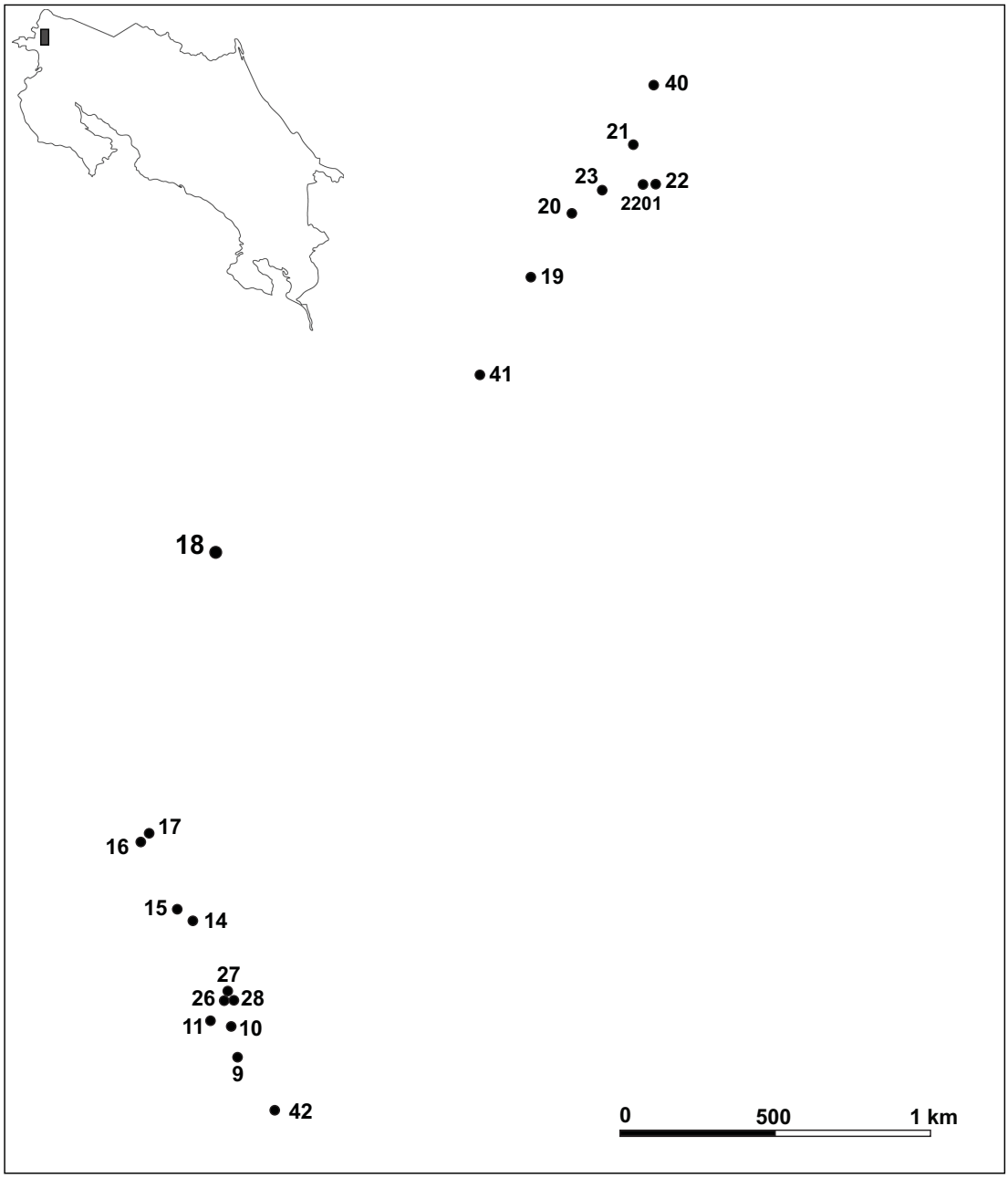


Figure 5.2: Proportion of paternity assignments ($\pm SE$) for all reproductive *G. sanctum* individuals in the Cuajiniquil population. Proportions are partitioned depending on the cluster location of the maternal tree: Northern cluster, Southern cluster or Isolated tree. ID: tree number. U: unknown pollen donor (i.e. gene flow). Location of paternal tree is shown below tree ID: Northern cluster (N), Southern cluster (S), Isolated tree (I). Tree #19 was omitted because it did not sire any progeny.

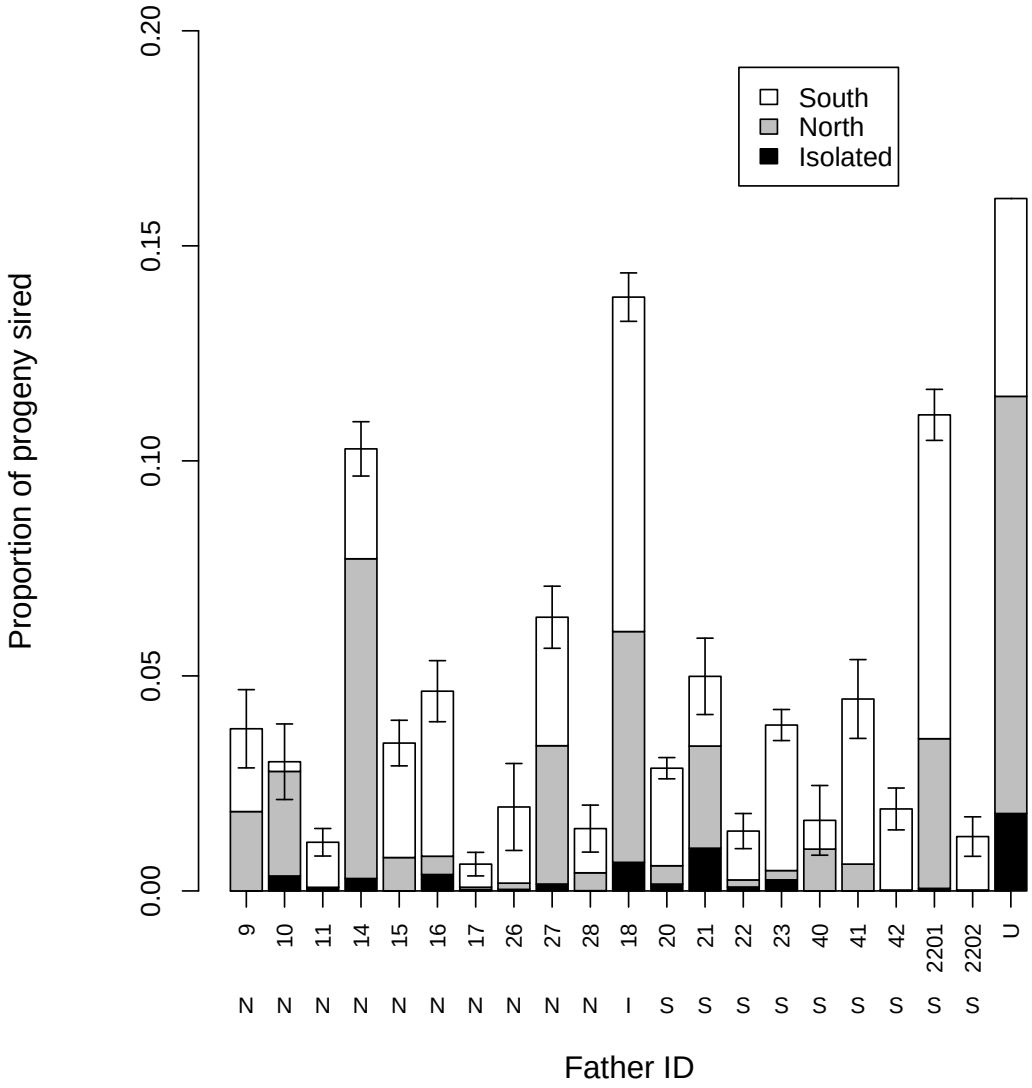


Figure 5.3: Histogram of pairwise distances between all reproductive adults in Cuajiniquil, Costa Rica.

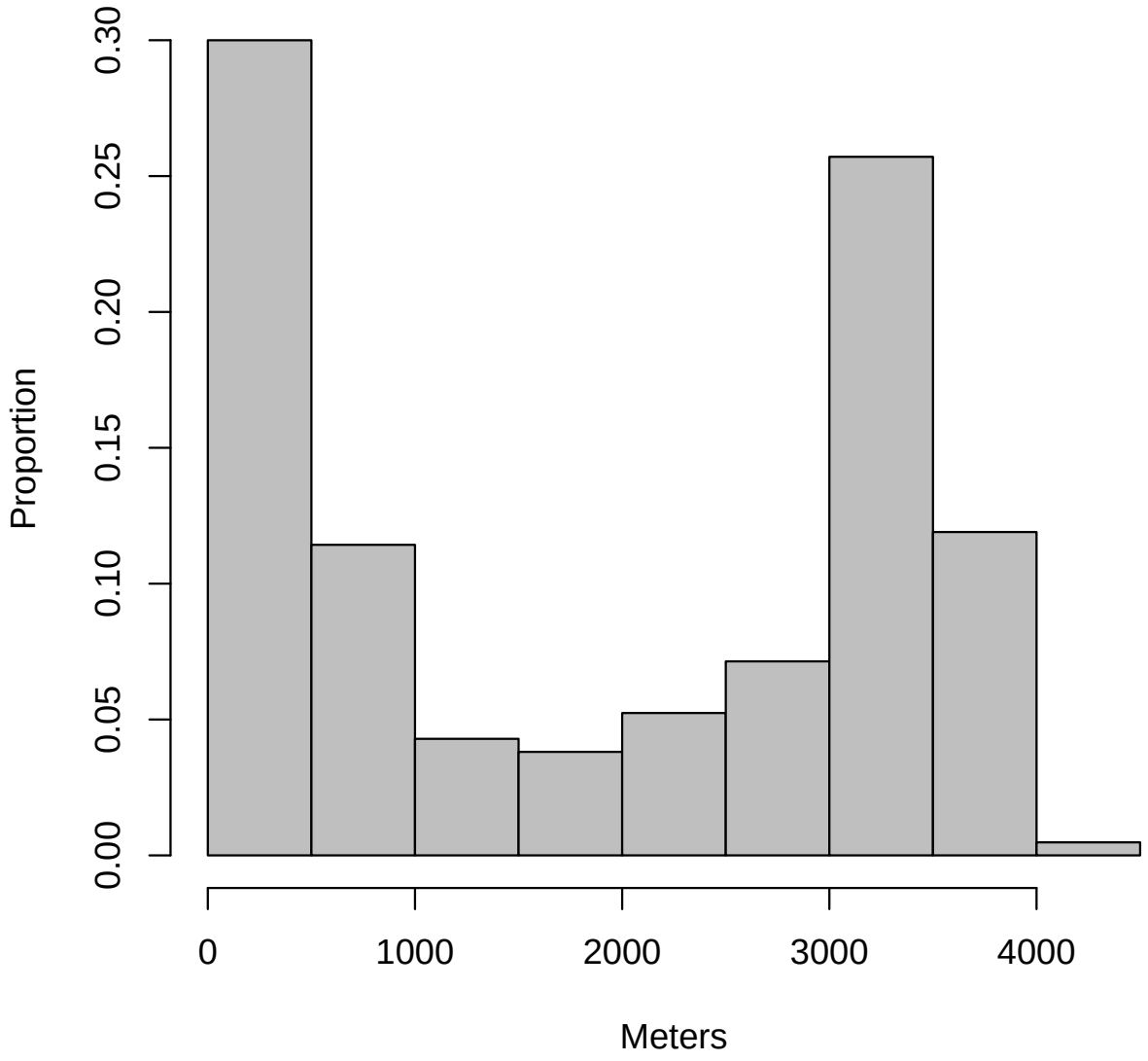


Figure 5.4: Proportion of pollen flow distances, estimated as the distance between pollen donor and maternal tree, weighted by the fractional score of the father. Dark bars show the proportion estimated from seeds with a single pollen donor.

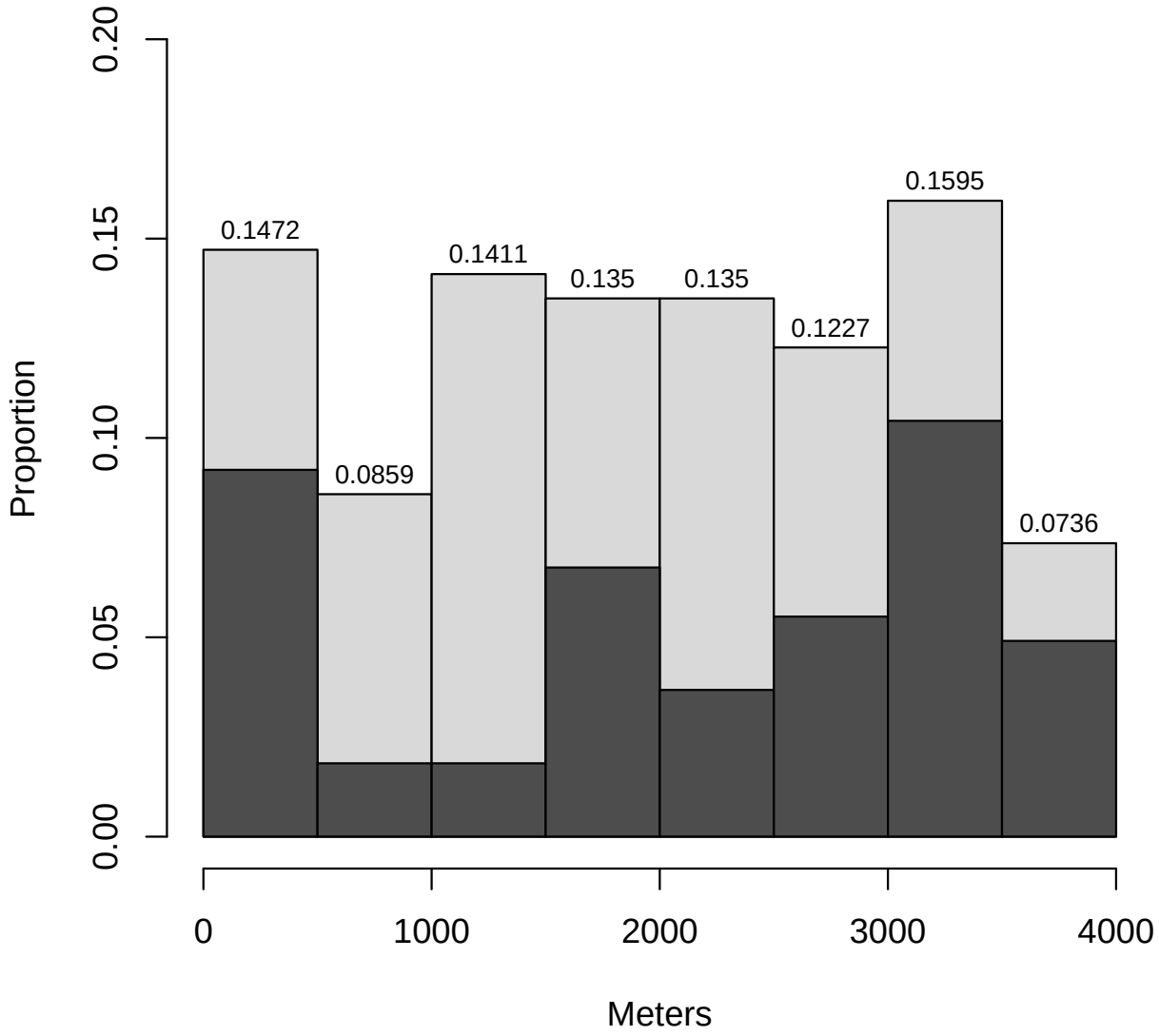
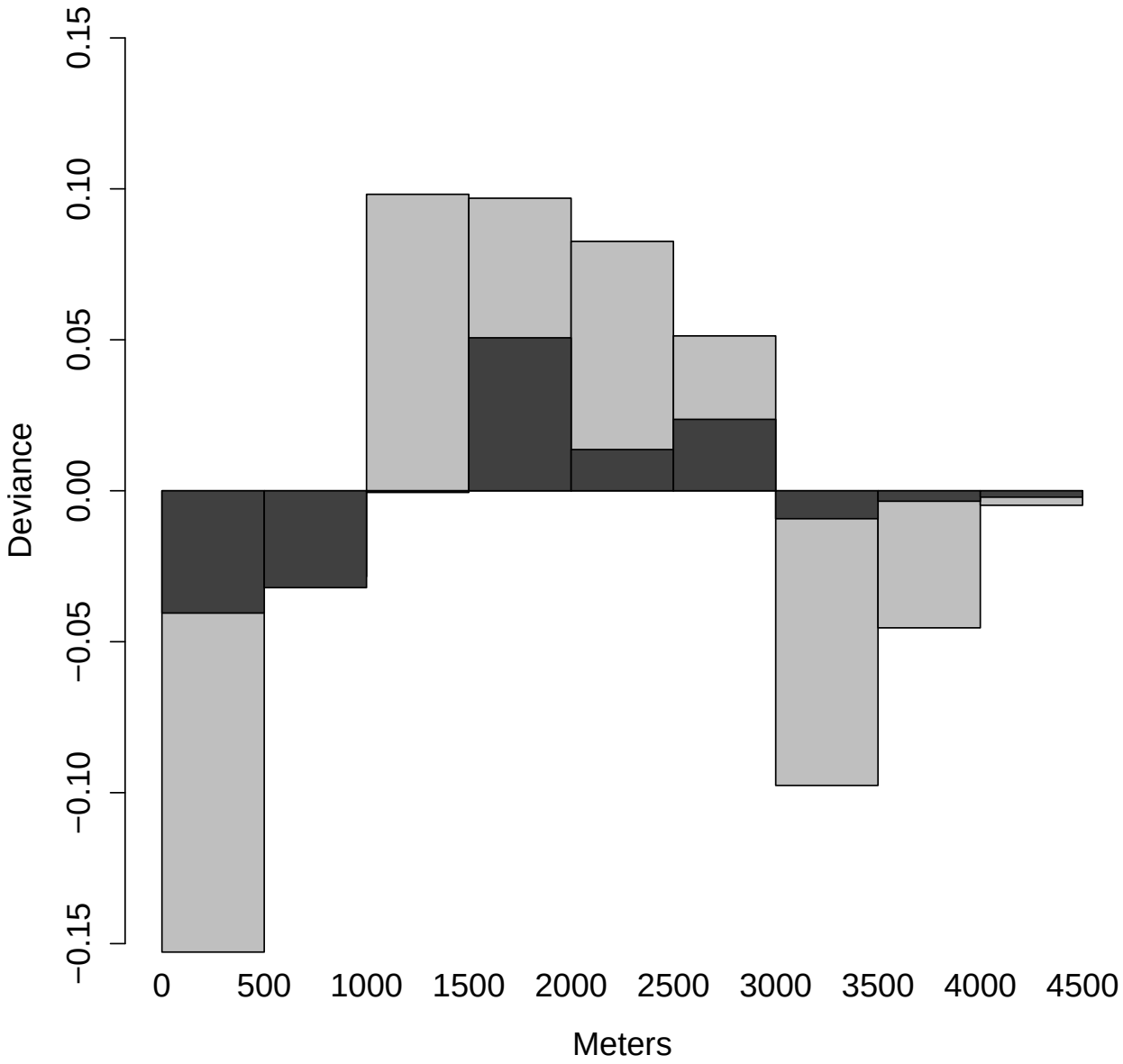


Figure 5.5: Deviations of pollen-flow distances from expectations of random mating among adults. Results for seed with a single pollen donor are shown in dark bars.



Chapter 6

Conclusions

This dissertation describes the reproductive biology and patterns of genetic diversity of the endangered tropical tree *Guaiacum sanctum*. This project provides information on the status of populations of this species by describing ecological and genetic parameters such as abundance, spatial distribution of individuals, genetic structure within and among populations, as well as direct measures of pollen and seed dispersal. Endangered species require detailed descriptions of life history traits and levels of genetic diversity, in order to develop appropriate management and conservation strategies, for the future survival of these species.

Populations of *G. sanctum* in Palo Verde National Park appear to be expanding given the large proportion of seedlings, saplings and juveniles, that were documented relative to the number of adults (Chapter 2) and relative to the size distributions observed in previous studies. Additionally, we demonstrated that all size classes are spatially aggregated. Previous work on tropical tree species has shown that spatial aggregation in the younger size classes is generally caused by localized seed dispersal, leading to the formation of family structure or fine-scale genetic structure (FSGS, Hamrick *et al.*, 1993a; Degen *et al.*, 2001; Lowe *et al.*, 2003). Nonetheless, when we examined patterns of genetic diversity within *G. sanctum* populations in PVNP, we found little evidence of FSGS (Chapter 3). A parent-pair analysis of the progeny found on three 0.25 ha plots revealed that bird mediated seed dispersal from outside the plots occurs commonly (48%), and over distances of at least 150 meters (Chapter 3). These results conflicted with our initial hypothesis that spatial aggregation was caused by localized seed dispersal. Further studies of the spatial distribution of *G. sanctum* seedlings, saplings and juveniles revealed that light availability due to canopy openings is an important factor determining the clumped patterns of recruitment (Chapter 2). Seedlings were more often found in closed canopy environments, which probably reduce mortality due to dessication. Similarly, juveniles are more common in open canopy environments, probably due to

the availability of light for growth (Itoh *et al.*, 1997). These results lead us to conclude that the observed patterns of spatial structure are dependent on forest gap dynamics, rather than seed dispersal patterns. In PVNP our results suggest that birds disperse mixtures of seeds from different maternal trees over large distances, which prevents the formation of FSGS.

Theory predicts that populations of endangered species should have lower levels of genetic diversity due to smaller effective population sizes and reduced gene flow (Hedrick and Miller, 1992; Frankham, 1996). Contrary to expectations, populations of *G. sanctum* in PVNP have high levels of genetic diversity at the intrapopulation level, even in the presence of apparent inbreeding due to moderate amounts of selfing (Chapter 3). We concluded that life history characteristics, as well as long distance seed dispersal, may be the proximate causes of the observed levels of genetic diversity in this population. These results suggest that populations of *G. sanctum* in PVNP have the potential for regeneration and restoration through colonization and expansion of extant populations into suitable habitats. Nonetheless, the continuing threats of habitat reduction due to deforestation may have detrimental consequences for *G. sanctum*, by reducing population sizes and disperser abundances which may hinder the ability for long-distance seed dispersal. Additionally, we showed that in PVNP *G. sanctum* has a mixed-mating breeding system ($t_m = 0.72$), which explains the significant levels of inbreeding observed in this population. Selfing levels were correlated with the density of reproductive individuals, indicating that future reductions in population sizes may negatively impact genetic diversity.

Results from the third chapter suggest that gene movement in *G. sanctum* may be a predominant factor shaping the genetic diversity of this species. Therefore, we determined (Chapter 4) patterns of genetic diversity within and among populations of *G. sanctum* in Costa Rica. Only seven populations were located, supporting the conclusion that this species has been extirpated from much of its natural distribution. Nonetheless, our results show that these populations possess unexpectedly high levels of genetic diversity for a species with very few, highly fragmented populations (Chapter 4). We demonstrated that a significant proportion of the genetic diversity is partitioned among populations located in two regions (North and South), separated by at least 70 km. In contrast, genetic differentiation among populations within regions was negligible, suggesting that inter-population gene flow may occur frequently at this spatial scale. These results lead us to

conclude that populations within a region, separated by $\sim$ 10Km from each other, have exchanged genes at rates high enough to significantly reduce genetic differentiation.

The evidence presented thus far suggests that *G. sanctum* has the potential for long distance gene dispersal. Nonetheless, our conclusions are mostly based on indirect measures of gene movement. Therefore, I documented pollen dispersal distances for this species using fractional paternity analyses (Chapter 5). Evidence is provided that indicates pollen is transported over several kilometers in fragmented populations of *G. sanctum*, supporting our indirect assessments of long-distance gene movement for this species. Additionally, these results concur with previous findings that show that insect pollinators can travel long distances, specially in fragmented or disturbed habitats (Nason *et al.*, 1996; Dick, 2001; White *et al.*, 2002). The most interesting result of this study was that isolated individuals play an important role in pollen movement over large distances, by effectively functioning as stepping stones between relict clusters. Janzen (1986) originally proposed that such isolated individuals were unlikely to be of any importance to the mating pool, which led him to term them the "living dead". Conversely, Hamrick (1994) proposed that isolated trees might serve as "stepping stones" between groups of adult trees facilitating gene movement among populations. Our findings are inconsistent with Janzen's "living dead" hypothesis and demonstrate that isolated individuals can be an important component in shaping genetic diversity within and among populations of tropical trees.

Overall, we conclude that *Guaiacum sanctum*, despite having small population sizes due to overexplotiation and habitat disruption, has high levels of genetic diversity as a consequence of long-distance pollen and seed dispersal. These results suggest that this endangered species has the potential for future adaptability, regeneration and population expansion. Our conclusions are important for the development of effective conservation and management practices, which strive to preserve extant levels of genetic diversity in this species. Additionally, we demonstrated that regeneration occurs within protected habitats (i.e. national parks, Chapter 2), but rarely occurs in disturbed areas (Chapter 5). These results emphasize the preservation of tropical dry forests to insure the long-term survival of this and other tropical tree species.

Bibliography

Achard, F., H. D. Eva, H.-J. Stibig, P. Mayaux, J. Gallego, T. Richards and J.-P. Malingreau. 2002. Determination of deforestation rates of the world's humid tropical forests. Science **297**: 999–1002.

Aldrich, P. R. and J. L. Hamrick. 1998. Reproductive dominance of pasture trees in a fragmented tropical forest mosaic. Science **281**: 103–105.

Aldrich, P. R., J. L. Hamrick, P. Chavarriaga and G. Kochert. 1998. Microsatellite analysis of demographic genetic structure in fragmented populations of the tropical tree *Symphonia globulifera*. Molecular Ecology **7**: 933–944.

Alvarez-Buylla, E. R., R. Garcia-Barrios, C. Lara-Moreno and M. Martinez-Ramos. 1996. Demographic and genetic models in conservation biology: Applications and perspectives for tropical rain forest tree species. Annual Review of Ecology and Systematics **27**: 387–421.

Apsit, V. J., J. L. Hamrick and J. D. Nason. 2001. Breeding population size of a fragmented population of a Costa Rican dry forest tree species. Journal of Heredity **92**: 415–420.

Armesto, J. J., J. D. Mitchell and C. Villagran. 1986. A comparison of spatial patterns of trees in some tropical and temperate forests. Biotropica **18**: 1–11.

Barot, S., J. Gignoux and J.-C. Menaut. 1999. Demography of a savanna palm tree: Predictions from comprehensive spatial pattern analyses. Ecology **80**: 1987–2005.

Bawa, K. S. 1974. Breeding system of tree species of a lowland tropical community. Evolution **28**: 85–92.

Bawa, K. S. 1990. Plant-pollinator interactions in tropical rain forests. Annual Review of Ecology and Systematics **21**: 399–422.

Bawa, K. S., S. H. Bullock, D. R. Perry, R. E. Coville and M. H. Grayum. 1985a. Reproductive-biology of tropical lowland rain-forest trees. 2. Pollination systems. American Journal of Botany **72**: 346–356.

Bawa, K. S., D. R. Perry and J. H. Beach. 1985b. Reproductive biology of tropical lowland rain-forest trees. 1. sexual systems and incompatibility mechanisms. American Journal of Botany **72**: 331–345.

Berg, E. E. and J. L. Hamrick. 1995. Fine-scale genetic structure of a turkey oak forest. Evolution **49**: 110–120.

Bissonette, J. and I. Storch. 2002. Fragmentation: Is the message clear? Conservation Ecology **6**: 14.

Bleher, B. and K. Bohning-Gaese. 2001. Consequences of frugivore diversity for seed dispersal, seedling establishment and the spatial pattern of seedlings and trees. Oecologia **129**: 385–394.

Bleher, B., R. Oberrath and K. Bohning-Gaese. 2002. Seed dispersal, breeding system, tree density and the spatial pattern of trees: a simulation approach. Basic and Applied Ecology **3**: 115–123.

Bohonak, A. J. 1999. Dispersal, gene flow, and population structure. Quarterly Review of Biology **74**: 21–45.

Boswell, G., N. Britton and N. Franks. 1998. Habitat fragmentation, percolation theory and the conservation of a keystone species. Proceedings of the Royal Society of London Series B-Biological Sciences **265**: 1921–1925.

Brook, B. W., D. W. Tonkyn, J. J. Q'Grady and R. Frankham. 2002. Contribution of inbreeding to extinction risk in threatened species. Conservation Ecology **6**: 1–16.

Bullock, S. H. 1985. Breeding systems in the flora of a tropical deciduous forest in Mexico. Biotropica **17**: 287–301.

Burnham, R. J. and A. Graham. 1999. The history of neotropical vegetation: New developments and status. Annals of the Missouri Botanical Garden **86**: 546–589.

Cascante, A., M. Quesada, J. A. Lobo and E. J. Fuchs. 2001. Effects of dry tropical forest fragmentation on the reproductive success and genetic structure of the tree *Samanea saman*. Conservation Biology **16**: 137–147.

Cespedes, M., M. V. Gutierrez, N. M. Holbrook and O. J. Rocha. 2003. Restoration of genetic diversity in the dry forest tree *Swietenia macrophylla* (Meliaceae) after pasture abandonment in Costa Rica. Molecular Ecology **12**: 3201–3212.

Chakraborty, R., T. R. Meagher and P. E. Smouse. 1988. Parentage analysis with genetic markers in natural populations. I. The expected proportion of offspring with unambiguous paternity. Genetics **118**: 527–536.

Charlesworth, B. and D. Charlesworth. 1999. The genetic basis of inbreeding depression. Genetic Research **74**: 329–340.

Chase, M. R., C. Moller, R. Kesseli and K. S. Bawa. 1996. Distant gene flow in tropical trees. Nature **383**: 398–399.

Chung, M. G., M. Y. Chung, G. S. Oh and B. K. Epperson. 2000. Spatial genetic structure in a *Newolitsea sericeae* population (Lauraceae). Heredity **85**: 490–505.

Chung, M. Y., B. K. Epperson and M. G. Chung. 2003a. Genetic structure of age classes in *Camellia japonica* (Theaceae). Evolution **57**: 62–73.

Chung, M. Y., J. D. Nason, B. K. Epperson and M. G. Chung. 2003b. Temporal aspects of the fine-scale genetic structure in a population of *Cinnamomum insularimontanum* (Lauraceae). Heredity **90**: 98–106.

Chung, M. Y., M. G. Chung, E. R. Myers, J. M. Chung, K. J. Kim, C. W. Park, B. Y. Sun and J. H. Pak. 2004. Spatial genetic structure in disturbed populations of *Quercus acutissima* (Fagaceae). Silvae Genetica **53**: 206–211.

CITES. 2000. Proposal to transfer *Guaiacum sanctum* from Appendix II to Apendix I of CITES. Speller & Sons, New York.

Clark, D. A. and D. B. Clark. 1984. Spacing dynamics of a tropical rain forest tree: Evaluation of the Janzen-Connell model. American Naturalist **124**: 769–788.

Clark, D. B. and D. A. Clark. 1996. Abundance, growth and mortality of very large trees in neotropical lowland rain forest. Forest Ecology and Management **80**: 235–244.

Collevatti, R. G., D. Grattapaglia and J. D. Hay. 2001. Population genetic structure of the endangered tropical tree species *Caryocar brasiliense*, based on variability at microsatellite loci. Molecular Ecology **10**: 349–356.

Condit, R., S. P. Hubbell and R. B. Foster. 1992. Recruitment near conspecific adults and the maintenance of tree and shrub diversity in a neotropical forest. American Naturalist **140**: 261–286.

Condit, R., R. Sukumar, S. Hubbell and R. Foster. 1998. Predicting population trends from size distributions: A direct test in a tropical tree community. American Naturalist **152**: 495–509.

Condit, R., P. S. Ashton, P. Baker, S. Bunyavejchewin, S. Gunatilleke, N. Gunatilleke, S. P. Hubbell, R. B. Foster, A. Itoh, J. V. LaFrankie, H. S. Lee, E. Losos, N. Manokaran, R. Sukumar and T. Yamakura. 2000. Spatial patterns in the distribution of tropical tree species. Science **288**: 1414–1418.

Connell, J. H. 1971. On the role of natural enemies in preventing competitive exclusion in some marine animals and in rain forest trees. *In* P. J. van der Boer and G. R. Gradwell (Eds.), Dynamics of Numbers in Populations. Center for Agricultural Publication and Documentation, Wageningen, pages 298–312.

Cortes, M. C. and J. H. Hunziker. 1997. Isozymes in *Larrea divaricata* and *Larrea tridentata* (Zygophyllaceae): a study of two amphitropical vicariants and autopolyploidy. Genetica **101**: 115–124.

Cuevas, E., D. M. Arias, C. A. Domnguez, R. A. Castillo and F. Molina-Freaner. 2006. The genetic structure of the gynodioecious *Kallstroemia grandiflora* (Zygophyllaceae): the role of male sterility and colonization history. Heredity **97**: 269–274.

Dayanandan, S., J. Dole, K. Bawa and R. Kesseli. 1999. Population structure delineated with microsatellite markers in fragmented populations of a tropical tree, *Carapa guianensis* (Meliaceae). Molecular Ecology **8**: 1585–1592.

Degen, B. and D. W. Roubik. 2004. Effects of animal pollination on pollen dispersal, selfing, and effective population size of tropical trees: A simulation study. Biotropica **36**: 165–179.

Degen, B., H. Caron, E. Bandou, L. Maggia, M. H. Chevallier, A. Leveau and A. Kremer. 2001. Fine-scale spatial genetic structure of eight tropical tree species as analysed by RAPDs. Heredity **87**: 497–507.

Denslow, J. S. 1987. Tropical rainforest gaps and tree species diversity. Annual Review of Ecology and Systematics **18**: 431–451.

Denslow, J. S. and T. Spies. 1990. Canopy gaps in forest ecosystems - an introduction. Canadian Journal of Forest Research **20**: 619–619.

Devlin, B., K. Roeder and N. C. Ellstrand. 1988. Fractional paternity assignment: theoretical development and comparison to other methods. Theoretical and Applied Genetics **76**: 369–380.

Dick, C. W. 2001. Genetic rescue of remnant tropical trees by an alien pollinator. Proceedings of the Royal Society of London Series B-Biological Sciences **268**: 2391–2396.

Dick, C. W., G. Etchelecu and F. Austelitz. 2003. Pollen dispersal of tropical trees (*Dinizia excelsa* : Fabaceae) by native insects and african honeybees in pristine and fragmented Amazonian rainforest. Molecular Ecology. **12**: 753–764.

Doligez, A., C. Baril and H. I. Joly. 1998. Fine-scale spatial genetic structure with nonuniform distribution of individuals. Genetics **148**: 905–919.

Duran, K. L., T. K. Lowrey, R. R. Parmenter and P. O. Lewis. 2005. Genetic diversity in Chihuahuan Desert populations of creosotebush (Zygophyllaceae : *Larrea tridentata*). American Journal of Botany **92**: 722–729.

Eguiarte, L. E., A. Burquez, J. Rodriguez, M. Martinez-Ramos, J. Sarukhan and D. Pinero. 1993. Direct and indirect estimates of neighborhood and effective population size in a tropical palm, *Astrocaryum mexicanum*. Evolution **47**: 75–87.

Ehrlich, P. R. and P. H. Raven. 1969. Differentiation of populations. Science **165**: 1228–1232.

Ellstrand, N. C. and D. R. Elam. 1993. Population genetic consequences of small population size: Implications for plant conservation. Annual Review of Ecology and Systematics **24**: 217–242.

Epperson, B. K. 1993. Spatial structure of genetic variation within populations of forest trees. New Forests **6**: 257–278.

Epperson, B. K. 2000. Spatial genetic structure and non-equilibrium demographics within plant populations. Plant Species Biology **15**: 269–279.

Epperson, B. K. and E. R. Alvarez-Buylla. 1997. Limited seed dispersal and genetic structure in life stages of *Cecropia obtusifolia*. Evolution **51**: 275–282.

Excoffier, L., G. Laval and S. Schneider. 2005. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online **1**: 47–50.

Falconer, D. S. and T. F. C. Mackay. 1996. Introduction to quantitative genetics. Longman, Essex, UK.

Forget, P. M., F. Mercier and F. Collinet. 1999. Spatial patterns of two rodent-dispersed rain forest trees, *Carapa procera* (Meliaceae) and *Vouacapoua americana* (Caesalpiniaceae) at Paracou, French Guiana. Journal of Tropical Biology **15**: 301–313.

Frankham, R. 1995. Conservation genetics. Annual Review of Genetics **29**: 305–327.

Frankham, R. 1996. Relationship of genetic variation to population size in wildlife. Conservation Biology **10**: 1500–1508.

Frankie, G. W., P. A. Opler and K. S. Bawa. 1976. Foraging behavior of solitary bees - implications for outcrossing of a neotropical forest tree species. Journal of Ecology **64**: 1049–1057.

Franklin, I. A. 1983. Why bees move among mass-flowering neotropical trees., chapter 1. Van Rostrand Reinhold, New York, pages 360–372.

Fuchs, E. J., J. A. Lobo and M. Quesada. 2003. Effects of forest fragmentation and flowering phenology on the reproductive success and mating patterns of the tropical dry forest tree *Pachira quinata*. Conservation Biology **17**: 149–157.

Gariepy, T. 1981. *Guaiacum* and the venereal pestilence. Journal of the History of Medicine and Allied Sciences **36**: 325–326.

Ge, X. J., Y. Yu, N. X. Zhao, H. S. Chen and W. Q. Qi. 2003. Genetic variation in the endangered inner Mongolia endemic shrub *Tetraena mongolica* Maxim. (Zygophyllaceae). Biological Conservation **111**: 427–434.

Gehring, J. L. and L. F. Delph. 1999. Fine-scale genetic structure and clinal variation in *Silene acaulis* despite high gene flow. Heredity **82**: 628–637.

Geist, H. J. and E. F. Lambin. 2002. Proximate causes and underlying driving forces of tropical deforestation. BioScience **52**: 143–150.

Gerber, S., S. Mariette, R. Streiff, C. Bodns and A. Kremer. 2000. Comparison of microsatellites and amplified fragment length polymorphism markers for parentage analysis. Molecular Ecology **9**: 1037–1048.

Gerber, S., P. Chabrier and A. Kremer. 2003. FaMoZ: a software for parentage analysis using dominant, codominant and uniparentally inherited markers. Molecular Ecology Notes **3**: 479–481.

Gerhardt, K. 1996. Effects of root competition and canopy openness on survival and growth of tree seedlings in a tropical seasonal dry forest. Forest Ecology and Management **82**: 33–48.

Gilbert, G. S., S. P. Hubbell and R. B. Foster. 1994. Density and distance-to-adult effects of a canker disease of trees in a moist tropical forest. Oecologia **98**: 100–108.

González-Rivas, B., M. Tigabu, K. Gerhardt, G. Castro-Marín and P. Odón. 2006. Species composition, diversity and local uses of tropical dry deciduous and gallery forests in Nicaragua. Biodiversity and Conservation **15**: 1509–1527.

Graham, C., J. Martinez-Leyva and L. Cruz-Paredes. 2002. Use of fruiting trees by birds in continuous forest and riparian forest remnants in Los Tuxtlas, Veracruz, Mexico. Biotropica **34**: 589–597.

Grau, H. R. 2000. Regeneration patterns of *Cedrela lilloi* (Meliaceae) in northwestern Argentina subtropical montane forests. Journal of Tropical Biology **16**: 227–242.

Greene, D. F., C. D. Canham, K. D. Coates and P. T. Lepage. 2004. An evaluation of alternative dispersal functions for trees. Journal of Ecology **92**: 758–766.

Hall, P., S. Walker and K. Bawa. 1996. Effect of forest fragmentation on genetic diversity and mating system in a tropical tree, *Pithecellobium elegans*. Conservation Biology **10**: 757–768.

Hamill, D. N. and S. J. Wright. 1986. Testing the dispersion of juveniles relative to adults, a new analytic method. Ecology **67**: 952–957.

Hamilton, M. 1999. Tropical tree gene flow and seed dispersal. Nature **401**: 129–130.

Hamrick, J. L. 1994. Genetic diversity and conservation in tropical forests. *In* R. M. Drysdale, S. E. T. John and A. C. Yapa (Eds.), Proceedings International Symposium on Genetic Conservation and Production of Tropical Forest Tree Seed. Asean-Canada Forest Tree Seed Centre, pages 1–9.

Hamrick, J. L. and V. J. Apsit. 2004. Breeding structure of neotropical dry-forest tree species in fragmented landscapes. *In* G. W. Frankie, A. Mata and S. B. Vinson (Eds.), Biodiversity

Conservation in Costa Rica: Learning the lessons in a seasonal dry forest. University of California Press, Berkeley, CA, pages 30–37.

Hamrick, J. L. and M. J. W. Godt. 1996. Effects of life history traits on genetic diversity in plant species. Philosophical Transactions of The Royal Society of London Series B-Biological Sciences **351**: 1291–1298.

Hamrick, J. L. and D. A. Murawski. 1990. The breeding structure of tropical tree populations. Plant Species Biology **5**: 157–165.

Hamrick, J. L. and D. A. Murawski. 1991. Levels of allozyme diversity in populations of uncommon neotropical tree species. Journal of Tropical Ecology **7**: 395–399.

Hamrick, J. L. and J. D. Nason. 1996. Consequences of dispersal in plants. *In* O. E. Rhodes, R. K. Chesser and M. H. Smith (Eds.), Population dynamics in ecological space and time. University of Chicago Press, Chicago, pages 203–236.

Hamrick, J. L., M. J. W. Godt and S. L. Sherman-Broyles. 1992. Factors influencing levels of genetic diversity in woody plant species. New Forests **6**: 95–124.

Hamrick, J. L., D. A. Murawski and J. D. Nason. 1993a. The influence of seed dispersal mechanisms on the genetic structure of tropical tree populations. Vegetatio **107**: 281–297.

Hamrick, J. L., D. A. Murawski and J. D. Nason. 1993b. The influence of seed dispersal mechanisms on the genetic-structure of tropical tree populations. Vegetatio **108**: 281–297.

He, F. L., P. Legendre and J. V. LaFrankie. 1997. Distribution patterns of tree species in a Malaysian tropical rain forest. Journal of Vegetation Science **8**: 105–114.

Hedrick, P. W. and S. T. Kalinowski. 2000. Inbreeding depression in conservation biology. Annual Review of Ecology and Systematics **31**: 139–162.

Hedrick, P. W. and P. S. Miller. 1992. Conservation genetics: Techniques and fundamentals. Ecological Applications **2**: 30–46.

Henle, K., K. Davies, M. Kleyer, C. Margules and J. Settele. 2004. Predictors of species sensitivity to fragmentation. Biodiversity and Conservation **13**: 207–251.

Holdrige, L. R. and L. J. Poveda. 1975. Árboles de Costa Rica, volume 1. Centro Científico Tropical, San José, Costa Rica.

Holsinger, K. and L. D. Gottlieb. 1991. Conservation of rare and endangered plants: Principles and prospects. *In* D. A. Falk and K. E. Holsinger (Eds.), Genetics and Conservation of Rare Plants. Oxford University Press, Oxford, UK, pages 195–208.

Howe, H. F. 1977. Bird activity and seed dispersal of a tropical wet forest tree. Ecology **58**: 539–550.

Howe, H. F. 1989. Scatter-dispersal and clump-dispersal and seedling demography - hypothesis and implications. Oecologia **79**: 417–426.

Howe, H. F. and J. Smallwood. 1982. Ecology of seed dispersal. Annual Review of Ecology and Systematics **13**: 201–228.

Hubbell, S. P. 1979. Tree dispersion, abundance, and diversity in a tropical dry forest. Science **203**: 1299–1309.

Hubbell, S. P. and R. B. Foster. 1986. Commonness and rarity in a neotropical forest: implications for tropical tree conservation. *In* M. E. Soul (Ed.), Conservation Biology: The Science of Scarcity and Diversity. Sinauer Associates, Inc., Sunderland, Massachusetts, pages 205–231.

Huenneke, L. 1991. Ecological implications of genetic variation in plant populations. *In* D. A. Falk and K. E. Holsinger (Eds.), Genetics and Conservation of Rare Plants. Oxford University Press, New York, pages 31–33.

Hufford, K. M. and J. L. Hamrick. 2003. Viability selection at three early life stages of the tropical tree, *Platypodium elegans* (Fabaceae, Papilionoideae). Evolution **57**: 518–526.

Husband, B. C. and D. W. Schemske. 1996. Evolution of the magnitude and timing of inbreeding depression in plants. Evolution **50**: 54–70.

Hutchinson, G. E. 1953. Concept of pattern in ecology. Proceedings of the Academy of Natural Science of Philadelphia **105**: 1–12.

Itoh, A., T. Yamakura, K. Ogino, H. S. Lee and P. S. Ashton. 1997. Spatial distribution patterns of two predominant emergent trees in a tropical rainforest in Sarawak, Malaysia. Plant Ecology **132**: 121–136.

Itoh, A., T. Yamakura, T. Ohkubo, M. Kanzaki, P. A. Palmiotto, J. V. LaFrankie, P. S. Ashton and H. S. Lee. 2003. Importance of topography and soil texture in the spatial distribution of two sympatric dipterocarp trees in a Bornean rainforest. Ecological Research **18**: 307–320.

Janzen, D. H. 1970. Herbivores and the number of tree species in tropical forests. American Naturalist **104**: 501–528.

Janzen, D. H. 1971. Euglossine bees as long-distance pollinators of tropical plants. Science **171**: 203–205.

Janzen, D. H. 1986. Blurry catastrophes. Oikos **47**: 1–2.

Janzen, D. H. 1988. Tropical dry forests: the most endangered major tropical ecosystem. *In* E. O. Wilson (Ed.), Biodiversity. National Academy Press, Washington, D.C., pages 130–137.

John, R., H. S. Dattaraja, H. S. Suresh and R. Sukumar. 2002. Density-dependence in common tree species in a tropical dry forest in Mudumalai, Southern India. Journal of Vegetation Science **13**: 45–56.

Jordano, P. 1983. Fig-seed predation and dispersal by birds. Biotropica **15**: 38–41.

Kalisz, S., J. D. Nason, F. M. Hanzawa and S. J. Tonsor. 2001. Spatial population genetic structure in *Trillium grandiflorum*: The roles of dispersal, mating, history and selection. Evolution **55**: 1560–1568.

Kenta, T., Y. Isagi, M. Nakagawa, M. Yamashita and T. Nakashizuka. 2004. Variation in pollen dispersal between years with different pollination conditions in a tropical emergent tree. Molecular Ecology **13**: 3575 – 3584.

Konuma, A., Y. Tsumura, C. T. Lee, S. L. Lee and T. Okuda. 2000. Estimation of gene flow in the tropical-rainforest tree *Neobalanocarpus heimii* (Dipterocarpaceae), inferred from paternity analysis. Molecular Ecology **9**: 1843–1852.

Korning, J. and H. Balslev. 1994. Growth rates and mortality patterns of tropical lowland tree species and the relation to forest structure in Amazonian Ecuador. Journal of Tropical Ecology **10**: 151–166.

Lambers, J. H. R., J. S. Clark and B. Beckage. 2002. Density-dependent mortality and the latitudinal gradient in species diversity. Nature **417**: 732–735.

Lande, R. and G. F. Barrowclough. 1987. Effective population size, genetic variation, and their use in population management. *In* M. E. Soule (Ed.), Viable Populations for Conservation. Cambridge University Press, Cambridge, UK., pages 87–123.

Latouche-Hall, C., A. Ramboer, E. Bandou, H. Caron and A. Kremer. 2004. Long-distance pollen flow and tolerance to selfing in a neotropical tree species. Molecular Ecology **13**: 1055–1064.

Laurance, W., L. Ferreira, J. R.-D. Merona and S. Laurance. 1998. Rain forest fragmentation and the dynamics of amazonian tree communities. Ecology **79**: 2032–2040.

Lemes, M. R., R. Gribel, J. Proctor and D. Grattapaglia. 2003. Population genetic structure of mahogany (*Swietenia macrophylla* King, Meliaceae) across the Brazilian Amazon, based on variation at microsatellite loci: implications for conservation. Molecular Ecology **12**: 2875–2883.

Lesica, P. and F. W. Allendorf. 1995. When are peripheral-populations valuable for conservation. Conservation Biology **9**: 753–760.

Levin, D. and H. Kerster. 1974. Gene flow in seed plants. Evolutionary Biology **7**: 139–220.

Lia, V., C. I. Comas, M. C. Cortes and J. H. Hunziker. 1999. Isozyme variation in *Larrea ameghinoi* and *Larrea nitida* (Zygophyllaceae): genetic diversity and its bearing on their relationship. Genetica **106**: 197–207.

Lieberman, D. and M. Li. 1992. Seedling recruitment patterns in a tropical dry forest in Ghana. Journal of Vegetation Science **3**: 375–382.

Loveless, M. D. and J. L. Hamrick. 1984. Ecological determinants of genetic structure in plant populations. Annual Review of Ecology and Systematics **15**: 65–95.

Loveless, M. D., J. L. Hamrick and R. B. Foster. 1998. Population structure and mating system in *Tachigali versicolor*, a monocarpic Neotropical tree. Heredity **81**: 134–143.

Lowe, A. J., B. Jourde, P. Breyne, N. Colpaert, C. Navarro, J. Wilson and S. Cavers. 2003. Fine-scale genetic structure and gene flow within Costa Rican populations of mahogany (*Swietenia macrophylla*). Heredity **90**: 268–275.

Lowe, A. J., D. Boshier, M. Ward, C. F. E. Bacles and C. Navarro. 2005. Genetic resource impacts of habitat loss and degradation; reconciling empirical evidence and predicted theory for neotropical trees. Heredity **95**: 255–273.

Lynch, M. and B. Walsh. 1998. Genetics and analysis of quantitative traits. Sinauer Sunderland, Ma.

Manchenko, G. 1994. Handbook of detection of enzymes on electrophoretic gels. CRC Press, Ann Arbor, MI.

Mandk, B., K. Bmov, V. Mahelka and I. Plackov. 2006. How much genetic variation is stored in the seed bank? a study of *Atriplex tatarica* (Chenopodiaceae). Molecular Ecology **15**: 2653–2663.

McLaren, K. P. and M. A. McDonald. 2003. The effects of moisture and shade on seed germination and seedling survival in a tropical dry forest in Jamaica. Forest Ecology and Management **183**: 61–75.

Meagher, T. and E. Thompson. 1986. The relationship between single parent and parent pair genetic likelihoods in genealogy reconstruction. Theoretical Population Biology **29**: 87–106.

Murawski, D. A. and J. L. Hamrick. 1991. The effect of the density of flowering individuals on the mating systems of nine tropical tree species. Heredity **67**: 167–174.

Murawski, D. A. and J. L. Hamrick. 1992. The mating system of *Cavanillesia platanifolia* under extremes of flowering-tree density: A test of predictions. Biotropica **24**: 99–101.

Murphy, P. G. and A. E. Lugo. 1986. Ecology of tropical dry forest. Annual Review of Ecology and Systematics **17**: 67–88.

Nason, J. D. and J. L. Hamrick. 1997. Reproductive and genetic consequences of forest fragmentation: Two case studies of neotropical canopy trees. Journal of Heredity **88**: 264–276.

Nason, J. D., E. A. Herre and J. L. Hamrick. 1996. Paternity analysis of the breeding structure of strangler fig populations: Evidence for substantial long-distance wasp dispersal. Journal of Biogeography **23**: 501–512.

Nason, J. D., E. A. Herre and J. L. Hamrick. 1998. The breeding structure of a tropical keystone plant resource. Nature **391**: 685–687.

Nathan, R. and H. C. Muller-Landau. 2000. Spatial patterns of seed dispersal, their determinants and consequences for recruitment. Trends in Ecology and Evolution **15**: 278–285.

Nei, M. 1972. Genetic distance between populations. American Naturalist **106**: 283–292.

Nei, M. 1975. Molecular population genetics and evolution. American Elsevier, New York.

Newman, D. and D. Pilson. 1997. Increased probability of extinction due to decreased genetic effective population size: experimental populations of *Clarkia pulchella*. Evolution **51**: 354–362.

Nichols, J. D., V. K. Agyeman, F. B. Agurgo, M. R. Wagner and J. R. Cobbinah. 1999. Patterns of seedling survival in the tropical African tree *Milicia excelsa*. Journal of Tropical Ecology **15**: 451–461.

Odum, E. 1983a. Basic ecology. Saunders College, London (UK).

Odum, H. 1983b. Systems Ecology: An Introduction. Wiley.

Okuda, T., N. Kachi, S. K. Yap and N. Manokaran. 1997. Tree distribution pattern and fate of juveniles in a lowland tropical rain forest, implications for regeneration and maintenance of species diversity. Plant Ecology **131**: 155–171.

Oldfield, S., C. Lusty and A. MacKinven. 1998. The World List of Threatened Trees. World Conservation Press, Cambridge.

Otterstrom, S. M., M. W. Schwartz and I. Velazquez-Rocha. 2006. Responses to fire in selected tropical dry forest trees. Biotropica **38**: 592–598.

Pacheco, L. F. and J. A. Simonetti. 2000. Genetic structure of a mimosoid tree deprived of its seed disperser, the spider monkey. Conservation Biology **14**: 1766–1775.

Palmiotto, P., S. Davies, K. Vogt, M. Ashton, D. Vogt and P. Ashton. 2004. Soil-related habitat specialization in dipterocarp rain forest tree species in Borneo. Journal of Ecology **92**: 609–623.

Parker, K. C., J. L. Hamrick, A. J. Parker and J. D. Nason. 2001. Fine-scale genetic structure in *Pinus clausa* (Pinaceae) populations: effects of disturbance history. Heredity **87**: 99–113.

Peakall, K. and P. E. Smouse. 2001. Spatial autocorrelation analysis of individual multiallele and multilocus genetic structure. Heredity **85**: 561–573.

Pither, R., J. Shore and M. Kellman. 2003. Genetic diversity of the tropical tree *Terminalia amazonia* (Combretaceae) in naturally fragmented populations. Heredity **91**: 307–313.

Premoli, A. C. and T. Kitzberger. 2005. Regeneration mode affects spatial genetic structure of *Nothofagus dombeyi* forests. Molecular Ecology **14**: 2319–2329.

Queller, D. C. and K. F. Goodnight. 1989. Estimating relatedness using genetic markers. Evolution **43**: 258–275.

Quesada, M. and K. E. Stoner. 2003. Threats to the conservation of tropical dry forests in Guanacaste, Costa Rica. *In* G. W. Frankie, A. Mata and S. B. Vinson (Eds.), Conservation biology in a tropical dry forest in Costa Rica: Learning the lessons in the Costa Rican dry forest. University of California Press, Berkeley, CA, pages 150–162.

Quesada, M., E. J. Fuchs and J. A. Lobo. 2001. Pollen load size, reproductive success and progeny kinship of natural pollinated flowers of the tropical dry forest tree, *Pachira quinata* (Bombacaceae). American Journal of Botany **88**: 2113–2118.

Quesada, M., K. Stoner, V. Rosas-Guerrero, C. Palacios-Guevara and J. Lobo. 2003. Effects of habitat disruption on the activity of nectarivorous bats (Chiroptera : Phyllostomidae) in a dry tropical forest: implications for the reproductive success of the neotropical tree *Ceiba grandiflora*. Oecologia **135**: 400–406.

Quirico, J. M. 1993. rboles maderables en peligro de extincin en Costa Rica. Museo Nacional de Costa Rica, San Jose, Costa Rica.

R Development Core Team. 2005. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0.

Ray, G. J. and B. J. Brown. 1995. Restoring Caribbean dry forests: Evaluation of tree propagation techniques. Restoration Ecology **3**: 86–94.

Reed, D. H., E. H. Lowe, D. A. Briscoe and R. Frankham. 2003. Inbreeding and extinction: Effects of rate of inbreeding. Conservation Genetics **4**: 405–410.

Ribeiro, P. J. and P. J. Diggle. 2001. geoR: a package for geostatistical analysis. R-NEWS **1**: 14–18.

Ripley, B. D. 1976. The second-order analysis of stationary point processes. Journal of Applied Probability **13**: 255–256.

Ripley, B. D. 1977. Modeling spatial patterns. Journal of the Royal Statistical Society, B. **39**: 172–212.

Ritland, K. 1989a. Correlated matings in the partial selfer *Mimulus guttatus*. Evolution **43**: 848–859.

Ritland, K. 1989b. Multilocus mating system program (MLTR) version 1.1. Technical report, Department of Botany, University of Toronto. Toronto.

Ritland, K. 2002. Extensions of models for the estimation of mating systems using n independent loci. Heredity **88**: 221–228.

Rocha, O. J. and G. Aguilar. 2001. Reproductive biology of the dry forest tree *Enterolobium cyclocarpum* (Guanacaste) in Costa Rica: a comparison between trees left in pastures and trees in continuous forest. American Journal of Botany **88**: 1607–1614.

Rossetto, M., R. Jones and J. Hunter. 2004. Genetic effects of rainforest fragmentation in an early successional tree (*Elaeocarpus grandis*). Heredity **93**: 610–618.

Rousset, F. 1997. Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. Genetics **145**: 1219–1228.

Russo, S. E. and C. K. Augspurger. 2004. Aggregated seed dispersal by spider monkeys limits recruitment to clumped patterns in *Virola calophylla*. Ecology Letters **7**: 1058–1067.

Sader, S. and A. Joyce. 1988. Deforestation rates and trends in Costa Rica, 1940 to 1983. Biotropica **20**: 11–19.

Sagar, R. and J. S. Singh. 2004. Local plant species depletion in a tropical dry deciduous forest of northern India. Environmental Conservation **31**: 55–62.

Sato, T., Y. Isagi, H. Sakio, K. Osumi and S. Goto. 2006. Effect of gene flow on spatial genetic structure in the riparian canopy tree *Cercidiphyllum japonicum* revealed by microsatellite analysis. Heredity **96**: 79–84.

Schaffer, B. and L. Mason. 1990. Effects of scale insect herbivory and shading on net gas-exchange and growth of a subtropical tree species (*Guaiacum sanctum* L). Oecologia **84**: 468–473.

Schnabel, A. and J. L. Hamrick. 1995. Understanding the population genetic structure of *Gleditsia triacanthos* L.: the scale and pattern of pollen gene flow. Evolution **49**: 921–931.

Schnabel, A., J. D. Nason and J. L. Hamrick. 1998. Understanding the population genetic structure of *Gleditsia triacanthos*: seed dispersal and variation in female reproductive success. Molecular Ecology **7**: 819–832.

Schupp, E. W. 1992. The Janzen-Connell model for tropical tree diversity: Population implications and the importance of spatial scale. American Naturalist **140**: 526–530.

Schupp, E. W. 1995. Seed seedling conflicts, habitat choice, and patterns of plant recruitment. American Journal of Botany **82**: 399–409.

Silla, F., S. Fraver, A. Lara, T. R. Allnutt and A. Newton. 2002. Regeneration and stand dynamics of *Fitzroya cupressoides* (Cupressaceae) forests of southern Chile's central depression. Forest Ecology and Management **165**: 213–224.

Slik, J. W. F. and K. A. O. Eichhorn. 2003. Fire survival of lowland tropical rain forest trees in relation to stem diameter and topographic position. Oecologia **137**: 446–455.

Smouse, P. E. and V. L. Sork. 2004. Measuring pollen flow in forest trees: an exposition of alternative approaches. Forest Ecology and Management **197**: 21–38.

Soltis, D. E., C. H. Haufler, D. C. Darrow and G. J. Gastony. 1983. Starch gel electrophoresis of ferns: a compilation of grinding buffers, gel and electrode buffers, and staining schedules. American Fern Journal **73**: 9–27.

Sork, V. L. and P. E. Smouse. 2006. Genetic analysis of landscape connectivity in tree populations. Landscape Ecology **21**: 821–836.

Sork, V. L., F. W. Davis, P. E. Smouse, V. J. Apsit, R. J. Dyer, J. F. Fernandez-M and B. Kuhn. 2002. Pollen movement in declining populations of california valley oak, *Quercus lobata*: where have all the fathers gone? Molecular Ecology **11**: 1657–1668.

Soul, M. E. and G. H. Orians. 2001. Conservation Biology: Research Priorities for the Next Decade. Island Press, London.

Soulé, M. E. 1986. Conservation Biology: The Science of Scarcity and Diversity. Sinauer Associates.

Souza, A. and F. Martins. 2004. Microsite specialization and spatial distribution of *Geonoma brevispatha*, a clonal palm in south-eastern Brazil. Ecological Research **19**: 521–532.

Sterner, R. W., C. A. Ribic and G. E. Schatz. 1986. Testing for life historical changes in spatial patterns of four tropical tree species. Journal of Ecology **74**: 621–633.

Streiff, R., A. Ducousso, C. Lexer, H. Steinkellner, J. Gloessl and A. Kremer. 1999. Pollen dispersal inferred from paternity analysis in a mixed oak stand of *Quercus robur* L. and *Q. petrae* Liebl. Molecular Ecology **8**: 831–841.

Tonsor, S. J., S. Kalisz, J. Fisher and T. P. Holtsford. 1993. A life-history based study of population genetic structure: seed bank to adults in *Plantago lanceolata*. Evolution **47**: 833–843.

Trapnell, D. W. and J. L. Hamrick. 2004. Partitioning nuclear and chloroplast variation at multiple spatial scales in the neotropical epiphytic orchid, *Laelia rubescens*. Molecular Ecology **13**: 2655–2666.

Turner, M. E., C. J. Stephens and W. W. Anderson. 1982. Homozygosity and patch structure in plant populations as a result of nearest-neighbor pollination. Proceedings of the National Academy of Science **79**: 203–207.

Ueno, S., N. Tomaru, H. Yoshimaru, T. Manabe and S. Yamamoto. 2006. Effects of canopy gaps on the genetic structure of *Camellia japonica* saplings in a Japanese old-growth evergreen forest. Heredity **96**: 304–310.

Vekemans, X. and O. J. Hardy. 2004. New insights from fine-scale spatial genetic structure analyses in plant populations. Molecular Ecology **13**: 921–935.

Voeks, R. A. 2004. Disturbance pharmacopoeias: Medicine and myth from the humid tropics. Annals of the Association of American Geographers **94**: 868–888.

Wang, J., W. G. Hill, D. Charlesworth and B. Charlesworth. 1999. Dynamics of inbreeding depression due to deleterious mutations in small populations: mutation parameters and inbreeding rate. Genet Res **74**: 165–178.

Weeden, N. F. and J. F. Wendell. 1989. Genetics of plant isozymes. *In* D. E. Soltis and P. S. Soltis (Eds.), Isozymes in Plant Biology. Dioscorides Press, Portland, OR, pages 46–72.

Weir, B. S. and C. C. Cockerham. 1984. Estimating F-statistics for the analysis of population structure. Evolution **38**: 1358–1370.

Wendelken, P. W. and R. F. Martin. 1987. Avian consumption of *Guaiacum sanctum* fruit in the arid interior of Guatemala. Biotropica **19**: 116–121.

Wendell, J. F. and N. F. Weeden. 1989. Visualization and interpretation of plant isozymes. *In* D. Soltis and P. Soltis (Eds.), Isozymes in Plant Biology, *Advances in Plant Sciences Series*, volume 4. Dioscorides Press, Portland, OR., pages 5–45.

Wheelwright, N. T. 1991. How long do fruit-eating birds stay in the plants where they feed? Biotropica **23**: 29–40.

White, G. M., D. H. Boshier and W. Powell. 2002. Increased pollen flow counteracts fragmentation in a tropical dry forest: An example from *Swietenia humilis* Zuccarini. Proceedings of the National Academy of Sciences of the United States of America **99**: 2038–2042.

Wilson, E. O. and T. Eisner. 1968. Lignumvitae - Relict island. Natural History **72**: 52–57.

Wright, S. 1946. Isolation by distance under diverse systems of mating. Genetics **31**: 39–59.

Wright, S. 1969. Evolution and the Genetics of Populations, Vol. 2. The Theory of Gene Frequencies. University of Chicago Press, Chicago, Ill.

Yamada, T. and E. Suzuki. 1997. Changes in spatial distribution during the life history of a tropical tree, *Scaphium macropodum* (Sterculiaceae) in Borneo. Journal of Plant Research **110**: 179–186.

Young, A., T. Boyle and T. Brown. 1996. The population genetic consequences of habitat fragmentation for plants. Trends in Ecology and Evolution **11**: 413–418.

Zuidema, P. A. and R. G. A. Boot. 2002. Demography of the brazil nut tree (*Bertholletia excelsa*) in the Bolivian Amazon: impact of seed extraction on recruitment and population dynamics. Journal of Tropical Ecology **18**: 1–31.

Appendix

Figure 1: Spatial genetic autocorrelograms for seedlings, saplings and juveniles (top to bottom) of *Guaiacum sanctum* in subplot 1, PVNP, Costa Rica (Chapter 3).

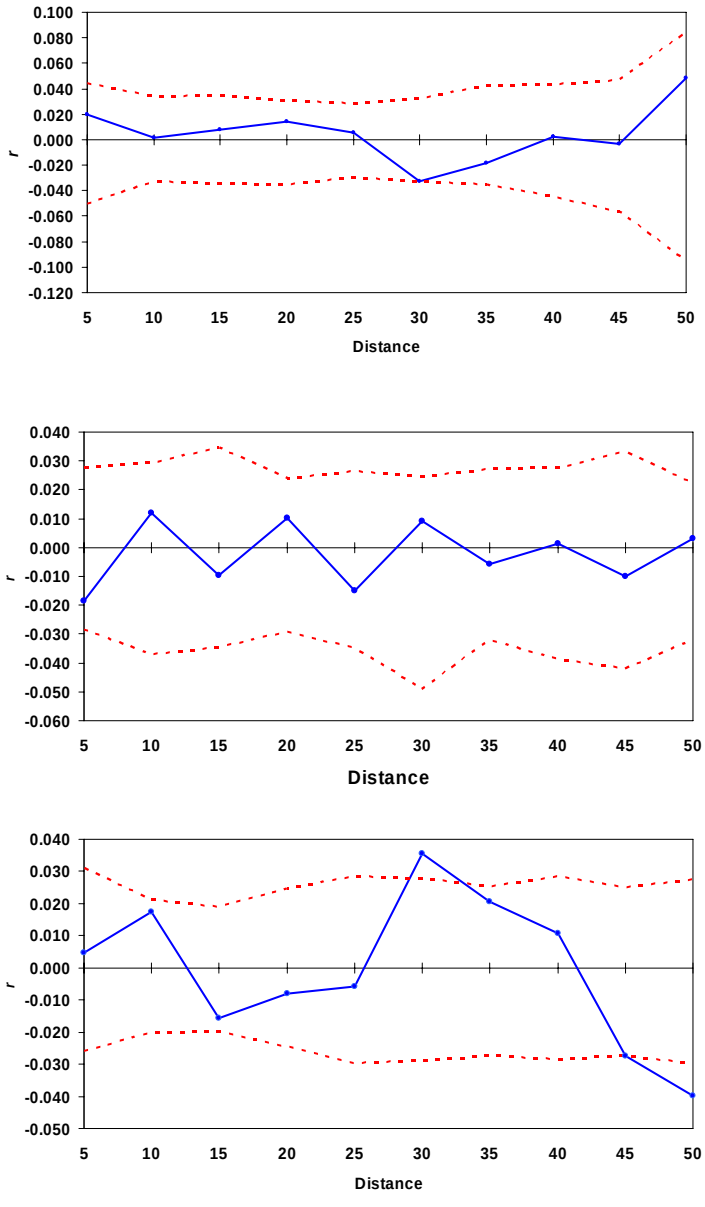


Figure 2: Spatial genetic autocorrelograms for seedlings, saplings and juveniles (top to bottom) of *Guaiacum sanctum* in subplot 2, PVNP, Costa Rica (Chapter 3).

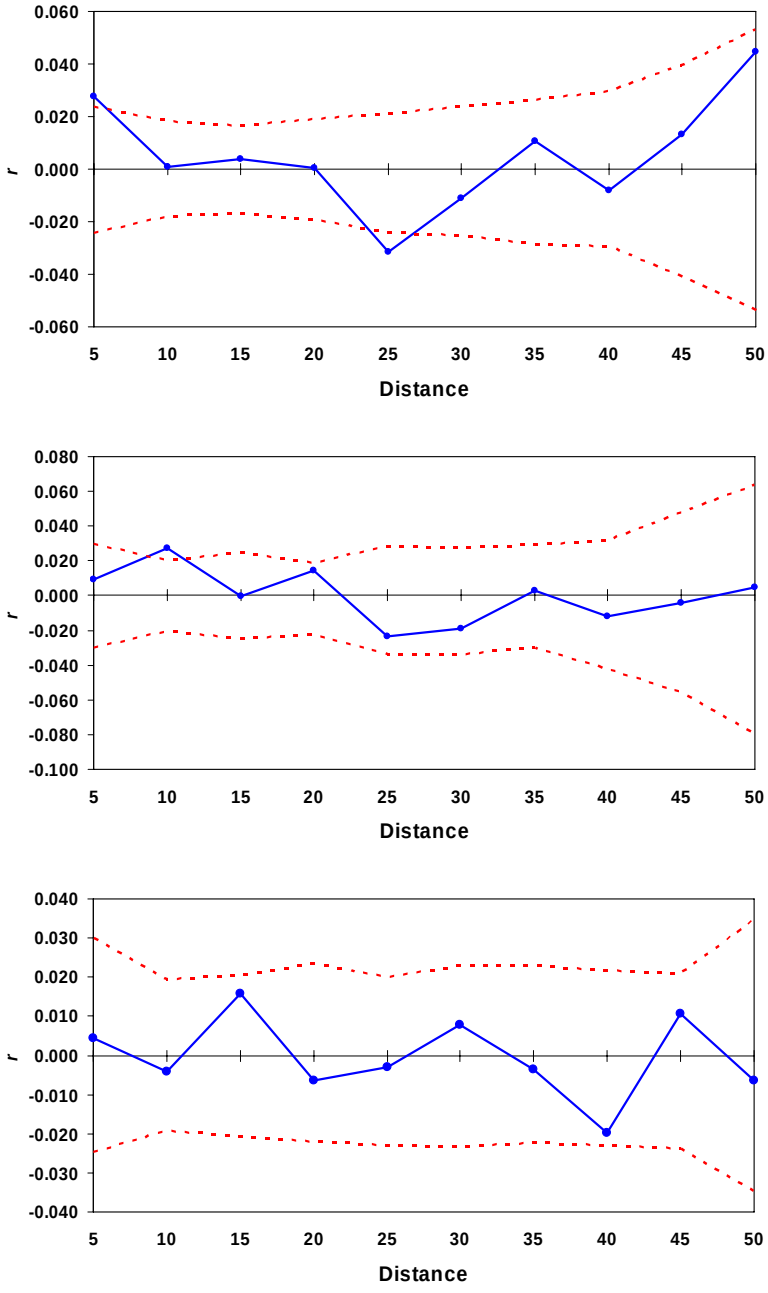


Figure 3: Spatial genetic autocorrelograms for seedlings, saplings and juveniles (top to bottom) of *Guaiacum sanctum* in subplot 3, PVNP, Costa Rica (Chapter 3).