

Breeding Population Size of a Fragmented Population of a Costa Rican Dry Forest Tree Species

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Pollen immigration can offset the effects of genetic drift and inbreeding in small populations. To understand the genetic consequences of forest fragmentation, estimates of pollen flow into remnant fragments are essential. Such estimates are straightforward for plants with singly sired, multiseeded fruits, since the pollen donor genotype for each fruit can be unambiguously reconstructed through full-sib genealogical analyses. Allozyme analyses were used to estimate pollen donor numbers from the progeny of fruits of the tropical dry forest tree *Enterolobium cyclocarpum* in a small (9.8 ha) fragmented population ($N = 11$) over three reproductive seasons (1994, 1995, and 1996). These analyses indicate that each tree receives pollen from many pollen donors. When data are pooled for the site, estimated maximum pollen donor pool sizes in all years exceed the number of individuals (56) in the 227 ha study area. Although unidentified pollen donors may be located as close as 250 m to the study trees, the number of unidentified pollen donors indicates that individuals in this forest fragment are part of a large network of reproductively active individuals.

Estimates of genetically effective population sizes are essential for predictions of the effects of genetic drift and inbreeding in small populations. In plant species with limited seed dispersal, pollen movement has the major influence on breeding population sizes (Hamrick and Murawski 1990). Determining the influence of pollen movement on effective population or neighborhood sizes requires detailed analyses of the breeding structure of populations (Hamrick and Nason 1996). Such analyses include the identity of pollen donors and their distance from the maternal plant. Thus mating patterns within populations, as well as pollen immigration into the population, must be quantified. Early studies followed pollinators (e.g., Levin and Kerster 1974) and concluded that most pollinations occurred among nearby individuals. Genetic markers, such as allozymes (Devlin et al. 1988; Godt and Hamrick 1993; Schnabel and Hamrick 1995) or microsatellites (e.g., Aldrich and Hamrick 1998; Chase et al. 1996) have been used to describe patterns of successful matings through the identification of paternal individuals. Such studies have shown that pollen can move considerable distances among plants within populations and that pollen gene flow rates between populations are often high (Broyles

et al. 1994; Dow and Ashley 1996; Hamrick et al. 1995; Schnabel and Hamrick 1995). Once the pollen donors are identified for an individual plant, it is possible to estimate the area they occupy, that is, the breeding population or neighborhood (Crawford 1984). When pollen is received from individuals outside the population, the effective breeding population may be larger than the area occupied by the recipient population (e.g., Nason et al. 1996, 1998). Effective breeding population sizes provide a useful ecological perspective on the spatial extent of plant breeding units and, indirectly, of pollinator behavior through genetically determined patterns of pollen deposition.

In plant species where seeds of individual fruits result from random mating events, each multiseeded fruit may have several pollen donors. In such species, estimates of pollen immigration rates or of relative male fertility improve with the number of seeds analyzed for a maternal individual, regardless of whether they are sampled from one or several fruits. In contrast, if a limited number of pollen donors is represented within a fruit (i.e., correlated mating), a single seed per fruit should be used to sample independent mating events. However, some plant groups, such as figs, milkweeds, orchids, and mimosoid

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legumes, have specialized pollination systems that usually result in fruits with a single pollen donor. These species represent special opportunities for paternity reconstruction, since by analyzing several full-sib progeny per fruit, the multilocus genotype of each pollen donor can be accurately inferred (e.g., Broyles and Wyatt 1990; Nason et al. 1996, 1998). Furthermore, if several polymorphic loci are available, each pollen donor within a local area should have a unique multilocus genotype, that is, the probability of paternal exclusion would be 100%.

The ability to identify a unique pollen donor for each fruit has several advantages over approaches that infer successful male gametes for individual seeds. First, since there is often overlap among potential pollen donors for the gametic genotypes they produce, analyses of full-sib progeny arrays greatly reduce paternal ambiguity within populations. Second, since full-sib paternity analyses greatly increase the probability of paternity exclusion, the ability to directly detect pollen immigration from outside the local population is improved, that is, cryptic pollen flow (Devlin and Ellstrand 1990) is greatly reduced. The main limitation of full-sib analyses is that the analysis of each mating event requires assaying several as opposed to a single offspring per fruit. It is usually feasible, however, to reconstruct paternity for enough singly-sired fruits to characterize the frequency distribution of pollen donor fertility for each maternal plant. Rarefaction procedures analogous to those used to estimate animal population sizes from capture-recapture data can then be used to estimate the total number of pollen donors within individual fruit crops from the observed distribution of pollen donors (Nason et al. 1998). By pooling the observed data across several reproductive adults, accurate estimates of population-level breeding parameters can be obtained.

In this study we use full-sib analyses of singly sired fruits to estimate the number of pollen donors for a small, fragmented population of the tropical dry forest tree *Enterolobium cyclocarpum* over three reproductive seasons (1994, 1995, and 1996). From pollen donor numbers, effective breeding population sizes for each of the three years are estimated.

Materials and Methods

Study Organism and Study Site

Enterolobium cyclocarpum (Jacq.) Griseb. (Mimosoideae: Fabaceae) is a hermaph-

roditic canopy tree commonly found throughout dry forest regions from central Mexico to northern Brazil (Janzen 1983). A large tree can produce hundreds of ear-shaped fruits that may contain as many as 22 seeds (Janzen 1981, 1983). Flowering usually occurs over a 3- to 4-week period toward the end of the dry season (March–April) and pollination is by hawkmoths, moths, bees, and beetles (Bawa K, personal communication; Frankie G, personal communication). Flowering is not completely synchronous, but there is usually considerable overlap in the flowering phenologies of individuals within populations (Apsit VJ, personal observation). Pollen grains are packaged into polyads of 32 associated monads (Guinet 1981), more than enough to fertilize the ovules of an individual flower. Genetic analyses have demonstrated that seeds in more than 99% of the pods are sired by a single pollen donor (Hamrick JL, unpublished data).

We selected the dry forest region of Guanacaste Province in northwest Costa Rica for our study area because the highly disturbed landscape is characterized by small, discrete, spatially isolated forest fragments containing small populations of *E. cyclocarpum*. The study site consisted of a core area containing trees from which fruits were sampled (designated “maternal” trees) surrounded by a border area in which conspecifics were mapped as potential pollen donors. The focal site for this study, FDL, is a 9.8 ha area containing 11 individuals (5 designated maternal trees and 6 additional potential pollen donors; Figure 1) located 7 km north of the town of Bagaces in a 1600 ha portion of the Stewart Ranch (10°33'N and 85°19'W). In addition to the FDL site, genotypes for 45 additional *E. cyclocarpum* occupying a 227 ha L-shaped area (Figure 1) of the Stewart Ranch were obtained. The nearest adult *E. cyclocarpum* within this area is approximately 300 m from the FDL site. Although trees have been identified at distances of 1.5 km from FDL on the east-west axis, the shorter north-south axis (450 m) indicates that 250 m is the greatest distance for unambiguous identification of pollen flow into FDL.

Sampling

Fifteen pods were obtained from each of the five designated maternal trees, if available, in 1994 and 1995. In 1996 only two of the five maternal trees produced fruit. Viable seeds were extracted from the pods, scarified with a file, and germinated in the Botany Department greenhouse facilities

at the University of Georgia. We germinated all the seeds of 15 pods from the 1994, 1995, and 1996 progeny arrays of each maternal tree in the FDL plot. Single seeds from a minimum of 15 pods of each of the 51 potential pollen donors were also germinated to infer adult genotypes in the absence of adult leaf tissue. Seedlings were harvested after 2–3 weeks and proteins were extracted from leaf tissue by pulverization in a few drops of “Camellia” buffer (Wendel and Parks 1982). The resulting slurry was absorbed onto 3 mm Whatman filter paper wicks and then stored at -70°C .

Electrophoretic Analysis

Twelve polymorphic loci were resolved for nine enzyme systems on 10% horizontal starch gels: system 4, *Isocitrate dehydrogenase* (*Idh1*) and *Phosphoglucosmutase* (*Pgm2*); a modified system 6, *Diaphorase* (*Dia1*), *Phosphoglucoisomerase* (*Pgi1* and 2); a modified system 8, *Aspartate aminotransferase* (*Aat1*), *Fluorescent esterase* (*Fe1*), and *Leucine aminopeptidase* (*Lap1*); and system 9, *Aconitase* (*Aco1* and 2) and *Malate dehydrogenase* (*Mdh1* and 2). The stain recipe for *Diaphorase* is from Cheliak and Pitel (1984), *Phosphoglucosmutase* is from Wendel and Weeden (1989), and the remaining seven enzyme stain recipes and the gel/electrode buffer designations are from Soltis et al. (1983).

Analytical Procedures

Pollen donor identification. A visual inspection of progeny arrays from each pod was made to ensure that the seeds from the pods were singly sired. Multilocus genotypes of the pollen donors were inferred from the genotypic arrays of a minimum of 6 (mean 12.1) seeds obtained from singly sired pods harvested from maternal individuals with known genotypes (Broyles and Wyatt 1990; Muona et al. 1991; Nason et al. 1996). Ambiguity can exist, for example, if both a mother and all the full-sib progeny within a fruit are homozygous for the same allele at a locus. Although it is possible that a heterozygous father produced this array of progeny genotypes, the probability of such an event is low (a maximum of $[0.5]^6$ or 0.0156) and we would designate the father as a homozygote at this locus. The probability of detecting only one of the two homozygous genotypes at a locus for which the mother and father are both heterozygous is higher but still low. With more progeny, these probabilities are lower. However, even with ambiguity at certain loci, the number

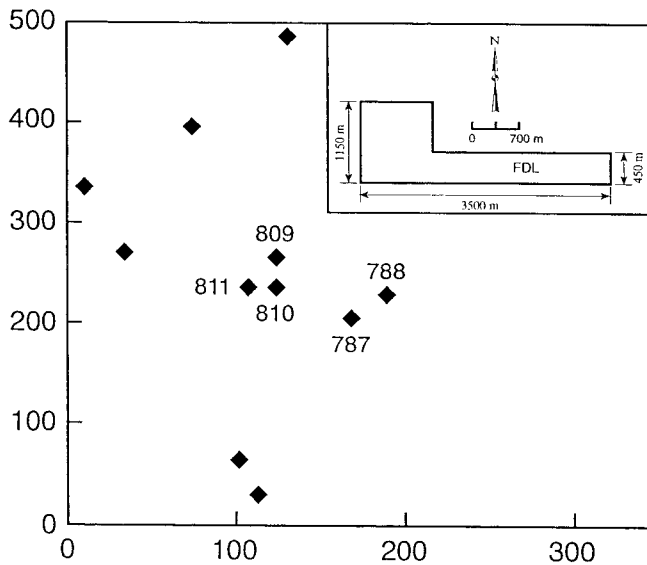


Figure 1. Map of the FDL site with the north-south orientation on the x axis. The five *E. cyclocarpum* designated as maternal individuals are numbered. The other six trees are included as additional potential pollen donors. Altogether there are 11 trees in 9.8 ha. The insert indicates the relative position of the FDL study site within the larger Stewart Ranch study area.

of available polymorphic loci made it possible to uniquely identify paternal genotypes. The resolving power of this procedure depends upon the number of polymorphic loci, the number of alleles at each polymorphic locus, and the evenness of allele frequencies. With $a(a + 1)/2$ possible genotypes per polymorphic locus (a = number of alleles) and 12 polymorphic loci, it is possible to distinguish more than two million unique genotypes. Based on the expected genotypes within our study site, the probability of any two individuals having the same multilocus genotype is 1.54×10^{-3} .

Pollen donor identities were determined for pods from seed parents with fruit in 1994, 1995, and 1996. A compilation of pollen donor identities generated a distribution of pollen donor success for the seed crops of each seed parent and for FDL when pooled over all seed parents. Comparisons of pollen donor genotypes with those of the 11 individuals within FDL provided estimates of the rates of pollen flow into FDL from outside this expanded area. We then included all genetically identified trees (56) within the 227 ha Stewart Ranch study site as potential pollen donors to estimate the rate of pollen immigration into FDL from outside of this expanded area.

Pollen donor number. The number of distinct pollen donor genotypes reconstructed from a sample of singly sired fruits provides a minimum estimate ($N_{d-\min}$) of the total number of pollen donors (N_d) siring offspring within a tree's fruit crop. The problem arises, then, when estimating N_d

from the observed distribution of pollen parents obtained from the sample of pods. Obviously the higher the proportion of the total fruit crop sampled, the more accurate an estimate the observed value $N_{d-\min}$ will be of N_d . However, since sampling an entire fruit crop or even a large proportion of the fruit crop of several adults is not feasible, estimation procedures must be employed to obtain accurate estimates of the total number of pollen donors. Intuitively, pollen donor distributions that feature considerable redundancy of pollen parents (i.e., a pollen donor is represented by several fruits) should give a lower estimate of N_d than a pollen donor array with little redundancy (i.e., each pod has a different pollen donor). The problem of estimating the total number of pollen parents from a sample distribution is analogous to that of estimating animal population sizes from capture-recapture data.

We therefore applied the estimation procedure of Burnham and Overton (1979) to calculate the total number of pollen donors contributing to an individual's fruit crop from the observed distribution of donor genotypes. This estimator was originally developed for recapture studies of marked populations and to estimate the number of species of a given taxonomic group within a community. Our application is therefore a logical extension of the Burnham and Overton model (Nason et al. 1996, 1998). For each fruit crop, the frequency distribution of donor genotypes was determined by full-sib paternity inference as described above. Total pollen donor number was then esti-

ated by applying the jackknife estimator of orders $k = 1$ to 5 to the data. We then tested the null hypothesis that there is no difference between the observed number of donors ($N_{d-\min} = \hat{N}_d^{(0)}$) and the expected value of the first-order jackknife estimate ($\hat{N}_d^{(1)}$), that is, test $\hat{N}_d^{(1)}$: $E[\hat{N}_d^{(1)} - \hat{N}_d^{(0)}] = 0$ versus the alternative hypothesis $H_a^{(1)}$: $E[\hat{N}_d^{(1)}] \neq 0$. If $H_0^{(1)}$ is rejected, $\hat{N}_d^{(1)}$ is interpreted as reducing the bias in the estimation of N_d and is preferred to $\hat{N}_d^{(0)}$.

Further reductions in the bias are also possible, however. As a result, the general procedure for choosing $\hat{N}_d^{(k)}$ involved testing the null hypothesis $H_0^{(k)}$: $E[\hat{N}_d^{(k+1)} - \hat{N}_d^{(k)}] = 0$ versus $H_a^{(k)}$: $E[\hat{N}_d^{(k+1)} - \hat{N}_d^{(k)}] \neq 0$ sequentially for $k \leq 4$ (Burnham and Overton 1979). We then chose $\hat{N}_d = \hat{N}_d^{(k)}$ such that $H_0^{(k)}$ was the first null hypothesis not rejected. For example, if $H_0^{(0)}$ and $H_0^{(1)}$ were rejected but $H_0^{(2)}$ was not, then we chose $\hat{N}_d^{(2)}$ as our estimate of N_d .

Higher-order jackknife estimates lead to successively greater reductions in the bias of the estimates of N_d , but at the cost of increasing sampling variance (Burnham and Overton 1979). Conversely, for any fixed value of k , the sampling variance will decrease as the sampling effort increases. Given the number of full-sib progeny that must be genotyped to accurately reconstruct the genotype of the paternal parent, no more than 15 singly sired fruit were assayed per tree. Thus, although the bias in the jackknife estimate may be relatively small, because of our small sample sizes the variance of the estimates is expected to be relatively large.

Results

Configuration of the FDL trees (Figure 1) met our requirements of a maternal core (trees 787, 788, 809, 810, and 811) surrounded by a border of six additional potential pollen donors at varying distances. Data from pods that produced at least six seedlings were included in this study. The vast majority of pods had considerably more seeds, however, for example, 783 seeds from 62 pods (12.6 seeds/pod) in 1994, 721 seeds from 56 pods (12.9 seeds/pod) in 1995, and 193 seeds from 18 pods (10.7 seeds/pod) in 1996.

Pollen Donor Number

Most pollen donors fathered a single pod, but a few sired two or three pods within an individual fruit crop (Table 1, Figure 2). Some pollen donors also sired fruits on several trees, hence the observed number of pollen donors summed over individual

Table 1. The number of singly-sired fruits analyzed (*N*), the observed number of unique pollen donor genotypes (OPD) inferred from full-sib progeny arrays, and the estimated total number of pollen donors (EPD) and standard error (SE) for five *E. cyclocarpum* maternal individuals of study site FDL over 3 years

Tree	1994			1995			1996		
	<i>N</i>	OPD	EPD (SE)	<i>N</i>	OPD	EPD (SE)	<i>N</i>	OPD	EPD (SE)
787	13	12	50 (15.8)	14	13	55 (16.4)	NF	NF	NF
788	14	11	36 (10.8)	12	9	15 (3.5)	5	5	IS
809	7	7	IS	15	14	60 (17.0)	NF	NF	NF
810	13	11	27 (7.3)	3	3	IS	NF	NF	NF
811	15	13	83 ^a	12	11	38 (11.2)	13	10	32 (10.2)
Plot	62	50	246 ^a	56	46	175 (30.8)	18	15	80 ^a

^a Minimum estimate.

NF = no fruit analyzed; IS = insufficient sample size and/or distribution to analyze.

See the Materials and Methods section for the protocol used to calculate the EPD.

trees is greater than the number of pooled pollen donors. For example, in 1994, 54 unique pollen donors were identified when each maternal tree was analyzed separately, but only 50 pollen donors were identified when fruit crops were pooled across the five trees. In some cases the number of unique pollen donor genotypes (*PD*) equaled the number of fruits analyzed (*N*), that is, tree 809 in 1994 ($N = PD = 7$), tree 810 in 1995 ($N = PD = 3$), and tree 788 in 1996 ($N = PD = 5$). Although such distributions do not allow estimates of the total number of pollen donors per tree (see above), they contribute data to the number of pooled pollen donors.

Most pollen donors sired a single pod within the pooled fruit crops during each of the three years (Table 1, Figure 2). The most pods attributed to the same pollen donor was five (by tree 787) in 1994 (three pods sired on tree 788 and two on tree 810). The next most successful pollen donor was tree 811, with four pods sired in 1994 (two each on trees 788 and 810) and four pods in 1995 (two each on trees 788 and 809). The greatest number of pods sired by the same pollen donor within an individual maternal tree was three (on tree 811 in 1994 and 1996, and on tree 788 in 1994). Multiple fruits within a maternal tree sired by the same pollen donor occurred most often on tree 788 (two pollen donors with multiple fruits in 1994 and three pollen donors with multiple fruits in 1995). Relatively few trees within the 227 ha Stewart Ranch study area contributed pollen to the FDL maternal trees during the study period: 6 of 50 pollen donors in 1994; 7 of 46 pollen donors in 1995; 4 of 15 pollen donors in 1996.

Pollen Immigration

Pods sired by genotypes different from the FDL trees represent pollen flow events. The proportion of pods representing pollen flow events provides minimum estimates of pollen immigration for individual trees and for the FDL site (Table 2). Estimates of pollen flow from beyond the FDL boundary for individual trees ranged from 64.2% to 100% in 1994, 66.6% to 100% in 1995, and 61.5% to 80.0% in 1996 (mean = 74.1%, 71.4%, and 69.2%, respectively). Estimates of pollen flow remained high (>60%; Table 2) when pollen donors within the 227 ha study area were included in the analyses.

Breeding Population Size

The Stewart Ranch study area, which includes three forest fragments and 14 iso-

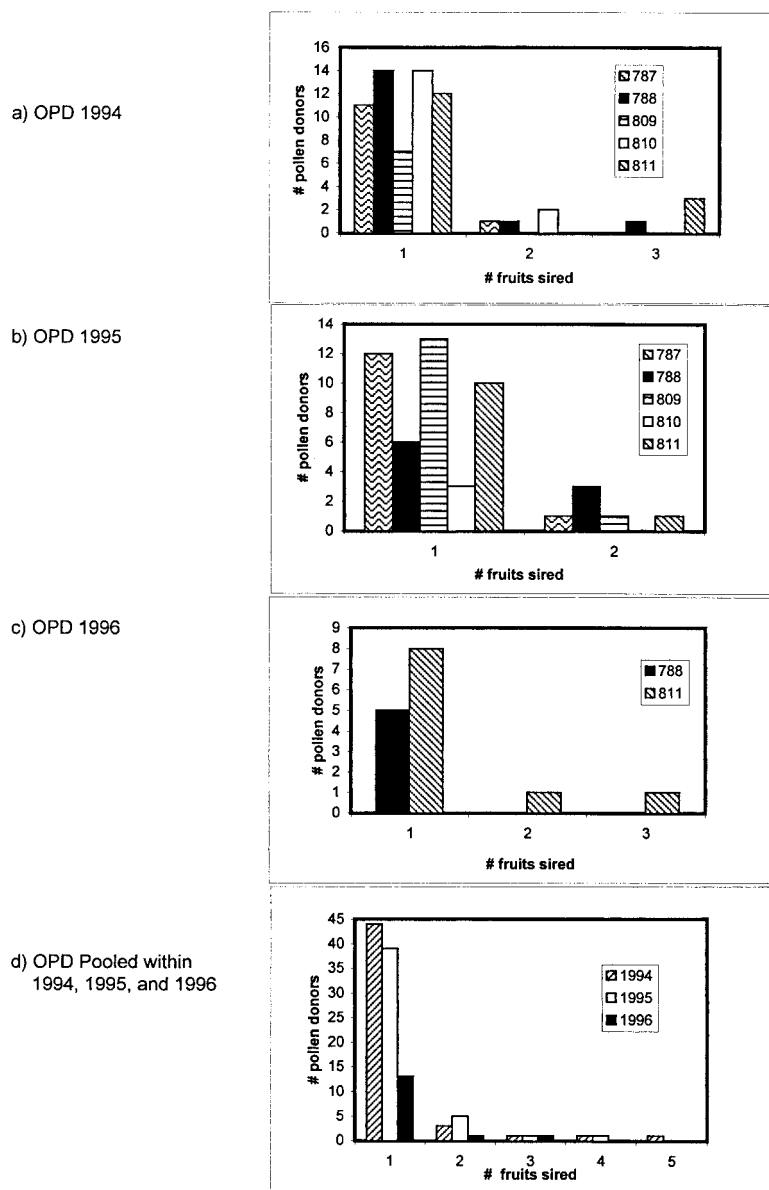


Figure 2. Observed frequency distribution of pollen donor genotypes (OPD) represented in the seed crops of each of five *E. cyclocarpum* maternal individuals in the FDL plot: (A) 1994; (B) 1995; (C) 1996; (D) pooled over FDL in 1994, 1995, and 1996. Pod numbers analyzed vary among trees and years.

Table 2. Estimates of pollen flow rates over two spatial scales for the five *E. cyclocarpum* FDL seed parents: from beyond the FDL site (FDL) and from beyond the 227 ha Stewart Ranch study area (STR)

Tree	1994		1995		1996	
	FDL	STR	FDL	STR	FDL	STR
787	76.9	61.5	92.8	92.8	NF	NF
788	64.2	64.2	66.6	66.6	80.0	60.0
809	100.0	100.0	73.3	73.3	NF	NF
810	69.2	69.2	100.0	66.6	NF	NF
811	100.0	93.3	100.0	91.6	61.5	61.5
Pooled	74.1	69.3	71.4	67.8	69.2	61.5

NF = no fruits analyzed due to the lack of a fruit crop in 1996.

lated *E. cyclocarpum*, covers approximately 227 ha and contains 56 adults, for an average density of one *E. cyclocarpum* per 4.05 ha. Breeding population areas were estimated for each maternal tree and for the whole plot by multiplying the observed (minimum estimate) and total estimated pollen donor numbers by the number of hectares per tree (4.05) (Table 3). Breeding areas of individual maternal trees estimated from the observed number of pollen donors all exceed the 9.8 ha area of the FDL plot, as do breeding size estimates when pollen donor numbers are pooled for FDL (Table 1). When maximum pollen donor population size estimates are pooled over the study plot, estimated neighborhood areas exceed the 227 ha of the Stewart Ranch study area in all three years. These areas produce radii of 1770 m, 1493 m, and 1010 m in 1994, 1995, and 1996, respectively. Thus pollen dispersal distances of 1500 m may be relatively common for *E. cyclocarpum*.

Discussion

Pollen donor identification by means of full-sib analyses indicates that a large number of individuals contribute pollen to the FDL trees and that pollen flow rates into the site are consistent over the 3 years of this study. Undoubtedly the large number of pollen donors observed is primarily due to the presence of trees at moderate distances (up to at least 2 km) from FDL that were outside the 227 ha study area. The location of FDL within this L-shaped study area allowed genetically unknown trees as close as 250 m to the FDL core site to go undetected. Unequal fruit numbers analyzed per tree make comparisons of pollen donor numbers among individual maternal trees difficult, but position within FDL does not seem to affect pollen donor numbers. For example,

Table 3. Estimates of the breeding population area (ha) containing the observed number of *E. cyclocarpum* pollen donors (OPD) and the area containing the estimated number of pollen donors (EPD) in 1994, 1995, and 1996

Tree	1994		1995		1996	
	OPD	EPD	OPD	EPD	OPD	EPD
787	49	202	53	223	NF	NF
788	45	146	36	61	20	IS
809	28	IS	57	243	NF	NF
810	45	109	12	IS	NF	NF
811	53	336	45	154	40	130
Pooled	202	996	186	709	61	324

Estimates were based on an average density of one tree per 4.05 ha estimated over the 227 ha Stewart Ranch study area.

NF = no fruit analyzed; IS = insufficient sample size and/or distribution to analyze.

tree 809 located in the middle of FDL received pollen from as many donors as tree 788 located on the edge. Estimated total pollen donor population sizes, if extrapolated from the few sampled fruits to the many hundreds or even thousands of fruits produced by a single tree, suggest large breeding population sizes for each maternal tree.

Genealogical reconstruction of pollen donor genotypes from full-sib progeny arrays removes the ambiguity inherent in standard paternity analyses and provides direct estimates of pollen immigration rates. Estimates of pollen movement based on the present analyses indicate substantial pollen immigration for all maternal trees. High pollen immigration rates may be due, in part, to the lengthy flowering period and overlap in flowering phenologies among individuals, especially toward the end of the dry season when resources are scarce and the availability of floral rewards may draw a variety of pollinators (but see Nason et al. 1996). Estimates of total pollen flow into FDL based on these analyses (74%, 71%, and 69%, respectively, for the three years) are, however, in general agreement with standard paternity analysis estimates of total pollen flow rates for the FDL plot in 1994 and 1995 (87% and 88%) (Apsit et al., in preparation). Similar analyses of five additional forest fragments located in Guanacaste Province also indicate that more than 75% of the pollinations were due to pollen immigration (Apsit et al., in preparation). Finally, full-sib analyses of a four-tree site in southern Costa Rica over a 2-year period indicated a pollen flow rate of 58% (Hamrick JL and Aldrich PR, unpublished data).

Our data indicate that breeding neighborhoods of individual maternal trees ex-

tend well beyond the FDL plot and that, far from being isolated, this remnant population is part of a large network of reproductively interacting individuals. Thus the landscape disturbance that has occurred in the area surrounding FDL has not produced barriers to pollen movement among forest fragments. In fact, there is some evidence that clusters of *E. cyclocarpum* adults located within relatively undisturbed forests may have smaller breeding populations than trees located in highly disturbed habitats (Hamrick JL, unpublished data).

Consistent with our results, James et al. (1998) demonstrated that a single *Cedrela odorata* (moth-pollinated) individual isolated by 300 m in a pasture set a full complement of outcrossed fruit. In addition, estimates of minimum estimated breeding population size ($N = 574$) and breeding population area (79.7 km²) for a single *Ficus popenoei* (wasp-pollinated) tree in Panama suggest that pollen is exchanged among many trees over a large area (Nason et al. 1996). In contrast, in the Coto Brus region of southern Costa Rica, two *Symphonia globulifera* trees (hummingbird-pollinated, bat dispersed) in a nearby pasture contributed nearly 50% of the seedlings established in a nearby fragment (Aldrich and Hamrick 1998). Thus species that share natural history traits (e.g., pollinator types and seed dispersal mechanisms) may have similar patterns of gene movement, while species with different natural history traits may have very different gene movement patterns in fragmented tropical landscapes.

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