

# Pollen dispersal of tropical trees (*Dinizia excelsa*: Fabaceae) by native insects and African honeybees in pristine and fragmented Amazonian rainforest

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## Abstract

Tropical rainforest trees typically occur in low population densities and rely on animals for cross-pollination. It is of conservation interest therefore to understand how rainforest fragmentation may alter the pollination and breeding structure of remnant trees. Previous studies of the Amazonian tree *Dinizia excelsa* (Fabaceae) found African honeybees (*Apis mellifera scutellata*) as the predominant pollinators of trees in highly disturbed habitats, transporting pollen up to 3.2 km between pasture trees. Here, using microsatellite genotypes of seed arrays, we compare outcrossing rates and pollen dispersal distances of (i) remnant *D. excelsa* in three large ranches, and (ii) a population in undisturbed forest in which African honeybees were absent. Self-fertilization was more frequent in the disturbed habitats (14%,  $n = 277$  seeds from 12 mothers) than in undisturbed forest (10%,  $n = 295$  seeds from 13 mothers). Pollen dispersal was extensive in all three ranches compared to undisturbed forest, however. Using a TWOGENER analysis, we estimated a mean pollen dispersal distance of 1509 m in Colosso ranch, assuming an exponential dispersal function, and 212 m in undisturbed forest. The low effective density of *D. excelsa* in undisturbed forest ( $\sim 0.1$  trees/ha) indicates that large areas of rainforest must be preserved to maintain minimum viable populations. Our results also suggest, however, that in highly disturbed habitats *Apis mellifera* may expand genetic neighbourhood areas, thereby linking fragmented and continuous forest populations.

**Keywords:** *Apis mellifera*, microsatellites, pollen flow, pollination, tropical rainforest, TwoGener analysis

Received 21 June 2002; revision received 20 November 2002; accepted 20 November 2002

## Introduction

Lowland tropical rainforests are unparalleled in both their species richness and in the complexity of their ecological interactions. In species-rich Amazonian forests nearly 300 species of trees may be found in a single hectare (Phillips *et al.* 1994; Romoleroux *et al.* 1997; De Oliveira & Mori 1999) and over 1000 species in 50 hectares (Condit *et al.* 2000). In such forests, where canopy trees typically occur in population densities of less than one individual per hectare (Hubbell & Foster 1983), self-fertilization is uncommon

because of the prevalence of dioecy and self-incompatibility mechanisms (Bawa *et al.* 1985), and high genetic loads that promote inbreeding depression (Alvarez-Buylla *et al.* 1996). Insects, especially bees, are the primary pollinators of most tropical rainforest trees (Bawa 1990). Bats and hummingbirds are also important pollinators but, in contrast with northern temperate forests, wind pollination is virtually absent.

Tropical forests may be experiencing the greatest challenge to their ecological resilience since the Cretaceous/Tertiary boundary [ $\sim 65$  million years ago (Ma)] when the impact of a meteor decimated most tropical forests (Morley 2000; Vajda *et al.* 2001) and disrupted important plant–insect interactions for several million years (Labandeira *et al.* 2002). The current deforestation crisis has nearly run

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to completion in several parts of the world, such as Indonesia and coastal Brazil. The Amazon basin harbours half of the world's remaining lowland rainforests, but is experiencing the highest absolute rates of deforestation, averaging 3–4 million hectares per year (INPE 2001). Amazonian deforestation usually produces a network of forest patches embedded in agricultural habitat or secondary vegetation, which may provide seed for forest regeneration. The conservation value of rainforest fragments depends on the ability of forest dwelling animals and plants to persist in them in spite of population bottlenecks, over-harvesting and demographic stochasticity (Lande 1988; Laurance *et al.* 2001).

Rainforest fragmentation can drive locally rare plants to extinction through area sampling effects (Hubbell & Foster 1983), secondary logging (Gullison *et al.* 1996) and edge effects (Laurance *et al.* 2002) that act on adult trees (Laurance *et al.* 2000) and seedlings (Bruna 2002). Forest fragmentation impinges on plant reproduction by altering the species composition and behaviour of pollinators (Aizen & Feinsinger 1994a; Law & Lean 1999; Steffan-Dewenter & Tschamtker 1999; Dick 2001a). Pollinator limitation can lower the fecundity of host plants (Levin 1995; Ghazoul *et al.* 1998; Robertson *et al.* 1999; Cunningham 2000) and increase levels of inbreeding (Murawski *et al.* 1994; Aldrich & Hamrick 1998; Lee 2000; Dick 2001a). In some cases, however, habitat disturbance seems to enhance pollinator activity and may even promote fecundity and gene flow (Young *et al.* 1996; Law & Lean 1999; Dick 2001a; Roubik 2002; White *et al.* 2002).

Many pollen flow studies of trees in Neotropical mosaic landscapes have found unexpectedly high levels of gene flow (reviewed in Nason & Hamrick 1997). This may be a result of the spatial dispersion of the remnant trees, which may cause pollinators to bypass neighbouring plants (Chase *et al.* 1996; Stacy *et al.* 1996). Or it may be because of shifts in pollinator composition, as when feral honeybees replace native pollinators in disturbed habitats

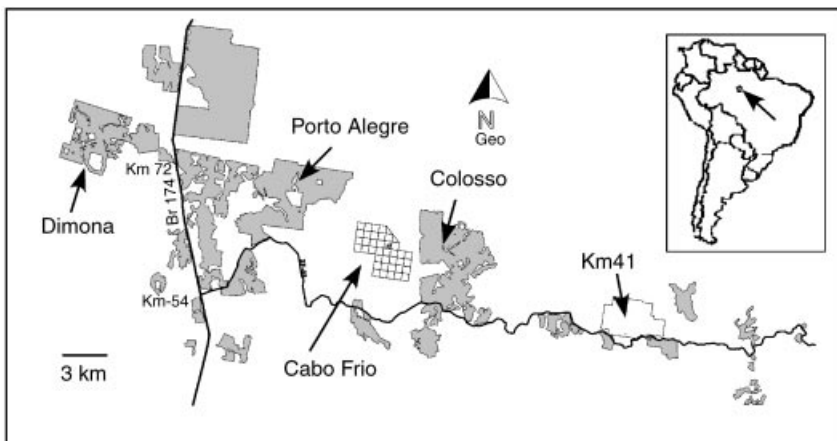
(Aizen & Feinsinger 1994b; Dick 2001a). Few studies have attempted to pinpoint the ecological causes of high gene flow in disturbed habitats, however, or provide comparisons of breeding patterns in continuous and fragmented populations.

In this study we used a TWOGENER analysis (Smouse *et al.* 2001) to estimate pollen dispersal in fragmented and contiguous forest populations of the Amazonian tree *Dinizia excelsa*. TWOGENER treats maternal trees as pollen traps (Sorensen 1972) and uses the differences in allele frequencies among progeny arrays to calculate the parameter  $\Phi_{FT}$ , which is analogous to Wright's  $F_{ST}$  and, in conjunction with an estimate of the local population density, can be used to estimate the average distance of pollen flow and the shape of the dispersal curve. A distinct advantage of TWOGENER over paternity inference is that the potential pollen donors need not be known. Our study populations differ in their spatial configurations, the kind of vegetation in which they are embedded, and the kinds of pollinators they attract. African honeybees (*Apis mellifera scutellata*) acted as the primary pollinators of trees in disturbed habitats during the study period, while undisturbed forest trees were visited exclusively by native stingless bees and small beetles (Dick 2001b). This experimental system allowed us to investigate the synergistic effects of habitat fragmentation and pollinator dynamics on pollen dispersal in these trees.

## Materials and methods

### Study site

The study was conducted in the reserves of the Biological Dynamics of Forest Fragments Project (BDFFP) (Lovejoy & Bierregaard 1990), located 70 km north of Manaus, Brazil (2°30'S, 60°W, Fig. 1). The BDFFP reserves are located in upland forest with a hilly topography that ranges from 50 m to 150 m in elevation. The reserves are spread over



**Fig. 1** Reserve system of the Biological Dynamics of Forest Fragments Project (2°30'S, 60°W). Adult *Dinizia excelsa* ( $\geq 40$  cm d.b.h.) were mapped in the three ranches shown (shaded), and in continuous forest (unshaded) of Cabo Frio, Km41 and the Ducke reserve (not shown, located ~70 km to south). The embedded white squares represent forest fragments.

three large cattle ranches (Dimona, Porto Alegre and Colosso), which contain isolated rainforest fragments of 1, 10 and 100 ha, in addition to gallery forests, abandoned pasture and actively grazed pastures. The BDFFP also maintains research sites in undisturbed forest (Cabo Frio and Km41) adjacent to the ranches. The undisturbed forests contain many large timber trees, with some individuals over 1000 years old (Chambers *et al.* 1998), and they harbour a high diversity of tree species, with over 1000 species [ $\geq 10$  cm diameter at breast height (d.b.h.)] documented in 67 ha (W. Laurance, personal communication). An intensive inventory of trees ( $\geq 10$  cm d.b.h.) in three single-hectare plots at the Km41 site found 513 species, from 181 genera and 58 families (De Oliveira & Mori 1999).

The ranches harbour several classes of secondary vegetation (Williamson & Mesquita 2001). Colosso was the most actively grazed ranch during the study period (1995–9), and contains mostly pastures and small patches of diverse secondary vegetation. In Dimona, the abandoned pastures have been burned frequently and support dense stands of the pioneer tree *Vismia* (Clusiaceae), which can propagate vegetatively (Mesquita *et al.* 2001). The third ranch, Porto Alegre, has no history of fire, and its abandoned pastures are dominated by the pioneer tree *Cecropia* (Cecropiaceae) which forms a nearly continuous canopy about 10 m in height. These vegetative matrices may affect pollinators in as yet undocumented ways. The *Vismia* in Dimona ranch, for example, produce copious resin that is used for nest building by native stingless bees, while *Cecropia*, pollinated by bats, produces few rewards for insect pollinators.

### Study species

*Dinizia excelsa* Ducke, an Amazonian endemic, is one of the largest South American trees, reaching 55 m in height (roughly 20 m above the mean canopy height) and 2 m in girth (Ducke 1922) (Fig. 2). It is an economically important species which accounts for about 50% of hardwood sales in central Amazonia (Barbosa 1990). With only a single species, *Dinizia* is considered one of the most isolated genera in the Legume family, occupying an intermediate phylogenetic position between the Mimosoid and Caesalpinoid subfamilies (Herendeen & Dilcher 1990). Fossilized leaves, flowers and pods with close affinities to *D. excelsa* have been discovered in Eocene deposits (c.a. 54 Ma) in the southeastern USA (Herendeen & Dilcher 1990), implying great age and a complex biogeographic history of the species.

*Dinizia excelsa* is diploid ( $2n = 28$ ) (Lewis & Elias 1981). The hermaphroditic flowers are small (calyx 1–1.5 mm), green-yellow, scented, and occur in terminal racemes (10–18 cm). Although individual flowers last only a few days,



Fig. 2 *Dinizia excelsa* (foreground) left standing as a shade tree in a pasture landscape. This tree is Col.06 (see also Fig. 3). Cattle can be seen near the base of the tree.

fresh flowers appear daily on different inflorescences, thus individual trees may bloom for up to one month. Field observations performed in 1995 indicated that most reproductive trees in the study sites experienced temporal overlap in flowering (Dick 2001b). The seeds of *D. excelsa* ripen after 9 months inside indehiscent, wind-dispersed pods, which contain an average of three seeds per pod. Parrots and macaws eat the unripe seeds, and many seeds are lost to predation by beetles (*Amblocerus* spp.) (Dick 2001b). *Dinizia excelsa* seedlings are able to establish and grow in abandoned pasture (Dick 2001a).

Canopy observations revealed that stingless bees (tribe Meliponini) are important pollinators of *D. excelsa* in the continuous and fragmented forests of the BDFFP (Dick 2001a,b). In forest fragments and pasture habitats of Colosso, however, African honeybees far outnumbered native bees and were often the only insect pollinators in pasture trees (Dick 2001a,b). Relatively few African honeybees were observed in the 100-ha fragments of Porto Alegre and Dimona, and they were absent from the densely flowering trees of Km41 during the observation periods, reflecting the preference of feral honeybees for disturbed habitats (Roubik 1989). Diverse small beetles (2–5 mm in length) were observed on the flowers of *D. excelsa*, but their movements were confined to individual inflorescences (Dick 2001b). Small beetles, which are important pollinators of some tropical trees (e.g. Armstrong & Irvine 1989) were absent from the isolated pasture trees in Colosso, suggesting that they may be vulnerable to habitat disturbance.

### Mapping

*Dinizia excelsa* are commonly left standing in pastures because of their value for both timber and shade. Adult trees ( $\geq 40$  cm d.b.h.) were surveyed and mapped in gallery forest, isolated reserves ('fragments') and continuous forest ('Cabo Frio' and 'Km 41') of the BDFFP, in addition to continuous forest at Reserva Ducke, located approximately 70 km south of the BDFFP. Surveys in the fragments were made along perpendicular transects at ~50-m intervals, while the continuous forest surveys followed a 100 × 100 m (Km41) and 500 × 500 m (Cabo Frio) network of trails. We used measuring tape to mark *x*-*y* co-ordinates to the nearest metre in Km41 and Colosso. We drew maps from satellite images to infer distances between remnant trees in Dimona and Porto Alegre.

### DNA extraction and genotyping

Leaves were collected from adult trees using a slingshot, or from recently fallen branches, and stored in silica gel prior to DNA extraction. The Km41 leaf samples were stored for several months in Brazil while permits were being processed. During this time, the DNA had become degraded enough to produce inconsistent genotypes, so we have excluded the Km41 adult genotypes from our analyses. To obtain seed arrays, we gathered over 50 indehiscent pods from the ground below each maternal tree. The seeds from each family (approximately 150 per family) were mixed in a plastic bag, from which 24 seeds were arbitrarily selected for genetic analyses. Two trees in Porto Alegre (PA2.1 and PA2.2) and two trees in Km41 (41.03 and 41.21) produced few pods and fewer than 24 seeds were available; nevertheless these seed arrays also showed high levels of genetic variation and represented half-sib relationships (see Table 1). Prior to DNA extraction, each seed was scarified with a hot dissecting needle and soaked overnight in 37 °C water to induce growth of the cotyledons. DNA was extracted from the expanding cotyledons of 596 seeds collected from 24 maternal trees (~24 seeds/tree) using the DNeasy kit (Qiagen corporation).

Genotypes were scored at five microsatellite loci using primers DE27, DE37, DE44, DE48 and DE54 (Dick & Hamilton 1999). Two additional primer pairs were developed (DE28 and DE53) to score a subset of progeny arrays. The clone sequence of DE28 contained the compound repeat CA<sub>15</sub>TA<sub>13</sub> (GenBank AY172333) and the size range of the polymerase chain reaction (PCR) product was 138–228 base pairs (bp). The DE53 clone contained the repeat CT<sub>23</sub>AT<sub>12</sub> (GenBank AF143985) and the alleles ranged in size from 118 to 146 bp. The primer sequences follow: DE28F, 5'-ATA TAG CTA CAC GCG TGC; DE28R, 5'-GCT ATA GAR ACC YAG AGG; DE53F, 5'-CAA GGG CCA

**Table 1** Progeny analysis illustrated with the seed family of tree PA2.1

Locus	37a	37b	44a	44b	48a	48b	53a	53b	54a	54b
<i>PA2.1A</i>	120	126	146	148	145	153	119	145	133	157
S01	120	126	<b>144</b>	146	145	<b>165</b>	<b>127</b>	145	133	<b>171</b>
S02	120	126	146	148	<b>133</b>	145	119	119	157	<b>171</b>
S03	<b>122</b>	126	<b>144</b>	146	145	<b>169</b>	119	<b>133</b>	<b>145</b>	157
S04	126	126	146	148	<b>133</b>	145	145	145	157	<b>171</b>
S05	126	126	146	148	<b>133</b>	145	119	<b>127</b>	133	<b>153</b>
S06	126	126	146	148	<b>133</b>	145	<b>127</b>	145	133	<b>151</b>
S07	126	126	<b>144</b>	146	<b>133</b>	145	n/a	n/a	<b>153</b>	157
S08	120	126	146	148	145	<b>165</b>	119	<b>127</b>	<b>153</b>	157

The italicized adult genotype may be inferred from the homozygous seed genotypes. Note the first allele of locus 37 could be either 120 or 126. The nonmaternal alleles in the seeds (in bold) were used to score outcross events. In this seed array, the loci DE27 and DE28 did not contain any nonmaternal alleles. The four distinct nonmaternal alleles for locus DE54, however, imply at least two different outcross pollen donors for this seed array. As this tree occurred in a 10-ha fragment with only one other adult tree (see Fig. 5) pollen must have been transported over at least 700 m through stands of secondary vegetation (*Cecropia* spp.) to reach the study tree.

AAG TGT ATA TTT G; DE53R, 5'-GGA ACT ACT TGA AGT CTC CC.

The PCR cocktail (10.0 µL total) contained 250 µM of each dNTP, 25 mM of MgCl<sub>2</sub>, 1.25 units of *Taq* polymerase (Qiagen Corporation), and 0.5 µM of each primer. The PCR was performed on an MJ Research PTC-200 thermal cycler using the following protocol: 5 min at 94 °C; 25 cycles of 45 s at 94 °C, 1 min at 55 °C, and 30 s at 72 °C; ending with 15 min at 72 °C. The amplification products were electrophoresed with a Rox 400 size standard (Applied Biosystems Incorporated, ABI) on an ABI 377 automated sequencer and later on an MJ Research Base Station. We ran the same PCR products on both machines to verify the consistency of allele size estimation. Allele sizes were scored using the program Cartographer (MJ Research).

### Outcrossing and paternity inference

The multilocus outcrossing rate ( $t_m$ ) was calculated directly for each maternal family as the proportion of seeds that contained nonmaternal alleles. This was done by visual inspection of the genotypes. The minimum number of sires for each seed family was estimated by dividing the number of paternal alleles at the most variable locus by two, because the species is diploid. The multilocus genotypes of seeds from the isolated tree PA2.2 are presented in Table 1 to illustrate how these calculations were performed. We also used a maximum likelihood

approach to estimate the outcrossing rate, based on the mixed mating system model of Ritland & Jain (1981) and implemented in the program MLTR (Ritland 2002). The mixed mating system model assumes that a portion  $t$  of a seed family is comprised of outcrossed seeds, with  $s$  ( $= 1 - t$ ) representing the proportion of selfed seeds, and that the pollen alleles are received in proportion to their frequencies in the population. We divided the seed arrays into two groups: (i) continuous forest, including seeds from Km41, Cabo Frio and Ducke Reserve, and (ii) disturbed habitats, including seeds from trees located in forest fragments and pastures in Colosso, Dimona and Porto Alegre ranches. We calculated standard errors for the multilocus outcrossing rate  $t_m$  using 100 bootstraps. In Colosso ranch, we used paternity inference to match seeds with fathers on the basis of multilocus segregation probabilities calculated by the program CERVUS 2.0 (Marshall *et al.* 1998). The results of the paternity inference at Colosso ranch are described in detail elsewhere (Dick 2001a).

#### TWOGENER analysis

We performed a TWOGENER analysis, as introduced by Smouse *et al.* (2001), on the progeny arrays of Km41 and Colosso ranch. The principle of this method is to estimate  $\Phi_{FT}$ , the differentiation of allelic frequencies among the pollen pools sampled by several females in the population. The relation between  $\Phi_{FT}$  and dispersal distance has been derived for given dispersal curves (Austerlitz & Smouse 2001), allowing the development of several estimates of pollen dispersal (Austerlitz & Smouse 2002). Some of the estimates are based on the global  $\Phi_{FT}$  measured on all the females of the population. These can only provide an estimate of the pollen dispersal distance ( $\delta$ ) assuming a dispersal curve and a density of reproducing adults ( $d$ ) in the landscape. We tested here the normal and exponential dispersal functions and used the observed adult density for each population. Also the pairwise  $\Phi_{FT}$  between all females in the population can be computed to design pairwise estimates that allow one to jointly infer several parameters. They assume that the pollen dispersal curve is an exponential power function with parameters  $a$  and  $b$ :

$$p(a, b; x, y) = \frac{b}{2\pi a^2 \Gamma(2/b)} \exp\left(-\left(\frac{\sqrt{x^2 + y^2}}{a}\right)^b\right), \quad (1)$$

where  $\Gamma$  is the classically defined gamma function (Abramowitz & Stegun 1964). The parameter  $b$  is the shape parameter affecting the tail of the dispersal function and  $a$  is a scale parameter homogeneous to a distance (Clark 1998). For  $b = 2$ , it corresponds to the bivariate normal distribution, and so the relation between  $a$  and the classical

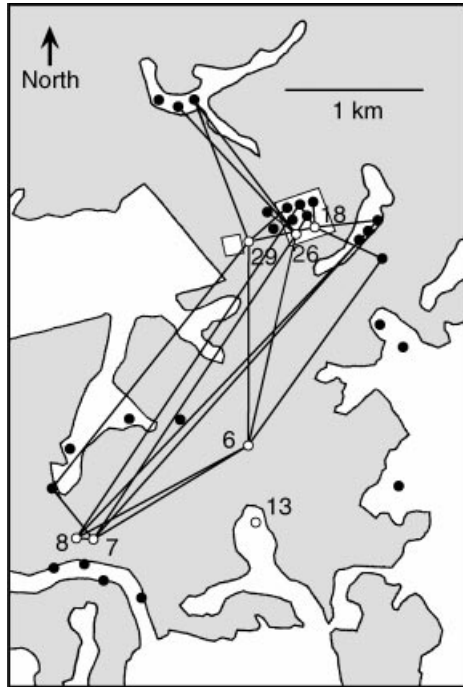
$\sigma$  parameter is the following:  $a = \sqrt{2}\sigma$ . For  $b = 1$ , it corresponds to the exponential distribution (so  $a = \gamma$ , where  $\gamma$  is the classical parameter of the exponential function). When  $b < 1$ , the dispersal kernel is fat-tailed (Clark 1998), i.e. the long-range decrease is slow (at least slower than an exponential of the distance). Conversely, when  $b > 1$  (for instance the Gaussian model) the dispersal is light-tailed, with a quick decrease of the dispersal function, implying few long-distance dispersal events. These pairwise estimates allow one to jointly estimate  $d$  and the dispersal parameters  $a$  and  $b$ . Some of them can be fixed at given values. Here we tried to estimate  $d$  and  $a$  for fixed  $b$ , i.e. for a given shape of the dispersal curve. We also tried the joint estimation of  $a$  and  $b$  for fixed  $d$ , as well as the joint-estimation of the three parameters. These methods will be described in greater detail in a later paper (S. Oddou-Muratorio, E. K. Klein & F. Austerlitz, in preparation). The pairwise analysis was performed only on the Km41 population as there were too few maternal families for Colosso. As pointed out by Austerlitz & Smouse (2002), the pairwise analysis yields more accurate estimates of  $\Phi_{FT}$  but requires larger sample sizes. We computed the 95% confidence interval of  $\Phi_{FT}$  by bootstrapping among loci (Goudet 1995; Weir 1996) with 15 000 replicates.

## Results

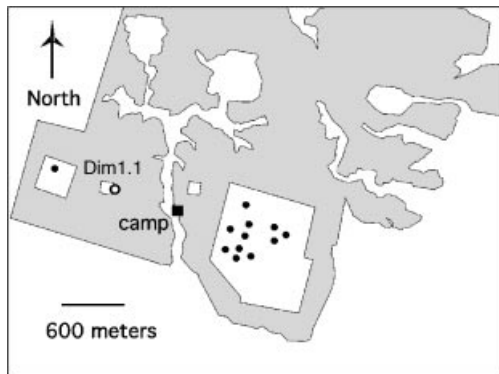
### Mapping

The surveys found a total of 184 adult *Dinizia excelsa* (d.b.h. = 40 cm). There were 43 individuals in the 1-, 10-, and 100-ha fragments, 25 in pasture and gallery forest, and 116 in the continuous forest sites (Figs 3, 4, 5, 6). The density of adult *D. excelsa* in the 232 ha of intensively surveyed forest was 0.17 trees/ha, roughly one tree per six hectares of intact forest. This is about half the density of *D. excelsa* ( $\geq 10$  cm d.b.h.) in the 67 inventoried hectares of the BDFFP reserves ( $\sim 0.3$  trees/ha; BDFFP unpublished data). The density of *D. excelsa* in the BDFFP inventory is close to the average population density of legume trees in the reserves (0.37 trees/ha;  $n = 127$  species; BDFFP unpublished data).

A clumped spatial pattern of *D. excelsa* adults is reflected in the varied abundance of individuals sampled in forest fragments of equal area. The 10-ha fragment at Colosso ranch contained 11 adults, in contrast with two adults in the Porto Alegre 10-ha fragment and a single adult in the Dimona 10-ha fragment. The spatial isolation of individuals in forest fragments and pasture was considerable. Pasture tree Col.06 (Fig. 2) was separated from its nearest neighbour by 600 m, and was the only reproductive individual in a circular area of at least 226 ha of pasture and sparse secondary vegetation. The Porto Alegre 10-ha fragment contained the only two individuals of *D. excelsa* in

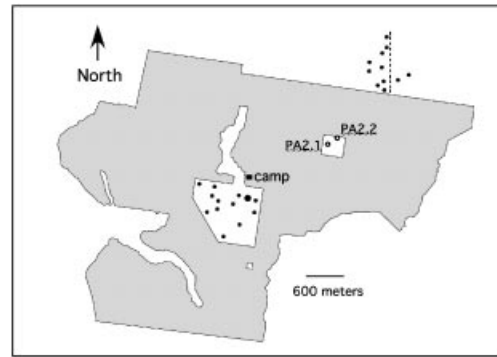


**Fig. 3** Pollen flow among remnant *Dinizia excelsa* (black and open circles) in Colosso Ranch as inferred from paternity analysis of seeds derived from maternal trees, represented as open circles. The shaded areas are actively grazed pasture or secondary vegetation. The white areas represent primary forest. Modified from Fig. 4 in Dick (2001a).



**Fig. 4** Detail of Dimona ranch showing mapped *Dinizia excelsa* adults (circles) in forest fragments (unshaded). The secondary vegetation (shaded) is dominated by dense stands of pioneer trees in the genus *Vismia*. Maternal tree Dim1.1 (open circle) was located adjacent to a 1-ha forest fragment, with over 600 m to its nearest neighbours in the 10-ha fragment. Paternity inference indicated that Dim1.1 received pollen from at least four different trees.

300 ha of *Cecropia*-dominated secondary forest, as the nearest *D. excelsa* were located at the edge of primary forest 700 m to the north (Fig. 5). One would expect to find about 30 adult trees in the same area in Km41.



**Fig. 5** Detail of the 10-ha and 100-ha forest fragments (unshaded) of Porto Alegre ranch, including a trail that extends into continuous forest in the upper right. The secondary vegetation (shaded) is comprised of dense stands of pioneer trees in the genus *Cecropia*, which form a canopy approximately 10 m in height. Paternity inference of PA2.1 and PA2.2 indicated that these trees received pollen from several trees, located either in the 100-ha or continuous forest.

**Table 2** Characteristics of the microsatellite loci used in this study

Locus	Clone motif	$k$	$n$	Allele size	$H_O$	$H_E$	Excl (2)
DE27	AAG <sub>8</sub>	6	546	112–118	0.540	0.462	0.187
DE37	AC <sub>20</sub>	12	569	118–138	0.655	0.752	0.543
DE44	GT <sub>13</sub>	8	567	140–150	0.549	0.578	0.363
DE48	GA <sub>27</sub>	36	564	120–160	0.756	0.945	0.884
DE54	CT <sub>39</sub>	29	519	118–166	0.757	0.902	0.802

Following each locus name: repeat motif of cloned DNA sequence, observed number of alleles ( $k$ ), sample size ( $n$ ), allele size range, observed heterozygosity ( $H_O$ ), expected heterozygosity ( $H_E$ ) and second-parent exclusion probability [Excl (2)].  $k$  and  $n$  represent seeds from all sites. The other values are derived from the seed arrays of Km41. The mean multilocus second parent exclusion probability was 0.995.

#### Microsatellite variation

Null alleles (Pemberton *et al.* 1995) were detected as genotype mismatches between mother/offspring pairs for the loci DE28 and DE53 when these samples were electrophoresed on the ABI 377. Although we were able to resolve most of the DE28 and DE53 alleles on the MJ Base Station, which can detect the faint signal of weakly amplifying alleles, we focused our comparative studies on the alleles from the five loci of Table 2. These five loci yielded 78 alleles with a mean of 13 alleles per locus just in the 240 genotyped seeds of Km41. The observed heterozygosity ( $H_O$ ) ranged from 0.540 (DE27) to 0.757 (DE54). The exclusion probabilities for individual loci ranged from 0.106 (DE27) to 0.793 (DE54) with a combined second parent exclusionary probability of 0.995. DE37 and DE54 showed a significant excess of homozygotes over

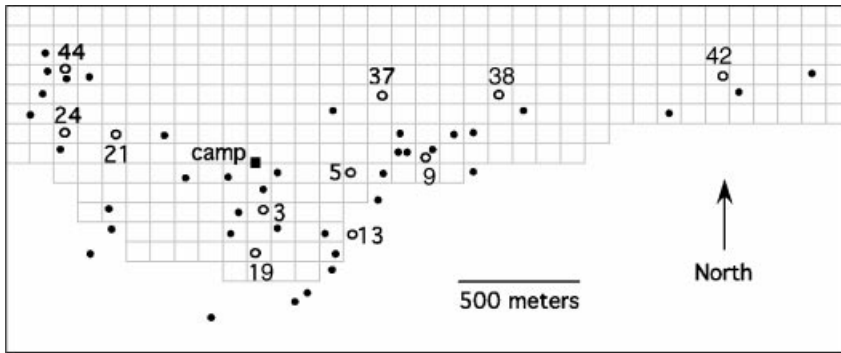


Fig. 6 *Dinizia excelsa* adults in the continuous forest reserve of Km41. The 100 m by 100 m grid is an established trail system. Open circles represent the maternal trees selected for progeny analyses.

Table 3 Diversity of microsatellite alleles in the *Dinizia excelsa* seed arrays

Maternal tree	Habitat	Seeds		Locus					Total alleles	Minimum sires
		<i>n</i>	<i>t<sub>m</sub></i>	DE27	DE37	DE44	DE48	DE54		
<i>Col.06 '95'</i>	Pasture	35	0.84	1	5	3	7	11	27	6
<i>Col.06 '93'</i>	Pasture	20	0.80	1	3	4	3	5	16	3
<i>Col.07 '95'</i>	Pasture	25	0.80	1	4	1	5	5	16	3
<i>Col.07 '93'</i>	Pasture	25	0.87	1	4	3	9	8	25	5
<i>Col.08</i>	Pasture	25	0.96	1	2	3	6	4*	16	3
<i>Col.29</i>	Pasture	25	0.80	0	4	3	7	1*	15	4
<i>Col.13</i>	Gallery	25	0.92	3	4	3	12	11	33	6
<i>Col.18</i>	10-ha	25	1.00	2	4	1	9	8	24	5
<i>Col.26</i>	10-ha	25	0.96	2	5	4	14	12	37	7
PA2.1	10-ha	8	1.0	0	1	1	3	4	9	2
PA2.2	10-ha	15	0.73	0	3	3	9	7	22	5
Dim1.1	1-ha	24	0.63	n/a	4	2	7	6	19	4
Duk 10	Forest	28	0.91	0	3	3	8	11	25	6
Duk.32	Forest	25	0.96	1	4	3	12	8	28	6
CF.26	Forest	25	1.00	1	4	0	7	5	17	4
41.03	Forest	14	0.93	0	6	4	4	2	16	3
41.05	Forest	24	1.00	0	2	2	8	9	21	5
41.09	Forest	24	0.96	1	5	2	9	7	24	5
41.13	Forest	25	0.90	1	4	3	8	9	25	5
41.19	Forest	24	0.75	0	4	3	7	6	20	4
41.21	Forest	10	0.60	0	2	1	3	1	7	2
41.24	Forest	24	0.88	0	4	2	7	6	19	4
41.37	Forest	24	0.92	1	4	2	8	10	25	5
41.38	Forest	24	0.75	1	3	3	3	3	13	2
41.42	Forest	24	1.0	0	3	2	10	8	23	5
41.44	Forest	24	1.0	0	3	4	9	5	21	3

*t<sub>m</sub>*, multilocus outcrossing rate. The numbers below each locus indicate the number of unique paternal alleles in the seed array, summed across loci under 'Total alleles' (see text). Seed arrays are from the 1995 flowering, except for *Col.06 '93'* and *Col.07 '93'* which are from 1993. Canopy observations were made on the trees indicated in italics. \*Sample < 10 seeds.

Hardy–Weinberg proportions, which may have resulted from null alleles carried in the pollen.

*Outcrossing rates and sire diversity*

The outcrossing rate (*t<sub>m</sub>*) of the progeny arrays (Table 3) ranged from 0.63 to 1.0 with the average across all seeds (*n* = 596) of 0.877 (523 cross-fertilized seeds), indicating that

*D. excelsa*, like most tropical trees that have been studied, is predominantly outcrossed but capable of self-fertilization (reviewed in Nason & Hamrick 1997). Significant differences in outcrossing rates were found among habitat types (*t*-test; *P* < 0.01); the mean outcrossing rate of 13 forest trees (*n* = 295 seeds) was 0.897, while those of pasture and forest fragments (*n* = 12 trees; 277 seeds) was 0.856. The values of *t<sub>m</sub>* calculated using the MLTR program were similar but

**Table 4** Adjustment of pollen dispersal kernel using pairwise pollen pool differentiation estimates for the Km41 population of *Dinizia excelsa*

Dispersal function	Fixed parameters			Estimated parameters				Error
	$d$ (trees/ha)	$a$ (m)	$b$	$\hat{d}$ (trees/ha)	$\hat{a}$ (m)	$\hat{b}$	$\hat{\delta}$ (m)	
Normal	0.17	—	2	—	203	—	180	0.0995606
Normal	—	—	2	0.128	284	—	206	0.0988038
Exponential	0.17	—	1	—	102	—	204	0.09906465
Exponential	—	—	1	0.100	130	—	260	0.0974929
Exponential power	0.17	—	—	—	80.6	0.877	213	0.0990395
Exponential power	—	—	0.877	0.0928	107	—	282	0.0971866

For each tested function, the effective tree density was either set to the observed tree density in the studied site (first line) or jointly estimated with other parameters (second line). The shape parameter  $b$  was either set to 2 (normal distribution) or to 1 (exponential distribution) or jointly estimated. The mean estimated pollen dispersal distance ( $\hat{\delta}$ ) is given in all cases.

slightly lower:  $0.875 (\pm 0.049)$  for undisturbed habitats and  $0.848 (\pm 0.044)$  for the disturbed habitats.

The paternal allele diversity in the seed arrays ranged from 13 nonmaternal alleles ( $n = 25$  seeds) for forest tree 41.38, to 37 alleles for Col.26, located in the 10-ha forest fragment of Colosso. Loci DE48 and DE54 provided the most information about sire diversity, with a maximum (for Col.26) of 14 and 12 alleles, respectively, indicating that the 25 seeds from this tree were sired by at least seven different pollen donors. Even the most isolated pasture tree (Col.06; see Fig. 2) was pollinated by at least six different trees in the 1995 seed array ( $n = 35$  seeds). The nearest flowering neighbour of Col.06, located 600 m away, was not one of the sires, and its next nearest neighbours were separated by over 1 km of pasture (Dick 2001a). The maternal trees in Km41, in contrast, had dozens of proximal mates yet their seed arrays never had more than five fathers.

#### Pollen flow in Porto Alegre and Dimona

The progeny analyses in Porto Alegre and Dimona ranches focused on trees in the isolated fragments. Neither ranch contained a high density of *D. excelsa* and there were few trees left standing in pastures. The two trees in the 10-ha fragment of Porto Alegre (Fig. 5) nevertheless captured a large number of foreign alleles. The seed array ( $n = 15$ ) of PA2.2 contained nine paternal alleles at locus DE48 and seven alleles at DE54, indicating at least five pollen donors. The nearest pollen donors were located in continuous forest separated from the 10-ha fragment by 600 m of dense *Cecropia*. In Dimona, the solitary tree Dim1.1, located on the edge of the 1-ha fragment, captured seven and six alleles at loci DE48 and DE54, indicating at least four different sires for this group of seeds. The nearest potential mate, situated in the 10-ha fragment, was separated by 600 m of pasture and *Vismia*-dominated secondary vegetation and all other potential mates were located over 1 km away. While the

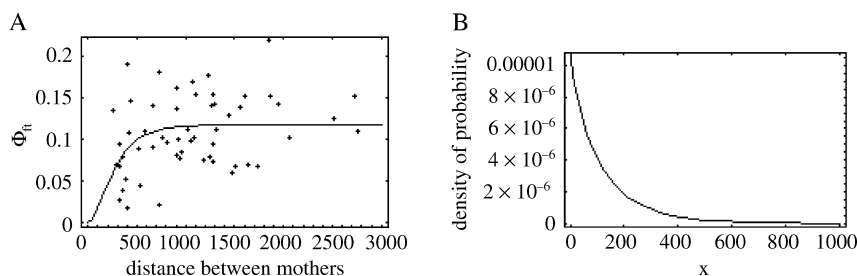
trees in the Porto Alegre fragment produced few seeds, Dim1.1 produced an estimated 10 000 seeds and had flowered intensely during this period. The number of hours spent observing flowers in the canopies of *D. excelsa* was not sufficient to assess the importance of African honeybee pollination in Dimona and Porto Alegre (Dick 2001b). It is clear from our genetic analyses, however, that there is potential for high levels of gene flow to solitary trees embedded in diverse kinds of secondary vegetation.

#### TWOGENER analysis

*Km 41 population.* The global estimate of  $\Phi_{FT}$  was 0.104 (95% confidence interval: 0.0853–0.115). Given that the average distance between the sampled females was 1076 m, this translated into an estimate of the dispersal distance  $d$  of 188 m assuming a normal pollen dispersal function and of 212 m assuming an exponential dispersal. The results of the pairwise estimates are given in Table 4 and the best fit is shown in Fig. 7. These estimates yielded concordant results with the global estimates for the normal and exponential distribution when the density was fixed. When the effective density was estimated jointly with the other parameters, it dropped from 0.17 trees/ha to 0.128 trees/ha for the normal dispersal model and 0.100 trees/ha for the exponential distribution. Also when the shape parameter ( $b$ ) was jointly estimated, with density fixed at 0.17 trees/ha, the estimated value was 0.877, thus slightly fat-tailed. For that dispersal distribution function, when density was jointly estimated with  $a$ ,  $b$  being fixed at this value, it was 0.0928 tree/ha. Thus in all cases, it indicated that the effective density was lower than the measured one. The simultaneous estimation of  $d$ ,  $a$  and  $b$  failed probably because of an insufficient quantity of data to estimate the three parameters jointly.

*Colosso population.* The global estimate of  $\Phi_{FT}$  was much lower:  $\Phi_{FT} = 0.00167$  (95% confidence interval: 0.0–0.0408),





**Fig. 7** Pairwise  $\Phi_{FT}$  for the seed arrays from Km 41 (A) along with the best fitting curve (see Table 4), with the corresponding dispersal functions (B).

with 1616 m as the mean distance between sampled mothers. Conversely, there was a much higher value for the estimate of the dispersal distance  $d$  of 1264 m if a normal dispersal function was assumed and 1509 m for the exponential function. Thus, it is clear that pollen dispersal was much more extensive in Colosso than in Km41.

## Discussion

The conversion of rainforest to cattle ranch severely altered the breeding structure of *Dinizia excelsa* by (i) destroying all but the largest individual trees left in pasture, (ii) producing fragments of primary forest that contained few reproductive trees and (iii) creating a mosaic pollination environment comprised of native and exotic bees. The small number of reproductive trees left in forest fragments applies to most of the low-density tree populations in this forest. The survival of adult trees in pastures, however, applies to relatively few canopy-emergent species that are useful to farmers for timber or shade. Two unexpected results of previous studies (Dick 2001a,b) were (i) the long distance pollination of *D. excelsa* by African honeybees, and (ii) the higher fecundity of trees visited by African honeybees, regardless of the habitat matrix. The latter result has been corroborated by a study in which coffee trees (*Coffea arabica*) visited by African honeybees produced > 50% seeds than coffee plants visited only by native bees (Roubik 2002). African honeybees are important pollinators for many plants because of their high densities, their social organization, and their proclivity for agricultural habitats. However, little is known about their effect on genetic processes of native plants (Huryn 1997).

### African honeybees

Honeybees (*Apis mellifera*) are a recent introduction to the Amazonian fauna. The African race *Apis mellifera scutellata* ('killer bees' in the popular press) was imported to southern Brazil in the early 1950s to breed a variety of honeybees that could endure a humid tropical climate and produce large quantities of honey. In 1956, 26 queens and about 200 males escaped from research apiaries near São Paulo and quickly spread, covering as much as 320 km/year and hybridizing with all of the European honeybee queens they

encountered. The hybrids retained African morphological and behavioural traits (Diniz & Malaspina 1996) and are thus still referred to as African honeybees. There were no *A. mellifera* of any kind in the Amazon basin prior to the African honeybee invasion (Roubik 1989). African honeybees were reported in the Manaus region in 1974 (Prance 1976). There are an estimated 50–100 million colonies in Latin America today (Winston 1992), with estimates of colony densities ranging from 10/km<sup>2</sup> (Taylor 1985) to 108/km<sup>2</sup> (Kerr 1971, in Michener 1975). African honeybees thrive in mosaic habitats apparently because of their eclectic choice of nesting sites and their utilization of primary forest, crop, and weedy plant species for nectar and pollen (Roubik 1989). Like *A. mellifera* introduced to other parts of the world, African honeybees visit about one third of local plant species, but intensively visit species that offer copious floral rewards (Menezes & Camargo 1991; Huryn 1997).

### Long-distance pollen flow and the breakdown of nearest-neighbour mating

The paternity inference revealed long distances of cross-pollination in addition to a breakdown of nearest-neighbour mating in the disturbed habitats. Both of these findings were unexpected, as published observations indicate that *A. mellifera* focus their foraging efforts on densely flowering plants and limit interplant movements to nearest neighbours (Levin & Kerster 1974). Deviations from nearest-neighbour pollination have been demonstrated in other studies of remnant trees in tropical pasture (Chase *et al.* 1996; White *et al.* 2002), but in these studies pollinators and floral phenology were not observed. Phenological data for *D. excelsa* indicate that nearest neighbours flowered in synchrony for at least one month (Dick 2001b) in all sites, yet the flowering neighbours in the pastures often did not mate with one another. This may result from the long distances between nearest potential mates in pasture. In a study of trees in undisturbed Panamanian forests, Stacy *et al.* (1996) found that most ( $\geq 90\%$ ) pollen movement occurred between nearest neighbours when trees were clustered, whereas pollen flow to isolated trees tended to violate the nearest-neighbour rule. The authors suggested that small bees and other insects may have difficulty in finding nearest

neighbours when flowering conspecific plants are broadly spaced. Thus there may be a threshold of distance over which nearest-neighbour mating breaks down.

#### *Outcrossing rates*

The multilocus outcrossing rate ( $t_m$ ) is a dynamic parameter for plants capable of self-fertilization, and it often varies with plant density (Murawski & Hamrick 1991; Franceschinelli & Bawa 2000). In this study  $t_m$  varied among habitat types, and there was a significant increase in self-fertilization in the disturbed habitats. Increased self-fertilization has been documented among trees left in pastures in Costa Rica (Aldrich & Hamrick 1998) and in selectively logged forests (Murawski *et al.* 1994; Lee 2000). High flowering intensity in these habitats, possibly because of increased light or nutrient availability, may promote within-crown pollinations as small insects do not need to fly among plants (Frankie & Haber 1983). Alternatively, the potentially greater availability of resources in disturbed habitats may result in fewer selective abortions of inbred ovules (Bawa & Webb 1984). Although inbreeding can result in the loss of progeny vigour, the demographic consequences of inbreeding in remnant populations of *D. excelsa* may be negligible. Remnant *D. excelsa* produced approximately three times more seeds per tree than did trees in undisturbed forest, and most of the seeds were cross-fertilized (Dick 2001a). Moreover, the slightly higher rate of self-fertilization among the hyper-dispersed remnant trees may be offset by lower rates of bi-parental inbreeding.

#### *Pollen dispersal and effective densities in the undisturbed forest*

The mean distance of gene flow in the Km41 population (212 m) is similar to estimates for other low-density tropical trees (reviewed in Nason & Hamrick 1997). Konuma *et al.* (2000), for example, using paternity inference, estimated the average distance of pollen flow in the tropical emergent tree *Neobalanocarpus heimii* to be 191.2 m ( $\pm 104.9$  m SD) with 663.6 m marking the longest pollination event. Estimates of mean dispersal using paternity inference are conservative, however, because most long-distance pollination is undetectable.

The low effective density of *D. excelsa* in the undisturbed forest—as few as a single reproductive tree per 10 hectares—may result from variation in reproductive success among the pollen donors. This result has important management implications. Discounting edge effects and clumped distribution patterns, roughly 5000 ha of forest is needed to maintain an effective population of  $N_e = 500$ , in which rare alleles or the additive genetic variation of complex traits are not lost through genetic drift (Franklin 1980). In the

fragmented habitats, African honeybees may mitigate the loss of genetic diversity by expanding genetic neighbourhood areas until rainforest regenerates in the abandoned pastures. Large pasture trees will play an important role as genetic stepping-stones, as indicated in the Colosso study. Moreover, pasture trees provide roosting sites for large frugivorous birds, such as parrots and macaws, and thereby serve as foci of regeneration for other plant species (Guevara & Laborde 1993) while seeding the abandoned pastures with their own genetically diverse offspring.

#### *Effects of African honeybees on other native plants*

We would like to stress that African honeybees are not a conservation panacea. African honeybees bypass plants with few floral rewards, and they may compete with native bees (Roubik 1978). African honeybees can reduce the viability of plants with specialized flowers, especially if significant quantities of floral resources are extracted without pollination. For example, 'buzz-pollinated' plants require sonic vibrations for pollen release (Buchmann 1983). Although honeybees consume nectar from buzz-pollinated plants, they are often not capable of pollinating them. The buzz-pollinated legume *Mimosa pudica* (Mimosaceae) in French Guiana, for example, suffered a 26% decline in seed set when about 74% of the visitors were African honeybees, compared to forest populations visited almost exclusively by native bees (Roubik 1996). There are many kinds of understudied rainforest plant that may become reproductively isolated in fragmented habitats. Canopy observations of *D. excelsa*, for example, suggest that stingless bees and small beetles do not cross open pastures. This observation indicates that plants pollinated exclusively by small beetles, such as trees in the nutmeg family (Myristicaceae), may be especially vulnerable to habitat fragmentation. Given the complexity of the ecological changes that accompany rainforest fragmentation, as shown in our study, we suggest that future genetic studies incorporate ecological observations, particularly of pollination and regeneration, and focus on species that seem most susceptible to reproductive isolation.

#### **Acknowledgements**

We thank the BDFFP for providing logistical support and the Manaus Free Trade Zone Authority (SUFRAMA) for permission to conduct the research. We would like to thank Heraldo Vasconcelos and Charles Clements for help in obtaining exportation permits, and Alaércio Marajó dos Reis and Marcela Santamaria for logistical help. We thank Peter Smouse, Kermit Ritland, Cyril Dutech and two anonymous reviewers for helpful comments, and Eldredge Bermingham and members of the Naos Molecular Laboratory i.e. Naos Molecular Laboratory for advice and support. This research was supported by the Smithsonian Institution's Molecular Evolution Program and Molecular Evolution fellowship (to C.D.) and by

a grant from the International Plant Genetics Resource Institute (IPGRD). This paper is number 393 in the BDFFP technical series.

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