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Partitioning nuclear and chloroplast variation at multiple spatial scales in the neotropical epiphytic orchid, *Laelia rubescens*

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Abstract

Insights into processes that lead to the distribution of genetic variation within plant species require recognition of the importance of both pollen and seed movement. Here we investigate the contributions of pollen and seed movement to overall gene flow in the Central American epiphytic orchid, *Laelia rubescens*. Genetic diversity and structure were examined at multiple spatial scales in the tropical dry forest of Costa Rica using nuclear (allozymes) and chloroplast restriction fragment length polymorphism (RFLP) markers, which were found to be diverse (allozymes, P = 73.3%; H_E = 0.174; cpDNA, H_E = 0.741). Nuclear genetic structure (F_{STn}) was low at every spatial scale (0.005–0.091). Chloroplast markers displayed more structure (0.073–0.254) but relatively similar patterns. Neither genome displayed significant isolation-by-distance. Pollen and seed dispersal rates did not differ significantly from one another (m_p/m_s = 1.40) at the broadest geographical scale, among sites throughout Costa Rica. However, relative contributions of pollen and seeds to gene flow were scale-dependent, with different mechanisms determining the dominant mode of gene flow at different spatial scales. Much seed dispersal is highly localized within the maternal population, while some seeds enter the air column and are dispersed over considerable distances. At the intermediate scale (10s to 100s of metres) pollinators are responsible for substantial pollen flow. This species appears capable of distributing its genes across the anthropogenically altered landscape that now characterizes its Costa Rican dry forest habitat.

Keywords: allozymes, cpDNA, gene dispersal, genetic structure

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Introduction

Plants accomplish gene dispersal during two independent life cycle stages, via seeds and pollen. Successful pollen and seed immigration and establishment into extant populations constitute gene flow events while seed dispersal and establishment into vacant sites constitute colonization (Levin 1981; Hamrick & Nason 1996). Because all natural plant populations originate in this manner, colonization is an important process shaping the genetic structure of species (Harper 1977; Slatkin 1977; Wade & McCauley 1988). Recognition of the two gene dispersal stages in plants is critical to understanding the distribution of genetic variation within and among populations (Levin 1981; Whitlock & McCauley 1990). Both pollen and seeds disperse biparentally inherited nuclear genes while maternally inherited gene movement depends exclusively on seed dispersal. In most angiosperms, the nonrecombinant chloroplast genome (cpDNA) is inherited maternally (Sears 1980; Corriveau & Coleman 1988; Mogensen 1996; Ennos *et al*. 1999). By comparing the distribution of genetic markers derived from nuclear (nDNA) and chloroplast genomes, the relative influence of pollen and seed dispersal on gene flow and genetic structure can be determined (Ennos 1994; McCauley 1995). If pollen and seed dispersal rates differ, cpDNA and nDNA should reveal different levels of spatial genetic structure (McCauley 1994; McCauley *et al*. 1996). Thus, the distribution of genetic diversity for these two distinct genomes can be used to infer the processes that produce genetic structure. While an increasing number of studies examine the relative contributions of pollen and

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seed movement to overall gene flow (e.g. El Mousadik & Petit 1996; Tarayre *et al*. 1997; Tomaru *et al*. 1998; Levy & Neal 1999; Heuertz *et al*. 2003; Latouche-Hallé *et al*. 2003), few have investigated plant species whose potential for long-distance seed dispersal may exceed their pollen dispersal potential (e.g. Comes & Abbott 1998; Squirrell *et al*. 2001; Cozzolino *et al*. 2003). Even fewer studies have documented the relative importance of seed and pollen dispersal at several spatial scales (e.g. McCauley 1994; Oddou-Muratorio *et al*. 2001).

Most orchids are animal-pollinated, with each capsule containing up to six million dust-like, wind-dispersed seeds (Arditti & Ghani 2000). While many seeds may settle within a few metres of the maternal plant (Trapnell *et al*. 2004), orchid seeds can be suspended in the air column and dispersed long distances (Dressler 1981; Arditti & Ghani 2000). For example, 13 years after the 1883 eruption of Krakatoa obliterated all plant life on the island, three orchid species had become established and within 50 years 35 orchid species had dispersed at least 40 km to colonize the island (Docters van Leeuwen 1936). Orchids are therefore of particular interest for studying relative contributions of pollen and seed dispersal because, unlike most angiosperms, their seeds have the potential to move considerable distances while pollen flow is limited by pollinator movement. As the planet's natural habitats become increasingly disturbed and fragmented, genetically diverse plant species with long-distance seed dispersal capabilities should be able to re-colonize extirpated populations.

Our objectives were twofold: first, to determine the relative importance of pollen and seed dispersal to gene flow in the epiphytic orchid, *Laelia rubescens* Lindley throughout its Costa Rican range; and second, to learn whether the relationship of pollen flow to seed migration is dependent on spatial scale. Our findings will provide insights into the mechanisms involved in gene migration and the maintenance of genetic diversity in this species. They will also indicate the ability of species with long-distance seed dispersal capabilities to preserve genetic diversity in highly fragmented landscapes. The seasonally dry tropical forest of Central America in which *L. rubescens* occurs is largely comprised of anthropogenically altered habitat. The ubiquitous nature and duration of disturbance experienced by dry tropical landscapes necessitate a better understanding of the mechanisms that shape the evolutionary trajectories of contemporary species. It is no longer sufficient to study exclusively species occupying pristine habitats, as disturbed landscapes now often represent the norm rather than the exception.

These objectives were addressed through examination of genetic structure of populations at multiple spatial scales using biparentally inherited nDNA and maternally inherited cpDNA. Based on the isolation-by-distance (IBD) model of gene flow (Wright 1943a,b) we predicted greater genetic structure of both nDNA and cpDNA at increasingly greater spatial scales. Maternally inherited marker variation is strongly geographically structured in a variety of plant taxa (Ennos 1994; El Mousadik & Petit 1996; Latta & Mitton 1997; Tomaru *et al*. 1998; Levy & Neal 1999; Sinclair *et al*. 1999). Furthermore, comparisons of the genetic structure of cpDNA and nDNA in angiosperm species have generally shown cpDNA variation to be more spatially structured than nDNA (Ennos 1994; El Mousadik & Petit 1996; McCauley *et al*. 1996). In hermaphroditic plants, the effective population size of maternally inherited markers is half that of biparentally inherited nDNA (Birky *et al*. 1983). Second, gene flow of maternally inherited cpDNA must be less than nuclear gene flow as nDNA can be dispersed by both pollen and seeds, whereas the maternally inherited genome is only dispersed via seeds (Petit *et al*. 1993; Ennos 1994). Previous studies have shown ratios of estimated pollen migration (m_p) to seed migration (m_s) ranging from 0.48 (*Anacamptis palustris*; Cozzolino *et al*. 2003) to 640 (*Fagus crenata*; Tomaru *et al*. 1998) for a variety of plant species (e.g. El Mousadik & Petit 1996; Comes & Abbott 1998; Levy & Neal 1999). Because orchid seeds are capable of considerable long-distance dispersal while pollen movement should reflect the more limited foraging range of the orchid's pollinators, we predicted that seed dispersal in *L. rubescens* would contribute extensively to gene movement and that the m_p/m_s ratio would be close to or less than 1.0.

Materials and methods

Study species

Laelia rubescens Lindley is a long-lived, neotropical, perennial epiphyte ranging from Mexico to Panama (Williams & Allen 1980) in dry habitats below 800 m (Mora de Retana & Atwood 1992). Its bisexual flowers are exclusively animal-pollinated, with hummingbirds as the primary agents (D. W. T., personal observation). Intraflower pollination is not possible, but pollination between flowers within the same genet (geitonogamy) occurs. Sticky pollen grains aggregate to form eight distinct pollinia per flower, each containing sufficient pollen to fertilize every ovule within a flower. As a result, fruit capsules contain seeds pollinated by a single pollen donor and represent full-sib progeny arrays. Each fertilization results in hundreds of thousands of tiny, wind-dispersed seeds. Orchid seeds possess no endosperm but instead have large internal air spaces that allow them to float in the air column (Arditti & Ghani 2000). Their size and morphology together facilitate long-distance dispersal. It has also been suggested that orchid seeds may be transported on birds' feathers and animal fur (Arditti & Ghani 2000). Seeds become almost completely inviable within a year (D. W. T., personal observation), making a substantial

seed bank unlikely. Once established on suitable substrate, *L. rubescens* grows clonally with each fleshy pseudobulb (inflated stem tissue) capable of producing one or two new pseudobulbs/year. Each pseudobulb bears one, and sometimes two, thick leathery leaves. Clusters can become large over time and possess 100 or more pseudobulbs. Each pseudobulb produces a single inflorescence with as many as 20 showy, pink flowers (Halbinger & Soto 1997; D. W. T., personal observation). Anthesis (January–March) is over an extended period during the dry season. A single inflorescence can produce up to 11 capsules (D. W. T., personal observation). There are $2n = 40$ chromosomes (Kamemoto 1950).

Study sites

Much of this work was conducted on three ranches located in the lower Tempisque River basin of Guanacaste Province in northwestern Costa Rica. *Laelia rubescens* grows on a variety of host trees (Trapnell & Hamrick, in preparation), occurring in habitats ranging from primary forests to highly, human-modified landscapes. In less disturbed forests *L. rubescens* is dispersed widely with relatively few individuals per tree. In open pasturelands, often one or more isolated shade trees with large spreading canopies were left. It is on these host trees that *L. rubescens* is most abundant, with 350 or more clusters. These trees support several other epiphytic species that occur in densities of a few individuals per tree. These include two orchids (*Brassavola nodosa* and *Encyclia fragans*), one bromeliad (*Tillandsia schiedeana*) and a cactus (*Hylocereus costaricensis*). Additional epiphytic taxa were observed among *L. rubescens* populations sampled in other parts of Costa Rica. The area is classified as a seasonally dry tropical forest characterized by semideciduous trees and a 6-month dry season (December–May).

Ranch populations are located at Porozol, Hacienda San Joaquin and Hacienda Solimar. A population is defined as all the clusters of *L. rubescens* growing within a tree. Haciendas San Joaquin and Solimar are large, privately owned cattle ranches characterized by isolated trees and small groups of trees in multiple pastures. Typical of the dry forest regions of Costa Rica (Sader & Joyce 1988), these two ranches were established during the mid-1950s. Pastures selected in Porozol are located on smaller ranches that share a similar disturbance history. For larger-scale geographical collections, 13 additional sites were sampled from throughout the range of *L. rubescens* in Costa Rica (Table 1).

Sampling

Leaf tissue was collected in duplicate from approximately 50 individuals per site from the 13 sites distributed throughout Costa Rica (Table 1). Within the lower Tempisque River basin the three ranches described above were sampled intensively (Table 1). Here duplicate leaf samples were obtained from 50 individuals per pasture from host trees located in four pastures at Porozol, four at Hacienda San Joaquin and three at Hacienda Solimar. Samples from trees within a pasture were pooled into a single within-pasture collection. All 50 samples were analysed for allozymes while a randomly selected subset of 24 samples was analysed for cpDNA haplotypes. For comparison with the geographically distributed samples, a single pasture within each ranch was selected randomly to be included in the analysis.

Enzyme extraction and electrophoresis

Leaf tissue samples were snap-frozen in liquid nitrogen within a few hours of collection and were stored in an ultra-cold dry shipper. Samples were sent to the University of Georgia where they were crushed in chilled mortars with a pestle, liquid nitrogen and a pinch of sea sand to disrupt cellular compartmentalization. Enzymes were extracted from the tissue with a polyvinylpyrrolidone-phosphate extraction buffer (Mitton *et al*. 1979). The resulting slurry containing crude protein extract was absorbed onto 4×6 mm wicks punched from Whatman 3 mm chromatography paper. Wicks were stored in microtest plates at −70 °C until used for electrophoresis. Wicks were placed in horizontal starch gels (10%) and electrophoresis was performed. Eleven enzyme stains in four buffer systems resolved 11 putative polymorphic and four monomorphic loci. Enzymes stained and loci identified (in parentheses) for each of the four buffer systems were: (1) system 6: alcohol dehydrogenase (ADH); (2) system 8-: aspartate aminotransferase (AAT1, AAT2), diaphorase (DIA1), fluorescent esterase (FE2) and triosephosphate isomerase (TPI1); (3) system 10: fluorescent esterase (FE1), and UTP-glucose-1-phosphate (UGPP1); and (4) system 11: isocitrate dehydrogenase (IDH1, IDH2), malate dehydrogenase (MDH1, MDH3), 6-phosphogluconate dehydrogenase (6-PGD), phosphoglucomutase (PGM2) and shikimic dehydrogenase (SKDH). All buffer and stain recipes were adapted from Soltis *et al*. (1983) except DIA (Cheliak & Pitel 1984) and UGPP (Manchenko 1994). Buffer system 8- is a modification of buffer system 8 as described by Soltis *et al*. (1983). Banding patterns were consistent with those expected for each enzyme system (Weeden & Wendel 1989).

Chloroplast markers

Frozen leaf tissue was placed with three Aldrich 3 mm borosilicate beads in an 1.7 mL centrifuge tube with a small hole in the lid. Samples were freeze-dried, the holes were sealed with self-adhesive stickers and samples were placed in a Retsch MM 300 bead mill for approximately 35 min.

Table 1 Location of sites used in this study

Sites	Abbreviation	Latitude	Longitude
Peñas Blancas	PB	11°11.8′	85°37.3′
Parque Nacional Santa Rosa	SR	10°50.1′	85°36.9′
Liberia	LB	10°44.8′	85°30.7′
Estación Experimental Horizontes UCR	HZ	10°41.0′	85°36.4′
Stewart Ranch	ST	10°31.9′	85°18.4′
Hacienda el Viejo	EV	10°23.3′	85°24.3′
Rio Limon	RL	10°21.6′	85°41.8′
Parque Nacional Palo Verde	PV	10°20.9′	85°20.0′
Porozol			
pasture 1	PZ-1	10°16.1′	85°12.2′
pasture 2	PZ-2	10°16.1′	85°11.3′
pasture 3	PZ-3	10°16.1′	85°11.1′
pasture 4	PZ-4	10°16.2′	85°10.9′
Hacienda San Joaquin			
pasture 1	SJ-1	10°15.9′	85°06.3′
pasture 2	SJ-2	10°16.1′	85°06.6′
pasture 3	SJ-3	10°16.2′	85°07.0′
pasture 4	SJ-4	10°16.3′	85°08.1′
Hacienda Solimar			
pasture 1	HS-1	10°16.3′	85°09.3′
pasture 2	HS-2	10°16.1′	85°09.7′
pasture 3	HS-3	10°17.4′	85°09.9′
Las Juntas	LJ	10°14.7′	84°59.6′
Hacienda La Ensenada	LE	10°08.8′	85°02.8′
Near Nicoya	NN	10°07.5′	85°26.7′
Orotina road	OR	09°54.4′	84°39.8′
Hacienda Doña Marta	DM	09°54.1′	84°37.5′

Total genomic DNA was extracted using the Qiagen DNeasy Plant Kit and used as a template to polymerase chain reaction (PCR) amplify two noncoding chloroplast regions. The first is an intergenic spacer approximately 680 base pairs (bp) long flanked by *trn*T and *trn*L (UAA) 5′ exon. The second region amplified is an approximately 650 bp long intron flanked by *trn*L (UAA) 5′ exon and *trn*L (UAA) 3′ exon. Primers used for these highly conserved flanking regions are described by Taberlet *et al*. (1991). The 30 μL PCR reactions consisted of 1 μL genomic DNA template, 15.0 μL Epicentre MasterAmp™ 2×PCR PreMixB, 13.1 μL HPLC grade water, 0.3 μL of each primer (10 μM) and 0.3 μL Applied Biosystems Ampli*Taq*® DNA polymerase. These were submitted to 94 °C for 4 min, 40 cycles of amplification (1 min 94 °C, 1 min 53 °C, 1 min 72 °C), 73 °C for 4 min, and 4 °C until samples could be removed and stored at 4 °C.

The PCR amplified intergenic spacer was digested with the restriction enzyme, *Mbo*I, in a 25 μL reaction consisting of 6.5 μL amplification product, 12 μL deionized water, 4.0 μL (20 units) New England BioLabs Inc. *Mbo*I enzyme, and 2.5 μL 10× NE buffer 3 provided with the enzyme. The reaction was topped with 12 μL of mineral oil and incubated at 37 °C for approximately 12 h. The intron PCR amplification product was divided for two separate restriction digests with *Alu*I and *Taq*αI. The first 25 μL reaction consisted of 6.5 μL amplification product, 14 μL deionized water, 2.0 μL (20 units) New England BioLabs Inc. *Alu*I enzyme, and 2.5 μL 10× NE buffer 2 provided with the enzyme. The reaction was topped with 12 μL of mineral oil and incubated at 37 °C for approximately 12 h. The second 25 μL reaction consisted of 6.5 μL amplification product, 12.5 μL deionized water, 1.0 μL (20 units) New England BioLabs Inc. *Taq*αI enzyme, 2.5 μL 10× NE buffer provided with the enzyme and 2.5 μL bovine serum albumin (BSA; 1 mg/mL dilution). After topping with 12 μL of mineral oil these samples were incubated at 65 °C for 7 h. Restriction fragments were separated by electrophoresis in Cambrex 4% NuSieve Reliant® gels at 100 V for 30 min with a Roche DNA Molecular Weight Marker V ladder (8–587 bp). The ethidium bromide tagged fragments were visualized with a Stratagene® Eagle Eye™ II.

Genetic analyses

Linkage disequilibrium between allozyme loci was tested using GENEPOP (Raymond & Rousset 1995). Allozyme diversity measures were estimated using a computer program designed by M. D. Loveless and A. F. Schnabel. Measures of genetic diversity were percentage of polymorphic loci,

P; mean number of alleles per polymorphic locus, AP; and genetic diversity, H_E ($= 1 - \sum p_i^2$, where p_i is the frequency of the ith allele; Nei 1973), which is the proportion of loci heterozygous per individual under Hardy–Weinberg expectations. Species values for these parameters were calculated by pooling data from all populations. Within population values were calculated for each population and then averaged across all populations.

Observed heterozygosity (H_O) was compared with Hardy–Weinberg expected heterozygosity (H_E) for each polymorphic locus in each population by calculating Wright's fixation indices using $F = (H_E - H_O)/H_E$ (Wright 1922). Deviations were tested for significance using $\chi^2 = F^2N(a-1)$; d.f. $= a(a-1)/2$ where N is the total number of individuals analysed and a is the number of alleles at the locus (Li & Horvitz 1953). Variation among populations ($F_{ST} = \theta$) was estimated according to Weir & Cockerham (1984) using FSTAT (Goudet 2001). Heterogeneity in allele frequencies among populations was tested by the χ^2 method of Workman & Niswander (1970). Genetic structure was determined for each spatial level. Chloroplast haplotypes were treated as alleles and were analysed similarly. F_{ST} values from allozyme and cpDNA markers (both markers were assayed from the same 24 samples per population) were compared following the protocol of Petit (1992) and Petit *et al*. (1992), whereby the ratio of pollen migration (m_p) to seed migration (m_s) was calculated by:

$$m_p/m_s = [2(1/F_{STc} - 1) - (1/F_{STn} - 1)]/(1 - 1/F_{STc})$$

where F_{STc} is cytoplasmic variation among sites and F_{STn} is nuclear variation among sites. Because this statistic reveals a relative relationship between F_{ST} values it should render the assumption (and possible violations of that assumption) of genetic and demographic equilibrium within populations less important. When pollen and seed migration rates are identical, chloroplast haplotypes in an outcrossing hermaphroditic plant with strict maternal chloroplast inheritance should show a smaller effective population size and three times as much fixation as the nuclear alleles, as F_{STn} approaches zero (Hamilton & Miller 2002). Under the null hypothesis of equal pollen and seed flow rates, the expected difference between chloroplast and nuclear F_{ST} values was calculated by:

$$F_{STc} = a_1F_{STn}/[a_2 + (a_1 - a_2)F_{STn}]$$

where $a_1 = 6$ and $a_2 = 2$ (Hamilton & Miller 2002). The significance of the difference between expected F_{STn}, due to different effective population sizes of the two genomes, and observed F_{STn} values was tested with a 95% confidence interval generated by bootstrapping procedures.

The relationship between the dispersal patterns of cpDNA and nDNA with increasing distance was examined by regressing multilocus estimates of pairwise differentiation among sites $[F_{ST}/(1 - F_{ST})]$ against the natural logarithm of distances (Rousset 1997). Pairwise estimates were obtained using FSTAT (Goudet 2001). A Mantel test of correspondence between this ratio and geographical distances was performed (Smouse *et al*. 1986) using NTSYS-PC (Rohlf 2000).

Hierarchical genetic structure can be estimated if subpopulations are grouped into progressively inclusive levels, i.e. each group at one level is 'nested' within the next higher level (Hartl & Clark 1997). Ideally, sampling is conducted in a balanced, nested design. However, the scope of such a balanced sampling design was prohibitive and intractable. Instead we develop similar insights by collecting data at several spatial scales [i.e. among geographical sites, among ranches, among pastures within ranches and among trees within pastures (Hacienda Solimar only)] and analysing the data for each spatial scale separately.

Results

Allozyme diversity

Eleven putative polymorphic and four monomorphic loci were resolved. Significant linkage disequilibrium was observed in 15 of 105 pairs of loci (14%; $P < 0.05$). While this reduces overall levels of nuclear diversity it should not influence the genetic structure statistics. All samples were used to assess allozyme diversity (mean of 50 samples/site at 16 sites for a total of 801 specimens). Genetic diversity for the species was high with 73% of the loci polymorphic, an average of 2.55 alleles per polymorphic locus (AP), and a mean genetic diversity (H_{ES}) of 0.174 (Table 2). Genetic variation among sites was low ($F_{ST} = 0.088$). One private allele was found in each of five sites (PB, SR, PV, LE and NN). Tests for heterogeneity in allele frequencies among populations of *L. rubescens* indicate that 82% (nine of 11 polymorphic loci) were significant ($P < 0.05$). Genetic diversity (H_E) values increased to the southeast (Table 2). The eight northwestern sites (PB, SR, LB, HZ, ST, EV, RL and NN) had a mean H_{ES} of 0.127, while the eight southeastern sites (PV, PZ, HS, SJ, LJ, LE, OR and DM) had a mean H_{ES} of 0.142. Jackknifing procedures (Weir 1996) indicated a significant difference in genetic diversity values between the two regions ($P = 0.001$).

At the population level *L. rubescens* displayed moderate levels of genetic diversity with mean values of $P_P = 44\%$, AP = 2.23, and $H_{EP} = 0.135$ (Table 2). Observed mean heterozygosity (H_{OP}) was 0.140. Fixation indices (F_{IS}) were significantly different ($P < 0.05$) from the Hardy–Weinberg expected values in 24% (25 of the 105) of the chi-square tests. Based on chance alone, one would expect 5% to be significantly different from the expected Hardy–Weinberg value. The mean fixation index (F_{IS}) across all polymorphic

Table 2 Summary of allozyme variation for 16 populations of *L. rubescens*. Variation is described by proportion of all loci that are polymorphic (P; if a second allele is observed at a locus it is considered polymorphic), mean number of alleles per polymorphic locus (AP), mean observed heterozygosity (H_O), and mean expected heterozygosity (H_E). The number of samples assayed is represented by *N*. Standard deviations are shown in parentheses. Gene frequency data are available from DWT upon request

Sites	*N*	*P* (%)	AP	H_O (SD)	H_E (SD)
PB	49	33.3	2.60	0.087 (0.037)	0.092 (0.049)
SR	46	53.3	2.25	0.149 (0.044)	0.134 (0.056)
LB	49	46.7	2.29	0.123 (0.044)	0.119 (0.055)
HZ	50	46.7	2.29	0.109 (0.040)	0.136 (0.061)
ST	12	23.1	2.00	0.083 (0.000)	0.083 (0.048)
EV	55	40.0	2.00	0.180 (0.041)	0.165 (0.056)
RL	51	46.7	2.29	0.181 (0.042)	0.153 (0.062)
PV	86	42.9	2.00	0.126 (0.029)	0.124 (0.049)
PZ-3	49	40.0	2.33	0.146 (0.041)	0.141 (0.056)
HS-2	60	46.7	2.29	0.139 (0.038)	0.135 (0.051)
SJ-2	50	40.0	2.17	0.158 (0.039)	0.149 (0.058)
LJ	50	42.9	2.17	0.120 (0.036)	0.115 (0.051)
LE	48	53.3	2.13	0.161 (0.046)	0.160 (0.059)
NN	53	53.3	2.25	0.147 (0.037)	0.131 (0.058)
OR	45	53.3	2.38	0.186 (0.046)	0.180 (0.061)
DM	48	46.7	2.29	0.156 (0.041)	0.135 (0.059)
Population mean	50	44.3	2.23	0.140 (0.010)	0.135 (0.014)
Species level	801	73.3	2.55	—	0.174

Table 3 Description of the 16 cpDNA haplotypes identified in *L. rubescens*. Recognizable inserts are designated with *ins*. Fourteen haplotypes were observed in the 16 geographical sites while haplotypes F and L were only observed in very low frequencies in the additional eight pastures surveyed within ranches for smaller spatial scale analysis. Their frequencies are not included here

		Number of fragments		
Haplotypes	Frequency	AB—*Mbo*I	CD—*Alu*I	CD—*Taq*αI
A	41.1%	3	2	3
B	0.3%	3	2	4
C	6.1%	3	3	3
D	7.6%	3	3	4
E	26.5%	3	4	3
F	—	3	4	4
G	3.4%	3	4	5
H	0.8%	3	3 *ins*	3
I	1.0%	3	3 *ins*	4
J	0.5%	3	4 *ins*	3
K	0.3%	3	4 *ins*	4
L	—	3	4 *ins*	5
M	10.3%	4	2	4
N	1.0%	4	3	4
O	0.8%	4	3	5
P	0.3%	4	4	4

loci was 0.087 suggesting that there is a slight excess of homozygotes in these populations.

A two-way Mantel test showed a positive but nonsignificant correlation ($r = 0.215$) between differentiation among populations (i.e. F_{ST}) and geographical distance. The maximum distance separating two sites was 180 km.

cpDNA diversity

When the PCR amplified intergenic spacer (AB region) was digested with *Mbo*I, either three or four fragments were observed. The PCR amplified intron (CD region) revealed either two, three or four fragments when digested with *Alu*I and three, four or five fragments when digested with *Taq*αI. Fragment sizes observed with *Alu*I digestions varied, suggesting the presence of an indel. There was no apparent correspondence between the number of fragments observed for the CD region with the two enzyme digestions. Sequence data indicate that the closest *Alu*I and *Taq*αI sites were separated by 16 bp, thus restriction fragment length polymorphism (RFLP) patterns of these two enzymes should not be related. Fourteen haplotypes were detected in the 368 individuals sampled for the geographical analyses (Table 3). Two additional haplotypes (F and L) were detected in the 184 samples genotyped in the more detailed survey of multiple pastures within ranches (Table 3). Based on observed variation there is a possible maximum of 40 haplotypes. There was polymorphism at every site sampled with two to six (mean of 3.75) haplotypes observed/site (Table 4). The five most common haplotypes (A, C, D, E and M) occurred in 92% of the samples (Table 3). Three sites had private haplotypes: sites SR and EV each had one private haplotype while NN had two. The geographical distribution of chloroplast haplotypes E and M revealed regional differentiation in Costa Rica. Haplotype M occurred in the eight most northwestern sites, excluding PB (SR, LB, HZ, ST, EV, PV, RL and NN) while haplotype E was detected in the 10 southeastern populations (ST, EV, PV, PZ, HS, SJ, LJ, LE, OR and DM; Fig. 1). Neither haplotype was observed in PB while both haplotypes were detected in three intermediate sites: ST, EV and PV (Fig. 1). A significant difference ($P = 0.001$) in haplotype diversity values between these two assemblages was shown with jackknifing. The mean H_{ES} was 0.608 for the eight northwestern populations and 0.477 for the eight southeastern sites. This is in contrast to the allozyme diversity values which were significantly higher in the southeastern assemblage. This variation among the two regions may represent two ancient assemblages that have recently come into contact. Interestingly, the area of contact is the driest in all of Costa Rica. These three populations (ST, PV and EV) had lower densities of *L. rubescens* and less capsule production than typically observed elsewhere. Perhaps climatic conditions of this

Table 4 Summary of cpDNA haplotype variation at two polymorphic regions for 16 populations of *L. rubescens*. The number of samples is represented by *N*. Variation is described by total number of haplotypes, effective number of haplotypes and haplotype diversity. Haplotype frequency data are available from DWT upon request

Sites	*N*	Total no. of haplotypes	Effective no. of haplotypes	Haplotype diversity
PB	24	4	2.80	0.642
SR	24	3	2.07	0.517
LB	23	6	3.81	0.737
HZ	24	2	1.49	0.330
ST	10	3	2.63	0.620
EV	24	4	2.23	0.552
RL	24	5	3.65	0.726
PV	23	5	2.92	0.658
PZ-3	24	2	1.18	0.153
HS-2	24	3	2.27	0.559
SJ-2	24	4	1.71	0.417
LJ	24	2	1.28	0.219
LE	24	6	5.05	0.802
NN	24	6	3.79	0.736
OR	24	2	1.70	0.413
DM	24	3	2.46	0.594
Population mean	23	3.75	2.57	0.542
Species level	368	14	3.86	0.741

region contribute to lower fruit set, hindering the dispersal of genes through this zone.

At the species level, the effective number of haplotypes per population was 3.86 and haplotype diversity was 0.741 (Table 4). Genetic variation among populations was moderate (F_{ST} = 0.254; Table 5). At the population level, the effective number of haplotypes was 2.57 and mean haplotype diversity was 0.542 (Table 4). There was a small but nonsignificant positive correlation between cpDNA dispersal patterns and geographical distance (r = 0.062). A negative but nonsignificant correlation occurred between pairwise F_{STn} and pairwise F_{STc} values (r = −0.118).

Hierarchical spatial genetic structure

Pollen movement was not significantly greater than seed flow at the broadest geographical scale (tens of kilometres) and at the most local scale among pastures within ranches (scale of hundreds of metres) for two of the three ranches (PZ and SJ; Table 5). However, pollen migration significantly exceeded seed flow rates among pastures in the lower Tempisque River basin and among the three ranches as well as among pastures within one ranch (HS; Table 5). At the broadest geographical level, which encompassed collections from throughout Costa Rica, the highest genetic structure (F_{STn} = 0.091, F_{STc} = 0.254) as well as the lowest ratio of pollen to seed movement (m_p/m_s = 1.40; Table 5) were observed. At the intermediate scale of the three ranches in the lower Tempisque River basin, less genetic structure was observed for both nuclear and chloroplast markers and higher m_p/m_s values. The proportion of total allozyme diversity due to variation among the 11 pastures sampled in the area (F_{STn}) was 0.036 (Table 5). Of that, 14% (0.005) resulted from variation among the three ranches and 86% (0.031) resulted from variation among pastures within ranches. For chloroplast markers 0.181 (F_{STc}) of the total diversity was observed among the 11 pastures of which 40% (0.073) resulted from variation among ranches and 60% (0.108) was due to variation among pastures within ranches. Among pastures within the Tempisque River basin, pollen migration significantly exceeded seed migration (m_p/m_s = 3.92; Table 5). Significantly higher pollen movement relative to seed flow was most important among the three ranches in the lower Tempisque River basin (m_p/m_s = 13.67; Table 5).

Table 5 Summary of the partitioning of nuclear (F_{STn}) and cpDNA (F_{STc}) variation among sites and the ratio of pollen movement to seed movement (m_p/m_s). A mean of 23 samples per site were assayed. [A]Includes data from the 13 geographical sites throughout Costa Rica as well as one pasture per ranch (PZ-3, HS-2 and SJ-2) within the lower Tempisque River basin region. [B]This represents the partitioning of varation among the 11 pastures sampled from the three ranches in the lower Tempisque River basin and [C]represents the genetic structure among the three ranches in which the pastures occur. [D]Allozyme data only were available at this level of the analysis, which shows genetic structure among 17 populations (i.e. trees) within one ranch (HS). *95% confidence intervals generated by bootstrapping indicate that m_p/m_s differs significantly from 1.0 (Hamilton & Miller 2002)

	F_{STn}	F_{STc}	m_p/m_s
Among all locations throughout Costa Rica[A]	0.091	0.254	1.401
Among pastures in lower Tempisque basin[B]	0.036	0.181	3.918*
Among ranches in lower Tempisque basin[C]	0.005	0.073	13.671*
Among pastures within Porozol	0.070	0.231	1.991
Among pastures within Hacienda San Joaquin	0.029	0.135	3.226
Among pastures within Hacienda Solimar	0.018	0.106	4.469*
Among trees within pastures at Hacienda Solimar[D]	0.033	—	—
Among trees within Hacienda Solimar[D]	0.051	—	—

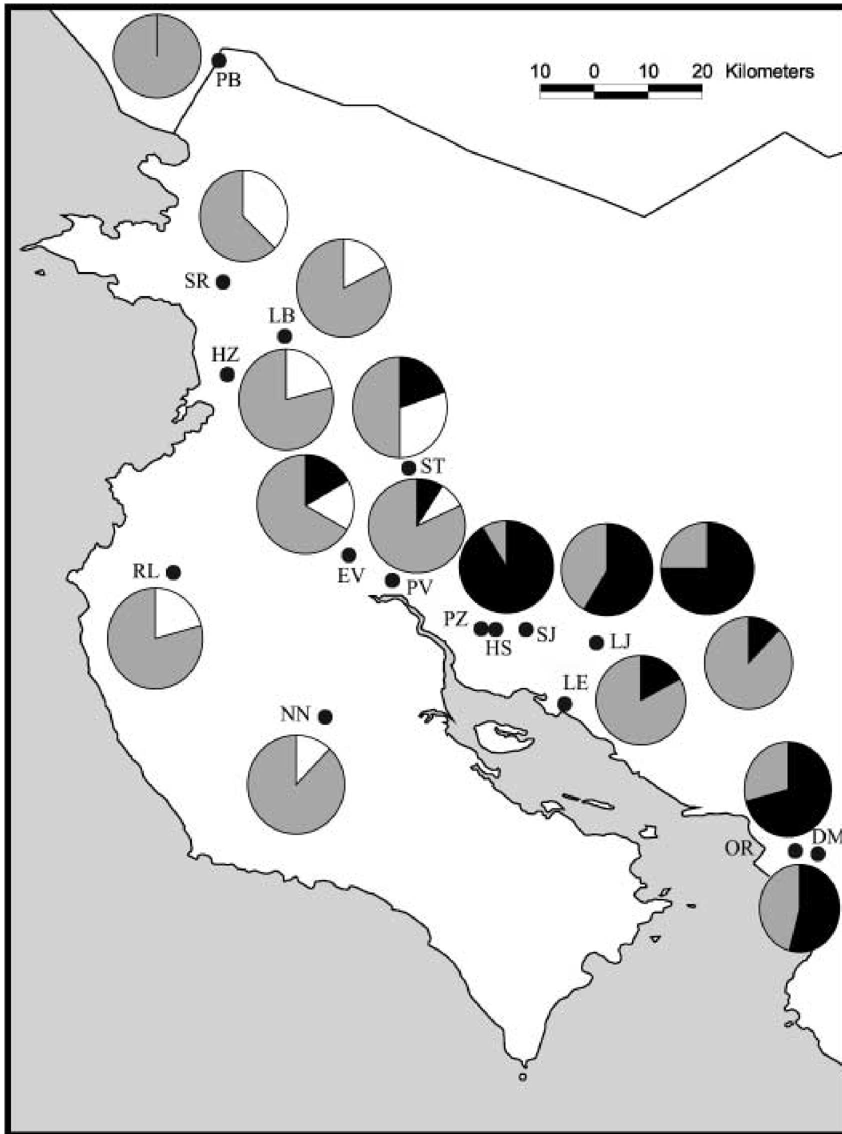

Fig. 1 Distribution of chloroplast haplotypes in Costa Rica. Two haplotypes in particular show a distinct southeast–northwest spatial pattern. Haplotype E (represented in black) was detected in the 10 southeastern sites while haplotype M (represented by white) was detected in the eight northwestern-most sites (with exception of PB). All other haplotypes have been pooled and are shown in grey.

Among pastures within ranches allozyme variation ranged from 0.018 to 0.070 (Table 5). For cpDNA, F_{STc} ranged from 0.106 to 0.231 (Table 5). In each case HS revealed the least genetic structure while PZ possessed the most. Similar pollen and seed flow rates were evident at PZ [(m_p/m_s = 1.99) and SJ (m_p/m_s = 3.23); Table 5]. Pollen movement was significantly greater than seed movement only at HS (m_p/m_s = 4.47; Table 5). Within HS a F_{STn} value of 0.051 was observed for 17 populations (i.e. trees) distributed in five pastures (Table 5). Of this, 35% (0.018) is among pastures and 65% (0.033; Table 5) is among trees within pastures. Chloroplast haplotype data were not available for all 17 populations. If nuclear variation among trees at HS is representative of variation among trees at other sites then 5% of the variation occurs among ranches, 39% occurs among pastures within ranches and 56% occurs among trees within pastures. However, the higher rates of pollen flow at HS may preclude such a comparison.

Discussion

Laelia rubescens is genetically diverse for both nuclear and cytoplasmic markers. Values of P and H_E for allozymes are higher in *L. rubescens* than the mean for animal-outcrossed plants with wind-dispersed seeds (Hamrick & Godt 1989). Partitioning of nuclear variation among sites (F_{STn} = 0.088) is also somewhat lower in *L. rubescens* than in outcrossed plant taxa with wind-dispersed seeds (G_{STn} = 0.101; Hamrick & Godt 1996) but similar to the mean for 16 orchid species (G_{STn} = 0.087; Hamrick & Godt 1996). Two terrestrial orchids for which the partitioning of nDNA and cpDNA markers is available (*Epipactis helleborine*; Squirrel *et al.* 2001 and

Anacamptis palustris; Cozzolino *et al*. 2003) had much higher G_{STn} (0.200 and 0.450, respectively) and G_{STc} values (0.506 and 0.670) in their native European range. The relatively low among-population variation observed in both genomes of *L. rubescens* is consistent with high levels of gene flow via both pollen (Trapnell & Hamrick, in review) and seeds and indicates that most of the species' genetic diversity occurs within individual populations.

Nuclear genetic structure was low at all spatial scales (F_{STn} = 0.005–0.091). While the F_{STc} values (0.073–0.254) were three to 15 times greater than the F_{STn} values for the same individuals, they displayed similar patterns at various spatial scales. These results and those of McCauley (1997), demonstrate that gene flow and genetic structure vary with scale, although less significantly for *L. rubescens* than for *Silene alba*. Highest genetic structure for both genomes was observed among the 16 geographically distributed sites (separated by tens of kilometres) situated throughout Costa Rica, although there was no significant correlation of pairwise F_{STn} and F_{STc} with distance between sites. The lack of an IBD effect may be due in part to the NW–SE disjunct distribution seen for both the allozyme and the cpDNA data (Fig. 1).

Interestingly, pollen and seed movement rates were not significantly different at the broadest geographical scale, suggesting that seed dispersal contributes substantially to overall gene flow on a scale of kilometres. However, pollen migration was significantly higher than seed flow at intermediate scales (among pastures in the lower Tempisque River basin and among the three ranches). The lowest genetic structure for both genomes and the strongest asymmetry in pollen and seed flow rates (m_p/m_s = 13.67) were observed among the three ranches. This indicates considerable pollen flow across spatial scales of 100s to a few 1000s of metres, which may reflect the foraging range and efficacy of the hummingbird pollinators. Pollinator effectiveness at this scale is supported by direct estimates of pollen movement (determined by paternity analysis of full-sib progeny arrays) beyond 1000 m (Trapnell & Hamrick, in review).

At the most local scale with both nuclear and chloroplast data, pollen and seed migration contributed equally to gene movement among pastures within two ranches (PZ and SJ). This observed symmetry between pollen and seed flow is consistent with highly localized seed dispersal demonstrated by the existence of significant fine-scale genetic structure at distances ≤ 0.45 m within populations (Trapnell *et al*. 2004). However, pollen was responsible for more gene flow than seeds among pastures within the third ranch (HS). Differences between HS and the other two ranches may be attributable to the observation that there were many more populations sampled at HS that were generally larger and more closely arranged. These factors would facilitate greater pollinator activity and increased pollen migration. For allozymes, the smallest spatial scale examined was among trees within pastures at HS (separated by 10s of metres) which had a higher F_{STn} value (0.033) than expected, suggesting that populations may be established by one or a few colonists. Subsequent population growth is accomplished, in part, by the deposition of seeds produced within the tree (Trapnell *et al*. 2004).

These data demonstrate that high levels of gene flow are achieved by different mechanisms at different spatial scales. Like McCauley (1997) we found scale-dependent relative contributions of pollen and seeds to gene migration. Much seed dispersal is highly localized within the maternal population (on a scale of centimetres, Trapnell *et al*. 2004). However, some seeds enter the air column and are dispersed over long distances (on a scale of kilometres). Long-distance seed migration is sufficiently common to minimize the importance of reduced cpDNA effective population size in structuring these populations. At the intermediate scale of 100s to a few 1000s of metres, hummingbird foraging behaviour facilitates substantial pollen flow, giving rise to a strong asymmetry between pollen and seed movement at this scale. Primary factors governing gene movement are gravity at the smallest scale, hummingbird behaviour at the intermediate scale and wind at the broadest spatial scale.

There are two models of geographical expansion that predict different spatial distributions of alleles (Ibrahim *et al*. 1996; Le Corre *et al*. 1997). One is the phalanx or diffusion expansion model that describes a steady 'wave front' expansion of the species resulting in a relatively homogeneous distribution of allele frequencies among populations. Alternatively, colonization involves occasional long-distance dispersal events followed by localized spreading. This would create a mosaic of allele frequencies and relatively higher F_{ST} values. The *L. rubescens* data suggest pioneer expansion whereby wind-blown seeds colonize novel sites. Once a site is colonized, localized seed dispersal from the original colonists coupled with occasional but consistent long-distance seed establishment results in a pattern of fine-scale genetic structure at very short distances and the maintenance of genetic diversity in the population. Pollen movement on the order of 100s of metres further homogenizes gene frequencies at this scale.

Seed dispersal is more important to gene flow in *L. rubescens* than most other plant taxa examined, with the exception of *A. palustris* (Cozzolino *et al*. 2003). Tree species with wind-pollination and gravity or animal-dispersed seeds tend to have the highest m_p/m_s ratios (e.g. *Fagus crenata*; m_p/m_s = 640; Tomaru *et al*. 1998). The low m_p/m_s values reported for orchids *A. palustris* (0.48), *L. rubescens* (1.40) and *E. helleborine* (1.43) are consistent with animal-mediated pollination and wind-dispersed seeds. Animal pollinators often have a restricted foraging range while wind-dispersed seeds are capable of moving considerable distances. Seeds

of an epiphyte such as *L. rubescens* have the added advantage of higher entry into the wind column, which should improve long-distance movement. The much lower F_{STn} and F_{STc} values for *L. rubescens* relative to those of *A. palustris* and *E. helleborine* support this expectation and indicate that *L. rubescens* has higher rates of both pollen and seed-mediated gene flow than these two terrestrial orchids.

Mating system, gene movement among populations and the spatial distribution of suitable habitat strongly influence a species' genetic structure. For *L. rubescens*, suitable habitat means trees with bark that is sufficiently coarse and possesses suitable mycorrhizae for the seeds to germinate and establish. Much of the seasonally dry tropical forest of Costa Rica has been highly disturbed for half a century, with substantial areas of habitat suitable for *L. rubescens* destroyed. Interestingly, populations of *L. rubescens* are much larger in these recently fragmented habitats than in undisturbed forests, where populations tend to be distributed in a highly disjunct manner. Direct measures of pollen flow showed lower rates of gene flow in these larger populations than in smaller populations that more closely resembled those in undisturbed forest (Trapnell & Hamrick, in review) which is contrary to the pattern discussed by Young *et al*. (1996). Because observed patterns of pollen and seed migration are based on the genetic structure of these young populations, these patterns represent contemporary processes. Relatively little partitioning of biparentally inherited nuclear and maternally inherited cytoplasmic markers was found. Our data show that *L. rubescens* has maintained high rates of gene flow among populations at spatial scales of a kilometer or more via both pollen and seed dispersal and that the relative importance of each dispersal mechanism varies with spatial scale. The data suggest that infrequent but evolutionarily important long-distance seed dispersal events are primarily responsible for reducing genetic structure at larger spatial scales while animal-mediated pollen dispersal plays the dominant role in homogenizing genetic diversity at more local scales. When taken together, pollen and seeds produce high rates of gene flow at all spatial scales, which make the tail of the gene dispersal curve much fatter than can either mechanism alone. We would therefore predict that *L. rubescens*'s life-history traits and its high levels of genetic diversity will allow it to persist in highly disturbed landscapes.

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This research is a part of Dorset Trapnell's doctoral dissertation. She uses molecular markers to understand evolutionary factors that shape patterns of genetic variation in natural populations and the consequences of habitat disturbance. This work reflects her deep interest in tropical epiphytes and particularly the Orchidaceae. Her dissertation adviser is Jim Hamrick, whose focus is on the genetics and evolution of natural plant populations with an emphasis on the genetic structure of populations and on those evolutionary factors that influence the development and maintenance of genetic structure; natural selection, the mating system and pollen and seed dispersal patterns.