RESEARCH PAPER

Heterozygote excess through life history stages in *Cestrum miradorense* Francey (Solanaceae), an endemic shrub in a fragmented cloud forest habitat

F. Reyes-Zepeda[1,2,3], J. González-Astorga[2] & C. Montaña[1]

1 Instituto de Ecología, A. C., Xalapa, Veracruz, México
2 Laboratorio de Genética de Poblaciones, Red de Biología Evolutiva, Instituto de Ecología, A. C., Xalapa, Veracruz, México
3 Present address: Instituto Tecnológico Superior de Tantoyuca, Tantoyuca, Veracruz, México

ABSTRACT

Comparisons of genetic diversity and population genetic structure among different life history stages provide important information on the effect of the different forces and micro-evolutionary processes that mould diversity and genetic structure after fragmentation. Here we assessed genetic diversity and population genetic structure using 32 allozymic loci in adults, seeds, seedlings and juveniles of eight populations of the micro-endemic shrub *Cestrum miradorense* in a highly fragmented cloud forest in central–eastern Mexico. We expected that due to its long history or rarity, this species may have endured the negative effects of fragmentation and would show moderate to high levels of genetic diversity. High genetic diversity (H_e = 0.445 ± 0.03), heterozygote excess (F_{IT} = −0.478 ± 0.034, F_{IS} = −0.578 ± 0.023) and low population differentiation (F_{ST} = 0.064 ± 0.011) were found. Seeds had higher genetic diversity (H_e = 0.467 ± 0.05) than the later stages (overall mean for adults, seedlings and juveniles H_e = 0.438 ± 0.08). High gene flow was observed despite the fact that the fragmentation process began more than 100 years ago. We conclude that the high genetic diversity was the result of natural selection, which favours heterozygote excess in all stages, coupled with a combination of a reproductive system and seed/pollen dispersal mechanisms that favour gene flow.

INTRODUCTION

Analysis of genetic variability among different stages of the life cycle provides vital information about the micro-evolutionary forces and processes that mould diversity and genetic structure in space and time (Álvarez-Buylla *et al.* 1996; Kalisz *et al.* 2001; Octavio-Aguilar *et al.* 2009). Effects of inbreeding depression, gene drift and microhabitat selection have been found in comparisons of adults, seeds, seedlings and juveniles (Conte *et al.* 2003; Lowe *et al.* 2005; Farwig *et al.* 2008). Increased genetic diversity due to genotype accumulation, along with modifications of seed longevity due to natural selection and damaging mutations have been found in studies of the temporal variation of genetic diversity in the seed bank of some species (Cabin *et al.* 1998; Honnay *et al.* 2008). When analysing the seeds produced in a single year (*e.g.* those collected from mother trees), the effects of gene flow and heterozygote excess on variation of genetic diversity between adults and seeds have been found (Eguiarte *et al.* 1992; Mandák *et al.* 2006; Mathiasen *et al.* 2007). Higher genetic diversity in adults than in their progeny has been found, particularly in long-lived species (Tonsor *et al.* 1993; González-Astorga & Castillo-Campos 2004; Fernández-M & Sork 2007; Mathiasen *et al.* 2007). However, it has also been reported that the seed stage is a reservoir of genetic diversity in some species (Álvarez-Buylla *et al.* 1996; Chung *et al.* 2003; Conte *et al.* 2003; Octavio-Aguilar *et al.* 2009).

Species with restricted distribution (*e.g.* endemics) tend to have less genetic diversity when compared to widely distributed species of the same genus (Cole 2003). However, not all endemic species have low levels of genetic variability: life history traits such as mating systems, and pollen and seed dispersal mechanisms can maintain genetic diversity at a relatively high level (Loveless & Hamrick 1984; Hamrick & Godt 1996). Honnay & Jacquemyn (2007) found that genetic diversity of naturally rare species (*i.e.* species with a long history of rarity) was less affected by habitat fragmentation than species that recently became rare (*e.g.* due to habitat fragmentation). These latter species lose much genetic diversity due to habitat fragmentation when the fragmented habitats cannot support the population numbers necessary for the mutation-drift equilibrium, or when between-fragment distances do not allow the gene flow necessary to recover the loss of rare alleles. These authors also indicate that obligate or mainly outcrossing species are more vulnerable to the loss of genetic variation through habitat fragmentation than self-compatible species.

Some meta-analyses have found that the negative effect of fragmentation varies depending on the life form and

disturbance intensity, and that those effects may be different in each life history stage (Lowe *et al.* 2005; Leimu *et al.* 2006). Aguilar *et al.* (2008) found that the negative effects on heterozygosis increase as the number of generations after fragmentation increase. These authors showed that the negative effects of fragmentation are stronger in an ecosystem that has been fragmented for more than 100 years than in one that has only been fragmented for 50 years.

Montane cloud forests are ecosystems with great biological richness that include several rare forms (Rzedowski 1996) and have suffered severe anthropogenic pressure, which has led to increasing fragmentation and isolation, with remnant forest patches increasing to form a matrix of vegetation archipelagos (Challenger 1998). The species that live on these forest remnants are ideal research subjects for the study of ecological and evolutionary effects of fragmentation in small, isolated and subdivided populations.

The genus *Cestrum* L. (Solanaceae) originates from the Americas and contains approximately 250 species distributed from subtropical parts of northern Mexico to northern Argentina. Phylogenetic studies suggest that during the Pliocene, high rates of diversification and speciation took place (Montero-Castro 2006), causing most of its species to become geographically restricted along mountain ranges and mostly in montane cloud forest, with a dozen species being widely distributed (Nee 2001). In Mexico, 14 species are found, and Veracruz is the state having the highest biodiversity of this genus, with 10 species, of which only four are endemic to cloud forests (*Cestrum elegans, C. endlicheri, C. fasciculatum* and *C. miradorense*; Castillo-Campos 2003). Phylogenetically, these species constitute one monophyletic group that underwent speciation ca. 3.1 million years ago (Montero-Castro *et al.* 2006).

Here we compared the diversity and genetic structure among four life history stages (*i.e.* adults, seeds, seedlings and juveniles) of *Cestrum miradorense* Francey, a naturally microendemic shrub. We expected that due to its long history of rarity, this species would show moderate to higher levels of genetic diversity than those reported for long-lived perennials of wider distribution that have become rare only recently after undergoing fragmentation.

MATERIAL AND METHODS

Study species

Cestrum miradorense is a shrub of 1–5 m in height, diploid and hermaphrodite, with terminal and axillary inflorescences and tetramerous or pentamerous tubular flowers (1.5 ± 0.03 cm long and 0.06 ± 0.02 cm wide (±SD), n = 50 flowers; F. Reyes-Zepeda, unpublished observations). The corollas open during the day and are creamy-white with violet mottling and are odourless; the fruits are blue-coloured berries that turn light purple when mature and are 10–15 mm in diameter (Nee 1986). Due to the lack of ecological and genetic studies in the species – this being the first study – its reproductive biology and pollinators are unknown. However, Montero-Castro (2006) mention that *Cestrum* species are pollinated by nocturnal moths and that they have out-crossed mating and self-incompatible reproductive systems, based on

the fact that several phylogenetically related species to *Cestrum* share these traits (Haber & Frankie 1989; Castro-Laportte & Ruiz-Zapata 2000; Aguilar & Galetto 2004). Similarly, the karyotype of *Cestrum* species has 2n = 2x = 16 chromosomes that are similar in size and shape, most of which are meta- or submetacentric except for one subtelocentric pair (Berg & Greilhuber 1993; Nunes *et al.* 2006). Montero-Castro *et al.* (2006) conclude that it is very likely that *C. miradorense* has the same number of chromosomes (*i.e.* 2n = 2x = 16) as the closely related species, *C. elegans*.

Only eight *C. miradorense* populations have been found (Fig. 1); all are small and show clumped distribution (245 ± 95 plants in 0.5 ha, n = 8 samples, one sample of 0.5 ha each, one in each population), usually growing along riverbanks and in very humid areas. It is not known whether seed banks are produced, but refrigerated seeds (collected in the summer of 2005 and 2006) had 50% germination by the summer of 2007 (F. Reyes-Zepeda, unpublished observations). Adults are able to resprout after disturbance (F. Reyes-Zepeda, unpublished observations).

Study sites

The species distribution is restricted to an area of ca. 100 km² located in the central part of the Mexican state of Veracruz (19°27′–19°35′ N, 96°56′–97°02′ W; Fig. 1). *C. miradorense* grows exclusively in cloud forests (Nee 1986), an ecosystem that covers around 0.8% of Mexican territory (Rzedowski 1996). The species has been found between 1250 and 1850 m a.s.l., in sites where the mean annual temperatures varies between 12 and 18 °C and total annual rainfall is between 1350 to 2200 mm (meteorological stations in Xalapa, 19°32′ N, 96°55′ W, 1390 m a.s.l, and Tembladeras, 19°30′ N, 97°07′ W, 2960 m a.s.l.) over the last 20 years.

The fragmentation of the region's cloud forest started more than a century ago, and over the past 30 years the process has intensified, causing the loss of over 70% of the original cover due to agricultural and cattle raising activities, as well as urban development (Challenger 1998; Williams-Linera *et al.* 2002). The only published work in the study region found that in 1990, over a 1325-km² area, the cloud forest cover was 426.9 km², and by 2003 this had dropped to 279.5 km², meaning a loss of 34.8% cover in 13 years (Muñoz-Villers & López-Blanco 2008). In digital orthophotos of the study area (INEGI 1995), a highly fragmented landscape is observed, with forest remnants of varying shapes and sizes within a matrix of different land-use regimes (Fig. 1). To the north of the 20-km² distribution area of the species, only about a 10% cover remains as undisturbed forest and it is on steep slopes of private properties within a matrix of induced pastures, urban development and fallow lands (Williams-Linera *et al.* 2002). To the south of the distribution area, there is higher forest cover where larger forest fragments are immersed in a matrix of coffee plantations, disturbed forest and zones of secondary vegetation (fallow land or 'acahuales' in Spanish). To the west, in a rugged mountain area, forest fragmentation is more important due to extensive wood exploitation and local disturbance that began in the 18th century (Gerez 1992) in the transition zone between cloud forest and pine–oak forests.
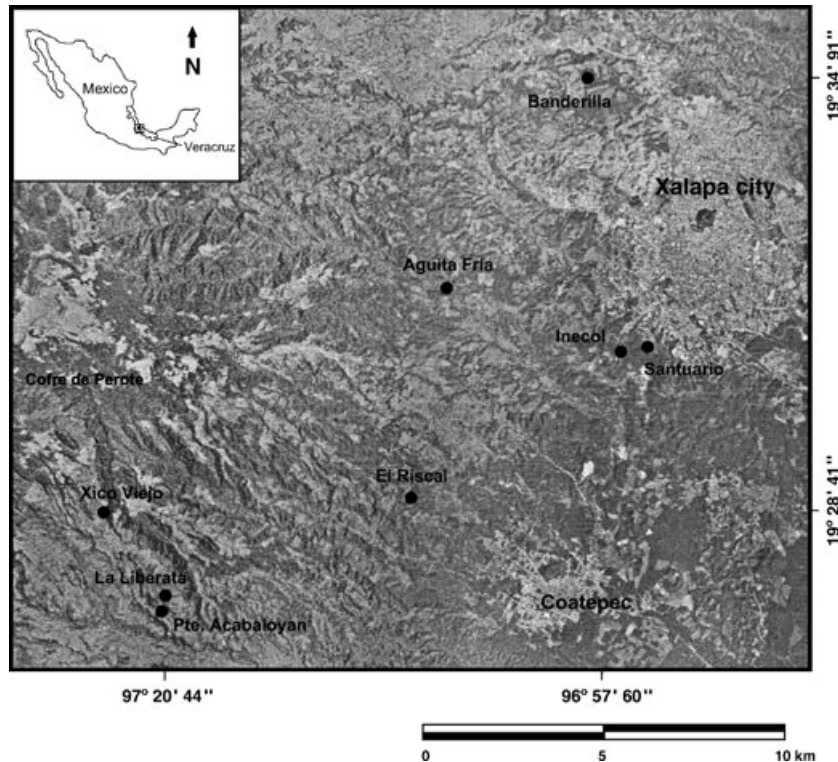
Fig. 1. Geographic distribution of eight *Cestrum miradorense* populations found in a fragmented cloud forest landscape in central-eastern Mexico. Population location is indicated by a dot. Cofre de Perote is the highest relief of the area (4282 m a.s.l.).

Sample collection

We collected young leaves from 18–30 individuals for all stages, except seeds, from the eight known population (696 samples in total). Samples were placed in plastic bags and transported in containers on ice. Later, they were frozen at −70 °C in a Revco ultra-freezer, where they remained until protein extraction was carried out. For each population, 10 mature fruits from 30 reproductive individuals were also collected; their seeds were allowed to germinate under controlled conditions (12-h day at 25 °C and 12-h night at 20 °C on filter paper that was kept saturated with water). The germination percentage varied between 81 and 85% (F. Reyes-Zepeda, unpublished observations). The seedlings obtained were raised for 2 months under the climate conditions of the local cloud forest and in soils collected in the same study sites where the seeds were collected. After that time, a random leaf sample from 30 seedlings per population (240 seedlings in total) was taken. The genetic characteristics of the tissue sampled from these seedlings were assumed to pertain to the seed stage.

Electrophoresis

Electrophoresis was performed using horizontal starch gels of 12% w/v (Müller-Strack 1998). Enzyme extraction was done by grinding 100 mg of leaf tissue from each individual with 250 µl of extraction buffer, as per Whalen & Caruso (1983). Fifteen allozymes were processed with two electrophoresis systems: Poulik (50 mA and 200 V) and L-histidine (30 mA

and 200 V) (Table 1). Gels were stained following the protocols of Wendel & Weeden (1989). The interpretation of zymograms (banded patterns on gels) was based on the number of chromosomes and the ploidy (2n = 2x = 16); furthermore, previous knowledge of the quaternary structure and number of loci of the enzymes studied was taken into account (Soltis & Soltis 1989).

Statistical analyses

Multilocus genotypes were used to estimate allele frequencies with the TFPGA 1.3 program (Miller 1997). The mean number of alleles per locus (A) was calculated, along with the polymorphic loci percentage (95% PL), mean observed heterozygosis (H_o) and mean expected heterozygosis (H_e) according to Hardy–Weinberg equilibrium (Hedrick 2000). For each locus, Hardy–Weinberg deviations were estimated with permutation tests. Linkage disequilibrium between all pairs of loci was tested using the software package FSTAT ver. 2.9.3 (Goudet 2001). Bonferroni correction for multiple testing was used for final estimate of statistical significance between comparisons of the LD test (Rice 1989). A nonparametric two-way Kruskal–Wallis ANOVA (Zar 2010), followed by multiple comparison Tukey tests, was used to identify differences in H_o and H_e among stages and populations (Zar 2010).

Genetic structure for each population and stage was estimated using *F*-statistics (Weir & Cockerham 1984). Deviations of F_{IT}, F_{IS} and F_{ST} from zero over all loci in each stage and population were tested using 95% confidence intervals

Table 1. Allozyme systems used in this study. Abbreviations based on IUBCN Enzyme Commission Number. *PK and L-H reference of buffer systems (gel/electrode) from Soltis & Soltis (1989).

allozyme	abbreviation	enzyme commission number	buffer*
6-phosphogluconate dehydrogenase	*6pgd1* and *6pgd2*	1.1.1.44	PK
aldolase	*Ald1, Ald2* and *Ald3*	4.1.2.13	PK
glucose-3-phosphate dehydrogenase	*G3pdh1, G3pdh2* and *G3pdh3*	1.2.1.12	PK
hexokinase	*Hk1* and *Hk2*	2.7.1.1	PK
isocitrate dehydrogenase	*Idh1, Idh2* and *Idh3*	1.1.1.41	PK
malate dehydrogenase	*Mdh1* and *Mdh2*	1.1.1.37	PK
menadione reductase	*Mnr1* and *Mnr2*	3.4.11.1	PK
phosphoglucose isomerase	*Pgi1* and *Pgi2*	5.3.1.9	PK
phosphoglucomutase	*Pgm1* and *Pgm2*	5.2.2	PK
shikimate dehydrogenase	*Sdh1* and *Sdh2*	1.1.1.25	PK
acid phosphatase	*Acph*	3.1.3.2	L-H
anodic peroxidase	*Apx1, Apx2* and *Apx3*	1.11.1.7	L-H
diaphorase	*Dia1* and *Dia2*	1.6.99	L-H
esterase	*Est*	3.1.1	L-H
malic enzyme	*Me1* and *Me2*	1.1.1.40	L-H

made on the basis of 5000 bootstrap replicates using the FSTAT software. A Kruskal–Wallis two-way ANOVA was applied, followed by multiple comparisons Tukey tests, to identify differences among stages and populations (Zar 2010).

The average gene flow among paired populations $N_m = (1/F_{ST}-1)/4$ was estimated on the basis of values for adults and seeds (Slatkin 1993). Despite the limitations of estimating N_m on the basis of F_{ST} (Allendorf & Luikart 2007: 222), it is considered important for genetic conservation, especially in fragmented environments, due to their susceptibility to rapid genetic changes. To determine whether there was geographic isolation, we regressed the number of migrants per population and the geographic distance with the Mantel test only for adults. Using the genetic distances of Nei (1972) and the UPGMA algorithm (unweighted pair group with arithmetic mean; Sneath & Sokal 1973), two dendrograms were built on the basis of genetic distances: one for populations by nesting, in each population, the information of the life history stages, and another for life history stages, where each life history stage of each population was a separate operational unit.

RESULTS

Genetic diversity

A total of 32 polymorphic loci were found in 15 alloenzymatic systems. In all cases, there were two alleles per locus. Adult, seedling and juvenile stages had 26 loci, while the seed stage had 32 (Table S1). The percentage of polymorphic loci was 99.6% in most stages and populations (Table 2). Most loci (72.2%) were in Hardy–Weinberg disequilibrium, although this was more marked during early than late stages (adults 62.5%, seeds 90.2%, seedlings 75.5%, juveniles 76.5%; Table S1). Linkage disequilibrium was observed in all stages (P < 0.0001 after Bonferroni correction).

Across the range of populations, observed mean heterozygosity was 0.707 ± 0.09 (range 0.521–0.822), while expected mean heterozygosity was 0.445 ± 0.03 (range 0.364–0.478). Seeds represented the life history stage with the highest genetic diversity (H_o = 0.783 ± 0.17 and H_e = 0.467 ± 0.05) when compared to adults (H_o = 0.670 ± 0.21 and H_e = 0.429 ± 0.09), seedlings (H_o = 0.693 ± 0.19 and H_e = 0.446 ± 0.07) and juveniles (H_o = 0.682 ± 0.19 and H_e = 0.438 ± 0.07) (Table 2). Both observed and expected heterozygosity differed among populations (Kruskal–Wallis test: $\chi^2_{0.05,7}$ = 21.7; P < 0.001 for H_o and $\chi^2_{0.05,7}$ = 23.4; P < 0.001 for H_e) and stages ($\chi^2_{0.05,3}$ = 14.3; P < 0.001 for H_o and $\chi^2_{0.05,3}$ = 12.7; P < 0.001 for H_e). Interaction between life history stages and populations was significant ($\chi^2_{0.05,7}$ = 16.7; P < 0.001 for H_o and $\chi^2_{0.05,7}$ < 14.3; P < 0.001 for H_e). The interaction between stages and populations showed that the between-population variability of H_o and H_e means was higher in adults (range 0.522–0.824 for H_o and 0.365–0.478 for H_e) than in seeds (range 0.736–0.823 for H_o and 0.455–0.478 for H_e), seedlings (range 0.639–0.815 for H_o and 0.447–0.487 for H_e) and juveniles (range 0.529–0.815 for H_o and 0.376–0.476 for H_e). Furthermore, in three populations, H_e values increased together with life cycle development, while in two other populations the opposite occurred (Fig. 2). The between-population multiple comparisons showed two groups of populations (P < 0.0001): a lower heterozygosity group (Xico Viejo, La Liberata and Puente Acabaloyan, H_e = 0.426 ± 0.04) as compared to a higher heterozygosity group (El Riscal, Agüita Fría, Inecol, Santuario and Banderilla, H_e = 0.457 ± 0.02). The between-stage multiple comparisons showed that seed heterozygosity was different from the other stages, in which there was no difference between them (P < 0.0001).

Genetic structure

The global inbreeding mean (F_{IT}) was −0.478 ± 0.034 (range −0.408 to −0.609), differing significantly among stages ($\chi^2_{0.05,2}$ = 56.0; P < 0.001) and from zero (P < 0.05 in all cases). Adults were observed to have higher F_{IT} values than the other stages (Fig. 3a). The local inbreeding mean (F_{IS}) was −0.578 ± 0.023 (range −0.540 to −0.667), which also showed differences among stages ($\chi^2_{0.05,2}$ = 42.3; P < 0.001) and from zero (P < 0.05 in all cases). It was clear that F_{IS} values increased from seeds to juveniles (Fig. 3b). Negative

Table 2. Genetic variability of four life history stages in eight populations of *Cestrum miradorense* growing in a fragmented landscape of cloud forests in central-eastern Mexico. Ni: average sample size; P: percentage of polymorphic loci; observed (H_o) and expected (H_e) heterozygosity.

population	stage	Ni	P	H_o	H_e
Xico Viejo	Adults	30	96	0.522 ± 0.26	0.365 ± 0.12
	Seeds	30	100	0.797 ± 0.20	0.465 ± 0.07
	Seedlings	30	100	0.699 ± 0.23	0.445 ± 0.08
	Juveniles	28	100	0.529 ± 0.25	0.376 ± 0.11
	Mean ± SD	29.5 ± 1.0	99 ± 2.0	0.636 ± 0.13	0.412 ± 0.05
La Liberata	Adults	30	100	0.535 ± 0.17	0.395 ± 0.09
	Seeds	30	100	0.763 ± 0.14	0.467 ± 0.03
	Seedlings	25	100	0.651 ± 0.17	0.436 ± 0.07
	Juveniles	30	100	0.649 ± 0.17	0.432 ± 0.08
	Mean ± SD	28.8 ± 2.5	100 ± 0.0	0.649 ± 0.09	0.433 ± 0.03
Puente Acabaloyan	Adults	30	96	0.596 ± 0.26	0.383 ± 0.13
	Seeds	30	100	0.823 ± 0.14	0.478 ± 0.03
	Seedlings	18	100	0.622 ± 0.23	0.418 ± 0.10
	Juveniles	25	100	0.687 ± 0.19	0.447 ± 0.07
	Mean ± SD	25.8 ± 5.7	99 ± 2.0	0.682 ± 0.11	0.432 ± 0.04
El Riscal	Adults	30	100	0.780 ± 0.15	0.465 ± 0.05
	Seeds	30	100	0.773 ± 0.13	0.469 ± 0.04
	Seedlings	30	100	0.685 ± 0.19	0.454 ± 0.11
	Juveniles	30	100	0.643 ± 0.22	0.420 ± 0.08
	Mean ± SD	30.0 ± 0.0	100 ± 0.0	0.720 ± 0.07	0.452 ± 0.02
Agüita Fría	Adults	30	100	0.824 ± 0.11	0.489 ± 0.02
	Seeds	27	100	0.790 ± 0.15	0.468 ± 0.05
	Seedlings	30	100	0.701 ± 0.13	0.448 ± 0.04
	Juveniles	30	100	0.815 ± 0.14	0.475 ± 0.04
	Mean ± SD	29.3 ± 1.5	100 ± 0.0	0.782 ± 0.06	0.468 ± 0.01
Inecol	Adults	30	100	0.652 ± 0.18	0.443 ± 0.07
	Seeds	30	100	0.777 ± 0.18	0.465 ± 0.06
	Seedlings	30	100	0.639 ± 0.16	0.432 ± 0.06
	Juveniles	30	100	0.646 ± 0.14	0.432 ± 0.05
	Mean ± SD	30.0 ± 0.0	100 ± 0.0	0.678 ± 0.07	0.443 ± 0.02
Santuario	Adults	30	96	0.655 ± 0.18	0.433 ± 0.09
	Seeds	30	100	0.803 ± 0.16	0.469 ± 0.05
	Seedlings	30	100	0.730 ± 0.21	0.454 ± 0.06
	Juveniles	30	100	0.680 ± 0.14	0.445 ± 0.05
	Mean ± SD	30.0 ± 0.0	99 ± 2.0	0.717 ± 0.07	0.450 ± 0.02
Banderilla	Adults	30	100	0.799 ± 0.15	0.468 ± 0.06
	Seeds	30	100	0.736 ± 0.22	0.455 ± 0,07
	Seedlings	30	100	0.815 ± 0.12	0.479 ± 0.03
	Juveniles	30	100	0.805 ± 0.11	0.476 ± 0.03
	Mean ± SD	30.0 ± 0.0	100 ± 0.0	0.789 ± 0.04	0.470 ± 0.01
	Global variation ± SD	29.2 ± 1.5	99.62 ± 1.18	0.707 ± 0.09	0.445 ± 0.03

values for both parameters indicate an excess of heterozygous genotypes in all populations and stages (Table 3). The average population differentiation (F_{ST}) was 0.064 ± 0.011, differing among stages ($\chi^2_{0.05,2}$ = 55.8; P < 0.001). The multiple comparisons showed that the F_{ST} of seeds and adults differed from all other stages (P < 0.0001), while it did not differ between seedlings and juveniles (P = 0.999). Seeds were observed to be the stage with a lower genetic differentiation (F_{ST} = 0.034 ± 0.008), while adults had the highest genetic structure (F_{ST} = 0.085 ± 0.015; Fig. 3c, Table 3).

Gene flow and genetic distance

Average gene flow (N_m) estimated for paired populations was lower in adults (N_m = 3.9 ± 3.3) than in seeds (N_m = 8.1 ± 3.3). In the former, the lowest value was found between

Xico Viejo and Puente Acabaloyan populations (3.13 km apart, N_m = 1.2) and the highest value was recorded between Agüita Fría and Banderilla populations (6.65 km apart, N_m = 16.9). In seeds, the lowest value was found between Xico Viejo and Inecol populations (15 km apart, N_m = 3.8) and the highest value was recorded between El Riscal and Banderilla populations (11.33 km apart, N_m = 18.8). The relationship between gene flow and geographic distance between paired populations of adults was not statistically significant (Mantel test: P = 0.9690; r = −0.3075).

Nei's genetic distance dendrogram among populations showed that the western populations Xico Viejo and La Liberata are separated from the central and eastern populations Inecol, Santuario, Agüita Fría, El Riscal and Banderilla; Puente Acabaloyan, also in a western location, is the most genetically distant population (Fig. 4). On the other hand,
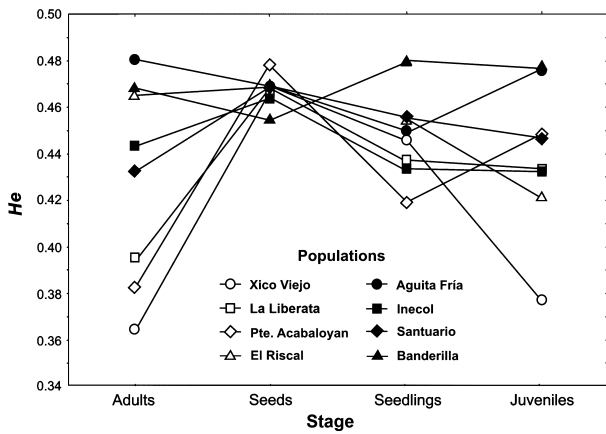
Fig. 2. Interaction among expected heterozygosis values (H_e) for stages and populations of *Cestrum miradorense*.
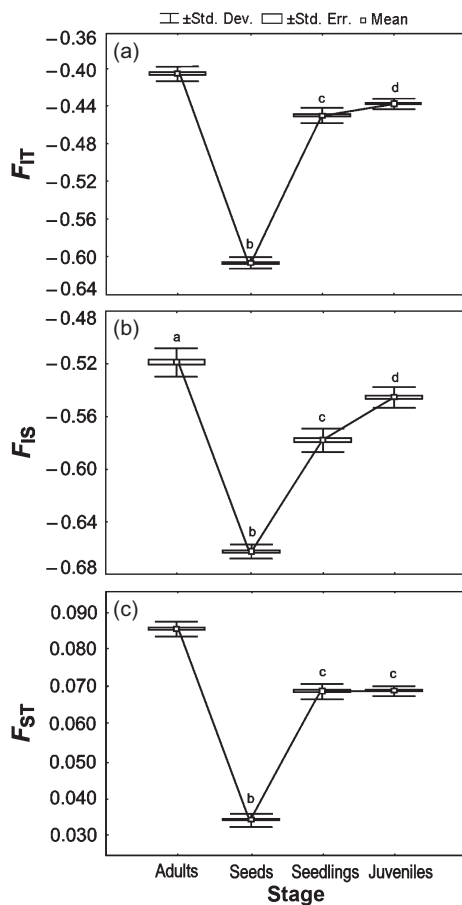

Fig. 3. Wright's *F* statistics for four *Cestrum miradorense* life history stages in eight populations found in a fragmented cloud forest landscape in central-eastern Mexico. Different letters show significant differences among classes (P < 0.001).

among stages, genetic distance revealed that seeds from all populations formed a single group (0.0451, dendrogram not shown).

DISCUSSION

Genetic diversity

Honnay & Jacquemyn (2007) suggest that habitat fragmentation affects genetic diversity of plant populations and that certain life history and ecological traits of plants can determine differential susceptibility to genetic erosion in fragmented habitats. They also propose that species with a long history of rarity are less susceptible to genetic erosion than species that recently became rare due to a decrease in population numbers and habitat fragmentation. *C. miradorense*, a micro-endemic species that lives in a highly fragmented landscape, showed surprisingly high levels of genetic diversity; higher than the average reported in Hamrick (2004) for 213 long-lived perennial plants (P = 47.9, H_e = 0.144) and very similar to that of other widely distributed Solanaceae, such as *Capsicum annuum* (P = 90.8, H_e = 0.445; Hernández-Verdugo *et al.* 2001).

Analysis of genetic diversity through different life history stages showed that seeds have higher genetic diversity than their parents, suggesting that natural selection favours heterozygous genotypes (Eguiarte *et al.* 1993; Conte *et al.* 2003). The heterozygote excess found in all stages and populations (cf. Eguiarte *et al.* 1992; González-Astorga *et al.* 2003; Fernández-M & Sork 2007) shows that natural selection is taking place. Furthermore, the fact that six of the loci in seeds were not found in seedlings, juveniles or adults (see Table S1; see also Tonsor *et al.* 1993; Álvarez-Buylla *et al.* 1996; Mandák *et al.* 2006; Honnay *et al.* 2008) is additional evidence of natural selection: when it acts on ecophysiological variables, natural selection can make the genes that code certain enzymes transcribe and translate differentially, due to various metabolic requirements as the life cycle evolves (cf. Abid *et al.* 2009). This can cause some loci to appear in early stages of the life cycle (*i.e.* seeds and seedlings) but not in later stages (*i.e.* juveniles and adults).

The origin of heterozygote excess has been poorly studied. Stoeckel *et al.* (2006) suggest that heterozygote excess revealed by negative F_{IS} has several potential causes, including self-incompatibility systems, outcross breeding and low effective population size (Ne). Studies of small or subdivided populations found that it is common to find few reproductive individuals involved in producing the next generation – and that there is often differential natural selection towards heterozygous individuals to avoid the effects of genetic drift (Lesica & Allendorf 1992; Luijten *et al.* 2000). *C. miradorense* preliminary demographic results from 2004 to 2007 confirm the existence of few reproductive individuals (*i.e.*, those bearing flowers and fruits) in the two populations for which demographic data are available: Xico Viejo (29 ± 6 SD plants out of n = 178 plants) and Santuario (66 ± 27 plants out of n = 324 plants; F. Reyes-Zepeda, unpublished observations). Byers & Meagher (1992) showed that despite small population sizes (<25 reproductive individuals), high genetic diversity could be maintained, as in *C. miradorense*.

Several authors have found that the mating system and the rarity status explained the highest proportion of variation in levels of genetic diversity among species (Hamrick & Godt 1996; Aguilar *et al.* 2008). In conditions of fragmentation, the genetic diversity of self-compatible species is less affected by decreasing population size than that of obligate outcrossing and self-compatible but mainly outcrossing species (Honnay & Jacquemyn

Table 3. Wright's F statistics for four life history stages of *Cestrum miradorense* in eight populations located in a fragmented cloud forest landscape in central-eastern Mexico.

	stage											
	adults			seeds			seedlings			juveniles		
loci	F_{IT}	F_{ST}	F_{IS}	F_{IT}	F_{ST}	F_{IS}	F_{IT}	F_{ST}	F_{IS}	F_{IT}	F_{ST}	F_{IS}
6pgd1	−0.402	0.087	−0.535	−0.609	0.033	−0.664	−0.447	0.071	−0.558	−0.445	0.067	−0.548
6pgd2	−0.406	0.088	−0.541	−0.607	0.035	−0.665	−0.450	0.071	−0.560	−0.437	0.070	−0.546
Acph	−0.401	0.087	−0.536	−0.608	0.035	−0.667	−0.451	0.067	−0.556	−0.441	0.068	−0.545
Ald1	−0.404	0.087	−0.538	−0.604	0.035	−0.663	−0.447	0.071	−0.557	−0.439	0.070	−0.548
Ald2	−0.405	0.088	−0.540	−0.603	0.035	−0.661	−0.442	0.071	−0.553	−0.440	0.070	−0.548
Ald3				−0.613	0.033	−0.669						
Apx1	−0.410	0.088	−0.546	−0.616	0.035	−0.675	−0.454	0.070	−0.564	−0.443	0.071	−0.553
Apx2	−0.414	0.084	−0.543	−0.611	0.035	−0.670	−0.457	0.068	−0.563	−0.437	0.071	−0.547
Apx3	−0.427	0.080	−0.551	−0.620	0.033	−0.676	−0.469	0.063	−0.569	−0.457	0.066	−0.559
Dia1	−0.410	0.087	−0.544	−0.608	0.035	−0.667	−0.440	0.071	−0.550	−0.439	0.070	−0.548
Dia2				−0.605	0.035	−0.663						
Est	−0.411	0.084	−0.540	−0.607	0.035	−0.666	−0.456	0.067	−0.561	−0.446	0.066	−0.548
G3pdh1	−0.400	0.088	−0.535	−0.606	0.035	−0.664	−0.453	0.069	−0.561	−0.438	0.071	−0.549
G3pdh2	−0.402	0.087	−0.535	−0.604	0.035	−0.662	−0.444	0.072	−0.555	−0.440	0.068	−0.546
G3pdh3				−0.616	0.034	−0.672						
Hk1	−0.422	0.078	−0.542	−0.610	0.035	−0.669	−0.468	0.061	−0.563	−0.446	0.066	−0.548
Hk2				−0.610	0.034	−0.666						
Idh1	−0.406	0.085	−0.537	−0.609	0.034	−0.665	−0.458	0.067	−0.562	−0.445	0.067	−0.548
Idh2	−0.400	0.087	−0.534	−0.611	0.034	−0.667	−0.444	0.069	−0.552	−0.440	0.070	−0.548
Idh3				−0.627	0.030	−0.676						
Mdh1	−0.419	0.082	−0.546	−0.610	0.032	−0.664	−0.452	0.070	−0.560	−0.445	0.069	−0.551
Mdh2	−0.405	0.086	−0.538	−0.616	0.032	−0.670	−0.443	0.071	−0.553	−0.435	0.070	−0.543
Me1				−0.612	0.033	−0.666						
Me2	−0.420	0.081	−0.546	−0.605	0.034	−0.662	−0.460	0.067	−0.565	−0.449	0.067	−0.554
Mnr1	−0.400	0.087	−0.534	−0.598	0.036	−0.657	−0.450	0.070	−0.558	−0.435	0.071	−0.544
Mnr2				−0.610	0.035	−0.669						
Pgi1	−0.420	0.079	−0.541	−0.607	0.035	−0.666	−0.461	0.064	−0.562	−0.451	0.064	−0.551
Pgi2	−0.415	0.081	−0.539	−0.597	0.036	−0.656	−0.447	0.068	−0.553	−0.441	0.068	−0.546
Pgm1	−0.403	0.087	−0.536	−0.612	0.035	−0.671	−0.450	0.071	−0.560	−0.440	0.070	−0.549
Pgm2	−0.399	0.087	−0.532	−0.607	0.036	−0.666	−0.439	0.071	−0.550	−0.434	0.070	−0.542
Sdh1	−0.406	0.086	−0.539	−0.607	0.035	−0.666	−0.456	0.068	−0.561	−0.437	0.070	−0.545
Sdh2	−0.403	0.086	−0.535	−0.607	0.035	−0.665	−0.439	0.071	−0.550	−0.438	0.070	−0.546
Mean	−0.408	0.085	−0.540	−0.609	0.034	−0.667	−0.451	0.069	−0.558	−0.441	0.069	−0.548
SD	0.038	0.015	0.023	0.032	0.008	0.026	0.039	0.013	0.025	0.026	0.010	0.017
95% Confidence Interval	−0.476	0.059	−0.581	−0.669	0.021	−0.715	−0.524	0.045	−0.605	−0.490	0.051	−0.579
	−0.332	0.116	−0.493	−0.544	0.050	−0.615	−0.373	0.095	−0.509	−0.389	0.088	−0.512

2007). Montero-Castro (2006) reported that *Cestrum* species are pollinated by nocturnal moths and have out-crossed mating and self-incompatible reproductive systems (Haber & Frankie 1989; Castro-Laportte & Ruiz-Zapata 2000; Aguilar & Galetto 2004). Taking into account the estimated heterozygosity, gene flow and the presence of hermaphroditic flowers, it is likely that *C. miradorense* has a self-incompatible and outcross breeding system. However, research on the reproductive biology is required to confirm this hypothesis.

Species of old or recent rarity may represent different time scales and origins of disturbance, which affect the genetic characteristics that species currently have. *C. miradorense,* is considered an old rare species (*i.e.* having a long history of rarity) because during the Pliocene, the genus *Cestrum* had high rates of diversification and speciation, causing most of its species to become geographically restricted along mountain ranges and inhabiting mostly montane cloud forest

(Montero-Castro 2006). The consequent size reduction of population numbers (and eventual bottlenecks) triggered the development of adaptations to the new habitats that allowed the current high genetic diversity found in *C. miradorense*, as proposed for other species by Honnay & Jacquemyn (2007). Similar results were reported in Moreira *et al.* (2010) for *Coccoloba cereifera* Schw. (Polygonaceae), a micro-endemic shrub of southeastern Brazil, restricted to an area of ca. 26 km², that has high genetic diversity ($H_e = 0.507$).

Fragmentation and genetic structure

It has often been reported that habitat fragmentation may have modified species genetic structure (Honnay & Jacquemyn 2007). However, it is important to determine the time elapsed and the number of generations that have occurred in the fragmented habitat. Aguilar *et al.* (2008) found that only frag-
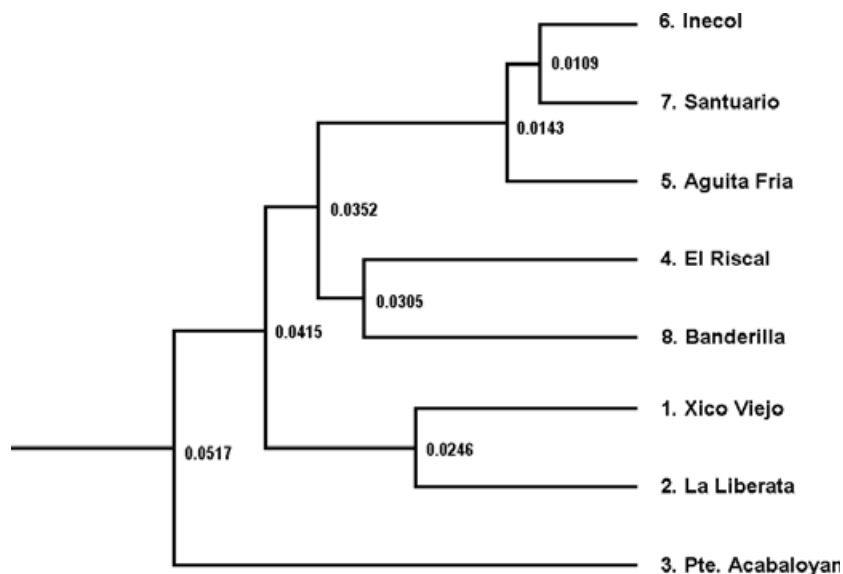
Fig. 4. Nei's genetic distances among eight populations of *Cestrum miradorense* growing in a fragmented landscape of cloud forest in central-eastern Mexico.

mented systems of at least 100 years old presented significantly stronger negative effects; whereas Lowe *et al.* (2005) mentioned that in tropical trees, it may take decades or centuries for the impact of fragmentation to appear, depending on factors such as breeding system, pollinator types and generation time. In the study area, habitat fragmentation began more than a century ago (Gerez 1992), and the longevity (L) of *C. miradorense* varied between 14.7 and 51.8 years while the generation time (μ_1) was 30 years. This demographic information was obtained, using the method described in Cochran & Ellner (1992), from the Santuario and Xico Viejo populations during the 2004–2007 period (F. Reyes-Zepeda, unpublished observations). This suggests that there have been at least three generations since the fragmentation process began in the central and eastern portion of the study area, and six generations in the western area according to fragmentation data available for these two areas (Gerez 1992; Williams-Linera *et al.* 2002).

The short time elapsed since the beginning of the fragmentation process also partially explains the high H_e and the low F_{ST} found in adults and seeds as compared to the average for 213 long-lived perennial plants ($H_e = 0.144$; $F_{ST} = 0.089$; Hamrick 2004). These results strongly suggest that *C. miradorense* has been able to maintain high levels of gene flow after fragmentation.

Some authors note that continuous habitat fragmentation gives rise to greater between-fragment pollination activity (*e.g.* Ward *et al.* 2005). Even though the pollinators of *C. miradorense* are unknown, we can assume that they are nocturnal moths, which have been consistently reported as the genus' main pollinator (Castro-Laportte & Ruiz-Zapata 2000; Aguilar & Galetto 2004; Montero-Castro 2006). Nocturnal moths promote intense pollen exchange, increasing the likelihood of higher gene flow among populations (Richards 1997).

Final considerations

For a long time it was assumed that rare species had lower genetic diversity than common ones, and that the genetic diversity of the former was more affected by habitat fragmentation (Young *et al.* 1996; Cole 2003). However, recent studies report that species with a long history of rarity are less susceptible to the erosion of genetic variation than common species or species than became rare only recently (Honnay & Jacquemyn 2007), underlining the importance of assessing the number of generations elapsed since population fragmentation began (Aguilar *et al.* 2008). We found that *C. miradorense*, a micro-endemic species restricted to a ca. 100 km² distribution area subjected to a fragmentation for more than 100 years ago, was able to maintain high levels of genetic diversity, both at population and life history stage levels, due to natural selection favouring heterozygote excess, and mating and pollen/seed dispersal systems that have allowed high gene flow.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Allele frequencies for 32 alloenzymatic loci in four life history stages (*i.e.* seeds, seedlings, juveniles and adults) of *Cestrum miradorense* in eight populations growing in a fragmented landscape of cloud forest in central-eastern Mexico.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

REFERENCES

Abid G., Silue S., Muhovski Y., Jacquemin J.M., Toussaint A., Baudoin J.P. (2009) Role of myo-inositol phosphate synthase and sucrose synthase genes in plant seed development. *Gene*, **439**, 1–10.

Aguilar R., Galetto L. (2004) Effects of forest fragmentation on male and female reproductive success in *Cestrum parqui* (Solanaceae). *Oecologia*, **138**, 513–520.

Aguilar R., Quesada M., Ashworth L., Herrerias-Diego Y., Lobo J. (2008) Genetic consequences of habitat fragmentation in plant populations: susceptible signals in plant traits and methodological approaches. *Molecular Ecology*, **17**, 5177–5188.

Allendorf F.W., Luikart G. (2007) *Conservation and the genetics of populations*. Blackwell Publishing, Oxford, 642 pp.

Álvarez-Buylla E.R., Chaos A., Piñero D., Garay A. (1996) Demographic genetics of a pioneer tropical tree species: match dynamics, seed dispersal, and seed banks. *Evolution*, **50**, 1155–1166.

Berg C., Greilhuber J. (1993) Cold-sensitive chromosome regions and heterochromatin in *Cestrum* (Solanaceae): *C. strigilatum*, *C. fasciculatum* and *C. elegans*. *Plant Systematics and Evolution*, **185**, 133–151.

Byers D.L., Meagher T.R. (1992) Mate availability in small populations of plant species with homomorphic sporophytic self-incompatibility. *Heredity*, **68**, 353–359.

Cabin R.J., Mitchell R.J., Marshall D.L. (1998) Do surface plant and soil seed bank populations differ genetically? A multi-population study of desert mustard *Lesquerella fendleri* (Brassicaceae). *American Journal of Botany*, **85**, 1098–1109.

Castillo-Campos G. (2003) *Biodiversidad de la selva baja caducifolia en un sustrato rocoso de origen volcánico en el centro del estado de Veracruz, México*. Thesis Doctoral. UAM. México, D.F.: 147 pp.

Castro-Laportte M., Ruiz-Zapata T. (2000) Biología floral y síndrome de polinización de cinco especies de *Cestrum* (Solanaceae). *Kurtziana*, **28**, 205–210.

Challenger A. (1998) *Utilización y Conservación de los Ecosistemas Terrestres de México: Pasado, Presente y Futuro*. CONABIO, Instituto de Biología, UNAM, Agrupación Sierra Madre, S.C. México, 847 pp.

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

Cochran M, Ellner S. (1992) Simple methods for calculating age-based life-history parameters for stage-structured populations. *Ecological Monographs*, **62**, 345–364.

Cole C.T. (2003) Genetic variation in rare and common plants. *Annual Review of Ecology, Evolution and Systematics*, **34**, 213–237.

Conte R., Nodari R.O., Vencovsky R., Sedrez dos Reis M. (2003) Genetic diversity and recruitment of the tropical palm, *Euterpe edulis* Mart., in a natural population from the Brazilian Atlantic forest. *Heredity*, **91**, 401–406.

Eguiarte L.E., Pérez-Nasser N., Piñero D. (1992) Genetic structure, outcrossing rate and heterosis in *Astrocaryum mexicanum* (tropical palm): implications for evolution and conservation. *Heredity*, **69**, 217–228.

Eguiarte L.E., Búrquez A., Rodríguez J., Martínez-Ramos M., Sarukhán J., Piñero D. (1993) Direct and indirect estimates of neighborhood and effective population size in tropical palm, *Astrocaryum mexicanum*. *Evolution*, **47**, 75–87.

Farwig N., Braun C., Bohning-Gaese K. (2008) Human disturbance reduces genetic diversity of an endangered tropical tree, *Prunus africana* (Rosaceae). *Conservation Genetics*, **9**, 317–326.

Fernández-M J.F., Sork V.L. (2007) Genetic variation in fragmented forest stands of the Andean oak *Quercus humboldtii* Bonpl. (Fagaceae). *Biotropica*, **39**, 72–78.

Gerez P. (1992) ¿Qué pasa en el Cofre de Perote? In: Boege E., Rodríguez H. (Eds), *Desarrollo y Medio Ambiente en Veracruz*. CIESAS, Instituto de Ecología y Friederich Ebert Stiftung, México, pp 151–157.

González-Astorga J., Castillo-Campos G. (2004) Genetic variability of the narrow endemic tree *Antirhea aromatica* Castillo-Campos & Lorence (Rubiaceae, Guettardeae) in a tropical forest of Mexico. *Annals of Botany*, **93**, 521–528.

González-Astorga J., Vovides A.P., Ferrer M.M., Iglesias C. (2003) Population genetics of *Dioon edule* Lindl. (Zamiaceae, Cycadales): biogeographical and evolutionary implications. *Biological Journal of the Linnean Society*, **80**, 457–467.

Goudet J. (2001) *FSTAT, A Program to Estimate and Test Genetic Diversities and Fixation Indices (Version 2.9.3)*. Available from http://www2.unil.ch/popgen/softwares/fstat.htm [accessed on 13 March 2011].

Haber W.A., Frankie G.W. (1989) A tropical hawkmoth community: Costa Rican dry forest Sphingdae. *Biotropica*, **21**, 155–172.

Hamrick J.L. (2004) Response of forest trees to global environmental change. *Forest Ecology and Management*, **197**, 323–335.

Hamrick J.L., Godt M.J.W. (1996) Effects of the history traits on genetic diversity in plants. *Philosophical Transactions of the Royal Society of London B*, **351**, 1291–1298.

Hedrick P.W. (2000) *Genetics of populations*, 2nd edition. Jones and Bartlett, Boston, MA, 553 p.

Hernández-Verdugo S., Luna-Reyes R., Oyama K. (2001) Genetic structure and differentiation of wild and domesticated populations of *Capsicum annuum* (Solanaceae) from Mexico. *Plant Systematics and Evolution*, **226**, 129–142.

Honnay O., Jacquemyn H. (2007) Susceptibility of common and rare plant species to genetic consequences of habitat fragmentation. *Conservation Biology*, **21**, 823–831.

Honnay O., Bossuyt B., Jacquemyn H., Shimono A., Uchiyama K. (2008) Can a seed bank maintain genetic variation in the above ground plant population? *Oikos*, **117**, 1–5.

INEGI (1995). *Aerial photographs. Scale 1:20000*. Instituto Nacional de Estadística, Geografía e Informática, INEGI. Mexico City, Mexico.

Kalisz S., Nasom J.D., Hanzawa F.M., Tonsor S.J. (2001) Spatial population genetic structure in *Trillium grandiflorum*: the role of dispersal, mating, history, and selection. *Evolution*, **55**, 1560–1568.

Leimu R., Mutikain P., Koricheva J., Fisher M. (2006) How general are positive relationships between plant population size, fitness and genetic variation? *Journal of Ecology*, **94**, 942–952.

Lesica P., Allendorf F.W. (1992) Are small populations of plants worth preserving? *Conservation Biology*, **6**, 135–139.

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

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

Luijten S.H., Dierick A., Oostermeijer J.G.B., Raijman L.E.L., Den Nijs H.C.M. (2000) Population size, genetic variation, and reproductive success in a rapidly declining, self-incompatible perennial (*Arnica montana*) in the Netherlands. *Conservation Biology*, **14**, 1776–1787.

Mandák B., Bímová K., Mahelka V., Plačková I. (2006) How much genetic variation is stored in the seed bank? A study of *Atriplex tatarica* (Chenopodiaceae). *Molecular Ecology*, **15**, 2653–2663.

Mathiasen P., Rovere A.E., Premoli A.C. (2007) Genetic structure and early effects of inbreeding in fragmented temperate forest of a self-incompatible tree, *Embothrium coccineum*. *Conservation Biology*, **21**, 232–240.

Miller M.P. (1997) *Tools for population genetic analyses (TFPGA) v. 1.3: a Windows program for the genetic data*. Computer Software Distributed by Author.

Montero-Castro J.C. (2006). *Filogenia Molecular de Cestrum L. sección Habrothamnus (Endl) Schtl. (Solanaceae): Un proceso de diversificación rápida*. Tesis de Doctorado, Doctorado en Ciencias Biológicas, UNAM. D.F.: 111 pp.

Montero-Castro J.C., Delgado-Salinas A., Luna E., Eguiarte L. (2006) Phylogenetic analysis of *Cestrum* section Habrothamnus (Solanaceae) based on plastid and nuclear DNA sequences. *Systematic Botany*, **31**, 843–850.

Moreira R.G., McCauley R.A., Cortés-Palomec A.C., Fernandes G.W., Oyama K. (2010) Spatial genetic structure of *Coccoloba cereifera* (Polygonaceae), a critical endangered microendemic species of Brazilian rupestrian fields. *Conservation Genetics*, **11**, 1247–1255.

Müller-Strack G. (1998) Isozymes. In: Karp A., Isaac P.G., Ingram D.S. (Eds), *Molecular tools for screening biodiversity. Plants and animals*. Chapman and Hall, London, pp 75–81.

Muñoz-Villers L.E., López-Blanco J. (2008) Land use/cover changes using Landsat TM/ETM images in a tropical and biodiverse mountainous area of central-eastern Mexico. *International Journal of Remote Sensing*, **29**, 71–93.

Nee M. (1986) *Solanaceae I. Flora de Veracruz*. Fascículo 49. INIREB. Xalapa, Veracruz, Mexico, 48 pp.

Nee M. (2001) An overview of *Cestrum*. In: Van den Berg R.G., Barendse G.W.M., van der Weerden G.M., Mariani C. (Eds), *Solanaceae V: advances in taxonomy and utilization*. Nijmegen University Press, Nijmegen, pp 109–136.

Nei M. (1972) Genetic distances between populations. *American Naturalist*, **106**, 283–292.

Nunes F.J., Fernandes T., Domingues T.J.M., Odete V. A., Laforga V. A. L. (2006) Karyotype differentiation of four *Cestrum* species (Solanaceae) based on the physical mapping of repetitive DNA. *Genetics and Molecular Biology*, **29**, 97–104.

Octavio-Aguilar P., González-Astorga J., Vovides A.P (2009) Genetic diversity by life history of *Dioon edule* Lindley (Zamiaceae, Cycadales). *Plant Biology*, **11**, 525–536.

Rice WR (1989) Analyzing tables of statistical tests. *Evolution*, **43**, 223–225.

Richards A.J. (1997) *Plant breeding systems*, 2nd edition. Chapman and Hall, London, 529 pp.

Rzedowski J. (1996) Análisis preliminar de la flora vascular de los bosques mesófilos de montaña de México. *Acta Botanica Mexicana*, **35**, 25–44.

Slatkin M. (1993) Isolation by distance in equilibrium and nonequilibrium populations. *Evolution*, **47**, 264–279.

Sneath P.H.A., Sokal R.R. (1973) *Numerical taxonomy*. W. H. Freeman, San Francisco, 573 pp.

Soltis D.E., Soltis P.S. (1989) *Isozymes in plant biology*. Dioscorides Press, Portland, OR, 504 pp.

Stoeckel S., Grange J., Fernández-Manjarrez J.F., Bilger I., Frascaria-Lacoste N., Maeriette S. (2006) Heterozygote excess in a self-incompatible and par-

tially clonal forest tree species –*Prunus avium* L. *Molecular Ecology*, **15**, 2109–2118.

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

Ward M., Dick C.W., Gribel R., Lowe A.J. (2005) To self, or not to self… A review of outcrossing and pollen-mediated gene flow in neotropical trees. *Heredity*, **95**, 246–254.

Weir D.S., Cockerham C.C. (1984) Estimating *F*-statistics for the analysis of population structure. *Evolution*, **38**, 1358–1370.

Wendel J., Weeden N. (1989) Visualization and interpretation of plant isozymes. In: Soltis D.E., Soltis P.S. (Eds) *Isozymes in plant biology*. Dioscorides Press, Portland, OR, pp 5–44.

Whalen M.D., Caruso E.E. (1983) Phylogeny in *Solanum* sect. Lasiocarpa (Solanaceae): congruence of morphological and molecular data. *Systematic Botany*, **8**, 369–380.

Williams-Linera G., Manson R.H., Isunza E. (2002) La fragmentación del bosque mesófilo de montaña y patrones de uso de suelo en la región oeste de Xalapa, Veracruz, México. *Madera y Bosques*, **8**, 73–79.

Young A.G., Boyle T., Brown A.H.D. (1996) The population genetic consequences of habitat fragmentation for plants. *Trends in Ecology and Evolution*, **10**, 413–418.

Zar J.H. (2010) *Biostatistical analysis*, 5th edition. Pearson Prentice Hall, Upper Saddle River, NJ, pp 214–218.